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THE LIPIDS

Volume I

CHEMISTRY

Volume II

BIOCHEMISTRY

*Digestion, Absorption,
Transport and Storage*

Volume III

BIOCHEMISTRY

*Biosynthesis, Oxidation, Metabolism,
and Nutritional Value*

THE LIPIDS

Their Chemistry and Biochemistry

HARRY J. DEUEL, Jr.

*Dean, Graduate School, and Professor of Biochemistry,
University of Southern California, Los Angeles*

Volume III: BIOCHEMISTRY

***Biosynthesis, Oxidation, Metabolism,
and Nutritional Value***

1957

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To my wife
GRACE C. DEUEL,
and to my many friends
all over the world.

HARRY J. DEUEL, Jr.

HARRY JAMES DEUEL, Jr., Ph.D.

1897-1956

Harry James Deuel, Jr., was born in St. Paul, Minnesota, October 15, 1897, the son of Harry James Deuel and Myrtle Lillian (Mouser) Deuel. After graduating from a local high school he entered Carleton College, where he displayed particular interest in science, doing his major work in chemistry. Before receiving his degree he accepted a position as Junior Chemist in the United States Department of Agriculture, Washington, D. C., which he held for two years, during which time he also did advanced work in his field as a student at George Washington University. His aptitude for research work, and his industry, became evident in the course of these years when he was an assistant to C. F. Langworthy. Six papers were published under their joint authorship during this period. At this time he also collaborated with Arthur D. Holmes in research reported in five papers. All of these earlier publications were in the field of the digestibility of various specific foods and classes of nutrients. The paper on "Digestibility of Certain Miscellaneous Vegetable Fats" (A. D. Holmes and H. J. Deuel, Jr., *J. Biol. Chem.*, 41, 227-235, 1920) was the first of what was to be a long and distinguished series of publications dealing with lipids.

The developing scientist evidently felt the need for more advanced study, and this led to his entrance into the Yale University Graduate School as a student for the doctorate under Professor Lafayette B. Mendel. This work was successfully completed in 1923, when he received the Doctor of Philosophy degree in Physiological Chemistry from Yale University. His doctoral dissertation was entitled "The Chemistry and Physiology of the Pyrimidines, Thymine and Uracil with Reference to Nucleic Acid Metabolism." To accomplish this work it was necessary for him to synthesize these pyrimidines in quantities sufficient to permit their administration to dogs for the metabolism study. While in the Yale Laboratory he also collaborated with the writer in "A Comparison of the Paths of Absorption with Respect to the Action of Secretin upon the Pancreas." This was made the basis of a report to the XIth International Physiological Congress held in Edinburgh in 1923, which was attended by both authors. This was the first of many such international meetings which Dr. Deuel attended over the many remaining years of his career.

As a result of these travels he came to know in a very personal and intimate way many distinguished colleagues in other countries, and was often able to introduce to them various American colleagues who were making their first trip to Europe. In this activity his personal qualities of friendship, interest in other people, and ingratiating manner made him a peculiarly effective ambassador and representative of American scientists.

One year after receiving his Ph.D. degree, Dr. Deuel married Grace Antoinette Cutting, a graduate student in History and Mathematics, Teachers' College, Columbia University. Thus began a long and happy married life during which his wife proved to be a most valuable aide in ways difficult to describe in words. She was his congenial companion on his many trips abroad. Their home was a place where faculty and students alike felt truly at ease in an atmosphere of friendliness and mutual interest. As one friend expressed it: "Here was a man who could carry out his official obligations with dignity and yet have time to make the shyest new foreign student feel at home." In this as well as in many other phases of his activities, the help of Mrs. Deuel was incalculable.

The first position which Dr. Deuel accepted on the advice of Professor Mendel was that of Assistant in Physiology in the Cornell University Medical College, New York City, under the distinguished Graham Lusk. He held this position for five years (1923-1928), and then was appointed Professor of Physiology and Head of the Department at the University of Maryland Medical School. During the first year of this appointment he was offered, and accepted, the post of Professor of Biochemistry in the newly reorganized School of Medicine at the University of Southern California, the position which he held for the rest of his career. For twenty years (1929-1949) he served as Head of the Department of Biochemistry. During this time, in recognition of the importance of nutritional biochemistry and of nutrition as a science in itself, the title of his position was changed to Professor of Biochemistry and Nutrition.

In 1949 Dr. Deuel was appointed Dean of the Graduate School at the University of Southern California. In this capacity he visited various institutions throughout the United States to see how their graduate schools were organized, and then directed the planning and operation of the school in his own institution. Because of his great personal drive and industry, he was able to accomplish all of this without sacrificing research and scholarly work of his own, shown by the fact that, as Dean, he not only continued to direct younger colleagues and graduate students in various research projects, but published many scientific papers, as well as the first

two volumes of the series entitled *The Lipids, Their Chemistry and Biochemistry*. The present volume completes this series.

Some idea of Dr. Deuel's scope of scientific activity can be gained from the fact that a complete list of publications which carry his name either as senior author or collaborator totals 276 items. The titles show that he maintained a wide breadth of view and interest. His experience as a teacher of physiology to medical students (with Graham Lusk at Cornell, and at the University of Maryland) was no doubt responsible in part for his continuing interest in such topics as carbohydrate metabolism, diabetes, and energy metabolism. More than 25 of his papers deal with these themes. Such topics of course naturally led to investigations in the field of lipids and their metabolism. This resulted in his conviction that somewhere a book or two should be available summarizing existing knowledge concerning the lipids. He therefore decided to undertake the writing of it as a personal assignment. Two of his volumes have already appeared. The present one represents the completion of this personally assigned and personally accepted responsibility.

As an active contributing scholar in his generation Dr. Deuel was elected to numerous professional organizations, only a few of which can be mentioned here: the American Physiological Society, American Society of Biological Chemists, American Chemical Society, Biochemical Society (London), Harvey Society (New York), the British Nutrition Society, and The American Institute of Nutrition. This latter society elected him President just before his death. He also served the American Institute of Nutrition as a member of the editorial board of its official publication, the *Journal of Nutrition*, for the usual term of four years (1946-1950). He was a member of the Board of Directors of Annual Reviews from 1946 on, and was elected to the presidency of the Board in 1953. In 1949 Dr. Deuel received the Borden Award. Through the Institute of Food Technologists he received the Monsanto Presentation Award in 1954. In January, 1956, during his year abroad on a Fulbright Lectureship, he was given an Honorary Medal by the University of Brussels. During this same year he attended six scientific meetings in England and continental Europe, and delivered numerous lectures before universities (Oxford, Cambridge, Edinburgh, Aberdeen, Dundee, Glasgow) and other professional groups, including the British Society of Nutrition.

As might be expected of a person as prominent as he was, Dr. Deuel was often called upon to serve his country, state, and community in various capacities. He was head of a Committee on Oilseeds and Fats under the

Dept. of Agriculture, and of other government committees concerned with nutrition.

The news of his passing evoked numerous expressions of sorrow and affection from many groups, his university circle, colleagues, students, and friends. It is fitting that this biography close with some passages taken from these many letters to Mrs. Deuel. "The life of a truly great and good man cannot be formalized in words. To be sure, scientific achievements, concrete contributions to the world's knowledge, awards of merit, and participation in scientific progress by publications, lectures, and laboratory research can be recorded. But the qualities of the spirit which make a human being stand out among his fellows, kindness and staunch defense of justice, the warmth of friendship, and the lovable personality of a man like Harry Deuel cannot be put into words."

"His passing has been a great loss not only to the University, to science, and to the community, but, also, to mankind wherever he touched. He was one of those invaluable persons whose moral code was never compromised by the trends of the day. The greatest loss, one feels, is to the youth who could have known him, for here was man with old-fashioned virtues who had not only achieved eminence but, also, the love and respect of all who knew him. He was one of the University of Southern California's greatest ambassadors."

One of his international students called him "the man with the golden heart."

GEORGE R. COWGILL

Yale University
New Haven, Connecticut

INTRODUCTORY EDITORIAL NOTE

On April 17, 1956, Dr. Harry J. Deuel, Jr., passed away following the recurrence of a serious illness for which he had been operated upon in 1954. After the operation he maintained as his chief professional objective the completion and publication of this third volume of his work *The Lipids, Their Chemistry and Biochemistry*. Before his death he had done most of the necessary writing. It remained for some of his colleagues and friends to see this written material through the press.

The entire text of this third volume had been completed by Professor Deuel several months before his return to the United States from England, where he was lecturing under the auspices of the United States Educational Commission in the United Kingdom, and on the continent, during the winter of 1955-1956.

During his last months in England the material of the latter half of the volume was being typed and checked in Los Angeles. These latter chapters were reviewed by Dr. Deuel's friends and associates before being forwarded to the publisher. For the most part, however, this material has been left unchanged so the volume is consistent with the style and organization of the first two volumes and in accord with the author's intentions. Certain minor corrections of details have been made to bring the material up to date beyond the time when it was originally written.

The bibliographic research and the technical work of preparing the manuscript for the publisher was carried out by Mrs. Margaret Ritter who fulfilled the same responsibilities for Volumes I and II.

Chapters VIII, IX, X, XI, XII, and XIV were reviewed by Dr. Roslyn B. Alfin-Slater and Dr. Ernest Geiger; Chapter XIV was also read by Dr. Wendell Griffith. Chapter XIII was revised by Dr. Raymond Reiser from an article written by Dr. Deuel and Dr. Reiser and previously published in *Vitamins and Hormones*, 13, 29-70, 1955. Dr. James Mead also made several suggestions for the revision of this chapter. Gratitude is hereby expressed to the Academic Press for permission to use the material forming a large part of this chapter of the present volume.

We would also like to thank Mrs. Joanne Forney, Mrs. Helen Rice, and Mrs. Marie Visser for typing the final manuscript, Dr. Lilla Aftergood for

preparing the indices, Dr. John Forney, Dr. Howard Hill, Dr. Norman T. Mattox, Dr. Sydney Rittenberg, Mr. Arthur Wells, and Dr. L. Zechmeister for checking some of the scientific details and nomenclature, and Dr. George R. Cowgill for preparing the biography.

Los Angeles, September, 1957

STANLEY R. TOWNSEND
—*for the Editorial Committee*

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THE LIPIDS

Volume III: BIOCHEMISTRY

*Biosynthesis, Oxidation, Metabolism,
and Nutritional Value*

CHAPTER 1

GENERAL CONSIDERATIONS OF LIPID DIGESTION, ABSORPTION, TRANSPORT, AND STORAGE

1. Introduction

The utilization of fats and other foodstuffs naturally falls into two distinct divisions. The first one is largely concerned with enzymatic changes of the foodstuff in the gastrointestinal tract by which the foodstuff is prepared for absorption. The absorption of such products from the gastrointestinal tract, together with their transport by way of the lymphatics or portal system and their preliminary deposition in the tissue, constitute closely related phases. These have been considered at some length in Volume II of *The Lipids*.

The second section into which the discussion of the biochemistry of fats and other foodstuffs is obviously divided includes the so-called intermediary metabolism of these substances. Although the term, intermediary metabolism, is usually considered to encompass all changes which take place from the time the substances under consideration are absorbed until their end-products are excreted in the urine, feces, expired air, or through the skin, the transformations which involve synthesis, oxidation, and intermediate changes of these substances actually comprise the chief importance of intermediary metabolism. These phases of intermediary metabolism of fats will be considered in the present volume.

The metabolism of the triglyceride fats, phospholipids, fatty acids, cerebroside, sterols, fat-soluble vitamins, and even hydrocarbons, displays many interrelationships. However, the metabolism of triglycerides, and that of phospholipids and of fatty acids, are so intimately connected that it is impossible to consider them separately. In the subsequent chapters on synthesis, oxidation, and intermediary changes, the above three groups of fats will be considered as a unit. Cerebroside and hydrocarbons will be dealt with separately, while the metabolic interrelations of

the sterols will constitute a separate chapter, as will also the discussion of the metabolism of the fat-soluble vitamins.

2. The Digestion and Absorption of Triglycerides, Phospholipids, and Fatty Acids in the Gastrointestinal Tract

There are two main theories to explain the mechanism by which fats are absorbed. According to one of these, the so-called Lipolytic Theory, which has been supported by Pflüger, Bloor, and most recently by Verzár, triglyceride fats are completely hydrolyzed in the intestine, by pancreatic lipase, to fatty acids and to glycerol. The bile acids are believed to aid in the absorption of the fatty acids by forming a soluble coordination compound, which is called a choleic acid. As soon as this is transported into the intestinal mucosa, the complex is broken down, and the bile acids are excreted into the lumen of the intestine to aid in the absorption of additional fatty acid molecules. The fatty acids thus set free in the intestinal mucosa combine directly with glycerol to yield triglycerides. It has been suggested that this synthesis involves the intermediate formation of the phospholipids, particularly that of lecithin. However, current evidence indicates that the rate of lecithin turnover in the intestinal mucosa is not sufficiently great to account for more than a fraction of the total fat passing through this intermediate stage. It is obvious that, with a total hydrolysis of the triglyceride molecules in the gut, followed by their resynthesis in the intestinal mucosa, a complete realignment in the orientation of the fatty acids in the triglyceride molecules may be expected, with the result that the newly synthesized fat may have properties which vary considerably from those of the ingested fat.

The second theory which has been proposed to explain the mechanism of fat absorption is known as the Particulate Theory. This hypothesis has been largely developed by Frazer and his colleagues. According to this concept, only a minimum hydrolysis of fat occurs in the gastrointestinal tract. The small amount of fatty acid and monoglycerides so formed, together with bile salts, stabilize the emulsion of the remaining triglyceride fat most effectively. It is believed that these fat droplets, which measure less than $0.5\ \mu$, are capable of passing directly into the cell. The small proportion of free fatty acid, monoglycerides, and diglycerides, are built up into triglycerides in the intestinal mucosa. Frazer is now of the opinion that the fat can be worked over in the intestinal mucosa to bring about a reorientation of the fatty acids in the glycerides. Moreover, Frazer

postulates that the triglycerides of the short-chain acids, such as tributyrin, are completely hydrolyzed in the gut.

Most of the discussion and experimentation on absorption has centered on the triglycerides. Until recently, it has generally been accepted that the phospholipids are partially or completely hydrolyzed in the gastrointestinal tract. This concept must now be modified in view of convincing evidence that a considerable proportion of the phospholipid may be absorbed without undergoing hydrolysis.

The mechanism of the absorption of fatty acids, when fed as such, is controversial. According to the Lipolytic Theory, it was believed that the free fatty acids were absorbed by the same mechanism as were those given in combination as triglycerides. The only exception is that the first step in the absorption process of the triglycerides, namely that of hydrolysis, is not required. On the other hand, Frazer presented some experimental evidence to the effect that the pathway of absorption and distribution of the fatty acids in the tissues differs according to whether triglyceride fats or the corresponding free fatty acids are fed. For a more complete discussion of the digestion and absorption of triglycerides, phospholipids, and fatty acids, the reader is referred to Chapters II and IV of Volume II, *The Lipids*.

3. The Role of the Lymph and Blood in the Transport of Triglycerides, Phospholipids, and Fatty Acids

Information is fragmentary as to the mechanism by which the fat particles pass from the epithelial cells of the intestinal wall to within the intestinal villi during the active phases of fat absorption. These epithelial cells lining the gut can store only a fraction of the total lipid which may be taken in with a single normal meal; it is therefore necessary for the fat droplets in these cells to be transferred to the lymph stream as rapidly as possible, to assure that absorption be a continuous process.

There are several theories as to how fat travels from the interior of the cell into the villus. The theory of Schafer postulates that the leucocytes carry out this transport by engulfing the fat droplets and then disgoring the fat into the lactels. Heidenhain suggests that the contraction of protoplasm accomplishes the transfer of the fat droplets to the lymphatics. A third theory (Loevenhart) postulates that the neutral fat is hydrolyzed at each cell boundary, and that the fatty acids and glycerol are resynthesized into neutral fat after passage through the cell membrane.

There are two pathways for transporting fat from the intestinal wall to the liver and tissues. The first of these involves the route *via* the lacteals

(in the villi), the intestinal lymphatics, the thoracic duct, and the blood stream. This is the principal pathway for fats, and practically complete recoveries of ingested fat can be obtained in the lymph if one collects this fluid from the thoracic duct. However, the fat appears in the lymph in fine droplets of neutral fat (chyle), irrespective of whether it has been ingested as neutral fat, diglycerides, monoglycerides, or fatty acids. The fine fat droplets are referred to as "chylomicrons." Ordinarily it is six hours before the maximum fat levels are obtained in the blood after the ingestion of a fatty meal; several additional hours elapse before the function of transporting the fat from the intestines to the tissues is completed, as indicated by the return of the blood lipids to the prefeeding levels. It is believed that phospholipids are transported along with the triglycerides *via* the lacteals, lymphatics, and the blood stream. They presumably aid in maintaining the finely-emulsified state of the fat. Free fatty acids are not ordinarily present in either the lymph or the blood stream, except in minimal quantities.

The second route available for fat transport from the intestines is by way of the portal system. This alternate pathway originates in the capillary network in each villus, from which it gradually builds up to the portal system through which the blood ultimately reaches the liver. Although this pathway is of little importance as a means of transporting the triglycerides and other lipid components, it is of major significance in the absorption of the water-soluble products arising from the digestion of proteins and carbohydrates. It is likewise believed that triglycerides with short-chain (hence water-soluble) fatty acids are transported by the portal route.

The blood stream is the ultimate route for the transfer of the lipids, not only from the intestine to the liver and to the storage depots, but also from the storage depots to the other organs, during fasting. Normally, the total lipids in the blood are present, on an average, to the extent of about 600 mg. %, of which the neutral fats comprise one-fourth. Fatty acids, which occur not only in combination with glycerol but also in the phospholipids and with cholesterol and vitamin A esters, account for approximately 350 mg. %. Some of the fats occur in combination with proteins in the form of the so-called lipoproteins. The level of blood lipids varies not only with the ingestion of food but with such physiologic factors as age, sex, race, pregnancy, lactation and time of day, with inanition, with over-nutrition, and with work, as well as with many pathologic conditions. For a more complete discussion of blood lipids, the reader is referred to Chapter V of Volume II, *The Lipids*.

4. The Deposition of Triglycerides and Phospholipids in the Tissues

According to the general consensus of opinion, neutral fats have a comparatively brief stay in the blood and tissues before they become oxidized or until they are incorporated into the depot fats. It has been suggested that the newly-absorbed fats are first carried to the liver, where they are worked over and remodeled to conform to the fatty acid pattern of the storage fat of the particular animal involved. However, the fat depots themselves must exert some power to modify the type of fat which they store. Otherwise, all storage fats in any one animal would be identical, and it is a well-known fact that there are marked variations in the physical constants of the depot fats from different parts of the animal body. The chief sites in which neutral fat is stored in the animal are in the muscle (intramuscular), around the ovaries (ovarian or genital), under the skin (subcutaneous), in the connective tissue around the kidney (perirenal), around the heart (pericardial), in the mesentery (mesenteric), and in the omentum (omental). This adipose tissue may contain as high a proportion as 90% of neutral fat. Although lipids are present in every organ of the body, they do not consist primarily of neutral fats, but rather of phospholipids, cholesterol, and cerebrosides. These latter lipids serve in these organs as essential structural components.

In the case of the non-herbivores, the depot fat originates, at least in part, from the ingested fats. This is proved by the demonstration of the presence of unusual fatty acids in the depot fats when fats containing non-physiologic fatty acids are fed. This effect can be particularly noted when the fat consumption is high. However, a considerable proportion of the fat deposits may likewise originate from newly-synthesized fat derived from carbohydrate, as well as from protein. It is believed that this is the chief source of the body fat in ruminants. This fact explains the relative constancy in the composition of the fat in this group of animals, and the difficulty of influencing its composition as the result of diet. Proof has been forthcoming that one site of synthesis of body fats from carbohydrates may be in the adipose tissue itself.

In addition to diet, a number of factors influence the site and quality of the fat deposited in the several depots. These include the species of the animal, age, sex, environmental temperature, and exercise. Fat deposition is greatly altered by ovariectomy, or by the administration of thyroxine.

The fat stored in the adipose tissues represents a dynamically active

component. Although the quantity of fat so stored remains fairly constant, it is now known that newly-absorbed fat, as well as newly-synthesized fat, is constantly being added to the depots, where it replaces a corresponding amount of fat which has been oxidized. It has been demonstrated that, even in situations in which a negative caloric balance obtains, some of the ingested fat is deposited in the adipose tissue, while a larger part is lost by its transfer from the adipose tissue to other organs, where it is oxidized. The reader is referred to Chapter VI (Volume II, *The Lipids*) for general factors concerned with fat storage in the animal body. Chapter VII of this earlier volume includes a review of the nature of the lipids stored in the several organs.

Under normal conditions phospholipids are not stored in the adipose tissue. They do, however, make up an appreciable proportion of the lipid in the intestinal wall, the liver, the kidney, and other organs. It is supposed that they have a function in the structure of the cells of these organs, and that they are concerned with intermediary metabolism and transport. Plasma phospholipids appear to be entirely synthesized in the liver. In such abnormal conditions as Niemann-Pick's disease, the deposition of sphingomyelin occurs in the reticuloendothelial system and in most organs; in another type of lipidosis, called Tay-Sachs' disease, the lipid deposited in the tissues is a ganglioside.

5. The Absorption, Transport, and Storage of Cerebrosides and Hydrocarbons

Little is known about the digestion and absorption of the cerebrosides. Since the normal diet of man contains only negligible amounts of cerebrosides, it is generally believed that the cerebrosides present in the tissues originate *in situ* from the hydrolysis products rather than that they are carried there from the intestinal tract. Moreover, since the cerebroside molecule is readily hydrolyzed by lipases, it would seem probable that they would not be absorbed without at least partial hydrolysis if they were present in the diet. Definite quantities of cerebrosides are present in the blood, but they are chiefly in the erythrocytes and leucocytes rather than in the plasma. Cerebrosides are not stored in the adipose tissue. Normally their main concentration is in the brain and spinal cord, although the liver, spleen, and related organs contain small amounts. In the abnormal condition known as Gaucher's disease, or reticular and histiocytic cerebrosidosis, cerebrosides are found in high concentrations in the liver, and especially in the spleen and other parts of the reticulo-

endothelial system. It is believed that, in this case also, the cerebroside originate at the site where they are deposited, rather than that they are carried to the latter organs from the brain. The chief argument for this hypothesis is the demonstration that the cerebroside in the brain is a galactolipid, while that in the spleen is largely, if not entirely, a glucolipid.

Hydrocarbons are absorbed to only a small extent by the higher animals. Certain species of animals, including man, are able to absorb β -carotene from the gastrointestinal tract, and to transport it to the liver and other tissues. It is believed that this is effected by means of a carotenoid-protein combination which is present in the blood. Mineral oil is another hydrocarbon which can be absorbed to some extent. It has been demonstrated that this substance accumulates in the liver. The mechanism by which such hydrocarbons are metabolized and ultimately removed from the tissues is not clear. For a complete exposition of the various phases of digestion, absorption, transport, and storage of the several lipids, the reader is referred to *The Lipids*, Volume II.

CHAPTER II

THE BIOSYNTHESIS OF TRIGLYCERIDES, PHOSPHOLIPIDS, AND FATTY ACIDS IN THE ANIMAL BODY

I. Introduction

Considerable evidence has accumulated to support the hypothesis that fat is readily synthesized in the body from non-fatty materials such as carbohydrates and proteins. Thus, it has been shown by the use of balance experiments that the amount of carbon unaccounted for in the excreta after the administration of starch is so great that it can be stored only in the form of fat. Moreover, the respiratory quotients observed in animals on a high-carbohydrate diet have been found in many cases to exceed unity—a condition which should obviously occur when an oxygen-rich substance (such as carbohydrate) is being converted to an oxygen-poor compound (such as fat). Probably the most convincing argument to prove the change of carbohydrate to fat in the animal is the demonstration of isotopic carbon in fats after isotopically tagged carbohydrates have been fed.

Proteins likewise are known to serve as a source of the triglycerides in the body. The same basic experimental procedures have been employed to demonstrate the change of protein $\rightarrow$ carbohydrate, as well as the complete transformation of protein $\rightarrow$ fat. Since the proof that carbohydrate can be changed to fat is extensive and convincing, the evidence that protein can yield carbohydrate is *a priori* proof that it can also be converted to fat.

Experiments have likewise demonstrated the complete protein-fat reaction. These procedures for establishing this change include the classical type of balance experiments, as well as the response of the R.Q. in dogs receiving large doses of protein; these confirm the fact that the non-protein moiety may be stored in the form of fat. The proof of the synthesis of fat from carbohydrate and protein has been detailed in Vol. II, *The Lipids* (pp. 538-553). The present chapter will be concerned chiefly with the intermediary changes in these transformations.

2. The Biosynthesis of Saturated Fatty Acids

The remarkable fact that practically all natural fats are composed of fatty acids with even numbers of carbons is convincing evidence that they are synthesized by the condensation of an intermediate containing an even number of carbon atoms. In fats such as butterfat, definite amounts of all of the even-numbered saturated fatty acids from butyric (C_4) to arachidic (C_{20}) occur. Since the differences in chain length are stepwise and amount to two carbons each, it is only natural to postulate that a two-carbon compound forms the building stone in the synthesis of the different fatty acids. As early as 1907, Raper¹ stated that "the formation of fatty acids in animals, from carbohydrates, . . . suggests that these acids are produced by the condensation of some highly reactive substance containing two carbon atoms and formed in the decomposition of sugar." The subject of the synthesis of fat from such two-carbon fragments as acetate has been reviewed by Bloch,^{2,3} by Kleinzeller,⁴ by Popják,⁵ and by Folley.⁶ Medes, Spirtes, and Weinhouse⁷ described a method for estimating the rate of fatty acid synthesis in liver slices, which consists in incubating slices of tissues with labeled acetyl precursors and comparing the specific activities of the acetoacetate and fatty acids.

(1) Possible Building Stones of Fatty Acids

a. Acetaldehyde as the Two-Carbon Fragment Required for Fatty Acid Synthesis. Acetaldehyde ($CH_3 \cdot CHO$) was first assumed to be an intermediate in the synthesis of fatty acids from glucose. Nencki⁸ discovered that two molecules of acetaldehyde were formed from each molecule of glucose when sugar was fermented by yeast; he further reported that these acetaldehyde molecules condensed in an alkaline medium to give aldol ($CH_3 \cdot CHOH \cdot CH_2 \cdot CHO$). It was suggested that this compound might be converted to butyric acid. This theory was further expanded by Magnus-Levy⁹ and by Raper¹ to explain the formation of long-chain acids as a re-

¹ H. S. Raper, *J. Chem. Soc.*, 91, 1831-1838 (1907).

² K. Bloch, *Physiol. Revs.*, 27, 574-620 (1947).

³ K. Bloch, *Cold Spring Harbor Symposia Quant. Biol.*, 13, 29-34 (1948).

⁴ A. Kleinzeller, *Advances in Enzymol.*, 8, 299-341 (1948).

⁵ G. Popják, *Fat Synthesis from Small Molecules*, in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia, No. 9, Cambridge Univ. Press, 37-51 (1952).

⁶ S. J. Folley, *Aspects of Fat Metabolism in the Ruminant, with Special Reference to the Biosynthesis of Milk Fat*, in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia, No. 9, Cambridge Univ. Press, 52-65 (1952).

⁷ C. Medes, M. A. Spirtes, and S. Weinhouse, *J. Biol. Chem.*, 205, 401-408 (1953).

⁸ M. Nencki, *J. prakt. Chem.* [2], 17, 105-124 (1878).

⁹ A. Magnus-Levy, *Arch. Anat. Physiol., Physiol. Abt.*, 1902, 365-368.

sult of successive condensations of a number of acetaldehyde molecules by a series of reactions similar to the aldol condensation. Although it is now known from experiments with isotopically marked acetaldehyde that this compound is not used directly for the synthesis of fatty acids,^{10,11} Brady and Gurin¹² demonstrated that the acetaldehyde carbon can serve as a source of fatty acid after first being converted to acetate.

b. Ethanol as the Two-Carbon Fragment Required for Fatty Acid Synthesis. Masoro and Abramovitch¹³ reported that ethanol can be converted to fatty acids by liver slices but not to any considerable extent by kidney slices; however, the kidney surpasses the liver in the rate at which it can utilize ethyl alcohol. It is not unexpected that the carbons of unoxidized ethanol might be converted to fatty acids, since it has been recognized for a number of years that the reverse change, namely the conversion of acetaldehyde to alcohol, can take place. Thus, Battelli and Stern¹⁴ demonstrated this reverse change as part of a Cannizzaro reaction which is mediated by an enzyme, aldehyde mutase. Neuberg and Windisch¹⁵ described the acetic acid fermentation in which two molecules of acetaldehyde react to give one molecule each of ethanol and acetic acid. The ethanol is then reconverted to acetaldehyde. Since acetaldehyde can be built into fatty acids, it is logical to suppose that it may be an intermediate in the conversion of ethanol to fatty acids.

c. Acetate as the Two-Carbon Fragment Required for Fatty Acid Synthesis. A number of investigations have pointed to acetate or to a closely related substance as the active intermediate in fat synthesis. Thus, the original observation of Embden and Oppenheimer,¹⁶ made in 1912, in which it was shown that acetoacetic acid appeared in the blood after the surviving liver of the dog was perfused with blood to which pyruvate had been added, led to the discovery by Loeb¹⁷ and by Friedmann¹⁸ that acetate was probably an intermediate, since it also gave rise to acetoacetate under similar experimental conditions. Smedley-MacLean and Hoffert¹⁹ were the first

¹⁰ J. W. Cornforth and G. Popják, Unpublished observations, 1952; cited by G. Popják in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia, No. 9, Cambridge Univ. Press, 1952, p. 37.

¹¹ G. L. Curran and D. Rittenberg, *J. Biol. Chem.*, **190**, 17-20 (1951).

¹² R. O. Brady and S. Gurin, *J. Biol. Chem.*, **189**, 371-377 (1951).

¹³ E. J. Masoro and H. Abramovitch, *Federation Proc.*, **12**, 94 (1953).

¹⁴ F. Battelli and L. Stern, *Compt. rend. soc. biol.*, **68**, 742-744 (1910).

¹⁵ C. Neuberg and F. Windisch, *Biochem. Z.*, **166**, 451-481 (1925).

¹⁶ G. Embden and M. Oppenheimer, *Biochem. Z.*, **45**, 186-206 (1912).

¹⁷ A. Loeb, *Biochem. Z.*, **47**, 118-126 (1912).

¹⁸ E. Friedmann, *Biochem. Z.*, **55**, 436-442 (1913).

¹⁹ I. Smedley-MacLean and D. Hoffert, *Biochem. J.*, **20**, 343-357 (1926).

to demonstrate experimentally that sterols and fatty acids could be accumulated in yeast cells grown on ethanol or acetate as the sole source of carbon, without the intermediate formation of carbohydrate. Steroid synthesis was inhibited by sulfite, while that of fatty acids continued to operate under such conditions. This finding could be interpreted to mean that acetaldehyde might be the direct intermediate in the synthesis of yeast sterols, while acetate was the precursor of the newly synthesized fatty acids. Sonderhoff and Thomas²⁰ reported that, when deuterio-acetate was metabolized by yeast, approximately ten times the quantity of isotopically labeled fatty acids was formed as compared with the amount of tagged carbohydrate. This indicates that carbohydrate is not an intermediate of acetate in the formation of fatty acids.

In addition to yeast cells, bacteria are likewise able to use acetate as a material for the synthesis of fatty acids. For example, Wood and associates²¹ reported that butyl alcohol was formed during the fermentation carried out in the presence of $\text{CH}_3\text{C}^{13}\text{OOH}$ by *Clostridium butylicum* and by *Cl. acetobutylicum*. In view of the fact that these organisms can reduce butyric acid to the alcohol, the following series of reactions was suggested:

This reaction is supported by the finding that C^{13} appeared in the 1 and 3 positions in butanol. Another example of synthesis of butyric acid by fermentation of acetate and of ethanol by bacteria is afforded by the results of Barker *et al.*,²² who employed *Cl. kluyverii*. When $\text{CH}_3\text{C}^{14}\text{OOH}$ was used, it was found that the butyrate formed contained approximately the same proportion of C^{14} on the carboxyl and β -carbon atoms. It was believed that the C^{14} was present on alternate carbon atoms in the caproate formed by means of this organism, since the carboxyl C^{14} accounted for only one-third of the isotopic carbon. Further information concerning the mechanism of synthesis is afforded by analysis of the caproic acid formed when fermentation occurred with normal ethanol and $\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{C}^{14}\text{OOH}$. The hexanoic acid was found to contain an isotopic carbon atom, but it was not located in the carboxyl group; this indicates that the active C_2 unit from ethanol must have condensed with the carboxyl of butyric acid.

Equally convincing proof that fatty acids originate from acetate has

²⁰ R. Sonderhoff and H. Thomas, *Ann.*, 530, 195-213 (1937).

²¹ H. G. Wood, R. W. Brown, and C. H. Werkman, *Arch. Biochem.*, 6, 243-260 (1945).

²² H. A. Barker, M. D. Kamen, and B. T. Bornstein, *Proc. Nat. Acad. Sci.*, 31, 373-381 (1945).

been adduced from animal experiments. Thus, Rittenberg and Bloch^{23,24} found that the fatty acids isolated from mice, which had been fed $\text{CD}_3\text{-C}^{13}\text{OOH}$, were labeled both with deuterium and with C^{13} , indicating that both carbons of the acetate had been used in the synthesis of fat. It is evident that the newly synthesized fat was formed by the condensation of a number of molecules of acetic acid. The whole acetate was used for this synthesis, as was indicated by the fact that only approximately 50 per cent of the carbon of the newly formed fatty acids contained C^{13} atoms.

There are two possible methods by which condensation of the acetate fragments might proceed. If the acetate having labeled carbon in the carboxyl group is added to an unlabeled acid by condensation with the carboxyl group of the latter, then only the new carboxyl group would consist of C^{13} , and the rest of the molecule would be isotope-free. On the other hand, if the tagged acetate adds to the ω -carbon of the new acid, the carboxyl will be unlabeled, and the C^{13} -atom will occur in the remainder of the new molecule.

In the case of long-chain acids, a number of investigators have proved that elongation occurs by the addition of an acetate molecule. The inter-conversion of palmitic and stearic acid by the addition or removal of C_2 units was demonstrated both by Schoenheimer and Rittenberg²⁵ and by Stetten and Schoenheimer,²⁶ using deuterium-labeled fatty acids. Klem,²⁷ and Stevens and Chaikoff²⁸ also proved that lauric (C_{12}) and myristic (C_{14}) acids could be elongated to palmitic and stearic acids; however, in none of these studies was it evident at which end of the fatty acid the extension occurred. Zabin²⁹ observed in both *in vitro* and *in vivo* that the lengthening of palmitic to stearic acid involves the addition of the methylene group of the acetate to the carboxyl group of palmitic acid. This result offers concrete evidence for the first of the two possible methods of addition of the acetate fragment in the synthesis of fatty acids.

Although most workers agree that acetate is the essential component in the synthesis of fatty acids, the fact remains that acetate as such is ineffective, and that some derivative of acetate possessing a greater reactive ability must be the so-called "active acetate." According to Bloch,²

²³ D. Rittenberg and K. Bloch, *J. Biol. Chem.*, **154**, 311-312 (1944).

²⁴ D. Rittenberg and K. Bloch, *J. Biol. Chem.*, **160**, 417-424 (1945).

²⁵ R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **120**, 155-165 (1937).

²⁶ De W. Stetten, Jr., and R. Schoenheimer, *J. Biol. Chem.*, **133**, 329-345 (1940).

²⁷ A. Klem, *Norske Videnskaps-Akad. Oslo*, Hvalrädets Skrifter, No. 27, 1-26 (1943).

²⁸ B. P. Stevens and I. L. Chaikoff, *J. Biol. Chem.*, **193**, 465-472 (1951).

²⁹ I. Zabin, *J. Biol. Chem.*, **189**, 355-359 (1951).

these activations are achieved in organic compounds by esterification or by anhydride formation; in the case of acetate, it might involve such compounds as acetyl chloride ($\text{CH}_3 \cdot \text{CO} \cdot \text{Cl}$), acetic anhydride ($(\text{CH}_3 \cdot \text{CO})_2 \cdot \text{O}$), ketene ($\text{CH}_2 : \text{CO}$), ethyl acetate ($\text{CH}_3 \cdot \text{CO} \cdot \text{OC}_2\text{H}_5$), or acetyl phosphate ($\text{CH}_3 \cdot \text{CO} \cdot \text{H}_2\text{PO}_4$).

(a) *Acetylphosphate as the Possible Precursor.* The formation of acetylphosphate along with formate by the phosphoroclastic splitting of pyruvate is an example of acetylation in the absence of free acetic acid; this occurs in certain bacterial systems.^{30,31} As recently as 1946, Lipmann³² had the impression that acetylphosphate might be the active form of acetate. However, Lipmann^{32,33} later showed that acetylphosphate was ineffective in the acetylation of aromatic amines; this reaction was effectively catalyzed by pigeon liver preparations.

Lynen showed that acetylphosphate³⁴ was unable to combine with oxaloacetic acid; it was also found that no citrate was formed.³⁵ Martius and Lynen³⁶ presented strong evidence that acetate is oxidized through the citric acid cycle, but that it must be "activated" before it can enter this cycle.^{36,37} Another reaction in which acetyl groups participate without the formation of the acetate molecule is afforded in the intermolecular transfer of acetyl groups from an N-acetyl amino acid to a free amino acid, as demonstrated in the intact animal.³⁸ However, this latter reaction is not one which has such general application as to be considered in the synthesis of fatty acids.

(b) *Coenzyme A and "Active Acetate."* The discovery that a coenzyme was involved in effecting the incorporation of the acetate molecule into new compounds was based upon a number of investigations. The interest was intensified in this field when it was realized that "active acetate" was involved not only in the synthesis of citric acid and the acetylation of sulfonamide and of choline, but also in the synthesis of acetoacetate, fatty acids, and the steroid nucleus. The early understanding of these relationships should be credited to Schoenheimer, as reported in the E. K. Dun-

³⁰ F. Lipmann, *J. Biol. Chem.*, **134**, 463-464 (1940).

³¹ M. F. Utter, F. Lipmann, and C. H. Werkman, *J. Biol. Chem.*, **158**, 521-531 (1945).

³² F. Lipmann, *Advances in Enzymol.*, **6**, 231-267 (1948); *Harvey Lectures*, **44**, 99-123 (1948).

³³ F. Lipmann, *Bacteriol. Revs.*, **17**, 1-16 (1953).

³⁴ F. Lynen, *Ber.*, **73**, 367-375 (1940).

³⁵ N. Neciullah, Thesis, Munich, 1941; cited by F. Lynen, *Harvey Lectures*, **48**, 210-244 (1952-1953), p. 212.

³⁶ C. Martius and F. Lynen, *Advances in Enzymol.*, **10**, 167-222 (1950).

³⁷ F. Lynen, *Ann.*, **552**, 270-306 (1942).

³⁸ K. Bloch and D. Rittenberg, *Federation Proc.*, **5**, 122-123 (1946).

ham lectures.³⁹ A considerable part of the historical development in this field has been reported by Lipmann³³ and by Lynen.⁴⁰

The first indication that a coenzyme existed was afforded by an experiment of Nachmansohn and Machado⁴¹ which involved the acetylation of choline in nerve. It was found that, when adenosine triphosphate (ATP) and a brain extract were added to the preparation, a vigorous acetylation occurred. The same system was found by Lipmann⁴² to be effective in promoting the acetylation of aromatic amines. Lipmann⁴² concluded that a pigeon liver preparation also provided a coenzyme. It was simultaneously observed by a number of investigators⁴³⁻⁴⁵ that an activator was needed in the acetylation of choline. Lipmann and co-workers⁴⁶ were the first to purify this coenzyme, isolated from hog liver, and to discover that it contained a pantothenic acid residue. It was named "coenzyme A" and will be abbreviated as CoA. It is also referred to as S-acetylpanthetheine and as acetyl LBF (*Lactobacillus bulgaricus* factor). It was proved shortly thereafter that CoA functions not only in the acetylation process but also in the synthesis of acetoacetate⁴⁷ and in that of citrate.^{48,49}

Through the work of Lipmann,^{32,33} it is becoming increasingly evident that the probable "active acetate," which is recognized as the fundamental building block in the metabolic patterns of animals, plants, and micro-organisms, is a combination of the acetyl group with CoA; this unit is referred to as "acetyl-CoA," and it is considered to be identical with active acetate in animal systems. The wide application of this compound is demonstrated by the fact that the acetyl-CoA prepared from yeast was shown to function⁵⁰ in the acetyl donor system (acetate, adenosinetriphosphate (ATP), CoA) in pigeon liver required for the acetylation of sulfanilamide.⁵¹

There are two mechanisms which can activate acetate.⁵² The first of

³⁹ R. Schoenheimer, *The Dynamic State of Body Constituents*, Harvard Univ. Press, Cambridge, 1942.

⁴⁰ F. Lynen, *Harvey Lectures*, 48, 210-244 (1952-1953).

⁴¹ D. Nachmansohn and A. L. Machado, *J. Neurophysiol.*, 6, 397-403 (1943).

⁴² F. Lipmann, *J. Biol. Chem.*, 160, 173-190 (1945).

⁴³ W. Feldberg and T. Mann, *J. Physiol.*, 104, 411-425 (1946).

⁴⁴ D. Nachmansohn and M. Berman, *J. Biol. Chem.*, 165, 551-563 (1946).

⁴⁵ M. A. Lipton and E. S. G. Barron, *J. Biol. Chem.*, 166, 367-380 (1946).

⁴⁶ F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle, and B. M. Guirard, *J. Biol. Chem.*, 186, 235-243 (1950).

⁴⁷ M. Soodak and F. Lipmann, *J. Biol. Chem.*, 175, 999-1000 (1948).

⁴⁸ J. R. Stern and S. Ochoa, *J. Biol. Chem.*, 179, 491-492 (1949).

⁴⁹ G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, 182, 213-228 (1950).

⁵⁰ F. Lynen, E. Reichert, and L. Rueff, *Ann.*, 574, 1-32 (1951).

⁵¹ N. O. Kaplan and F. Lipmann, *J. Biol. Chem.*, 174, 37-44 (1948).

⁵² A. D. Welch and C. A. Nichol, *Ann. Rev. Biochem.*, 21, 633-686 (1952).

these, the acetylphosphate system, can yield acetylphosphate by means of bacterial enzymes. The second mechanism for acetate activation is the ATP-acetate system; the formation of active acetate in mammalian and in avian tissues requires the mediation of ATP. Apparently acetylphosphate is not formed in the case of the second type of activated acetate, although there may be an intermediary formation of a phosphorylated form of CoA. For a further discussion, see the section on CoA on page 27.

As regards the synthesis of fat from carbohydrate, Rittenberg and Bloch²⁴ suggested that the intermediate compound, pyruvate, might not necessarily pass through the stage of acetate, but that it might be directly employed for the synthesis of fatty acids; it was suggested that a pre-

liminary formation of acylpyruvic acid, $\text{CH}_3\text{C} \begin{array}{l} \nearrow \text{OH} \\ \text{---} \text{COOH} \\ \searrow \text{R} \end{array}$, might occur,

and that this compound would then be directly decarboxylated. The net result would be the same as if a molecule were directly added, but an acetate molecule would not actually be set free. It has also been suggested by Anker⁵³ that the units used for fatty acid synthesis might not invariably be identical.

In spite of the uncertainty as to the identity of acetate and the fat precursor from carbohydrate, a number of experiments indicate that this identity exists. For example, French and Popják⁵⁴ proved that, when carboxyl- C^{14} acetate, C^{14} -glucose, or C^{14} -starch was fed to lactating rabbits, the labeled fatty acids obtained in the milk six hours later were remarkably similar. Moreover, the same amount of radioactive acetyl was incorporated into *p*-amino benzoic acid, irrespective of whether the normal rabbit was fed glucose or acetate containing C^{14} . This also indicates that acetate in an intermediate of glucose, regardless of whether the site of transformation is the mammary gland (as in the lactating animal) or extramammary tissue (as in the normal rabbit).

Further evidence for the assumption that acetate is the intermediate in the glucose $\rightarrow$ fat transformation has likewise been recorded by Popják.⁵ If the course of synthesis of the fatty acids involves the following changes, as seems probable, Glucose $\rightarrow$ Pyruvate $\rightarrow$ Acetate $\rightarrow$ Fatty acid, then the incorporation of C^{14} should be identical, irrespective of whether this isotope is introduced into the α - or β -position of pyruvate, or into either the carboxyl or the methyl group of acetate. Popják⁵ proved that such is the case; moreover, it was shown, on the one hand, that the position of

⁵³ H. S. Anker, *J. Biol. Chem.*, 176, 1337-1352 (1948).

⁵⁴ T. H. French and G. Popják, *Biochem. J.*, 49, iii-iv (1951).

C¹⁴ was the same following the administration of C¹⁴-carboxylacetate and α -C¹⁴-pyruvate, and, on the other hand, after that of α -C¹⁴-acetate and β -C¹⁴-pyruvate. In the first case, the octanoic acid, which was synthesized, was labeled on the odd carbons while, in the second case, the acid was labeled on the even carbons. These results are supported by the findings of Anker⁵³ that the carboxyl group of the acid synthesized after the feeding of α -C¹⁴-pyruvate has twice the concentration of isotope which obtains in the whole molecule. Moreover, when the radio C is introduced into the carboxyl group of pyruvic acid, it does not appear in newly synthesized fat.⁵³ All these observations are in harmony with the concept that acetate is the building stone for fatty acids, and that it arises from pyruvate (and hence from carbohydrate) by decarboxylation of this keto acid. Ponticorvo *et al.*⁵⁵ demonstrated that 20 per cent of the isotopic carbon fed as acetate was incorporated into the fatty acids of growing rats.

(2) *The Site of Biosynthesis of Fatty Acids from Acetate*

The liver has usually been considered to be the chief organ in which fat synthesis takes place. Rittenberg and Bloch²⁴ were the first to demonstrate that fatty acids could be synthesized in the liver from the acetate building stones. The experiments of Pihl and Bloch⁵⁶ indicated that labeled acetate was incorporated into the triglyceride fats in the isolated liver of the rat; the rate at which acetate was incorporated into the triglycerides was greater than that of its incorporation into the phospholipids. Chernick and co-workers⁵⁷ likewise noted, on the basis of experiments with rat liver slices, that fat synthesis from glucose occurs chiefly in the liver. Masoro *et al.*⁵⁸ confirmed the fact that the liver is the principal site of lipogenesis.

However, in spite of the commanding role of the liver in fat synthesis, this function can be assumed by many other tissues. Fat synthesis has been demonstrated in surviving slices of rat kidney and diaphragm as well.⁵⁷ Labeled acetate is incorporated in the fatty acids in the mucosa of the gastrointestinal tract of rabbits.⁵⁹ The utilization of acetate is reported to be especially marked in lung tissue. Chernick and associates⁶⁰ showed that rat arterial wall can effect a synthesis of fatty acids from acetate, as well

⁵⁵ L. Ponticorvo, D. Rittenberg, and K. Bloch, *J. Biol. Chem.*, **179**, 839-842 (1949).

⁵⁶ A. Pihl and K. Bloch, *J. Biol. Chem.*, **183**, 431-439 (1950).

⁵⁷ S. S. Chernick, E. J. Masoro, and I. L. Chaikoff, *Proc. Soc. Exptl. Biol. Med.*, **73**, 348-352 (1950).

⁵⁸ E. J. Masoro, I. L. Chaikoff, and W. G. Dauben, *J. Biol. Chem.*, **179**, 1117-1125 (1949).

⁵⁹ G. Popják and M. L. Beeckmans, *Biochem. J.*, **47**, 233-238 (1950).

⁶⁰ S. S. Chernick, P. A. Srere, and I. L. Chaikoff, *J. Biol. Chem.*, **179**, 113-118 (1949).

as a new formation of phospholipid when phosphate is present. The synthesis of fat from carbohydrate is also a function of adipose tissue itself, although acetate has not been shown to be an intermediate in this case.⁶¹ However, the addition of pyruvate or of acetate to retroperitoneal adipose tissue of rats was shown by Haugaard and Marsh⁶² to elevate the R.Q. above unity; this phenomenon is interpreted as indicative of fat synthesis. Sperry and co-workers⁶³ reported that, when octanoic acid-1-C¹⁴ was perfused through rat brains, all lipid fractions except cholesterol contained C¹⁴.

Of all the extrahepatic tissues which bring about fat synthesis, mammary tissue has been shown to be the most important. This is particularly true in the case of ruminants, especially during the period of lactation. Employing a direct approach to the problem, Zelter⁶⁴ reported that the administration of acetate to a lactating cow increased lipid production in the mammary gland, while butyric acid caused no significant change in lipogenesis, unless given in conjunction with pyruvic acid or acetate.

The ability of the mammary tissue to synthesize fatty acids from acetate varies with the species. On the one hand, Folley and French^{65,66} found that surviving udder slices of the lactating cow, goat, and sheep actively utilize acetate as the sole substrate with an R.Q. exceeding unity. These data were logically interpreted to mean that the ruminant mammary gland is able to convert acetate into fatty acids; the short-chain acids in the newly synthesized fat would represent an intermediate stage in the synthesis of long-chain acids.

On the other hand, acetate cannot be used for fat synthesis by the mammary tissue of such non-ruminants as the rat, mouse, rabbit, or guinea pig,^{65,67} unless glucose is simultaneously present. This is in line with the earlier results of Bloch and Kramer,⁶⁸ who demonstrated that acetate could not be employed by liver slices for fat synthesis unless glucose was also in the medium. Fat synthesis can readily be demonstrated in slices of lactating rat udder when glucose is the substrate.⁶⁹ Acetate utilization in sheep mammary tissue is likewise increased by this monosaccharide.⁷⁰

⁶¹ E. Wertheimer and B. Shapiro, *Physiol. Revs.*, **28**, 451-464 (1948).

⁶² N. Haugaard and J. B. Marsh, *J. Biol. Chem.*, **194**, 33-40 (1952).

⁶³ W. M. Sperry, R. M. Taylor, and H. L. Meltzer, *Federation Proc.*, **12**, 271-272 (1953).

⁶⁴ Z. Zelter, *Compt. rend.*, **231**, 1574-1576 (1950); **234**, 567-569 (1952).

⁶⁵ S. J. Folley and T. H. French, *Biochem. J.*, **46**, 465-473 (1950).

⁶⁶ S. J. Folley and T. H. French, *Nature*, **163**, 174-175 (1949).

⁶⁷ S. J. Folley and T. H. French, *Biochem. J.*, **45**, 117-125 (1949).

⁶⁸ K. Bloch and W. Kramer, *J. Biol. Chem.*, **173**, 811-812 (1948).

⁶⁹ J. H. Balmain, S. J. Folley, and R. F. Glascock, *Nature*, **169**, 447-449 (1952).

⁷⁰ J. H. Balmain, S. J. Folley, and R. F. Glascock, *Biochem. J.*, **52**, 301-306 (1952).

Popják and Tietz⁷¹ reported that, in addition to glucose, pyruvate, oxaloacetate, α -ketoglutarate, and succinate stimulate fatty acid synthesis from acetate, but to a lesser extent than does glucose. Oxaloacetate had the greatest effect, next to glucose, and, in addition, it inhibited the oxidation of acetate. The other substrates either had no effect or stimulated the oxidation of acetate. Homogenates of mammary tissue, like slices, were found incapable of bringing about fatty acid synthesis when acetate alone or with ATP was added as the only substrate. Glucose alone or with ATP also failed to promote lipogenesis from acetate by mammary homogenates. The system producing most fat from acetate under such conditions was composed of acetate, oxaloacetate, and ATP, together with homogenates of the mammary gland.

Although the evidence cited above for the lipogenic activity of mammary tissue is largely indirect, since it is based upon physiologic indices such as R.Q., there is considerable direct proof of this reaction. Thus, Cowie *et al.*⁷² were able to demonstrate the synthesis of isotopically labeled fatty acid in the perfused isolated bovine udder, while negative results were obtained with sodium bicarbonate-C¹⁴. *In vivo* tests on the pregnant rabbit by Popják and Beeckmans⁷³ likewise showed that glyceride fatty acids are synthesized by the udder even before milk production begins. The later work of Popják and associates⁷³⁻⁷⁶ on intact animals furnishes further direct evidence of the fat synthesis from acetate in the udder. Since, after injection of radioacetate, the specific activities of the blood plasma were of a much lower order than those of the volatile fractions of milk fat, the synthesis of the fatty acids must occur in the udder. Independent confirmation of this reaction was obtained by Kleiber and co-workers⁷⁷ in the case of the cow, and by French and Popják,⁵⁴ who used rabbits. McClymont,^{78,79} using cows and sheep, arrived at similar conclusions by the use of a different technic. The disappearance of acetate from the mammary gland was established by demonstrating arteriovenous differences in acetate concentration across the udder of 2 to 6 mg. %, depending upon the acetate level in the arterial blood.^{78,79}

⁷¹ G. Popják and A. Tietz, *Biochem. J.*, **54**, xxv (1953).

⁷² A. T. Cowie, W. G. Duncombe, S. J. Folley, T. H. French, R. F. Glascock, L. Massart, G. J. Peeters, and G. Popják, *Biochem. J.*, **49**, 610-615 (1951).

⁷³ G. Popják and M. L. Beeckmans, *Biochem. J.*, **46**, 547-558 (1950).

⁷⁴ G. Popják, S. J. Folley, and T. H. French, *Arch. Biochem.*, **23**, 508-510 (1949).

⁷⁵ G. Popják, T. H. French, and S. J. Folley, *Biochem. J.*, **46**, xxviii-xxix (1950).

⁷⁶ G. Popják, T. H. French, and S. J. Folley, *Biochem. J.*, **48**, 411-416 (1951).

⁷⁷ M. Kleiber, A. H. Smith, and B. Tolbert, *Abst. Commun. XVIIIth Physiol. Congr.*, Copenhagen, Aug., 1950, p. 304.

⁷⁸ G. L. McClymont, *Biochem. J.*, **45**, i-ii (1949).

⁷⁹ G. L. McClymont, *Australian J. Agr. Research*, **2**, 158-180 (1950).

All of the above results, which illustrate the lipogenic activity of the mammary glands, are uniform in indicating that milk fatty acids originate largely in the udder itself, and that they are not merely excreted by this organ after having been synthesized in the liver and carried to the mammary gland in the blood stream. Moreover, the experimental data suggested that the short-chain acids are not degradation products of oleic acid, as postulated in the Hilditch theory, but rather arise by synthesis from acetate.

The conclusion is inescapable that acetate is an intermediate in the synthesis of fat in the animal body. Moreover, not only the liver, but many other extrahepatic tissues are able to bring about this synthesis of fat. Mammary tissue has a particularly high capacity for fat production from acetate, especially in the case of the ruminants. Although udders of the non-ruminants lack the capacity to form fatty acids from acetate in the absence of glucose, they do develop this facility when the monosaccharide is present.

(3) *The Mechanism of Synthesis of Fatty Acids from Acetate*

The synthesis of the fatty acids appears to be the result of successive additions of two-carbon units to the carboxyl end of the fatty acids. Such a condensation has been repeatedly demonstrated in the synthesis of the C₄ acids (especially acetoacetic acid), and considerable evidence for its occurrence in longer chain acids is now available.

a. The Synthesis of Acetoacetate from Two-Carbon Fragments. In studying the origin of the acetoacetate from long-chain fatty acids, considerable data have accumulated to indicate that the primary reaction involves the formation of a two-carbon compound which is condensed to yield the four-carbon ketone body acid, acetoacetic acid.

The earliest proof that a condensation of two acetate molecules could take place, with the resultant formation of acetoacetate, was that of Loeb,¹⁷ and also of Friedmann,¹⁸ who reported the formation of acetoacetate after the liver had been perfused with acetate. This reaction has been confirmed in liver slices by Jowett and Quastel,⁸⁰ by Leloir and Muñoz,⁸¹ and by Weinhouse and co-workers in liver,⁸² and in kidney slices,⁸³ with the labeled compound. Soodak and Lipmann⁴⁷ demonstrated a synthesis of acetoacetate when acetate, pigeon liver extract, and ATP were incubated.

⁸⁰ M. Jowett and J. H. Quastel, *Biochem. J.*, **29**, 2159-2180 (1935).

⁸¹ L. F. Leloir and J. M. Muñoz, *Biochem. J.*, **33**, 734-746 (1939).

⁸² S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **153**, 698-690 (1944).

⁸³ G. Medes, S. Weinhouse, and N. F. Floyd, *J. Biol. Chem.*, **157**, 751-752 (1945).

CoA is apparently the active component of the liver extract. Monguio⁸⁴ and MacKay *et al.*⁸⁵ obtained similar data with ordinary acetate in the intact or fasting animal, while Swendseid and associates,⁸⁶ using C¹³-acetate, also reported acetoacetate synthesis in fasting animals.

When octanoic acid containing a C¹³-labeled carboxyl group was fed, Weinhouse and associates⁸⁷ found a considerable amount of C¹³ present in the carbonyl group of the acetoacetate. This would indicate that the octanoic acid was degraded into two-carbon units which would again be combined in a random manner to form acetoacetate. The following two possibilities might explain the results:

According to reaction (1), all the acetoacetate would be of synthetic origin while, with reaction (2), the acetoacetate would be a mixture of "primary" (non-synthetic) and synthetic acetoacetate molecules. The four terminal carbon atoms would be a direct source of acetoacetate. However, in later studies with carboxyl-labeled butyric acid, Medes *et al.*⁸⁸ noted an unequal distribution of C¹³ between the carboxyl and the carbonyl groups of acetoacetate. The acetoacetate could not have been formed by a random combination of two equivalent C₂ molecules; it is postulated that the newly formed acetoacetate is in equilibrium with acetate as follows:

A similar rapid interconversion of acetoacetate and acetate obtains in the liver⁸⁹ and in the kidney,⁹⁰ but it is not known whether or not the splitting of acetoacetate proceeds rapidly enough to account for a redistribution of the isotope as indicated above. Lehninger⁹¹ reported that the reverse reaction proceeds slowly in muscle mince.

The primary formation of acetoacetate is also indicated in the *in vivo*

⁸⁴ J. Monguio, *Klin. Wochschr.*, **31**, 1116-1120 (1934).

⁸⁵ E. M. MacKay, R. H. Barnes, H. O. Carne, and A. N. Wick, *J. Biol. Chem.*, **135**, 157-163 (1940).

⁸⁶ M. E. Swendseid, R. H. Barnes, A. Hemingway, and A. O. Nier, *J. Biol. Chem.*, **142**, 47-52 (1942).

⁸⁷ S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **155**, 143-151 (1949).

⁸⁸ G. Medes, S. Weinhouse, and N. F. Floyd, *J. Biol. Chem.*, **157**, 35-41 (1944).

⁸⁹ S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **158**, 411-419 (1945).

⁹⁰ G. Medes, N. F. Floyd, and S. Weinhouse, *J. Biol. Chem.*, **162**, 1-9 (1946).

⁹¹ A. L. Lehninger, *J. Biol. Chem.*, **143**, 147-157 (1942).

experiments of Morehouse,⁹² and in those of Morehouse and Deuel.⁹³ When caproate labeled with deuterium in the β, γ -positions was fed to fasting rats, the resulting β -hydroxybutyrate isolated from the urine had twice the isotope content of that of the same product prepared from the urine after α - β -dideutero-caproate had been fed. If the acids were split into three C_2 fragments, one would expect a similar proportion of deuterium in the β -hydroxybutyrate in both cases. On the other hand, if the caproate breaks down by β -oxidation to form "primary" acetoacetate, and the ketone bodies are formed only in part by recondensation of acetate units, the higher isotope content of the β, γ -, as compared with that of the α, β -compound, would be expected.

On the basis of results obtained with partially purified bacterial phosphotransacetylase, Stadtman, Doudoroff, and Lipmann⁹⁴ postulated that two activated C_2 units are used in the synthesis of acetoacetate, with the aid of an enzyme system present in pigeon liver. These workers are of the opinion that 2 moles of acetylphosphate are first converted to 2-acetyl-CoA molecules by transacetylase. It was suggested that, following this, the pigeon liver enzyme catalyzes the head-to-tail condensation of these molecules.

According to Lynen,⁴⁰ the acetoacetate synthesis involves a modified Claisen condensation. After combination of two molecules of acetyl-CoA, one molecule of the thiol alcohol-CoA splits off, leaving acetoacetyl-CoA, which, on hydrolysis, yields acetoacetate. This is shown in the equation on the following page.

In the scheme suggested by Lynen,⁴⁰ acetyl-CoA functions to bring about the synthesis of acetoacetate, while the reverse reaction is catalyzed by the thiol alcohol $HS \cdot CoA$. This combines with acetoacetyl-CoA on the carbonyl group, and splits it by the reaction described as "thiolysis." The split occurs between the methylene carbon and the carbonyl carbon, whereby the hydrogen attaches to the methylene group and the sulfur, with the rest of the coenzyme molecule, combines with the carbonyl carbon.

One must conclude that, although the animal has the power to condense acetyl groups to yield acetoacetate, this reaction accounts for only a portion of the acetoacetate normally produced in the animal, inasmuch as the latter compound can also be formed directly from 4-, 6-, or even 8-carbon acids. The acetoacetate must in all probability be in equilibrium with acetate

⁹² M. G. Morehouse, *J. Biol. Chem.*, 129, 769-779 (1939).

⁹³ M. G. Morehouse and H. J. Deuel, Jr., *Proc. Soc. Exptl. Biol. Med.*, 45, 96-98 (1940).

⁹⁴ E. R. Stadtman, M. Doudoroff, and F. Lipmann, *J. Biol. Chem.*, 191, 377-382 (1951).

The Role of Acetyl-CoA in the Synthesis and Hydrolysis of Acetoacetic Acid⁴⁰

molecules, and hydrolysis and resynthesis are continually taking place. For a further discussion of acetoacetate formation as related to oxidation, the reader is referred to Chapter III (page 77).

However, acetoacetate must likewise be regarded as an intermediate product in the synthesis of long-chain acids from acetate. Thus, Chen, Chapman, and Chaikoff⁹⁵ found that the carbonyl carbon of acetoacetate is readily incorporated into long-chain fatty acids by the liver of the normal fed rats. The capacity for conversion of acetoacetate into fat was found to be lost in the livers from normal fasted rats and from fed diabetic rats. The injection of insulin into diabetic rats restored their ability to synthesize long-chain fatty acids from acetoacetate.

b. The Synthesis of Long-Chain Fatty Acids from Acetate. There are a number of types of experimental evidence to prove that fatty acids having chains longer than four carbons are synthesized by successive condensations of two-carbon fragments. For example, Rittenberg and Bloch,²⁴ in 1945, demonstrated that the long-chain fatty acids, synthesized by mice and rats after the feeding of labeled acetate, had the label uniformly

⁹⁵ R. W. Chen, D. D. Chapman, and I. L. Chaikoff, *J. Biol. Chem.*, **205**, 383-393 (1953).

distributed throughout the molecule. A similar result was obtained with liver slices incubated with labeled acetate, as reported by Brady and Gurin.⁹⁶

The most convincing evidence of the stepwise nature of the synthetic process in the formation of fatty acids has come from the experiments of the Popják group⁹⁷ on lactating goats. After 5 mc. of carboxyl-labeled

FIG. 1. Relationship between the specific activity and the chain length of the glyceride fatty acids in the milk of a goat injected with 5 mc. of carboxyl-labeled C^{14} acetate. Experimental period I represents the results of analyses of samples of milk fat collected from 1 to 2 hours after the acetate injection. Experimental period II involves analyses of samples collected from 12 to 48 hours after the acetate injection. The symbol Δ^9 over the last columns indicates oleic acid.⁹⁷

C^{14} acetate had been injected into a goat, the milk fatty acids were found to contain increasing specific activities in a stepwise ratio from butyric to palmitic (hexadecanoic) acids; on the other hand, the results on stearic (octadecanoic) and oleic (9-octadecenoic) acids are entirely out of line with those of the lower acids. A summary of the data from these experiments is given in Figure 1.

⁹⁶ R. O. Brady and S. Gurin, *J. Biol. Chem.*, **186**, 461-469 (1950).

⁹⁷ G. Popják, T. H. French, G. D. Hunter, and A. J. P. Martin, *Biochem. J.*, **48**, 612-618 (1951).

It would thus appear that the relationship between specific activity and chain length of the fatty acids is in some way related to the mechanism of fatty acid synthesis. On analysis of the butyrate, caproate, and caprylate formed in the udder of the goat after the injection of $\text{CH}_3\cdot\text{C}^{14}\text{OONa}$, it was found⁹⁷ that the C^{14} was present exclusively in carbons 1, 3, and 5, and that the isotope was completely absent from carbons 2, 4, 6, and 8. These results are especially convincing in indicating that the synthesis of the long-chain acid results from successive additions of the methyl groups of acetic acid fragments to the carboxyl group of the partly synthesized acids.

The results of Popják, French, and Folley⁷⁶ are likewise of interest in this connection. The milk fatty acids obtained four hours after the injection of 5 mc. of C^{14} -carboxyl-labeled acetate were separated and isolated into four fractions, namely: (1) steam volatile and soluble, (2) steam volatile and insoluble, (3) nonvolatile and liquid, and (4) non-volatile and solid. Although all four fractions showed maximum specific activities three to four hours after the injection, those of the two volatile fractions were much higher than were those of the non-volatile fractions. This experiment proves that the formation of new milk fat proceeds from the shorter acids to longer-chain acids rather than in the reverse direction. Further support for this hypothesis is afforded by the demonstration that acetate and butyrate are normal components of animal fat; James and Popják⁹⁸ were able to demonstrate the presence of acetic and butyric acids in small amounts in the distillate of glyceride fatty acids of rabbit liver.

c. The Elongation of the Fatty Acid Chains with Acetate. The fact that long-chain fatty acids can be elongated or shortened by two-carbon fragments was first demonstrated by the classical experiments of the Schoenheimer group. Thus, Stetten and Schoenheimer,²⁶ using deuterio-palmitic acid, proved that it could be converted to deuteriostearic acid in rats, as well as into deuteriopalmitleic acid. On the other hand, Schoenheimer and Rittenberg,²⁵ using mice, had proved earlier with the deuterium technic that stearic acid could be shortened by two carbons to yield palmitic acid. A similar phenomenon was demonstrated somewhat later by Zabin²⁹ in both *in vivo* and *in vitro* tests. In the latter experiments, it was proved that the elongation of palmitic acid to stearic acid resulted from the addition of an acetate unit to the carboxyl group of the palmitic acid.

Klem²⁷ demonstrated that lauric (dodecanoic) acid can be lengthened

⁹⁸ A. T. James and G. Popják, Unpublished observations, 1952; cited by G. Popják in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia, No. 9, Cambridge Univ. Press, 1952, p. 39.

to palmitic and stearic acids, while Stevens and Chaikoff,²⁸ as well as Anker⁹⁹ proved that myristic (tetradecanoic) acid could likewise be changed to palmitic or stearic acid. In these tests also, it was demonstrated that the new palmitic acid originated by the condensation of the methyl group of acetate with the carboxyl group of the longer-chain acid.⁹⁹ Finally, Brady and Gurin⁹⁶ demonstrated that carboxyl-labeled butyric, caproic, and caprylic acids are converted by liver slices to long-chain fatty acids. Apparently, these acids are largely cleaved into two-carbon fragments which are employed as the building stones.

Popják *et al.*⁹⁷ have presented further evidence that a four-carbon acid can serve as the primary building stone for the acetate condensation to form long-chain acids. It was shown that the specific activity of the carboxyl carbon in caproic acid was 2.5 times that of carbons 3 and 5. This was best explained by the assumption that only 40 per cent of the butyric acid used for caproate synthesis was formed from acetate, while 60 per cent was derived from another non-isotopic C₄ compound. Although the non-isotopic precursor might be butyric acid itself, which was absorbed from the blood, or a substance converted by mammary tissue into butyric acid, it was suggested that the four-carbon primary building stone was β -hydroxybutyric acid; it is known that this substance is present in the blood of lactating ruminants in considerable amounts, and that it can be readily absorbed by the udder.¹⁰⁰

Further indication that acetic acid may add to medium-length fatty acid to produce an elongation has been reported by Dauben and co-workers¹⁰¹ who used intact mice. When carboxyl-labeled acetate was injected into mice four hours before they were sacrificed, it was found that the first and third carbons of the sixteen-carbon acids were about equally labeled whereas, in the eighteen-carbon acids, the carboxyl carbon was labeled to about three times that of the third carbon. It was found that the tail end of the molecule was not equally labeled in this short test.

Not only is acetate employed for the synthesis of the saturated fatty acids, but it may likewise be directly concerned with the synthesis of unsaturated acids. Thus, Mead and coworkers^{102,103} reported that arachidonic acid containing labeled carbon could be obtained from the liver lipids of rats after they were fed acetate containing C¹⁴.

⁹⁹ H. S. Anker, *J. Biol. Chem.*, **194**, 177-182 (1952).

¹⁰⁰ J. C. Shaw and C. B. Knodt, *J. Biol. Chem.*, **133**, 287-292 (1941).

¹⁰¹ W. G. Dauben, E. Hoerger, and J. W. Petersen, *J. Am. Chem. Soc.*, **75**, 2347-2351 (1953).

¹⁰² J. F. Mead, G. Steinberg, and D. R. Howton, *Federation Proc.*, **12**, 244 (1953).

¹⁰³ G. Steinberg, D. R. Howton, and J. F. Mead, *Univ. Calif. (Los Angeles) School Med., Atomic Energy Project No. 91120-10007*, May 27, 1953.

(4) *The Role of Coenzyme A in Fat Synthesis*

Reactions which involve the synthesis of fatty acids certainly must entail the intermediation of enzymes. CoA has also been identified as a factor important in the transfer of acetyl groups, and hence in fatty acid synthesis. The same co-factor is required for many enzymatic reactions involving C_2 compounds, which, in addition to acetylation of sulfonamides and choline, includes the synthesis of acetoacetate and citrate, as well as the oxidation of pyruvate and acetaldehyde. In 1945, Lipmann⁴² first called attention to the necessity of a soluble co-factor to explain the enzymatic acetylation of sulfanilamide which was effected by pigeon liver extracts. Subsequently, Lipmann and co-workers¹⁰⁴ were able to obtain a partially purified product containing the active co-factor; this was given the name "coenzyme A" (CoA).

a. Coenzyme A in the Transfer of Two-Carbon Intermediates. It is now widely recognized that CoA is able to bring about a dual activation of the acetate molecule. In the transfer of acetyl groups, which is involved in condensation reactions such as occur in the synthesis of acetoacetate, fatty acids, or citrate, the methyl carbon of the acetate is activated. On the other hand, in reactions such as the acetylation of aromatic amines or of choline, CoA activates the carboxyl group of acetate. Lipman^{32,33} reviewed the role of CoA as understood in 1946, 1948, 1953 and 1955,¹⁰⁵ while Ochoa *et al.*¹⁰⁶ summarized the later work on the crystallization and function of the condensing enzyme. The latter worker has also recorded the unique function of this coenzyme, not only in the mechanisms involved in the transfer of two-carbon units, but also in the activation and transfer of other acyl groups. Other recent reviews on the functional aspects of CoA are those of Stern,¹⁰⁷ of Welch and Nichol,⁵² and especially that of Lynen.⁴⁰

Acetyl-CoA (active acetate) is a key substance in acetyl transfer. It occupies a central position between the acetyl donor and the acetyl acceptor system; Welch and Nichol⁵² pictured this relationship as shown in Figure 2, while Lynen *et al.*⁵⁰ suggested the intermediate role of acetyl-CoA as illustrated in Figure 3.

The probability that acetyl-CoA is the intermediate carrier between the acetyl-donor and the acetyl-acceptor systems is strengthened by the

¹⁰⁴ F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle, and B. M. Guirard, *J. Biol. Chem.*, **167**, 869-870 (1947).

¹⁰⁵ F. Lipmann, *Am. Scientist*, **43**, 37-47 (1955).

¹⁰⁶ S. Ochoa, J. R. Stern, and M. C. Schneider, *J. Biol. Chem.*, **193**, 691-702 (1951).

¹⁰⁷ J. R. Stern, *Phosphorus Metabolism*, **1**, 264-271 (1951).

(a) *The Function of Phosphotransacetylase.* Phosphotransacetylase is an enzyme which activates the acetyl group in acetylphosphate (Figure 2), both for methyl transfer in the synthesis of citrate, and for carboxyl reactions in acetylation.⁵² This enzyme has been detected by Stadtman¹⁰⁹ only in extracts of the *Clostridium kluyverii*. It requires potassium and ammonium ions for its activity.¹¹⁰ Stern and co-workers¹¹¹ explain the dual action of acetyl-CoA as due to the fact that it may exist as an equilibrium of tautomeric forms as follows:

(b) *The Role of the Condensing Enzyme.* The so-called condensing enzyme functions in the reversible reaction:¹⁰⁶

When citrate and phosphate are combined with the condensing enzyme and with highly purified phosphotransacetylase, acetylphosphate is formed. The condensing enzyme, which is widely distributed in animal tissues, was purified by Stadtman,¹¹⁰ as well as by Stern, Ochoa, and collaborators^{106,111-113} and, in fact, has been separated in crystalline form.¹¹³

b. The Mechanism of Transfer of Acyl Groups by Coenzyme A. The reaction by which acetyl-CoA functions to bring about oxidation with long-chain acids indicates that the co-factor can condense with acyl groups, as well as with acetate, to form a condensing compound. This either requires ATP or it functions by the simultaneous mediation of active acetate. Thus, Chantrenne¹¹⁴ demonstrated the synthesis of hippuric acid from glycine and benzoic acid in the presence of CoA and ATP. According to Barker,¹¹⁵ the activation of butyric acid with acetyl-CoA follows the following pathway:

The activation of the higher fatty acids by this method has been demonstrated in the case of extracts of the anaerobe, *Clostridium kluyverii*.¹¹⁵

¹⁰⁹ E. R. Stadtman, *Federation Proc.*, **11**, 291 (1952).

¹¹⁰ E. R. Stadtman, *J. Biol. Chem.*, **196**, 527-534, 535-546 (1952).

¹¹¹ J. R. Stern, B. Shapiro, E. R. Stadtman, and S. Ochoa, *J. Biol. Chem.*, **193**, 703-720 (1951).

¹¹² J. R. Stern and S. Ochoa, *J. Biol. Chem.*, **191**, 161-172 (1951).

¹¹³ J. R. Stern, S. Ochoa, and F. Lynen, *J. Biol. Chem.*, **198**, 313-321 (1952).

¹¹⁴ H. Chantrenne, *J. Biol. Chem.*, **189**, 227-233 (1951).

¹¹⁵ H. A. Barker, *Phosphorus Metabolism*, **1**, 204-245 (1951).

c. **The Chemical Nature of Coenzyme A.** Lipmann and his colleagues¹⁰⁴ first reported the presence of the component of the vitamin B complex, pantothenic acid, in their partially purified preparation of CoA. In fact, Kaplan and Lipmann⁵¹ found that the pantothenic acid in the tissues behaved like CoA. In the investigation of the pantothenic acid content of a partially purified CoA preparation, Hegsted and Lipmann¹¹⁶ showed that a parallelism exists between pantothenic acid content and CoA activity. It was later reported that the CoA concentration in the liver of ducks decreased quite rapidly to 40% of the normal activity when the fowl was placed on a diet deficient in pantothenic acid.¹¹⁷

Novelli and co-workers¹¹⁸ prepared pantothenic acid by treatment of CoA with an intestinal phosphatase, followed by treatment with an extract of acetone-dried pigeon liver. This destroys the enzymic action of CoA. In a later publication of the Lipmann group,⁴⁶ the presence of pantothenic acid in CoA was again confirmed.

Adenylic acid has likewise been identified as a component of the CoA molecule.⁴⁶ Another important group is the sulfur-containing amino compound which is believed to be mercaptoethylamine.^{119,120} Pantothenic acid is linked to this sulfur-containing compound through the carboxyl group of β -alanine of pantothenic acid.^{46,121} Pantothenic acid is connected with adenylic acid by a pyrophosphate bridge,¹²² which probably involves the γ -hydroxyl of pantothenic acid.^{123,124} A third phosphate group occurs on the ribose of the adenylic acid as a monoester¹²⁵ in position 2' or 3'. The acetyl mercaptan group in acetyl-CoA was further identified by an absorption maximum at 232μ , which is the absorption value previously recorded by Sjöberg¹²⁶ and by Stadtman;¹²⁷ the latter author later followed the acetylation of CoA spectrometrically by measuring the increase in density at 240μ due to the formation of the thioester linkage.¹²⁸ On the

¹¹⁶ D. M. Hegsted and F. Lipmann, *J. Biol. Chem.*, **174**, 89-92 (1948).

¹¹⁷ R. E. Olson and N. O. Kaplan, *J. Biol. Chem.*, **175**, 515-529 (1949).

¹¹⁸ G. D. Novelli, N. O. Kaplan, and F. Lipmann, *J. Biol. Chem.*, **177**, 97-107 (1949).

¹¹⁹ W. H. DeVries, W. M. Govier, J. S. Evans, J. D. Gregory, G. D. Novelli, M. Soodak, and F. Lipmann, *J. Am. Chem. Soc.*, **72**, 4838 (1950).

¹²⁰ E. E. Snell, G. M. Brown, V. J. Peters, J. A. Craig, E. L. Wittle, J. A. Moore, V. M. McGlohon, and O. D. Bird, *J. Am. Chem. Soc.*, **72**, 5349-5350 (1950).

¹²¹ G. M. Brown, J. A. Craig, and E. E. Snell, *Arch. Biochem.*, **27**, 473-475 (1950).

¹²² G. D. Novelli, N. O. Kaplan, and F. Lipmann, *Federation Proc.*, **9**, 209 (1950).

¹²³ J. Baddiley and E. M. Thain, *Chemistry & Industry*, **1951**, No. 17, 337-338.

¹²⁴ J. Baddiley and E. M. Thain, *J. Chem. Soc.*, **1951**, 2253-2258.

¹²⁵ J. Baddiley and E. M. Thain, *J. Chem. Soc.*, **1951**, 3421-3424.

¹²⁶ B. Sjöberg, *Z. physik. Chem.*, **B 52**, 209-221 (1942).

¹²⁷ E. R. Stadtman, *J. Biol. Chem.*, **203**, 501-512 (1953).

¹²⁸ E. R. Stadtman, *J. Cellular Comp. Physiol.*, **41**, suppl. 1, 89-107 (1953).

basis of the work by Lipmann, Baddiley, Novelli, Kaplan, and himself,¹²⁹⁻¹³² Lynen⁴⁰ postulated the following structure for CoA. The molecular weight of this structure of 767 agrees well with an earlier estimation of Novelli,¹³³ who calculated it as 766. Acetyl-CoA has been synthesized by King and co-workers¹³⁴ and independently by Baddiley and Thain.¹³⁵

Structure of Acetyl Coenzyme A (Acetyl-CoA)⁴⁰

(a) *Different Natural Forms of Coenzyme A.* Pantetheine is the simplest and most common form of CoA. In this compound, the sulfur is present in the reduced ($-SH$) form. Snell and co-workers^{120,121} demonstrated that the growth factor for *Lactobacillus bulgaricus* (LBF) is identical with pantetheine. Pantetheine was shown by Snell *et al.*¹²⁰ to be synthesized from pantothenic acid by certain bacteria. However, it was found that LBF is more reactive than is the corresponding amount of pantothenic acid. At least seven forms of LBF have been identified, by paper chromatography and by bioautographic technics,¹³⁶ in culture filtrates of *Bacillus megatherium* (rod-shaped bacilli from water, soil, cabbage, *etc.*); these were pre-

¹²⁹ G. D. Novelli, J. D. Gregory, R. M. Flynn, and F. J. Schmetz, *Federation Proc.*, **10**, 229-230 (1951).

¹³⁰ F. Lynen and E. Reichert, *Angew. Chem.*, **63**, 47-48 (1951).

¹³¹ J. Baddiley, E. M. Thain, G. D. Novelli, and F. Lipmann, *Nature*, **171**, 76 (1953).

¹³² T. P. Wang, L. Shuster, and N. O. Kaplan, *J. Am. Chem. Soc.*, **74**, 3204-3205 (1952).

¹³³ G. D. Novelli, *Phosphorus Metabolism*, **I**, 414-417 (1951).

¹³⁴ T. E. King, C. J. Stewart, and V. H. Cheldelin, *Science*, **117**, 439 (1953).

¹³⁵ J. Baddiley and E. M. Thain, *Science*, **117**, 439-440 (1953).

¹³⁶ C. L. Long and W. L. Williams, *J. Bacteriol.*, **61**, 195-202 (1951).

sumably synthesized by the bacteria. Moreover, Brown and Snell¹³⁷ prepared a large number of synthetic compounds with LBF activity, as measured by *Lactobacillus helveticus* 80 (present in Swiss cheese), by treating pantethine with sodium sulfide, cysteine, glutathione, and several thiols.

Numerous derivatives of CoA exist in which the sulfur is in reduced form. In addition to the combination of CoA with the acetyl group, there are combinations with various fatty acids (propionyl, butyryl, and other acyl groups), with dicarboxylic acids (succinyl) and with the acetoacetyl group. Wakil and Mahler^{138,139} and Kornberg and Pricer^{140,141} proved that the linkage of the higher fatty acids with CoA is analogous to that between the acetyl group and CoA.

Pantethine is the name for the oxidized form of CoA which may be represented as CoAS-SCoA. It is not active until present in the reduced form. Any of several mixed disulfides may exist.

d. Reactions of the Thiol Group of Coenzyme A. The thiol or mercaptan group is the functional part of the CoA molecule, just as it is in the case of cysteine or in reduced glutathione. Most of the studies on this group have been carried out with acetyl-CoA, which gives the typical reactions of acetyl mercaptans. Thus, acetyl-CoA obtained in partially purified form from yeast^{50,130} was shown to give a color reaction with sodium nitroprusside which is characteristic of an acetyl mercaptan.¹⁴² Acetyl-CoA also exhibits lability toward mercury salts;⁴⁰ this is another characteristic of an acetyl mercaptan. However, the —SH group is protected by the acetate group when acetyl-CoA is exposed to iodoacetate.¹⁴² The latter compound has a destructive effect on the unprotected —SH group. After mild hydrolysis of the acetyl-CoA, which results in the liberation of the —SH group, the coenzyme loses its power to catalyze the acetylation of sulfanilamide.⁵⁰

e. The Distribution of Coenzyme A. Coenzyme A was first prepared from liver, and this tissue is the most reliable source of the coenzyme. However, Kaplan and Lipmann⁵¹ have shown that it is widely distributed in animal tissues in microorganisms, and in higher plants. One of the main sources of this co-factor is yeast.⁵¹ Novelli and Lipmann^{49,143} demonstrated that yeast can synthesize CoA from pantothenic acid when the

¹³⁷ G. M. Brown and E. E. Snell, *Proc. Soc. Exptl. Biol. Med.*, **77**, 138–140 (1951).

¹³⁸ S. Wakil and H. R. Mahler, *Federation Proc.*, **12**, 285 (1953).

¹³⁹ H. R. Mahler, S. J. Wakil, and R. M. Bock, *J. Biol. Chem.*, **204**, 453–468 (1953).

¹⁴⁰ A. Kornberg and W. E. Pricer, Jr., *J. Am. Chem. Soc.*, **74**, 1617 (1952).

¹⁴¹ A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **204**, 329–343 (1953).

¹⁴² F. Lynen, *Ann.*, **574**, 33–37 (1951).

¹⁴³ G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **171**, 833–834 (1947).

solution is aerated in a glucose-phosphate medium. Tissues and micro-organisms which use acetate groups for the synthesis of fatty acids, acetoacetate, and cholesterol have a much higher content of CoA than do tissues, such as muscle, in which there is little fat synthesis and in which most of the acetate is utilized by way of the tricarboxylic acid cycle. One would expect mammary tissue to be especially high in this component.

(5) *The Role of Enzymes in the Biosynthesis of Fat*

Presumably not only the coenzyme but also an enzyme system is required for fat synthesis. Brady and Gurin¹⁴⁴ described a preparation from the homogenate of pigeon liver which possessed the ability to cause the incorporation of C¹⁴-labeled acetate into long-chain fatty acids. In an extension of their earlier work, Brady and Gurin¹⁴⁵ have prepared a water-soluble enzyme system from pigeon liver which is capable of effecting this synthesis. This reaction can likewise occur in liver homogenates, as well as in washed mitochondria suspended in particle-free supernatant fluid. The efficiency of the water-soluble system in converting acetate to long-chain fatty acids is approximately equal to that of the homogenate. The synthesis occurs under anaerobic conditions, but the fatty acid oxidation is negligible. In this system, acetyl-CoA appears to be a less efficient precursor of fatty acid than is free acetate. The synthetic process occurring under anaerobic conditions is stimulated by Mg⁺⁺, diphosphopyridine nucleotide (DPN), cytochrome C, and especially by citrate, while Ca⁺⁺ and F⁻ are inhibitory.

Bucher¹⁴⁶ also prepared cell-free homogenates from rat liver which were capable of incorporating labeled acetic acid into long-chain fatty acids and cholesterol. These results have been confirmed by Dituri and Gurin,¹⁴⁷ who also reported that the addition of citrate brought about a twenty-fold increase in lipogenesis.

Green and co-workers¹⁴⁸ isolated a soluble enzyme (or a mixture of enzymes) from pig heart muscle which catalyzes a reversible conversion of acetoacetate to acetyl-CoA in the presence of succinyl-CoA. It is suggested that the activation of acetoacetate is brought about by a replacement reaction with succinyl-CoA, probably leading to the formation of

¹⁴⁴ R. O. Brady and S. Gurin, *Arch. Biochem.*, **34**, 221-222 (1951).

¹⁴⁵ R. O. Brady and S. Gurin, *J. Biol. Chem.*, **199**, 421-431 (1952).

¹⁴⁶ N. L. R. Bucher, *J. Am. Chem. Soc.*, **75**, 498 (1953).

¹⁴⁷ F. Dituri and S. Gurin, *Arch. Biochem. Biophys.*, **43**, 231-232 (1953).

¹⁴⁸ D. E. Green, D. S. Goldman, S. Mii, and H. Beinert, *J. Biol. Chem.*, **202**, 137-150 (1953).

acetoacetyl-CoA which, in turn, is cleaved into two molecules of acetyl-CoA. Coon and co-workers¹⁴⁹ have proposed a mechanism for the synthesis of acetoacetate and its breakdown in heart muscle.

(6) *The Role of Hormones in the Biosynthesis of Fat*

a. The Effect of Insulin. Insulin has a profound effect on the rate of synthesis of fat. Although the extent of lipogenesis from acetate in rat liver slices was increased several times when pyruvate was added to the substrate, Bloch and Kramer⁶⁸ reported that it was still further augmented when insulin was also introduced into the medium. These results are interpreted as indicating that insulin must be concerned with the utilization of pyruvate for the synthesis of fatty acids. The addition of glucose or oxaloacetate augmented fat synthesis from acetate to a somewhat lesser degree than did pyruvate. When insulin was added to a glucose-containing medium, increased lipogenesis also occurred.

Although Brady and Gurin⁹⁶ reported that the addition of insulin to a medium containing acetate increased the rate of its incorporation into long-chain fatty acids, no increased lipogenesis from acetate was caused by this hormone if glucose was absent. Masri *et al.*¹⁵⁰ noted that insulin by itself was inactive in promoting lipogenesis from acetate in liver slices obtained from rats which had fasted for eighteen hours. Haugaard and Stadie¹⁵¹ confirmed the stimulatory effect of crystalline insulin on the incorporation of acetate into the liver fatty acids of the rat.

The quantitative aspects of the insulin effect have been recorded by Felts, Chaikoff, and Osborn.¹⁵² It was found that only 2% of the acetate became incorporated into the fatty acids in surviving liver slices from alloxan-diabetic rats. On the other hand, when these slices were treated with insulin, the incorporation of C¹⁴ from acetate into the fatty acids was found to range between 6 and 23%. Conversely, the oxidation of acetate was also markedly reduced in liver slices from insulin-treated rats. The diabetic liver was found to oxidize two to four times as much of the carboxyl-carbon of acetate as it did of the methyl carbon. The introduction of insulin reduced the oxidation of formate-C¹⁴ to C¹⁴O₂, and concomitantly increased its incorporation into fatty acids. However, the actual amounts of formate-C¹⁴ isolated as fatty acids were quite small. Balmain

¹⁴⁹ M. J. Coon, J. R. Stern, A. del Campillo, and S. Ochoa, *Federation Proc.*, **12**, 191 (1953).

¹⁵⁰ M. S. Masri, I. Lyon, and I. L. Chaikoff, *J. Biol. Chem.*, **197**, 621-624 (1952).

¹⁵¹ E. S. Haugaard and W. C. Stadie, *J. Biol. Chem.*, **200**, 753-757 (1953).

¹⁵² J. M. Felts, I. L. Chaikoff, and M. J. Osborn, *J. Biol. Chem.*, **193**, 557-562 (1951).

and associates¹⁵³ likewise noted a stimulation of fat formation caused by insulin in slices from lactating mammary gland from rat, rabbit, and mouse as studied with C¹⁴ and tritium. Tritium was found to offer a more sensitive method for the study of lipogenesis than did C¹⁴.

It is known that the development of hyperglycemia in diabetes is largely to be ascribed to the inability of the animal to convert carbohydrate to fat. Stetten and Boxer¹⁵⁴ were the first to demonstrate that the rat, when rendered diabetic with alloxan, largely loses its capacity to utilize glucose for lipogenesis. In their later work, Chernick and associates¹⁵⁵ reported that the conversion of C¹⁴-labeled glucose to fatty acids was reduced to practically zero in liver slices from alloxan-diabetic rats. However, when the diabetic rats were pretreated with insulin for five days, the defect in lipogenesis from carbohydrate was completely corrected, even in the case of a rat which had suffered from diabetes for as long as 150 days.¹⁵⁶

The extent to which acetate and lactate are used for lipogenesis in diabetes has been shown to be a function of the type of carbohydrate which is simultaneously being metabolized. Thus, Baker, Chaikoff, and Schusdek¹⁵⁷ reported that hepatic lipogenesis in liver slices from alloxan-diabetic rats was greatly depressed when glucose was fed. In some cases, the extent of fat formation was reduced to 10% of the normal. The livers of these rats had almost completely lost their capacity to convert glucose to fatty acids. In contradistinction to these results, the administration of fructose restored the ability of the liver to convert acetate or lactate to fatty acids; however, fructose could not correct the defective lipogenesis observed in the presence of glucose. The action of fructose differs from that of insulin in this latter respect. Whereas insulin is able to restore the lipogenic capacity of the diabetic liver completely, not only in the case of acetate and lactate but also in that of glucose, fructose cannot function with the latter metabolite. It is believed that fructose exerts its effect by affording a continuous source of metabolites for the glycolytic system; these may be required to maintain lipogenesis in the liver.

Insulin likewise promotes fat synthesis in mammary tissue. Several investigators^{158,159} reported that crystalline insulin exerts a marked potentiating effect upon fat synthesis in slices of mammary tissue from

¹⁵³ J. H. Balmain, S. J. Folley, and R. F. Glascock, *Nature*, **168**, 1083 (1951).

¹⁵⁴ De W. Stetten, Jr., and G. E. Boxer, *J. Biol. Chem.*, **156**, 271-278 (1944).

¹⁵⁵ S. S. Chernick, I. L. Chaikoff, E. J. Masoro, and E. Isaef, *J. Biol. Chem.*, **186**, 527-534 (1950).

¹⁵⁶ S. S. Chernick and I. L. Chaikoff, *J. Biol. Chem.*, **186**, 535-542 (1950).

¹⁵⁷ N. Baker, I. L. Chaikoff, and A. Schusdek, *J. Biol. Chem.*, **194**, 435-443 (1952).

¹⁵⁸ J. H. Balmain, T. H. French, and S. J. Folley, *Nature*, **165**, 807-808 (1950).

¹⁵⁹ J. H. Balmain and S. J. Folley, *Biochem. J.*, **49**, 663-670 (1951).

non-ruminants (mouse, rat, and rabbit), as judged by its influence on the R.Q. Glucose is required in the medium. Acetate is also converted to fat in the presence of glucose. The threshold concentration of insulin required to initiate this reaction was found to be only approximately 0.1 μ g. (0.0022 I.U./ml.).

The action of insulin in effecting fat synthesis is also a function of species. Thus, although insulin can stimulate fat synthesis in mammary tissue of the non-ruminants, it is unable to effect a similar augmentation in udder slices of ruminants such as sheep. The effect of species on lipogenesis, as demonstrated by the use of the R.Q., has been confirmed by the employment of labeled substrates. Thus, Balmain and co-workers⁷⁰ reported that increased fat synthesis occurred with mammary slices of the rat when insulin was added to the medium, as judged from the C¹⁴-fatty acids synthesized after C¹⁴-carboxyl-labeled acetate and unlabeled glucose, respectively, were used. The effect of insulin in fat formation was confirmed later by the use of tritium in the medium.¹⁵³ Finally, it was likewise found by the use of isotope experiments that fat synthesis from acetate or from glucose and acetate in the udder of sheep is uninfluenced by insulin; this confirms the R.Q. studies. Insulin, by itself, was shown to be inactive in stimulating lipogenesis in mammary tissue.

b. The Effect of the Hormones of the Anterior Lobe of the Pituitary. Brady, Lukens, and Gurin¹⁶⁰ are of the opinion that neither insulin nor the anterior pituitary hormones are required for the synthesis of fatty acids from acetate by the liver. It is suggested that the pituitary secretes a principle which inhibits lipogenesis either directly, or indirectly through the mediation of some other endocrine organ. Insulin appears to act as an antagonist to an inhibitory substance of this nature. Normal fatty acid synthesis requires an appropriate balance between the insulin and the hypophyseal activity.

The results of a number of investigations are readily understood if one accepts the premise that fat synthesis depends upon the balance between insulin and the anterior pituitary hormone. Thus, Brady *et al.*¹⁶¹ noted that pancreatectomy inhibited lipogenesis, while hypophysectomy and adrenalectomy produced an augmented fat synthesis. Hepatic lipogenesis proceeded at a normal rate in liver slices from depancreatized-hypophysectomized cats; the rate was variable in hepatic tissue from depancreatized-adrenalectomized animals. It is claimed¹⁶¹ that growth hormone prevents lipogenesis in Houssay cats (depancreatized and hypophysec-

¹⁶⁰ R. O. Brady, F. D. W. Lukens, and S. Gurin, *Science*, **113**, 413-415 (1951).

¹⁶¹ R. O. Brady, F. D. W. Lukens, and S. Gurin, *J. Biol. Chem.*, **193**, 459-464 (1951).

tomized); this hormone has also been shown to inhibit the incorporation of deuterium into fatty acids of rats *in vivo*.¹⁶²

c. The Effect of Adrenal Hormones. The data on the effect of the adrenocortical hormones on lipogenesis have been somewhat contradictory. In Houssay cats, the administration of cortisone reduced lipogenesis.¹⁶¹ Welt and Wilhelmi¹⁶² also reported that ACTH (adrenocorticotrophic hormone, which stimulates the production of cortisone) likewise decreases fatty acid synthesis. On the other hand, Altman *et al.*¹⁶³ found that, when cortisone and insulin were given concomitantly, they exerted a synergistic effect upon lipogenesis in perfused rat liver, without affecting the synthesis of cholesterol. Epinephrine has been shown to depress the incorporation of acetate into fatty acids.¹⁵⁰

d. The Effect of the Thyroid Gland. Spirtes, Medes, and Weinhouse¹⁶⁴ reported that the rate of oxidation of acetate was 30 to 70% higher in the livers of hyperthyroid rats than in those of normal rats, while the level of oxidation was found to be reduced to below normal in athyroid animals. No differences in acetoacetate synthesis from acetate were observed between the normal and the abnormal animals. On the other hand, although fatty acid synthesis was as high or higher in liver slices from hyperthyroid rats as in those from normal animals, only a negligible fatty acid synthesis obtained in the livers of athyroid rats. The latter situation was similar to that which occurs in fasting normal animals.¹⁶⁴

(7) *Factors Other Than Hormones Affecting the Biosynthesis of Fatty Acids in Animals*

a. Fetal Synthesis. Fat synthesis in the embryo varies markedly from that occurring in older animals. Thus, in the case of pregnant rabbits, Popják and Beeckmans¹⁶⁵ found a greater synthesis of fatty acids in fetuses than in their mothers. It was also noted that the rate of cholesterol synthesis in the fetus was highest in the liver, while the bulk of the fatty acid formation occurred in the extrahepatic tissues.¹⁶⁵ However, the highest rate of fatty acid synthesis was in the mammary tissue of the mother.¹⁶⁶

b. Nutritional Status. The nutritional condition has likewise been

¹⁶² I. D. Welt and A. E. Wilhelmi, *Yale J. Biol. Med.*, **23**, 99-111 (1950).

¹⁶³ K. I. Altman, L. L. Miller, and C. G. Bly, *Arch. Biochem. Biophys.*, **31**, 329-331 (1951).

¹⁶⁴ M. A. Spirtes, G. Medes, and S. Weinhouse, *XIX Intern. Physiol. Congress*, Montreal, Aug.-Sept., 1953, *Abst.*, 789-790, *J. Biol. Chem.*, **204**, 705-713 (1953).

¹⁶⁵ G. Popják and M. L. Beeckmans, *Biochem. J.*, **44**, xxxvi-xxxvii (1949).

¹⁶⁶ G. Popják and M. L. Beeckmans, *Biochem. J.*, **44**, xxxvii (1949).

shown to be an important factor in fat synthesis. Boxer and Stetten¹⁶⁷ were the first to prove that newly synthesized fatty acids are reduced by restriction of the food intake. Thus, Medes and co-workers,¹⁶⁸ basing their conclusions upon the behavior of tissue slices of rats, found that lipogenesis from acetate was greatly impaired by fasting or by undernutrition. The highest rates of fatty acid synthesis from acetate were observed in the tissues from rats which had previously been fasted or had undergone food restriction, and which had then been heavily fed for some time prior to the removal of the tissues. Although lipogenesis does occur in extrahepatic tissues, Medes and her co-workers reported that the rates of synthesis were considerably below those in the liver. Lyon, Masri, and Chaikoff¹⁶⁹ reported further experiments on the effect of the nutritional state upon the rate of fat synthesis from acetate. Whereas 25% of the acetate added to the liver slices from rats which had been fed *ad libitum* was converted to fatty acids, and 25% was oxidized, the average recoveries of fatty acids from the liver slices of rats fasted six, twelve, or eighteen hours before sacrifice were only 25, 12, and 3%, respectively. The amount of acetate oxidized remained at figures varying between 20 and 30%. It is suggested that the glycolytic and oxidative enzyme systems remained intact in the fasted animals, while the ability to synthesize fat from acetate had been lost, for the most part. The lipogenic ability was rapidly restored when glucose was given orally. The administration of a casein hydrolysate was also shown to stimulate lipogenesis, but to a lesser extent than did glucose; the feeding of corn oil had no effect in restoring the capacity to synthesize fats. Coniglio and co-workers¹⁷⁰ observed marked impairment of the fatty acid synthesis in the whole rat subjected to inanition. Van Bruggen *et al.*¹⁷¹ also found about one-third less fatty acid synthesis in rats fasted 120 hours than in unfasted animals.

The effect of inanition on the synthesis of fatty acids from glucose is the same as its effect on lipogenesis from acetate. Masoro *et al.*¹⁷² also reported that the conversion of C¹⁴-labeled glucose to fatty acids was reduced by a twenty-four-hour fast to 10% of that noted when a standard diet was given, although the rate of oxidation to C¹⁴O₂ was not altered. Likewise,

¹⁶⁷ G. E. Boxer and De W. Stetten, Jr., *J. Biol. Chem.*, **153**, 607-616 (1944).

¹⁶⁸ G. Medes, A. Thomas, and S. Weinhouse, *J. Biol. Chem.*, **197**, 181-191 (1952).

¹⁶⁹ I. Lyon, M. S. Masri, and I. L. Chaikoff, *J. Biol. Chem.*, **186**, 25-32 (1952).

¹⁷⁰ J. G. Coniglio, C. E. Anderson, and C. S. Robinson, *J. Biol. Chem.*, **198**, 525-532 (1952).

¹⁷¹ J. T. Van Bruggen, T. T. Hutchens, C. K. Claycomb, W. J. Cathy, and E. S. West, *J. Biol. Chem.*, **204**, 389-394 (1952).

¹⁷² E. J. Masoro, I. L. Chaikoff, S. S. Chernick, and J. M. Felts, *J. Biol. Chem.*, **185**, 845-856 (1950).

the administration of a carbohydrate-free diet composed entirely of protein or of protein and fat resulted in a marked depression in hepatic lipogenesis. The ingestion, for three days, of a diet in which carbohydrate was the sole source of calories did not influence hepatic lipogenesis, although this function was somewhat reduced in animals maintained on an exclusively carbohydrate regimen for ten days. In this case, the decrease in fat synthesis might be related to an insulin deficiency, inasmuch as the insulin content of the pancreas is believed to be due in large part to the amount of available carbohydrate in the diet.^{171,173} A variation in the effect of insulin in producing lipogenesis in the livers from fasted and from fed rats has already been reported. Haugaard and Stadie¹⁷⁴ were likewise able to correlate the glycogen content of the liver of rats with their ability to synthesize fatty acids from acetate.

c. X-Irradiation. Whole body x-irradiation has been shown to have a profound effect on the rate of conversion of acetate-1-C¹⁴ to fatty acid C¹¹ by liver slices. The inhibition of the conversion of acetate to fatty acids in fasting rats was shown by Lerner *et al.*¹⁷⁵ to be removed by exposing the rat to whole body irradiation. Liver slices obtained forty-eight to seventy-two hours after x-irradiation with 2500 *r*, formed more than twice as much fatty acid as did slices from fed rats, and ten times as much as slices from fasted control rats. In the case of previously fasted rats, x-irradiation was shown to raise the rate of lipogenesis to two-thirds of that of the normally fed rat.

Altman *et al.*¹⁷⁶ reported a somewhat similar effect exerted by x-irradiation on bone marrow. In this case, the rate of synthesis of fat from acetate by bone marrow homogenates was doubled shortly after x-irradiation. However, it had returned to a normal value within forty-eight hours, after which it dropped to 58% below preirradiation values at the end of 158 hours. The synthesis of unsaturated fatty acids was the factor primarily affected by x-irradiation.

d. Species. The variation in the insulin effect on lipogenesis as related to species, which has already been discussed, is only one phase of this problem. Thus, Balmain *et al.*¹⁷⁷ reported that rat liver slices incorporated about eight times as much glucose carbon and one-sixth as much acetate carbon in fatty acids as did liver slices from sheep. Whereas sheep slices

¹⁷³ R. E. Haist, *Physiol. Revs.*, **24**, 409-444 (1944).

¹⁷⁴ E. S. Haugaard and W. C. Stadie, *J. Biol. Chem.*, **199**, 741-744 (1952).

¹⁷⁵ S. R. Lerner, W. L. Warner, and C. Entenman, *Federation Proc.*, **12**, 85 (1953).

¹⁷⁶ K. I. Altman, J. E. Richmond, and K. Salomon, *Biochem. et Biophys. Acta*, **7**, 460-465 (1951).

¹⁷⁷ J. H. Balmain, S. J. Folley, and R. F. Glascock, *Biochem. J.*, **53**, xxvi-xxvii (1953).

used the same amount of glucose for fat synthesis, irrespective of whether or not acetate was also present, rat slices transformed more glucose to fat in the absence of acetate than was the case when the latter compound was present.

A species difference has likewise been noted in the effect of x-rays. Thus, while treatment with x-rays augmented fat synthesis in the rat liver, it was without effect in this organ in the mouse.¹⁷⁵ This species difference in response to lipogenesis is in agreement with the observation that no increase in size of the liver occurs in the mouse following x-ray treatment, while this organ is enlarged in the rat.

e. The Effect of Vitamins. Vitamins of the B-complex are the ones primarily concerned with fat synthesis. Of these, thiamine is believed to play one of the most important roles. Thus, McHenry and Gavin,^{178,179} on the basis of earlier work of Ansbacher *et al.*,¹⁸⁰ and of Bender and Supplee,¹⁸¹ reported that thiamine, riboflavin, and rice polishings (vitamin B₆) exert associated effects upon the amount of body fat laid down by rats and pigeons.¹⁷⁹ Thiamine was shown to be the most important of the three vitamins.¹⁷⁹ Boxer and Stetten¹⁶⁷ likewise showed that rats on an unrestricted diet formed more fat than did those which received a thiamine-free diet *ad libitum*. However, when the fat formation of control rats receiving a complete diet but restricted in quantity to that consumed by the rats on the thiamine-deficient regimen was compared no difference in the fat content of the rats was noted. Boxer and Stetten¹⁶⁷ conclude that the failure of rats on a thiamine-deficient diet to form fat is attributable chiefly to the diminished food intake, rather than to any specific action of the vitamin. Gruber¹⁸² was unable to demonstrate any variation in fat synthesis in pigeons which were force-fed, irrespective of whether or not the birds received thiamine.

Carter and Phizackerley¹⁸³ reported that a deficiency of pyridoxine in the rat results in a decrease of the total lipid content of the body, and a diminution in the amount of neutral fat in the liver. The proportion of fat in the tissues can be restored on the pyridoxine-free diet by feeding this foodstuff. These workers conclude that the results are consistent with the view that the deficiency of pyridoxine involves an inability on the part of the rat to convert carbohydrate to fat.

¹⁷⁸ E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **125**, 653-660 (1938).

¹⁷⁹ E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **128**, 45-49 (1939).

¹⁸⁰ S. Ansbacher, G. C. Supplee, and R. C. Bender, *J. Nutrition*, **11**, 401-409 (1936).

¹⁸¹ R. C. Bender and G. C. Supplee, *J. Am. Chem. Soc.*, **59**, 1178-1182 (1937).

¹⁸² M. Gruber, *Biochim. et Biophys. Acta*, **7**, 480-481 (1951).

¹⁸³ C. W. Carter and P. J. R. Phizackerley, *Biochem. J.*, **49**, 227-232 (1951).

Curran¹⁸⁴ reported that the rate of fatty acid synthesis was slightly increased in the biotin-deficient rat; the increased rate of synthesis was believed to be related to the inanition accompanying biotin deficiency rather than to the deficiency itself.

Because of the importance of pantothenic acid as a component of the CoA molecule, it is obvious that a deficiency in this vitamin would have a profound effect on fatty acid synthesis. In pantothenic acid deficiency in ducks, CoA is present in the liver to the extent of only 40% of the normal amount.¹¹⁷ Although definite effects of CoA deficiency have not been reported, other intermediary reactions such as acetylation (see page 28) and fat oxidation (see page 86) are markedly altered under such conditions.

f. The Effect of Experimental Diabetes. Lipogenesis has been studied in alloxan diabetes and in pancreatic diabetes. Stetten and Boxer¹⁵⁴ first reported that the depression in lipogenesis was the major metabolic defect in alloxan diabetes in the rat. Subsequently, Brady and Gurin¹⁸⁵ found that the synthesis of long-chain acids from acetate could not be effected in the livers of rats with alloxan diabetes. According to Van Bruggen *et al.*,¹⁸⁶ a decrease in the incorporation of acetate in liver slices from alloxan-diabetic rats occurs, comparable to that obtained on fasting. After a fast lasting twenty hours, the decrease in lipogenesis was less in the slices from diabetic rats than in those of normal rats. Cholesterol synthesis was not similarly affected.

The same failure in synthesis of acetate obtained in depancreatized cats; however, the synthesis of cholesterol from acetate was not found to be inhibited, except in prolonged and severe diabetes. The lipogenic action could not be restored by the addition to the medium of insulin, glucose, fructose-6-phosphate, oxaloacetate, or α -ketoglutarate. The accumulation of fat in the liver in diabetes is therefore attributed mainly to increased transport from extrahepatic tissues, rather than to an increased rate of synthesis *in situ*.

g. The Effect of Glycerol. Glycerol was found to stimulate the utilization of acetate more than that of glucose. This throws open to question the hypothesis that glycerol stimulates lipogenesis by removing fatty acids, in the form of glycerides, from the site of synthesis. It also indicates that, although the prolipogenic action of insulin is closely related to the utilization of carbohydrate, it is doubtful whether or not this action is mediated through the formation of glycerol from glucose.¹⁷⁷

¹⁸⁴ G. L. Curran, *Proc. Soc. Exptl. Biol. Med.*, **75**, 496-498 (1950).

¹⁸⁵ R. O. Brady and S. Gurin, *J. Biol. Chem.*, **187**, 589-596 (1950).

¹⁸⁶ J. T. Van Bruggen, T. T. Hutchens, and P. Yamada, *Federation Proc.*, **12**, 283 (1953).

3. The Biosynthesis of Unsaturated Fatty Acids

Although the theory of Leathes and Meyer-Wedell¹⁸⁷ that desaturation is the first step in fatty acid catabolism has been widely accepted since it was propounded in 1909, it remained for Schoenheimer and Rittenberg¹⁸⁸ to offer the first experimental proof for this reaction in the animal body. In their classical experiments, it was shown that unsaturated fatty acids could originate in the mouse from tagged saturated fatty acids. Although it was not proved in the early work that only monoethenoid acids are involved in the synthesis, Bernhard and Schoenheimer¹⁸⁹ convincingly demonstrated that the rat was unable to synthesize either the diethenoid acid, linoleic, or the triethenoid acid, linolenic, from carbohydrate, although the synthesis of large amounts of palmitic and stearic acids could be shown. Stetten and Schoenheimer²⁶ demonstrated the synthesis of palmitoleic acid from palmitic acid in rats. Anker⁹⁹ reported that, after carboxyl-labeled- C^{14} -myristic acid was fed to rats, myristoleic acid, $CH_3 \cdot (CH_2)_3 \cdot CH : CH \cdot (CH_2)_7 \cdot C^{14}OOH$, could be isolated from the unsaturated fatty acid fraction of the carcass. Reiser and co-workers¹⁹⁰ suggest that, although the hen can synthesize minimum amounts of polyethenoid fatty acids, oleic acid replaces the polyunsaturated acids in the neutral fat of the eggs produced by hens on a fat-free ration. This must indicate the synthesis of oleic acid from saturated acids by the hen under these conditions. Although a number of polyunsaturated acids are synthesized by hens on a fat-free regimen, Reiser¹⁹¹ produced unsaturated fatty acid deficiency in this species. His results are further discussed by Holman.¹⁹²

Essential fatty acids cannot be produced *de novo* in any common species of animal; however, there is evidence that one essential acid can be transformed to another. Thus, Mead and associates,^{102,103} using rats, demonstrated the synthesis of arachidonic acid which resulted from the addition of labeled acetate to the carboxyl group of linoleic acid, followed by removal of hydrogen from specific linkages.

Polyunsaturated acids can be synthesized by the plant. Thus, Painter¹⁹³ noted that, although unsaturated acids may be synthesized by desaturation during oil deposition in the flaxseed, most of it is preformed, and hence is directly synthesized.

¹⁸⁷ J. B. Leathes and L. Meyer-Wedell, *J. Physiol.*, **38**, xxxviii-xl (1909).

¹⁸⁸ R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **113**, 505-510 (1936).

¹⁸⁹ K. Bernhard and R. Schoenheimer, *J. Biol. Chem.*, **133**, 707-712 (1940).

¹⁹⁰ R. Reiser, B. Gibson, M. J. Carr, and B. G. Lamp, *J. Nutrition*, **44**, 159-175 (1951).

¹⁹¹ R. Reiser, *J. Nutrition*, **42**, 319-323 (1950).

¹⁹² R. T. Holman, *Proc. Third Conf. Research, Council on Research, Am. Meat Inst.*, Chicago, March, 1951, pp. 1-10.

¹⁹³ E. P. Painter, *Arch. Biochem.*, **5**, 337-348 (1944).

4. The Disposition of Newly Synthesized Fatty Acids

The fate of newly synthesized fatty acids has recently received much attention.^{56,59,194-197} The incorporation of isotopic carbon from C¹⁴-labeled acetate was found to proceed more rapidly in the neutral fat of the liver of the intact rat than in the phospholipid fraction. This indicated, according to Pihl and Bloch,⁵⁶ that phospholipids are not obligatory intermediates in fat synthesis. These results are at variance with those obtained in the earlier studies of Barrett *et al.*,¹⁹⁸ in which a higher proportion of D-labeled fatty acids were found in the phospholipids of the liver than in the neutral fat of this organ after deuterio-linseed oil had been fed. According to the experiments of the former investigators,⁵⁶ in all the internal organs other than the liver, including mesenteric fat, the isotope concentrations were found to be higher in the phospholipids than in the neutral fat fraction. The distribution of biosynthesized fatty acids between the neutral fat and the phospholipid fractions was similar in fed animals and in those fasted for as long as 72 hours.¹⁹⁷ The ratio between these fractions varied only slightly from 1.0; in the intestine, however, the phospholipid fatty acids were the more heavily labeled. Since the proportionate ratio between the intramuscular and the subcutaneous fat differed, it was concluded that they represent separate types of lipid. The neutral fat fatty acids of the intramuscular tissue were found to have a higher activity than did those in the phospholipid fraction, whereas the opposite situation obtained in the case of the subcutaneous tissue.¹⁹⁷ While the relative activities of the saturated and unsaturated fatty acids of fed and of fasted rats were similar in the liver, in the case of the carcass fat, the saturated fatty acids were found to be more heavily labeled when the animals had been fasted.¹⁹⁷ Moreover, Bloch³ reported that acetate carbon is incorporated at a much faster rate into saturated than into unsaturated acids by rat liver slices.

5. The Biosynthesis of Glycerol

Because of the close relationship between glycerol and glucose, both from a structural and from a metabolic standpoint, the synthesis of the

¹⁹⁴ G. Ehrlich and H. Waelsch, *J. Biol. Chem.*, **163**, 195-202 (1946).

¹⁹⁵ P. V. Harper, W. B. Neal, Jr., and G. R. Hlavacek, *Metabolism*, **2**, 69-80 (1953).

¹⁹⁶ J. T. Van Bruggen, T. T. Hutchens, C. K. Claycomb, and E. S. West, *J. Biol. Chem.*, **200**, 31-37 (1953).

¹⁹⁷ J. G. Coniglio, C. E. Anderson, and C. S. Robinson, *Am. J. Physiol.*, **177**, 69-72 (1954).

¹⁹⁸ H. M. Barrett, C. H. Best, and J. H. Ridout, *J. Physiol.*, **93**, 367-381 (1938).

glycerol moiety in the triglyceride molecule has not been considered by most biochemists as posing any particular difficulty.

The earlier literature furnishes considerable information to indicate that glycerol is readily transformed to glucose in the animal body. Since the reactions involved in such transformations are reversible, it is believed that this proof likewise constitutes evidence for the reverse reaction, namely glucose $\rightarrow$ glycerol.

As early as 1902, Cremer¹⁹⁹ demonstrated that as much as 40% of ingested glycerol could be accounted for as "extra glucose" in the urine of the phlorhizinized dog; Lüthje²⁰⁰ obtained similar results with depancreatized dogs. On the other hand, Chambers and Deuel²⁰¹ were able to recover practically all of the administered glycerol in the urine of fasting phlorhizinized dogs as "extra glucose." It is now generally considered that this transformation is a quantitative one.

The glycerol $\rightarrow$ glucose reaction has been directly demonstrated by another procedure, namely by the isolation of an increased glycogen deposition following the administration of the trihydric alcohol. This conversion was first reported by Catron and Lewis,²⁰² and was confirmed by Shapiro.²⁰³ Although Deuel and associates²⁰⁴ were unable to demonstrate a conversion of the glycerol of common fats to glycogen in rats, they did prove that the glycerol of triglycerides such as triacetin, tributyrin, tricaproin, and tri-caprylin, which cannot be deposited in the fat depots, gives rise to glycogen.

The conversion of glycerol to glucose has been indicated by a number of indirect procedures. Thus, Voegtlin *et al.*²⁰⁵ found that glycerol will replace glucose in relieving the hypoglycemic and other toxic effects of insulin injections. On the other hand, Mann and Magath²⁰⁶ were unable to maintain the blood sugar level of the hepatectomized dog by the administration of glycerol, except for a transitory period; hence, it was considered that the liver is essential for the storage of glucose and the transformation of glycerol to glucose.

¹⁹⁹ M. Cremer, *Münch. med. Wochschr.*, 49, 944 (1902).

²⁰⁰ H. Lüthje, *Deut. Arch. klin. Med.*, 80, 98-104 (1904).

²⁰¹ W. H. Chambers and H. J. Deuel, Jr., *J. Biol. Chem.*, 65, 21-29 (1925).

²⁰² L. F. Catron and H. B. Lewis, *J. Biol. Chem.*, 84, 553-559 (1929).

²⁰³ I. Shapiro, *J. Biol. Chem.*, 108, 373-387 (1935).

²⁰⁴ H. J. Deuel, Jr., J. S. Butts, H. Blunden, C. H. Cutler, and L. Knott, *J. Biol. Chem.*, 117, 119-129 (1937).

²⁰⁵ C. Voegtlin, E. R. Dunn, and J. W. Thompson, *Am. J. Physiol.*, 71, 574-582 (1924-1925).

²⁰⁶ F. C. Mann and T. B. Magath, *Arch. Internal Med.*, 30, 73-84; 171-181 (1922); 31, 797-806 (1923).

Another procedure for demonstrating the convertibility of glycerol to glucose is by proving that it reduces ketonuria. Only substances which are convertible to glucose are believed to have a ketolytic effect.²⁰³ Thus, Hirschfeld,²⁰⁷ Satta,²⁰⁸ and Lang²⁰⁹ reported that the ingestion of glycerol by diabetic or by normal fasting human subjects is followed by a decrease in ketonuria. Moreover, Shapiro,²⁰³ employing the procedure of exogenous ketonuria with the fasting rat, demonstrated that glycerol reduced the excretion of ketone bodies to the same extent as did an isodynamic amount of glucose.

Not only has the experimental evidence left little doubt that a quantitative conversion of glycerol to glucose or to glycogen can take place in normal animals, but the proof of the opposite reaction is equally convincing. Freeman and Friedemann²¹⁰ demonstrated that, after the feeding of oleic acid to dogs, the acid was recovered exclusively as triglyceride in the lymph; this result was interpreted as a direct proof of the synthesis of glycerol in the gut wall.

The appearance of glycerol arising from glucose in the process of glycolysis in tissues and in the fermentation with yeast offers another example of the glucose $\rightarrow$ glycerol transformation. Thus, West and Todd²¹¹ suggest that, in the breakdown of glycogen and glucose in the liver, the Harden-Young ester (fructose-1,6-diphosphate) gives rise to dihydroxyacetone phosphate; the latter compound can be converted to α -glycerol phosphate (and hence to glycerol) or to 3-phosphoglyceric aldehyde. The latter substance is not ordinarily the main source of glycerol, although it can be converted to this polyhydric alcohol. The sequence of reactions which result in glycerol production is shown on the following page.

In the fermentation of glucose with yeast, small amounts of glycerol are produced, in addition to ethyl alcohol.²¹¹ In this instance, the immediate source of the glycerol is believed to be 3-phosphoglyceric aldehyde, which is converted to α -glycerol phosphate by diphosphopyridine nucleotide (DPN) under the influence of α -glycerophosphate dehydrogenase. When α -glycerophosphate is acted on by phosphatase, free glycerol is formed.

Experiments with lactating animals have provided additional evidence that glucose can serve as the mother substance of glycerol. Under such conditions, the synthesis of glycerol apparently takes place in the mam-

²⁰⁷ F. Hirschfeld, *Z. klin. Med.*, **28**, 176-209 (1895); **31**, 212-278 (1897).

²⁰⁸ G. Satta, *Beitr. Chem. Physiol. Pathol.*, **6**, 376-391 (1905).

²⁰⁹ R. M. Lang, *Biochem. J.*, **9**, 456-478 (1915).

²¹⁰ S. Freeman and J. E. Friedemann, *J. Biol. Chem.*, **108**, 471-478 (1935).

²¹¹ E. S. West and W. R. Todd, *Textbook of Biochemistry*, Macmillan, New York, 1955, p. 965.

The Breakdown of Fructose-1,6-Diphosphate to Form Glycerol under Anaerobic Conditions (DPN is diphosphopyridine nucleotide)²¹¹

mary gland itself. Thus, French and Popják⁵⁴ reported that the specific radioactivities of glycerol isolated from milk glycerides secreted six hours after the administration of C¹⁴-glucose and C¹⁴-acetate, respectively, in equiradioactive doses to lactating rabbits were much higher in the first case. In fact, the specific activity of the glycerol was of the same order as that of the lactose isolated from the same milk sample. An analysis of these results indicated that between 50 and 100% of the glycerol excreted in the triglyceride fraction of milk fat had been newly synthesized in the udder.⁵⁴

Although blood glucose is, in all probability, the main precursor of glycerol in mammary tissue, acetate can likewise serve to a limited extent in a similar capacity. Under these circumstances, acetate acts indirectly as a source of lactose. It is known that acetate can be incorporated into the lactose molecule.⁷² Moreover, the glucose and lactose present in the milk of the lactating rabbit after the administration of labeled acetate were shown by French, Popják, and Malpress²¹² to have identical specific activities. Popják *et al.*²¹³ found that the specific radioactivities of glycerol and lactose, when plotted against time, indicate a relationship between the curve of a reaction product (glycerol) and that of its immediate precursor (lactose). However, Folley^{6,214} is of the opinion that glycerol

²¹² T. H. French, G. Popják, and F. H. Malpress, *Nature*, **169**, 71 (1952).

²¹³ G. Popják, R. F. Glascock, and S. J. Folley, *Biochem. J.*, **52**, 472-475 (1952).

²¹⁴ S. J. Folley, *Biochem. J.*, **51**, xv (1952).

is derived, not from lactose, but from a hexose (glucose) which is in equilibrium with lactose. Thus, glucose originating from the newly-formed lactose which, in turn, owes its origin to acetate, would serve as the mother substance of glycerol.

However, Balmain and associates⁷⁰ reported that, although the mammary tissue of the living animal has the capacity to utilize acetate as a source of glycerol, this conversion cannot occur in the mammary slices of the sheep or rat *in vitro*. Under such conditions, with labeled acetate in the substrate, the glycerol isolated from the newly-synthesized fat was in all cases non-radioactive. Thus, such isolated mammary tissue, although it can effect a synthesis of fatty acids from acetate, cannot bring about a conversion to glucose under the conditions so far investigated.

While the foregoing evidence clearly indicates that glycerol is derived from glucose in the mammary gland, the question which is still unanswered is whether or not the rate of fat synthesis may be conditioned by the speed of glycerol formation. It has been suggested that, once the fatty acid is synthesized, it is "trapped" by being converted to the triglyceride. Thus, it will immediately be removed from the site of synthesis. If a plentiful supply of glycerol is present in mammary tissue, a maximal amount of glyceride formation is to be expected under such conditions, both because the synthesis is not delayed by lack of constituents, and particularly because the rate of synthesis of fatty acids is augmented by the rapid removal of the end-product from the arena of activity.

An answer to the question as to whether or not the speed of fat synthesis is conditioned by the glycerol currently available has been given by Balmain and Folley.¹⁵⁹ These workers demonstrated that glycerol may be a limiting factor in triglyceride synthesis in rat mammary tissue. When glycerol was added to a system consisting of the mammary slices metabolizing glucose plus acetate, a stimulation of R.Q., Q_{O_2} , and acetate uptake similar to that obtained with insulin was noted. Balmain and associates⁷⁰ subsequently demonstrated that the addition of glycerol to mammary slices containing labeled acetate increased the incorporation of the acetate into fat. A similar stimulation of fat synthesis by glycerol was reported in sheep udder when acetate was used, but not when both acetate and glucose were present.

It is now believed¹⁵⁹ that the increased fat synthesis resulting from the addition of insulin in the presence of glucose is explained by the additional glycerol thus made available as a result of the effect of the hormone on glucose. The fact that the fat synthesis occurs with glycerol in rat mammary tissue without an increase in glucose uptake is in harmony with this

viewpoint. Moreover, Balmain and Folley¹⁵⁹ found that the glycerol and insulin effects are not additive; this would also support the thesis that the action of insulin is related to a stimulation of glycerol synthesis. Although this explanation does not seem to apply to sheep udder, in which no stimulation obtains when glycerol is added to tissue metabolizing acetate plus glucose, it is possible that glucose can be broken down rapidly enough in the udder of this species so that glycerol is present in maximal concentration without the intervention of insulin.

6. The Rate and Extent of Biosynthesis of Fatty Acids and of Glycerol from Acetate and Glucose

The rate of synthesis of fatty acids from acetate is fairly rapid. Pihl, Bloch, and Anker,²¹⁵ making use of labeled acetic acid, demonstrated that the synthesis of saturated acids had a half-life of one day in the liver, and of sixteen to seventeen days in the carcass. The corresponding periods for unsaturated fatty acid (oleic) were one and two and nineteen to twenty days, respectively. Although acetic acid is capable of furnishing every carbon of long-chain fatty acid molecules,^{24,215} the maximal concentration of isotope in the liver fatty acids was found to be about 25% of that of acetyl groups; this would indicate that at least one-fourth of the carbon of fatty acids is furnished by acetic acid.²¹⁵

In experiments on rabbits, Popják, Hunter, and French²¹⁶ demonstrated that, within six hours, 30 to 70% of the short-chain milk fat acids originated from acetate, and about 25% from carbohydrate. However, only one-fifth to one-tenth of these long-chain fatty acids were synthesized in the mammary gland from either of these sources.²¹⁶ On the other hand, 65 to 95% of the glycerol moiety of fat was derived from carbohydrate in the mammary gland over a six-hour period. It is concluded that the glycerol fraction of fat is more rapidly metabolized by the animal than is the fatty acid part. In contradistinction to this Popják *et al.*²¹⁶ noted that about 20% of the body acetate used for acetylation of *p*-aminobenzoic acid was derived from glucose in fully-fed lactating rabbits.

7. The Biosynthesis of Phospholipids

Phospholipids can be synthesized with relative ease in the animal. Thus, a number of investigators demonstrated that animals develop normally when their diet contains no preformed phospholipids and all the phos-

²¹⁵ A. Pihl, K. Bloch, and H. S. Anker, *J. Biol. Chem.*, **183**, 441-450 (1950).

²¹⁶ G. Popják, G. D. Hunter, and T. H. French, *Biochem. J.*, **54**, 238-247 (1953).

phorus is given in the inorganic form.²¹⁷⁻²¹⁹ The tissues of these animals were later shown to contain the normal amounts of phospholipids. Moreover, it has long been known that the phospholipids of the chick embryo increase during the incubation of the egg; thus, Hevesy *et al.*²²⁰ demonstrated that the phospholipids in the chick embryo are not derived from the yolk, but are synthesized by the embryo itself. The subject of the synthesis and origin of phospholipids has been reviewed by Sinclair,^{221,222} by Bloor,²²³ and by Chaikoff.²²⁴

(1) *Methods for the Demonstration of Phospholipid Biosynthesis in vivo*

In addition to the balance experiments as proof of phospholipid synthesis, direct evidence has been adduced by the use of a number of newer procedures. London²²⁵ employed a unique method developed by himself, by which it is possible to obtain blood from internal blood vessels of normal, intact animals. He showed that the phospholipid content in the blood leaving the liver was greater than that in the blood entering this organ.

The use of unnatural fatty acids has afforded another method for the study of the synthesis of phospholipids. One of these procedures involves the use of elaidic acid, the *trans*-isomer of oleic acid, which is a foreign fatty acid.²²⁶ However, it behaves *in vivo* exactly as if it were a naturally occurring fatty acid. Elaidic acid can be metabolized when fed in large amounts; it is stored mainly in the fat depots by the rat.^{227,228} After being deposited in the adipose tissues, it disappears slowly.²²⁹ Kohl^{227,228} reported that the rate of disappearance of elaidic acid is 72 mg./hr. in rats fed elaidin alone, and 35 mg./hr. in animals which received the elaidin along with protein and sugar. It is not known whether or not the elaidic acid is transformed in the body by the process of desaturation, or by hydrogenation, or to oleic acid by isomerization. However, the last procedure probably

²¹⁷ J. P. Gregersen, *Z. physiol. Chem.*, **71**, 49-99 (1911).

²¹⁸ E. V. McCollum, J. G. Halpin, and A. H. Drescher, *J. Biol. Chem.*, **13**, 219-224 (1912).

²¹⁹ R. H. A. Plimmer, *Biochem. J.*, **7**, 43-80 (1913).

²²⁰ G. C. Hevesy, H. B. Levi, and O. H. Rebbe, *Biochem. J.*, **32**, 2147-2155 (1938).

²²¹ R. G. Sinclair, *Physiol. Revs.*, **14**, 351-403 (1934).

²²² R. G. Sinclair, *Biol. Symposia*, **5**, 82-98 (1941).

²²³ W. R. Bloor, *Physiol. Revs.*, **19**, 557-577 (1939).

²²⁴ I. L. Chaikoff, *Physiol. Revs.*, **22**, 291-317 (1942).

²²⁵ E. S. London, *Ergeb. Physiol.*, **26**, 320-369 (1928).

²²⁶ R. G. Sinclair, *J. Biol. Chem.*, **111**, 515-526 (1935).

²²⁷ M. F. F. Kohl, *J. Biol. Chem.*, **126**, 709-719 (1938).

²²⁸ M. F. F. Kohl, *J. Biol. Chem.*, **126**, 721-729 (1938).

²²⁹ M. F. F. Kohl, *J. Biol. Chem.*, **126**, 731-736 (1938).

does not occur, since no good evidence for a *trans* $\rightarrow$ *cis* isomerization of the fatty acids has been demonstrated *in vivo*.

Miller and Burr²³⁰ investigated the synthesis of phospholipids by the use of fats containing fatty acids having characteristic absorption spectra. These experiments were performed with tung oil, which is made up largely of elaeostearic acid; the latter acid contains three conjugated double bonds.²³¹ Acids having conjugated bonds can readily be detected because of their characteristic absorption spectra. Fatty acids such as linoleic acid, which do not have conjugated double bonds, have a low ultraviolet absorption. However, Miller *et al.*²³¹ demonstrated that linoleate can be converted to a conjugated acid having approximately 100 times the absorption coefficient of the original non-conjugated dienoic acid.²³² It was shown by Barnes and co-workers²³³ that conjugated fatty acids were incorporated into both the neutral fat and the phospholipid fraction of the intestinal mucosa, although in this case the synthesis of neutral fat proceeded more rapidly than did that of phospholipid.

Since the development of the knowledge of isotopes, the incorporation of labeled fatty acids into the phospholipid molecule has been used by a number of workers as a method of studying the origin of these compounds. Cavanagh and Raper²³⁴ employed a partially deuterated linseed oil and followed the incorporation of the labeled fatty acids into the phospholipid of the liver, brain, kidney, blood plasma, and blood corpuscles of rats. Barrett, Best, and Ridout,¹⁹⁸ and Sperry *et al.*²³⁵ likewise employed this procedure to demonstrate the synthesis of phospholipids and fatty acids. Among the workers who have followed the formation of phospholipids by using fatty acids containing C¹⁴, Stevens and Chaikoff²⁸ may be cited. Although Levy²³⁶ was unable to demonstrate the presence of C₁₁, C₁₂, or C₁₄ acids in the phospholipids after feeding the triglycerides containing these acids, Stevens and Chaikoff²⁸ reported that lauric (C₁₂) and myristic (C₁₄) acids containing C¹⁴-labeled carboxyl groups were incorporated into the phospholipids.

In addition to introducing the label into the fatty acid, tagging of other components of the phospholipid molecule has been carried out. Inorganic

²³⁰ E. S. Miller and G. O. Burr, *Proc. Soc. Exptl. Biol. Med.*, **36**, 726-729 (1937).

²³¹ E. S. Miller, R. H. Barnes, J. P. Kass, and G. O. Burr, *Proc. Soc. Exptl. Biol. Med.*, **41**, 485-489 (1939).

²³² R. H. Barnes, E. S. Miller, and G. O. Burr, *Am. J. Physiol.*, **126**, 427 (1939).

²³³ R. H. Barnes, E. S. Miller, and G. O. Burr, *J. Biol. Chem.*, **140**, 233-240 (1941).

²³⁴ B. Cavanagh and H. S. Raper, *Biochem. J.*, **33**, 17-21 (1939).

²³⁵ W. M. Sperry, H. Waelsch, and V. A. Stoyanoff, *J. Biol. Chem.*, **135**, 281-290 (1940).

²³⁶ M. Levy, *Arch. sci. physiol.*, **4**, 337-343 (1950).

phosphate containing radioactive phosphate (P^{32}) has been most widely employed. Hevesy and Hahn,²³⁷ and, three years earlier, Artom *et al.*,²³⁸ reported that P^{32} was incorporated into the phospholipids in both intestine and liver, and possibly also in the kidney.

In addition to phosphorus as a tag, the nitrogen isotope has been employed to trace choline synthesis. Thus Stetten²³⁹⁻²⁴¹ used N^{15} in investigating the fate of choline and ethanolamine. The incorporation of labeled choline into lecithin, and of tagged ethanolamine into phosphatidylethanolamine, could readily be demonstrated. Arsenocholine, which contains arsenic in place of the nitrogen in ordinary choline, was used by Welch^{242,243} to follow the *in vivo* synthesis of liver and brain lecithin. Phospholipid synthesis from methionine and choline²⁴⁴ has been followed by using compounds in which the methyl group is tagged with C^{14} .

(2) The Site of Biosynthesis of Phospholipids

a. Liver. The liver has long been considered to be the main organ in which phospholipid synthesis takes place. Since London²²⁵ first demonstrated this fact in 1928, several different tests have been employed. For example, many workers^{237,238,245-248} found that P^{32} , given as inorganic phosphate (Na_2HPO_4), is incorporated into phospholipid in the liver. Thus, it was observed that a rapid increase of phospholipid- P^{32} occurred in the liver after the feeding of inorganic salt, and that this was followed by a decrease during the later periods. According to Fishler *et al.*,²⁴⁸ no synthesis of phospholipid takes place in the hepatectomized dog. Maximum concentrations of phospholipids were found in the livers of normal rats and mice²⁴⁹ within ten hours after the injection of $Na_2HP^{32}O_4$. Phospho-

²³⁷ G. Hevesy and L. Hahn, *Kgl. Danske Videnskab. Selskab, Biol. Medd.*, **15**, No. 5, 1-60 (1940).

²³⁸ C. Artom, G. Sarzana, C. Perrier, M. Santangelo, and E. Segrè, *Arch. intern. physiol.*, **45**, 32-39 (1937).

²³⁹ De W. Stetten, Jr., *J. Biol. Chem.*, **138**, 437-438 (1941).

²⁴⁰ De W. Stetten, Jr., *J. Biol. Chem.*, **140**, 143-152 (1941).

²⁴¹ De W. Stetten, Jr., *J. Biol. Chem.*, **142**, 629-633 (1942).

²⁴² A. D. Welch, *Proc. Soc. Exptl. Biol. Med.*, **35**, 107-108 (1936).

²⁴³ A. D. Welch and M. S. Welch, *Proc. Soc. Exptl. Biol. Med.*, **39**, 7-9 (1938).

²⁴⁴ J. A. Stekol, S. Weiss, P. T. Hsu, and P. Smith, *Federation Proc.*, **12**, 274-275 (1953).

²⁴⁵ C. Artom, G. Sarzana, and E. Segrè, *Arch. intern. physiol.*, **47**, 245-276 (1938).

²⁴⁶ I. Perlman, S. Ruben, and I. L. Chaikoff, *J. Biol. Chem.*, **122**, 169-182 (1937-1938).

²⁴⁷ C. Entenman, S. Ruben, I. Perlman, F. W. Lorenz, and I. L. Chaikoff, *J. Biol. Chem.*, **124**, 795-802 (1938).

²⁴⁸ M. C. Fishler, C. Entenman, M. L. Montgomery, and I. L. Chaikoff, *J. Biol. Chem.*, **150**, 47-55 (1953).

²⁴⁹ H. B. Jones, I. L. Chaikoff, and J. H. Lawrence, *J. Biol. Chem.*, **128**, 631-644 (1939); **133**, 319-327 (1940).

lipid synthesis likewise occurred in rats deprived of kidneys and gastrointestinal tract but with intact livers.²⁵⁰

When the rate of turnover of phospholipids in the liver was determined by the procedure of Hevesy and Hahn,²³⁷ it was calculated that 19% of the total phospholipid in the liver was renewed within four hours. According to the data of Chargaff, alone and with co-workers,^{251,252} the rate of lecithin formation in the liver exceeds that of cephalin. Although similar results were obtained in experiments in which the time interval exceeded one day, Hevesy and Hahn²³⁷ noted that the cephalin turnover occurred more rapidly in the four-hour tests. Hunter²⁵³ reported that sphingomyelin accounted for only a small fraction of the new liver phospholipid formed, as determined by the use of P³².

The administration of choline was found by Entenman and co-workers²⁵⁴ to increase the rate of synthesis and turnover of the choline-containing phospholipids in the liver of the dog; on the other hand, the specific activity of the non-choline-containing phospholipids was not increased, but was actually depressed. According to the report of Stekol *et al.*,²⁴⁴ differences can be noted in the utilization of the methyl group from methionine and from choline for phospholipid synthesis. After methionine-C¹⁴H₃ was injected, the period of decreasing choline-C¹⁴ content in the liver was accompanied by a simultaneous increase in carcass choline-C¹⁴H₃ up to 240 hours. When choline-C¹⁴H₃ itself was injected, the gradual decrease of choline-C¹⁴ in the liver was not associated with a concomitant increase in tissue choline. However, the level of choline reached in the carcass following the injection of choline was considerably higher than that after methionine had been administered. The authors suggest that, although there is no doubt about the chemical identity of the choline-C¹⁴ in the phospholipids formed after the administration of choline or methionine, there is a possibility of the nonidentity of the phospholipid molecules into which the intact choline-C¹⁴ or the choline synthesized from methionine-C¹⁴ enter.

Fishler and associates²⁵⁵ were the first to demonstrate the *in vitro* formation of phospholipids in liver slices, a finding which was rendered possible

²⁵⁰ B. A. Fries, S. Ruben, I. Perlman, and I. L. Chaikoff, *J. Biol. Chem.*, **123**, 587-593 (1938).

²⁵¹ E. Chargaff, *J. Biol. Chem.*, **128**, 587-595 (1939).

²⁵² E. Chargaff, K. B. Olson, and P. F. Partington, *J. Biol. Chem.*, **134**, 505-514 (1940).

²⁵³ F. E. Hunter, *Proc. Soc. Exptl. Biol. Med.*, **46**, 281-282 (1941).

²⁵⁴ C. Entenman, I. L. Chaikoff, and H. D. Friedlander, *J. Biol. Chem.*, **162**, 111-118 (1946).

²⁵⁵ M. C. Fishler, A. Taurog, I. Perlman, and I. L. Chaikoff, *J. Biol. Chem.*, **141**, 809-818 (1941).

by the use of radiophosphate in the medium. Simultaneously with the formation of new phospholipid, catabolism of the phospholipid formed was shown to occur. The net result was a decline in total liver phospholipid, while phospholipid P^{32} was being laid down. Taurog *et al.*²⁵⁶ reported that oxygen was required for the maintenance of the synthetic reaction, while it was inhibited to the extent of 90% when cyanide or hydrogen sulphide was present, and under anaerobic conditions. The state of tissue organization was also found to be important in the *in vitro* synthesis of phospholipids; the ability to effect phospholipid synthesis was completely lost in liver homogenates.²⁵⁵ On the other hand, Ackerman and co-workers²⁵⁷ were unable to demonstrate the synthesis of phospholipid in rat liver slices, incubated with L-methionine and 2-dimethylaminoethanol, unless the activity of choline oxidase was inhibited with KCN. Under the latter condition, a net synthesis of 0.2 to 0.6 mg. of choline per g. of liver could be observed. In the absence of an inhibitor, choline was oxidized to betaine before it could be incorporated into the phospholipid molecule.

b. Intestine. The intestine offers a second site for the synthesis of phospholipids. Thus, it has been demonstrated that phospholipid containing P^{32} is rapidly deposited in the gastrointestinal tract after the administration of phosphate containing isotopic P.^{237, 245-247, 250, 251} Fries *et al.*²⁵⁰ reported that the small intestine is much more active in effecting phosphorylation than is the stomach or the large intestine. Similar variations in phospholipid synthesis have been noted in different parts of the gastrointestinal tract of birds. The greatest rate of synthesis was noted in the small intestine; this level exceeded that in the gizzard, proventriculus, cecum, and colon.²⁴⁷ When the P^{32} was given orally, the specific activity of phospholipid P^{32} in the intestine was shown to exceed that in the liver.²⁴⁵ Proof that the route of administration is concerned with the high activity noted in the intestine is furnished by the fact that a higher value for intestine phospholipid P^{32} was noted when the labeled inorganic phosphate was given orally than when it was administered subcutaneously.²⁵⁰ In harmony with the results on the liver, the incorporation of P^{32} occurs more rapidly in the cephalin fraction than in that of lecithin during the first four hours;²³⁷ however, at longer intervals, the situation is reversed.²⁵¹

c. Kidney. Considerable evidence exists that the kidney is likewise able to effect a synthesis of phospholipid. However, Artom *et al.*²³³ and Perlman and associates²⁴⁶ are in agreement that the rate of phospholipid synthesis is slower in the kidney than in the small intestine or liver. More-

²⁵⁶ A. Taurog, I. L. Chaikoff, and I. Perlman, *J. Biol. Chem.*, **145**, 281-291 (1942).

²⁵⁷ C. J. Ackerman, M. J. Burns, and W. D. Salmon, *Federation Proc.*, **12**, 165 (1953).

over, Artom *et al.*^{238,245,258} reported that the specific activity of kidney phospholipid is lower than that of the liver and small intestine during the early period following the administration of P^{32} . On the other hand, Fishler and associates, in unpublished observations cited by Chaikoff,²²⁴ reported that the deposition of P^{32} in the kidney phospholipids of the dog was less than that in the liver, but more than the amount in the small intestine at six hours. After ninety-eight hours, the concentrations in all these tissues were essentially the same.

The ability of the kidney to effect the synthesis locally rather than merely to withdraw it from the blood has been amply proved. Thus, the kidney of the hepatectomized dog was found by Fishler *et al.*²⁴⁸ to contain newly formed phospholipid, despite the fact that none was present in the plasma; the quantities of radiophospholipid were similar in the kidneys of normal and of liverless dogs.

However, the most cogent argument for the thesis that the kidney cells themselves possess the ability to synthesize phospholipid is the fact that kidney slices, like liver slices, can incorporate labeled phosphate into the phospholipid in the presence of oxygen, although this capacity is reduced to 10% of the normal value when the kidney slices are kept under nitrogen. However, unlike liver homogenates,²⁵⁵ those prepared from kidney are still able to form new phospholipid.²⁴⁸

d. Muscle. The rate of synthesis of phospholipids in skeletal muscle, as determined in experiments in which P^{32} was used as a tracer, is slower than that exhibited by small intestine, liver, or kidney tissue.^{237,247,249,259} The slow rate of synthesis of phospholipid under these conditions may be related to the rate of entrance of inorganic phosphorus into the muscle cell.^{237,260,261} Entenman and co-workers²⁴⁷ reported that the phospholipid P^{32} content of leg and breast muscle of the bird was the same six and twelve hours after the injection of P^{32} , but that cardiac muscle incorporated more P^{32} than did other types of muscle. Bloor and Snider²⁶² noted that the phospholipid content of muscle paralleled the activity of the muscle, but the reason for this is unexplained. Artom²⁶³ and Friedlander *et al.*²⁶⁴ observed that administered P^{32} is deposited in muscle phospholipids at an in-

²⁵⁸ C. Artom, G. Sarzana, C. Perrier, M. Santangelo, and E. Segrè, *Nature*, **139**, 836-837 (1937).

²⁵⁹ G. Hevesy, *Ann. Rev. Biochem.*, **9**, 641-662 (1940).

²⁶⁰ J. F. Manery and W. F. Bale, *Am. J. Physiol.*, **132**, 215-231 (1941).

²⁶¹ G. Hevesy and O. Rebbe, *Acta Physiol. Scand.*, **1**, 171-182 (1940).

²⁶² W. R. Bloor and R. H. Snider, *J. Biol. Chem.*, **107**, 459-470 (1934).

²⁶³ C. Artom, *J. Biol. Chem.*, **139**, 953-961 (1941).

²⁶⁴ H. D. Friedlander, I. Perlman, and I. L. Chaikoff, *Am. J. Physiol.*, **132**, 24-31 (1941).

creased rate. In some cases, the rate of synthesis was doubled in the denervated muscle, as contrasted with the corresponding contralateral intact muscle. Although a decrease in phospholipid content has been observed in inanition²⁶⁵ and in muscular atrophy and muscular dystrophy,²⁶⁶ the changes reported for denervated muscles occurred before any atrophy had occurred. Hevesy and Smedley-MacLean²⁶⁷ noted an increased turnover rate for phospholipids in the muscles of rats maintained on a fat-deficient diet.

e. Brain. The rate of synthesis of phospholipids would appear to be slowest in the intact brain. Before radioisotope techniques were available, McConnell and Sinclair²⁶⁸ reported that brain incorporates elaidic acid into the lecithins and cephalins at a slower rate than do muscle and liver. With the advent of P^{32} as an indicator of phospholipid synthesis, many investigations, including those of Hevesy and Hahn,^{237,269} Artom *et al.*,²⁴⁵ and the Chaikoff group^{246,250,270,271} have confirmed the earlier opinion as to the sluggishness of the synthesis of phospholipids in the brain. Some workers^{237,260,271-273} attributed this retarded phospholipid synthesis in brain to a slow penetration of the labeled phosphate into the brain and into its extracellular spaces. In the experiments of Changus, Chaikoff, and Ruben,²⁷⁰ incorporation of P^{32} into brain phospholipids occurred at a progressive rate for as long as 200 hours following the administration of the isotopic phosphate. After the maximum content of P^{32} phospholipid was attained, it was retained for a prolonged period; as long as 800 hours after the administration of P^{32} the brain still contained 70% of the amount which had been noted at the 200-hour interval.²⁷⁰ Cephalin and sphingomyelin were found to be more readily replaced than was lecithin.^{237,252}

The ability of the brain to synthesize phospholipids is dependent upon age. Thus, the maximum incorporation of P^{32} in the whole brain phospholipids of the rat was shown by Fries *et al.*^{271,274} to occur between birth and the age at which the animal attained a weight of 50 g. This is also the period of maximum myelinization of the brain.²⁷⁵ In their later work,

²⁶⁵ C. Ciaccio, *Arch. farmacol. sper.*, **24**, 231-254 (1917).

²⁶⁶ W. R. Bloor, *J. Biol. Chem.*, **119**, 451-465 (1937).

²⁶⁷ G. C. Hevesy and I. Smedley-MacLean, *Biochem. J.*, **34**, 903-905 (1940).

²⁶⁸ K. P. McConnell and R. G. Sinclair, *J. Biol. Chem.*, **118**, 131-136 (1937).

²⁶⁹ L. Hahn and G. Hevesy, *Skand. Arch. Physiol.*, **77**, 148-157 (1937).

²⁷⁰ G. W. Changus, I. L. Chaikoff, and S. Ruben, *J. Biol. Chem.*, **126**, 493-500 (1938).

²⁷¹ B. A. Fries and I. L. Chaikoff, *J. Biol. Chem.*, **141**, 479-485 (1941).

²⁷² W. E. Cohn and D. M. Greenberg, *J. Biol. Chem.*, **123**, 185-198 (1938).

²⁷³ H. B. Jones, I. L. Chaikoff, and J. H. Lawrence, *Am. J. Cancer*, **40**, 243-250 (1940).

²⁷⁴ B. A. Fries, C. Entenman, G. W. Changus, and I. L. Chaikoff, *J. Biol. Chem.*, **137**, 303-310 (1941).

²⁷⁵ J. B. Watson, *Animal Education*, Univ. Chicago Press, Chicago, 1903.

Fries and Chaikoff²⁷¹ reported that the highest P^{32} recovery in all divisions of the central nervous system was on the day of birth. However, the age at which the maximum turnover rate of the brain phospholipids was observed varied in the different parts of the central nervous system.²⁷⁶ During the period from birth until the rat reached the weight of 50 g. phospholipid activity was highest in the spinal cord, after which the relative activity of the forebrain, cerebellum, and medulla increased steadily. By the time the animals had attained weights of 200 or 300 g., the phospholipid turnover in these tissues sometimes exceeded that in the cord.^{271,276}

The synthesis of phospholipid in the brain is influenced by the degree of activity. Thus, Torda²⁷⁷ reported that convulsive agents, such as pentylenetetrazole, physostigmine salicylate, or electro-shock, significantly reduced the specific activity of brain phospholipids. On the other hand, the administration of adrenocorticotropin (ACTH) restored the synthetic capacity of the brain to form phospholipids. This indicates that the phospholipid turnover is influenced by hormones.

The earlier observations on the intact animal do not indicate whether or not brain tissue possesses the capacity to synthesize phospholipids *per se*. The answer to this problem has been arrived at by the use of techniques similar to those employed to prove this point in the case of other tissues. Thus, on the basis of *in vitro* tests, Fries and co-workers²⁷⁸ showed that surviving brain slices were able to incorporate P^{32} into phospholipids when the slices were immersed in a Ringer-bicarbonate solution containing radioactive phosphate. Brain homogenates were able to synthesize phospholipids, although the rate was slower than in the organized tissues such as slices. Dawson²⁷⁹ found that the rate of synthesis of phospholipids in the brain homogenates from guinea pigs was dependent upon the extent of oxidative phosphorylation, and was controlled by it. Moreover, the phospholipids synthesized were confined to certain components of the cephalin fraction (phosphatidylserine and diphosphoinositide), while practically no new formation of lecithins, sphingomyelin, or phosphatidylethanolamine could be observed.

In contradistinction to the activity of homogenates of the brain in effecting syntheses,²⁸⁰ those from the liver are entirely inactive in the synthesis of phospholipids, although the slices of the latter organ are very effective in promoting this reaction.²⁵⁵ The rate of phospholipid synthesis

²⁷⁶ B. A. Fries, G. W. Changus, and I. L. Chaikoff, *J. Biol. Chem.*, **132**, 23-34 (1940).

²⁷⁷ C. Torda, *Federation Proc.*, **12**, 145 (1953).

²⁷⁸ B. A. Fries, H. Schachner, and I. L. Chaikoff, *J. Biol. Chem.*, **144**, 59-66 (1942).

²⁷⁹ R. M. C. Dawson, *Biochem. J.*, **53**, viii (1953).

²⁸⁰ H. Schachner, B. A. Fries, and I. L. Chaikoff, *J. Biol. Chem.*, **146**, 95-103 (1942).

in the brain is accelerated if glucose, mannose, galactose or fructose is present under aerobic conditions.²⁸⁰ Peripheral nerves such as the sciatic nerve of the dog are also able to convert phosphate to phospholipids.²⁷⁸

f. Blood Plasma and Cells. According to Hevesy and collaborators,^{230,274} the newly formed phospholipid present in plasma after the administration of P^{32} is actually formed in the liver and possibly in other organs. According to an unpublished study of Fishler referred to by Chaikoff,²²⁴ the liver was found to be the tissue which first assumed the highest level of phospholipid- P^{32} following the administration of the isotope. However, ninety-eight hours thereafter practically identical values were noted for liver and plasma. Moreover, when P^{32} was injected intravenously immediately after hepatectomy, no phospholipid- P^{32} could be recovered from the plasma as late as six hours after the operation, although the kidney and small intestine presented values for newly formed phospholipid at the same level as that in unoperated dogs. These data strongly suggest that the liver is the sole site for the synthesis of plasma phospholipids, although they do not absolutely exclude the possibility that other organs may serve to a limited extent in this capacity. Entenman *et al.*²⁵⁴ also interpreted their data on the effect of ingested choline on the synthesis of choline-containing phospholipids in the liver as evidence for the fact that plasma phospholipids are formed in this organ.

The incorporation of P^{32} into blood cell phospholipids has been shown by Fishler (cited by Chaikoff²²⁴) and by others^{237,281} to be much slower than into plasma. Fishler noted that, whereas it required thirty-six hours to reach a maximum P^{32} incorporation into the phospholipids of plasma, forty-eight hours elapsed before measurable amounts of P^{32} were present in the cells; from 200 to 300 hours were required before maximum levels of radiophospholipids were obtained in the cells. The highest level of phospholipid- P^{32} present in the formed elements of the blood never exceeds 25% of the amount found in the plasma. Hevesy and Aten²⁸¹ reported that, in the case of the laying bird, the renewal of the corpuscle phospholipid was only one-third of that in the plasma. The specific activity of phospholipid phosphorus in the blood cells of man²⁸¹ was shown to reach a level of 50% of that in the plasma eight days after the injection of P^{32} .

g. Fetus. According to Popják,^{282,283} phospholipids can readily be synthesized in the liver of the fetus. It was found that the specific activi-

²⁸¹ G. Hevesy and A. H. W. Aten, Jr., *Kgl. Danske Videnskab. Selskab, Biol. Medd.* 14, No. 5, 1-38 (1939).

²⁸² G. Popják, *Nature*, 160, 841-842 (1947).

²⁸³ G. Popják, *Biochem. J.*, 42, xi (1948).

ties of phospholipid- P^{32} extracted from fetal livers and from the placentae of pregnant rats, rabbits, and guinea pigs, two to six hours after the administration of Na_2HPO_4 labeled with P^{32} , was higher in all cases than it was in the maternal plasma. It is reasoned that, if the fetal phospholipids had been derived from the performed maternal substances by placental transmission only, the specific activity of the fetal phospholipids should not exceed that of the maternal plasma phospholipids. These data can only be interpreted to mean that the labeled phospholipids present in the fetal liver were synthesized *in situ*. They are also in harmony with the report of Gortner²⁸⁴ that the highest phospholipid content is to be found in the very young pig fetus.

On the other hand, the proportion of newly synthesized phospholipids extracted from tissues other than liver and placenta was less than in the corresponding maternal tissues. This may be related to the poor penetration of the labeled Na_2HPO_4 . When labeled phosphate was injected directly into the uterus, all the tissues contained newly synthesized phospholipids, while the maternal blood contained no labeled phospholipids. The rate of phospholipid turnover is higher in the fetal liver and placenta than in the maternal liver, which is the most active site of phospholipid synthesis in the mother. The rate of turnover is slower in the other fetal tissues, but it is equal to that in the maternal liver.

h. Egg. It has long been recognized that the rate of turnover of phospholipid is much increased in the laying bird. Thus, Entenman *et al.*²⁴⁷ found that the rate of synthesis of phospholipids in the blood and oviduct was markedly augmented in the hen parallel with egg production. Phospholipid- P^{32} was shown to be deposited in the egg yolk at an early period after the feeding of isotopic phosphorus.²⁴⁷ Hevesy and Hahn²⁸⁵ are of the opinion that the bulk of the yolk phospholipids are obtained from the plasma, which in turn obtains them from their primary site of synthesis, namely from the liver. However, the rate of synthesis of "free" lecithin and cephalin in egg yolk is the same as that of the "combined" phospholipids in the vitellin fraction.²⁸⁶

i. Bone. Claassen, Vogels and Wöstmann²⁸⁷ demonstrated that the femur and tibia of the white rat are able to synthesize phospholipids. This was proved by the fact that the specific activity due to P^{32} was higher in the bone marrow than it was in the blood plasma. Moreover,

²⁸⁴ W. A. Gortner, *J. Biol. Chem.*, **159**, 135-143 (1945).

²⁸⁵ G. Hevesy and L. Hahn, *Kgl. Danske Videnskab. Selskab, Biol. Medd.*, **14**, No. 2, 1-39 (1938).

²⁸⁶ E. Chargaff, *J. Biol. Chem.*, **142**, 505-512 (1942).

²⁸⁷ V. Claassen, R. J. Vogels, and B. Wöstmann, *Nature*, **169**, 191-192 (1952).

the fact that bone marrow is capable of synthesizing phospholipids, as demonstrated by *in vitro* tests as well as by *in vivo* experiments, is convincing evidence of the independent synthesis in the bone marrow.

j. Neoplastic Tissue. The rate at which phospholipid formation occurs in malignant tissue has been shown to be high, resembling that in normal liver, kidney, or small intestine.^{249,259,288} However, in contradistinction to the behavior of tissues from normal animals, the maximal deposition of P^{32} in phospholipid may last as long as forty to fifty hours. The turnover rates of lecithin in rat carcinosarcoma 256 were reported to be somewhat higher than those of cephalin.²⁸⁸ Moreover, the rate of incorporation of P^{32} into phospholipids varies with the type of tumor. Thus, Jones *et al.*^{249,273} reported that lymphosarcomas and mammary carcinomas are much more active than is sarcoma 180 or lymphoma.

(3) Factors Affecting the Biosynthesis of Phospholipids

In addition to the factors already discussed, such as tissue and age, a number of more general conditions are concerned with the rate of synthesis of phospholipids.

a. The Effect of Diet. The presence of fat in the gastrointestinal tract has been shown to increase phospholipid synthesis in the small intestine.²⁴⁵ Moreover, an increased phospholipid turnover was found to be associated with the action of choline in sweeping out fat from the liver of rats on a high-fat diet.²⁸⁹ However, in the case of the liver, the ratio of phospholipid P to N was found to be constant irrespective of the protein or fat content of the diet, or whether or not the liver was normal, fatty, or fibrotic.²⁹⁰ The same constancy in the ratio of phospholipid to nucleoprotein synthesized in the liver was also shown by Cornatzer *et al.*²⁹¹ to exist under a wide variety of conditions. However, Campbell and Kosterlitz²⁹² found that, whereas the desoxyribonucleic acid content of the liver, and its turnover in rats, are not affected by variations in the protein, fat, or choline content of the diet, the phospholipid P content is determined mainly by protein intake, to a small extent by the dietary fat, and not to any degree by the choline in the diet. The ratio of phospholipid P:protein N was found to be constant over a wide range of protein intake. The only situ-

²⁸⁸ F. L. Haven, *J. Nat. Cancer Inst.*, **1**, 205-209 (1940).

²⁸⁹ I. Perlman and I. L. Chaikoff, *J. Biol. Chem.*, **127**, 211-220 (1939).

²⁹⁰ W. E. Cornatzer, J. P. Davison, and D. G. Gallo, *Federation Proc.*, **12**, 191-192 (1953).

²⁹¹ W. E. Cornatzer, D. G. Gallo, and J. P. Davison, *XIX Intern. Physiol. Congr.*, Montreal, Aug.-Sept., 1953, *Abst.*, 282-283.

²⁹² R. M. Campbell and H. W. Kosterlitz, *Biochim. et Biophys. Acta*, **8**, 664-679 (1952).

ation in which a constancy in synthesis between phospholipid P and nucleoprotein P was absent was in the case of rats which were receiving a 25% casein diet supplemented with 1% of thiourea.²⁹¹

b. The Effect of Ingested Cholesterol. It has long been known that an antagonism exists between cholesterol and phospholipid metabolism. Thus, when fatty livers are produced by cholesterol,²⁹³⁻²⁹⁷ the excess fat deposition in the liver can be prevented by choline.^{296,297} Perlman and Chaikoff²⁸⁹ found that an inverse relation exists between the rate of fat deposition in the liver and the speed of phospholipid exchange, following the administration of choline. In a later study, these workers²⁹⁸ found that cholesterol depressed the phospholipid exchange in rat livers. The effect was observed as early as thirty hours after the feeding of cholesterol was initiated. Choline stimulated the rate of phospholipid turnover in cholesterol-fed rats. In fact, the acceleration of phospholipid production induced by choline was more pronounced in the cholesterol-fed rat than in the fat-fed animal. These results were confirmed by Clément and co-workers.²⁹⁹ When rats received a high cholesterol diet for ten months, a significant decrease in turnover occurred. When they were again given a normal diet, fatty livers disappeared, but the phospholipid turnover did not return to normal.

c. The Effect of the Adrenal Glands. Verzář and Laszt³⁰⁰ suggested that the reason why adrenalectomy interferes with fat absorption is because the adrenal glands are concerned with phosphorylation. However, most investigations have failed to support this hypothesis. Thus, Stillman, as cited by Chaikoff,²²⁴ found no difference in the rate at which P³² was incorporated into the phospholipids of the liver and small intestine of rats, irrespective of whether or not both adrenal glands had been excised. Moreover, Barnes and collaborators^{301,302} showed that the rate at which tagged fatty acids were incorporated into phospholipids was not influenced by adrenalectomy. These results indicate that the synthesis of phospholipids occurs normally after the removal of the adrenal glands, not only as

²⁹³ R. Schönheimer and D. Yuasa, *Z. physiol. Chem.*, **180**, 5-16 (1929).

²⁹⁴ R. Okey, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1003-1005 (1933).

²⁹⁵ W. M. Sperry and V. A. Stoyanoff, *J. Nutrition*, **9**, 131-155 (1935).

²⁹⁶ C. H. Best and J. H. Ridout, *J. Physiol.*, **78**, 415-418 (1933).

²⁹⁷ C. H. Best, H. J. Channon, and J. H. Ridout, *J. Physiol.*, **81**, 409-421 (1934).

²⁹⁸ I. Perlman and I. L. Chaikoff, *J. Biol. Chem.*, **128**, 735-743 (1939).

²⁹⁹ G. Clément, E. LeBreton, M. Pascaud, and M. Tubiana, *Compt. rend.*, **236**, 412-415 (1953).

³⁰⁰ F. Verzář and L. Laszt, *Biochem. Z.*, **276**, 11-16 (1935); **278**, 396-400 (1935).

³⁰¹ R. H. Barnes, E. S. Miller, and G. O. Burr, *J. Biol. Chem.*, **140**, 241-246 (1941).

³⁰² R. H. Barnes, E. S. Miller, and G. O. Burr, *J. Biol. Chem.*, **140**, 247-253 (1941).

regards the incorporation of phosphate (which involves the phosphate-glycerol and the phosphate-choline bonds), but also in the incorporation of fatty acids into the molecule (glycerol-fatty acid bonds). The importance of ACTH in phospholipid synthesis in brain was noted by Torda.²⁷⁷

d. The Effect of Phlorhizin. Since the classical demonstration by von Mering³⁰³ that the injection of the glycoside, phlorhizin, into a normal animal is followed almost immediately by a profuse glycosuria, it has repeatedly been demonstrated that its action is exclusively renal. Thus, Deuel *et al.*³⁰⁴ and Nash³⁰⁵ reported that this drug had no direct effect upon the carbohydrate metabolism of nephrectomized dogs.

The renal effect is now known to result from the inability of the glucose to be reabsorbed in the kidney tubules. The absence of reabsorption has been attributed to the fact that phosphorylation is required to bring about absorption, and that phlorhizin prevents this.³⁰⁶ However, both Lundsgaard³⁰⁷ and Lambrechts³⁰⁸ reported that the concentration of phlorhizin necessary to produce glycosuria is much less than that required to poison phosphatases.

On the other hand, Wilbrandt and Laszt³⁰⁹ reported that iodoacetate, which is known to interfere with phosphorylation, also inhibits the absorption of glucose. Moreover, Beck³¹⁰ was able to confirm these findings in regard to phosphorylation; he used phlorhizin, which is much less toxic than is the iodoacetate. When animals received phlorhizin, absorption of glucose from the gastrointestinal tract was decreased. Moreover, this investigator demonstrated that the accumulation of hexose-phosphate in the intestinal mucosa of rats which had received phlorhizin was not comparable to that observed in the normal animals. Thus, if phlorhizin inhibits phosphorylation of sugars, it would be expected that it might retard the synthesis of phospholipids in a similar manner.

However, Weissberger³¹¹ did not observe any inhibition in the rate at which P³² was incorporated into phospholipids in the liver, kidney, or small

³⁰³ J. von Mering, *Verhandl. Kong. inn. Med.*, 5, Wiesbaden, 185-189 (April, 1886).

³⁰⁴ H. J. Deuel, Jr., H. E. C. Wilson, and A. T. Milhorat, *J. Biol. Chem.*, 74, 265-298 (1927).

³⁰⁵ T. P. Nash, Jr., *J. Biol. Chem.*, 83, 139-155 (1929).

³⁰⁶ J. P. Peters and D. D. Van Slyke, *Quantitative Clinical Chemistry*, 2nd ed., vol. I, Williams & Wilkins, Baltimore, 1946, p. 256.

³⁰⁷ E. Lundsgaard, *Biochem. Z.*, 264, 209-220; 221-227 (1933); *Skand. Arch. Physiol.*, 72, 265-270 (1935).

³⁰⁸ A. Lambrechts, *Compt. rend. soc. biol.*, 122, 468-471 (1936).

³⁰⁹ W. Wilbrandt and L. Laszt, *Biochem. Z.*, 250, 398-417 (1933).

³¹⁰ L. V. Beck, *J. Biol. Chem.*, 143, 403-415 (1942).

³¹¹ L. H. Weissberger *J. Biol. Chem.*, 139, 543-550 (1941).

intestine of the intact rat after phlorhizin had been injected in a single 65 mg. dose. This finding was confirmed by Stillman and Fries (cited by Chaikoff²²⁴), who used much larger doses of the drug. Moreover, DiLuzio and Zilversmit³¹² found no change in the liver phospholipid of the phlorhizinized dog, in spite of the fact that the level of plasma phospholipids was elevated. The turnover of phospholipids in the liver and plasma was increased over that of the controls. These observations support the conclusion that phlorhizin does not inhibit the incorporation of phosphate into the phospholipid molecule in the intact animal. On the other hand, according to Taurog (cited by Chaikoff²²⁴), phlorhizin reduced the extent of incorporation of P³² into the phospholipids of surviving liver slices.

e. The Effect of Pancreatectomy. After the removal of the pancreas, a marked stimulation of the rate of phospholipid synthesis obtains in the plasma. Harper and co-workers³¹³ observed an increase in the rate of turnover of plasma phospholipids in diabetic dogs after the feeding of raw pancreas. However, Chaikoff *et al.*³¹⁴ reported that no change in the rate of phospholipid turnover in depancreatized dogs occurred after insulin had been withdrawn, provided that the level of blood phospholipid remained constant. In later work, Zilversmit and DiLuzio³¹⁵ noted a marked increase in phospholipid synthesis in the liver and plasma of depancreatized dogs; slightly smaller increases of phospholipid synthesis were observed in the kidney and small intestine. The variation in rate of synthesis of phospholipid was only slight, or absent, in the muscle of diabetic dogs while, in the brain, the new formation of phospholipids proceeded at an identical rate in normal and in depancreatized dogs.³¹⁵ These results are interpreted as indicating that phospholipids are involved in the increased fat metabolism in the diabetic dog.

f. The Effect of Lipotropic Agents and of Related Compounds. The ability of certain compounds to prevent the accumulation of fat in the liver is believed to be related to their stimulating effect upon phospholipid synthesis. Substances which increase the total amount of available choline are especially active in augmenting the rate of synthesis of phospholipids. Thus, it has been shown that choline itself,²⁸⁹ betaine,³¹⁶ and methionine,³¹⁷ all of which are potent lipotropic agents, stimulate phos-

³¹² N. R. DiLuzio and D. B. Zilversmit, *Am. J. Physiol.*, **170**, 472-476 (1952).

³¹³ P. V. Harper, Jr., W. B. Neal, Jr., and G. R. Rogers, *Proc. Soc. Exptl. Biol. Med.*, **68**, 363-369 (1948).

³¹⁴ I. L. Chaikoff, D. B. Zilversmit, and C. Entenman, *Proc. Soc. Exptl. Biol. Med.*, **68**, 6-9 (1948).

³¹⁵ D. B. Zilversmit and N. T. DiLuzio, *J. Biol. Chem.*, **194**, 673-683 (1952).

³¹⁶ I. Perlman and I. L. Chaikoff, *J. Biol. Chem.*, **130**, 593-600 (1939).

³¹⁷ I. Perlman, N. Stillman, and I. L. Chaikoff, *J. Biol. Chem.*, **133**, 651-659 (1940).

pholipid synthesis as measured by the incorporation of P^{32} into the phospholipid molecules. Their effectiveness is in proportion to the quantity of material administered,^{289,316} and to the relative lipotropic activities of the substances in question. Thus, betaine is less effective in preventing fat accumulation in the liver than is choline.^{243,318} In doses up to 50 mg. per rat, its capacity to stimulate phospholipid synthesis is also less than that of choline.³¹⁶ Artom and Cornatzer³¹⁹ reported that choline, administered alone, exerted less stimulatory effect upon the formation of intestinal phospholipids than did choline and fat given simultaneously.

The administered choline apparently speeds up phospholipid synthesis by being available as one of the essential building stones. Stetten²³⁹ proved that labeled choline finds its way into the phospholipid molecule, while Welch and Welch^{242,243} reported that arsenocholine is incorporated into the lecithin molecule to yield arsenolecithin.

On the other hand, Artom and Swanson³²⁰ reported somewhat contradictory experimental results on the stimulatory effect of choline on phospholipid synthesis. Although this base did, to a certain extent, increase the rate of synthesis of phospholipids in liver slices from rats previously maintained on low-protein diets, choline actually inhibited phospholipid synthesis in liver slices from rats previously on a high-protein regimen. In the absence of added choline, the rate of phospholipid synthesis as measured by P^{32} uptake was lower in liver slices from rats which had received a stock diet or one containing 25% of casein than in livers from animals which had received a high-protein regimen. Since oxidation is required for the incorporation of inorganic phosphate into liver slices, it is suggested that the depressed rate of phosphate incorporation in liver slices from rats on protein-low diets is related to a decrease in cytoplasmic proteins which reduces the proportion of oxidative enzymes.

The increased phospholipid synthesis was observed in many cases, even though the total phospholipid in the liver showed no measurable change. Since the phospholipid content of the liver is relatively uniform, the stimulatory effect of choline on phospholipid synthesis can be observed only when labeled compounds are employed.²⁹⁸

Perlman and collaborators^{317,321} made the interesting observation that, not only did methionine stimulate phosphorylation, but cystine and cysteine brought about the same reaction. Of these three sulfur-containing

³¹⁸ A. P. Platt, *Biochem. J.*, **33**, 505-511 (1939).

³¹⁹ C. Artom and W. E. Cornatzer, *J. Biol. Chem.*, **165**, 393-394 (1946).

³²⁰ C. Artom and M. A. Swanson, *J. Biol. Chem.*, **193**, 473-480 (1951).

³²¹ I. Perlman, N. Stillman, and I. L. Chaikoff, *J. Biol. Chem.*, **135**, 359-364 (1940).

amino acids, only methionine is lipotropic. On the other hand, such amino acids as glycine, alanine, serine, tyrosine, proline, glutamic acid, and asparagine, did not augment phospholipid turnover as measured by the incorporation of P^{32} into these molecules.³²¹ Moreover, taurine, creatine, di-(β -hydroxyethyl) sulfoxide, and sarcosine were entirely inactive in stimulating phospholipid synthesis.³²¹

Ethanolamine was found by Stetten²⁴⁰ to stimulate phospholipid synthesis in adult rats, although aminoethylphosphate was reported by Chargaff and Keston³²² to be less effective than labeled inorganic phosphate in effecting this synthesis. Artom and Cornatzer³²³ observed that choline stimulated the synthesis of choline phospholipids only. However, ethanolamine, methylethanolamine, and dimethylethylethanolamine, which also increased phospholipid synthesis, produced this effect on both the choline-containing and the non-choline-containing phospholipid fractions. According to a later report by these workers,³²⁴ other agents were found to stimulate phospholipid synthesis; these included diethanolamine ($(HOCH_2 \cdot CH_2)_2NH$), triethanolamine ($(HOCH_2 \cdot CH_2)_3N$), ethylethanolamine ($HOCH_2 \cdot CH_2NHC_2H_5$), diethylethanolamine ($HOCH_2 \cdot CH_2N(C_2H_5)_2$), triethylcholine ($(C_2H_5)_3NCH_2 \cdot CH_2OH$), and ethylamine ($C_2H_5NH_2$). The opinion is expressed that the stimulating action of lipid phosphorylation in the liver requires a lower degree of structural specificity than do other biological reactions mediated by the same substance. On the other hand, Morton and Schwartz³²⁵ reported that the thyrotropic hormone of the pituitary (TSH) causes an increase in thyroid lipids by 184%, as determined by *in vitro* studies, whereas there was no evidence of any increase in P^{32} in the kidney or liver lipids.

(4) *The Biosynthesis of the Nitrogenous Bases of Phospholipids*

Although the component fatty acids of phospholipids, as well as the glycerol and phosphoric acid, ordinarily pose no difficulty in the biosynthesis of the several phospholipids, since they usually occur in the tissues in adequate amounts, the absence or low concentration of the nitrogen-containing component at the site of synthesis may under some conditions be a limiting factor in this new formation of phospholipids.

a. Ethanolamine. Ethanolamine, $HOCH_2 \cdot CH_2NH_2$, which is a necessary component of the phosphatidylethanolamine molecule, is also an

³²² E. Chargaff and A. S. Keston, *J. Biol. Chem.*, **134**, 515-522 (1940).

³²³ C. Artom and W. E. Cornatzer, *J. Biol. Chem.*, **176**, 949-959 (1948).

³²⁴ W. E. Cornatzer and C. Artom, *J. Biol. Chem.*, **178**, 775-787 (1949).

³²⁵ M. E. Morton and J. R. Schwartz, *Science*, **117**, 103-104 (1953).

ethanolamine and of dimethylethanolamine to choline, D-methionine is considerably more efficient as a methyl donor than is the L- form of this amino acid. Ethanolamine is also methylated by betaine, or by other lipotropic substances, acting as methyl donors. However, the betaine molecule is not converted as a whole to choline.³³⁴

Barrenscheen and Skudrzyk³³⁵ confirmed the fact that ethanolamine and serine are the methyl acceptors, and that a lyo-enzyme, liver pherage, brings about the methylation by methionine. The enzyme system is active in liver homogenate; it was found to be inhibited by ascorbic acid. Methioninesulfoxide ($\text{CH}_3\text{S}:\text{OCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$) was found to be the first intermediary product formed by aerobic action on methionine. Arnstein³³⁶ likewise demonstrated that serine, methanol, sodium formate, and glycine all give rise to choline labeled both in the methyl groups and in the ethanolamine residue. Only carboxyl-labeled glycine failed to transfer the label to choline; this is interpreted as indicating that glycine is converted to ethanolamine *via* serine, under which condition the carboxyl group of glycine is lost. On the other hand, Weinhouse, Millington, and Strassman³³⁷ demonstrated that the methylene carbon of glycine can give rise to the choline methyl group.

Some information as to the intermediates involved in the methylation of ethanolamine can be obtained from the experiments of Horowitz³³⁸ with certain strains of the red bread mold, *Neurospora crassa*, which have lost their capacity for effecting a synthesis of choline. One variant of *Neurospora* was shown to have lost its power to synthesize choline at a stage preceding monomethylaminoethanol ($\text{CH}_3\cdot\text{NH}\cdot\text{CH}_2\cdot\text{CH}_2\text{OH}$), while the block in the second strain followed this compound. These results prove that monomethylaminoethanol is a normal intermediate in the synthesis of choline by *Neurospora*, and presumably by higher species.

Stekol *et al.*³³⁹ reported that the incorporation of the methyl group of methionine and of betaine into tissue choline was reduced in folic acid deficiency. On the other hand, a deficiency of vitamin B₁₂ had no effect on transmethylation. When vitamin B₆ (pyridoxine) was deficient, the incorporation of the methyl group from methionine into choline was reduced,

³³⁴ V. du Vigneaud, S. Simmonds, J. P. Chandler, and M. Cohn, *J. Biol. Chem.*, **165**, 639-648 (1946).

³³⁵ H. K. Barrenscheen and I. Skudrzyk, *Z. physiol. Chem.*, **284**, 228-236 (1949).

³³⁶ H. R. V. Arnstein, *Biochem. J.*, **47**, xviii-xix (1950).

³³⁷ S. Weinhouse, R. H. Millington, and M. Strassman, *J. Am. Chem. Soc.*, **73**, 1421-1423 (1951).

³³⁸ N. H. Horowitz, *J. Biol. Chem.*, **162**, 413-419 (1946).

³³⁹ J. A. Stekol, S. Weiss, P. Smith, and K. Weiss, *J. Biol. Chem.*, **201**, 299-316 (1953).

and the transfer of the β -carbon of serine into both moieties of choline was also curtailed. These results do not necessarily prove that folic acid, vitamin B₁₂, or pyridoxine is involved in the enzymatic systems concerned with transmethylation. It is suggested that a decreased synthesis of the appropriate methyl acceptors may be the causative factor in the decrease in transmethylation.

c. Sphingosine. Sphingosine ($\text{CH}_3(\text{CH}_2)_{12}\cdot\text{CH}\cdot\text{CH}\cdot\text{CHOH}\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2\text{OH}$) is the normal nitrogenous component in sphingomyelin molecules. Zabin and Mead^{340,341} demonstrated that this compound is synthesized in the rat mainly from the acetate molecule. When carboxyl-labeled acetate was administered to weanling rats, the sphingosine isolated from the brain and carcass had the same specific activity as the fatty acids in the sphingosine fraction. These authors believe that, since carbons 1 and 2 contained no C¹⁴, the results rule out a biosynthesis of sphingosine by condensation of a C₁₆ acid with ethanolamine; it is more probable that serine is utilized in this biosynthesis.³⁴⁰

(5) Intermediates in Phospholipid Synthesis

In the synthesis of the phospholipid molecule, Chaikoff²²⁴ suggested several possibilities. Although it is not possible to identify the intermediate compounds in the transformation of inorganic phosphorus to lecithin, glycerophosphate and phosphorylcholine might be expected to act as intermediates; in the case of cephalin, glycerophosphate and phosphoryl-ethanolamine would be the logical intermediates. Chaikoff²²⁴ further stated that it is improbable that a single pathway exists for the conversion of inorganic phosphate to phospholipid. It is suggested that compounds such as glycerophosphate, phosphorylcholine, phosphorylaminoethanol, diglycerides, and triglycerides may all be present; moreover, the sequence in which the several intermediates combine with each other need not be orderly.²²⁴ Chargaff²⁸⁶ has indicated that phosphoproteins may serve as intermediates in phospholipid synthesis.

According to Taurog (cited by Chaikoff²²⁴), glycerophosphate and phosphorylcholine were found to be incorporated into the liver and kidney phospholipids, both *in vitro* as slices and *in vivo* in the intact animal. Zilversmit *et al.*,³⁴² on the basis of data from the same laboratory, later concluded that, of the two metabolites cited above, only glycerophosphoric

³⁴⁰ I. Zabin and J. F. Mead, *Federation Proc.*, **12**, 294-295 (1953).

³⁴¹ I. Zabin and J. F. Mead, *J. Biol. Chem.*, **205**, 271-277 (1953).

³⁴² D. B. Zilversmit, C. Entenman, and I. L. Chaikoff, *J. Biol. Chem.*, **176**, 193-208 (1948).

acid should be considered as a precursor of liver phospholipids. Likewise, Popják and Muir³⁴³ isolated what they consider to be the precursors of lecithin and cephalin from liver; these have been identified mainly as α - and β -glycerophosphates. Moreover, when lecithin or cephalin, labeled with P^{32} , was injected into rabbits, glycerophosphate, which was isolated from the placenta and liver, had the next highest specific activity after the phospholipids. It may be concluded that glycerophosphate is a precursor of phospholipids, and that this reaction is reversible. Chevallier and Schneider³⁴⁴ also postulated that glycerophosphate is an intermediate in phospholipid synthesis. Finally, there is considerable evidence that enzyme systems exist in animal tissues which can catalyze the combination of fatty acids with glycerophosphate to form phosphatidic acids.³⁴⁵⁻³⁴⁷ In fact, Kornberg and Pricer³⁴⁵ reported that L- α -glycerophosphate is esterified by two moles of a higher fatty acid, with a concomitant conversion of two moles of ATP to inorganic pyrophosphate and adenosine-5-phosphate in the presence of a liver enzyme preparation and catalytic amounts of CoA.

There is considerable evidence that phosphorylcholine is not an intermediate in lecithin synthesis. Although Inukai and Nakahara³⁴⁸ isolated phosphorylcholine from normal beef liver, it is possible that the appearance of this ester might be attributed to isolation procedures. Riley,³⁴⁹ on the basis of extensive experiments on rats, was unable to find any evidence that phosphorylcholine is utilized as a unit in phospholipid synthesis; he suggested rather that cephalin, and perhaps lecithin and sphingomyelin, evolve from more labile complex intermediates which are in rapid equilibrium with simple precursors. Kennedy,³⁵⁰ also, has presented data which indicate that phosphorylcholine is not an intermediate in lecithin synthesis. When choline-methyl- C^{14} was incubated with mitochondria derived from rat liver homogenates, the mitochondrial phospholipid rapidly became radioactive. In contradistinction to these results, no significant incorporation of phosphorylcholinemethyl- C^{14} into mitochondrial lecithin occurred. Kennedy³⁵⁰ states that the incorporation of free choline into the lecithin fraction of enzyme granules evidently does not involve phosphorylcholine.

³⁴³ G. Popják and H. Muir, *Biochem. J.*, **45**, xi (1949); **46**, 103-113 (1950).

³⁴⁴ A. Chevallier and R. Schneider, *Compt. rend. soc. biol.*, **146**, 1135-1137 (1952).

³⁴⁵ A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **204**, 345-357 (1953).

³⁴⁶ A. Kennedy, *Federation Proc.*, **13**, 241 (1954).

³⁴⁷ R. M. C. Dawson, *Biochim. et Biophys. Acta*, **14**, 374-379 (1954).

³⁴⁸ F. Inukai and W. Nakahara, *Proc. Imp. Acad. Tokyo*, **11**, 260-261 (1935).

³⁴⁹ R. F. Riley, *J. Biol. Chem.*, **153**, 535-549 (1944).

³⁵⁰ E. P. Kennedy, *J. Am. Chem. Soc.*, **75**, 249-250 (1953).

Phosphorylethanolamine (ethanolamine phosphoric acid), likewise, is believed not to be in the direct pathway in the synthesis of phosphatidylethanolamine (cephalin). Norman and Dawson³⁵¹ found that the rate of incorporation of P³² into ethanolamine phosphoric acid in rat brain is 100 times greater than that into phosphatidylethanolamine. Unless the turnover of the latter fraction is exceedingly heterogeneous, one may assume that the ethanolamine phosphoric acid is formed by a pathway which is independent of phosphatidylethanolamine catabolism.

Another suggestion is that both glycerophosphate and phosphorylcholine are first hydrolyzed into their component units and then reconstructed into a new phospholipid molecule. In fact, Popják and Muir³⁴³ suggest that the synthetic reaction is reversible. However, there are several facts which argue against the hydrolysis and resynthesis theory. In the first place, α -glycerophosphate is hydrolyzed extremely slowly by animal phosphatase.³⁵² Moreover, phosphorylcholine has also been shown by Welch and Welch²⁴³ to be resistant to liver phosphatases. They are thus protected from destruction by the liver.

In another communication, Ansell and Norman³⁵³ postulated that glycerylphosphorylethanolamine (GPE) may be an intermediate in the *in vivo* synthesis of phosphatidylethanolamine in the brain. Ducet³⁵⁴ likewise suggested that glycerylphosphorylcholine (GPC) may be intermediate in the synthesis of phosphatidylcholine in plants; he based this assumption upon the widespread distribution of GPC in the plant kingdom. Moreover, Schmidt and co-workers³⁵⁵ demonstrated the presence of free GPC in aqueous extracts of animal tissues, and Campbell and Work,³⁵⁶ Walker,³⁵⁷ and Ansell and Norman³⁵³ isolated GPE from similar sources. These findings would appear to render this hypothesis plausible. However, Dawson,³⁵⁸ on the basis of a comparison of the specific radioactivity of liver phosphatidylcholine with that of GPC, and of the radioactivity of phosphatidylethanolamine with that of GPE, concluded that GPC and GPE cannot act as intermediates in the synthesis of their respective phospholipids. On the other hand, it is postulated that they may serve as products of catabolism of these phospholipids, since liver phosphatidylcholine

³⁵¹ J. M. Norman and R. M. C. Dawson, *Biochem. J.*, **54**, 396-399 (1953).

³⁵² J. J. Rae, H. D. Kay, and E. J. King, *Biochem. J.*, **28**, 143-151 (1934).

³⁵³ G. B. Ansell and J. M. Norman, *Biochem. J.*, **54**, viii-ix (1953); **55**, 768-774 (1953).

³⁵⁴ G. Ducet, *Bull. soc. chim. biol.*, **31**, 52-56 (1949).

³⁵⁵ G. Schmidt, L. Hecht, P. Fallot, L. Greenbaum, and S. J. Thannhauser, *J. Biol. Chem.*, **197**, 601-609 (1952).

³⁵⁶ P. N. Campbell and T. S. Work, *Biochem. J.*, **50**, 449-454 (1952).

³⁵⁷ D. M. Walker, *Biochem. J.*, **52**, 679-683 (1952).

³⁵⁸ R. M. C. Dawson, *Biochem. J.*, **57**, xv (1954); **59**, 5-8 (1955).

satisfies the radioactive requirements as the precursor of GPC, and similarly liver phosphatidylethanolamine fulfils the isotopic conditions essential for its function as the precursor of GPE. According to the available evidence, the best theoretical explanation of phospholipid synthesis is that glycerophosphate combines with two fatty acid molecules to form the phosphatidic acid before it combines with the nitrogenous base. The last step in the synthesis of phospholipids, therefore, would involve the combination of the phosphatidic acid with choline to form lecithin, with ethanolamine to produce phosphatidylethanolamine (cephalin), and probably likewise with serine to give rise to phosphatidylserine. Dawson³⁵⁸ has interpreted his experimental findings in this manner.

It is not entirely clear to what extent energy-rich phosphate bonds, as described by Lipmann, are involved in the synthesis of phospholipids. Thus, neutral fat (R) might react with phosphate to yield phosphatidic acid, which contains an energy-rich bond (designated as $\sim$), as follows:

Under these conditions, the addition of choline, ethanolamine, or serine to the phosphatidic acid would complete the phospholipid molecule. The energy required for phosphorylation of lipids under such conditions would be that necessary to provide the energy-rich phosphate bonds. As soon as such compounds were formed, the remaining synthesis could take place spontaneously. In conditions which prevent the generation of high-energy bonds, such as under anaerobic conditions or in the presence of respiratory inhibitors, no $\text{R} \sim \text{HPO}_4$ is formed, and phospholipid synthesis is blocked.

In a study *in vitro* of the synthesis of phospholipids from inorganic phosphate by isolated rat liver mitochondria, Kennedy³⁵⁹ noted that a continuance of synthesis required the maintenance of oxidative phosphorylation. The rate of synthesis was found to be approximately doubled by the addition of free glycerol; it is believed that α -glycerol phosphate is an important intermediate in the synthesis of the phospholipid. Nygaard³⁶⁰ has likewise shown that ATP is involved in the condensation of fatty acid and lysolecithin to form lecithin; the enzymes involved are presumably located in the mitochondria.

³⁵⁹ E. P. Kennedy, *J. Biol. Chem.*, **201**, 399-412 (1953).

³⁶⁰ A. P. Nygaard, *Arch. Biochem. Biophys.*, **43**, 493-494 (1953).

CHAPTER III

THE OXIDATION AND METABOLISM OF TRIGLYCERIDES, FATTY ACIDS, AND GLYCEROL IN THE ANIMAL BODY

1. Introduction

Under normal conditions, fats are completely oxidized in animals to carbon dioxide and water; approximately nine Calories of heat are liberated per g. of fat broken down. It is now known that the fatty acids and glycerol have different pathways of oxidation. In the case of the fatty acids, the degradation involves a series of reactions by which the fatty acid chain is broken down by two carbon units at a time. There is increasing evidence that the processes of synthesis and oxidation of fatty acids represent reversible phases of the same reaction. The understanding of the subject of fat oxidation has been greatly facilitated by the fact that, under conditions of fasting, or in such metabolic disorders as diabetes, the ketone bodies are excreted without being oxidized to carbon dioxide and water. It is generally agreed that glycerol is first converted to glucose, after which its metabolic fate parallels that of glucose.

The general subject of fat oxidation has been reviewed by Breusch¹ and by Stadie,² while Lehninger³ summarized the work on enzymic oxidation of fatty acids.

2. The Oxidation of Triglycerides and Fatty Acids

(1) *Methods and Theories of Fat Oxidation*

Several theories have been advanced to explain the mechanism of oxidation of fats. These are all concerned with the oxidation of fatty acids, since the preliminary change in the oxidation of triglycerides prob-

¹ F. L. Breusch, *Advances in Enzymol.*, 8, 343-423 (1948).

² W. C. Stadie, *Physiol. Revs.*, 25, 395-441 (1945).

³ A. L. Lehninger, *Enzymic Oxidation of Fatty Acids*, in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia, No. 9, Cambridge Univ. Press, 66-79 (1952).

ably involves their hydrolysis to fatty acids and glycerol. As mentioned earlier, the metabolism of glycerol is apparently the same as that of glucose. On the other hand, the oxidation of fatty acids is quite specific, and represents true fat oxidation.

Although the different hypotheses for fat oxidation vary, they are not necessarily mutually exclusive. It is possible that the several types of reactions upon which the theories are based may occur concomitantly, and that no single explanation will include all of the different reactions which are possible in the degradation of fatty acids.

a. β -Oxidation Theory. According to the β -oxidation theory, fatty acids are oxidized on the carbon atom-3 (β -carbon), which results in the formation of a β -keto acid. Following this, a split occurs in the molecule, with the result that an acetic acid molecule originates, and an acid remains which has two less carbon atoms than the original acid. This process is repeated until the long-chain acid is shortened to butyric acid, propionic acid (in the case of odd-chain acids) or to acetic acid. Butyric acid may be oxidized directly to acetoacetate and be excreted as such if the host is fasting or is diabetic. On the other hand, acetic acid may be the terminal compound in a normal animal, and this can then be utilized for energy *via* the Krebs cycle. Under conditions of fasting or diabetes, acetic acid may be rebuilt to acetoacetic acid, after which it will be partially or wholly excreted in the urine as a component of the ketone bodies. Odd-chain fatty acids are not ordinarily found as components of natural fats; however, when they are administered, they are broken down to propionic acid. This latter acid is then converted to sugar; the resulting compound subsequently follows the usual paths of metabolism of the glucose molecule.

The first experimental evidence of the phenomenon of β -oxidation in fatty acids was adduced in the classical experiments of Knoop⁴ in 1905. Prior to this time, the nature of the intermediate compounds of fatty acids could not be ascertained, inasmuch as the acid under study was completely oxidized, or was excreted unchanged. Knoop conceived the idea of coupling the acid residue with a benzene group, since under these conditions an incombustible residue remained which was excreted in the urine.

It had long been known that, after benzoic acid was given, it was detoxicated by conjugation with glycine, and that the resulting hippuric acid was excreted in the urine as shown on the next page:

⁴ F. Knoop, *Beitr. chem. Physiol. Pathol.*, 6, 150-162 (1905).

The Detoxication of Benzoic Acid with Glycine

It is now known that not only may glycine be used for the detoxication of benzoic acid, but glucuronic acid may also serve as a second line of defense, as follows:

The Detoxication of Benzoic Acid with Glucuronic Acid

It was likewise demonstrated by Salkowski and Salkowski,⁵ in 1879, and later by Knoop, that phenylacetic acid is not oxidized, but that it is also coupled with glycine to yield phenaceturic acid, which is excreted in the urine.

The Detoxication of Phenylacetic Acid with Glycine, with the Formation of Phenaceturic Acid

Thierfelder and Sherwin⁶ reported that, in the case of man, phenylacetic acid is conjugated with glutamine to form phenacetylglutamine.

When the phenyl-substituted fatty acids with aliphatic chains longer than that of acetic acid were fed, they were oxidized either to benzoic or to phenylacetic acid. This derivative was then conjugated with glycine or glucuronic acid, and was subsequently excreted in the urine. Apparently

⁵ E. Salkowski and H. Salkowski, *Ber.*, 12, 653-655 (1879).

⁶ H. Thierfelder and C. P. Sherwin, *Ber.*, 47, 2630-2634 (1914); *Z. physiol. Chem.*, 94, 1-9 (1915).

these acids were shortened two carbons at a time, rather than by a single carbon atom. Thus, phenylpropionic acid ($\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$) was converted to benzoic acid rather than to phenylacetic acid.⁵ Had the latter situation obtained, phenaceturic acid would have been excreted; actually hippuric acid was the end-product. When phenylbutyric acid ($\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$) was fed, it was converted to phenylacetic acid, which was subsequently eliminated as phenaceturic acid. Here again, the rupture of the aliphatic fatty acid must have proceeded two carbons at a time. Had the degradation occurred by single carbon units, then phenylpropionic acid would have originated from phenylbutyric acid; the former acid is known to be oxidized to benzoic acid, which is conjugated with glycine to appear in the urine as hippuric acid. However, no hippuric acid was present in the urine after the administration of phenylbutyric acid, but only phenaceturic acid. Finally, Knoop⁴ demonstrated that, when phenylvaleric acid ($\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$) was fed, it was oxidized to benzoic acid and was excreted as hippuric acid. It was postulated that phenylvaleric acid is converted to phenylpropionic acid, which is oxidized further to benzoic acid.

Embden and co-workers^{7,8} demonstrated that the normal aliphatic fatty acids undergo β -oxidation in the same way as do the phenyl-substituted acids. Thus, when surviving livers were perfused with blood containing hexanoic (caproic), octanoic (caprylic), or decanoic (capric) acids, acetoacetic acid accumulated in the perfusate. On the other hand, acetoacetate could not be demonstrated in increased amounts in the blood after odd-numbered acids had been added to the perfusing fluid.

Dakin⁹⁻¹³ further supported the β -oxidation theory on the basis of a number of experiments in which intermediate products of the phenyl-substituted fatty acids were recovered from the reaction mixture or from the urine, and identified. Thus, when phenylpropionic acid was fed, or injected, not only was it possible to isolate hippuric acid from the urine, but β -phenyl- β -hydroxypropionic acid ($\text{C}_6\text{H}_5\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{COOH}$), benzoyl-acetic acid ($\text{C}_6\text{H}_5\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH}$), and acetophenone ($\text{C}_6\text{H}_5\cdot\text{CO}\cdot\text{CH}_3$) were also obtained. Intermediate compounds of other phenyl-substituted

⁷ G. Embden, H. Salomon, and F. Schmidt, *Beitr. chem. Physiol. Pathol.*, **8**, 129-155 (1906).

⁸ G. Embden and A. Marx, *Beitr. chem. Physiol. Pathol.*, **11**, 318-322 (1908).

⁹ H. D. Dakin, *J. Biol. Chem.*, **4**, 77-89 (1908).

¹⁰ H. D. Dakin, *J. Biol. Chem.*, **4**, 91-100 (1908).

¹¹ H. D. Dakin, *J. Biol. Chem.*, **4**, 227-233, 419-435 (1908).

¹² H. D. Dakin, *J. Biol. Chem.*, **5**, 173-185, 303-309 (1908).

¹³ H. D. Dakin, *J. Biol. Chem.*, **6**, 203-219, 221-233 (1909).

fatty acids in which a partial oxidation at the β -carbon atom had occurred were likewise isolated. According to Dakin,¹³ when phenylvaleric acid ($\text{C}_6\text{H}_5 \cdot (\text{CH}_2)_4 \cdot \text{COOH}$) was oxidized, the side chain was degraded by the removal of the four carbons in two pairs, which he suggested might be termed "successive beta-oxidation." The benzoic acid resulting from the oxidation of phenylvaleric acid was also shown by Dakin to be excreted as hippuric acid.

Dakin⁹ observed that, when ammonium butyrate was oxidized *in vitro* with H_2O_2 under conditions approaching those in the body, acetoacetate and acetone were end-products. On the other hand, when the reaction temperature was higher, the acetoacetate was decarboxylated to acetone. In longer-chain fatty acids,¹² the terminal products were aldehydes having one less carbon atom than the original acid.

In later work, Dakin¹⁴ was able to isolate a number of intermediate compounds of caproic acid ($\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$) from the perfusate, after perfusing a surviving liver with Ringer solution containing this acid. The intermediates included:

2-hexenoic acid ($\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{CH} \cdot \text{COOH}$),

β -hydroxycaproic acid ($\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{COOH}$),

β -ketocaproic acid ($\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH}$).

In addition to the aforementioned acids, acetoacetic acid and β -hydroxybutyric acid were always present. When 2-hexenoic acid, β -hydroxycaproic acid, or β -ketocaproic acid was fed, acetoacetate and β -hydroxybutyrate were formed, as well as some of the other intermediates.

Further support for the β -oxidation theory can be deduced from the results with unsaturated and hydroxy acids. As early as 1842, Erdmann and Marchand¹⁵ disclosed that cinnamic acid ($\text{C}_6\text{H}_5 \cdot \text{CH} \cdot \text{CH} \cdot \text{COOH}$) was eliminated as hippuric acid by the organism, while v. Graebe and Schultzen¹⁶ and Dakin¹³ confirmed this fact and demonstrated, possibly erroneously, that mandelic acid ($\text{C}_6\text{H}_5 \cdot \text{CHOH} \cdot \text{COOH}$), in contradistinction to phenylacetic acid, could also give rise to hippuric acid. On the other hand, Quick¹⁷ noted that the benzoic acid formed after phenylpropionic acid or after the corresponding α,β -unsaturated acid (cinnamic) was given was conjugated largely with glucuronic acid.^{17,18} The ratio of benzoate excreted as glucuronate to that as hippurate was 3:1. In sharp contrast to

¹⁴ H. D. Dakin, *J. Biol. Chem.*, **56**, 43-51 (1923).

¹⁵ O. L. Erdmann and R. F. Marchand, *J. prakt. Chem.*, **26**, 494-498 (1842).

¹⁶ C. v. Graebe and O. Schultzen, *Ann. Chem. Pharm.*, **142** [n.s.66], 345-350 (1867).

¹⁷ A. J. Quick, *J. Biol. Chem.*, **77**, 581-593 (1928).

¹⁸ A. J. Quick, *J. Biol. Chem.*, **80**, 515-526 (1928).

this finding, Quick¹⁷ reported that phenylacetic acid, when administered as such, or when formed after the feeding of phenylbutyric acid or of phenylisocrotonic acid ($\text{C}_6\text{H}_5\cdot\text{CH}:\text{CH}\cdot\text{CH}_2\cdot\text{COOH}$), is conjugated with glucuronic acid and with glycine in the ratio of 1:2. In the later study, Quick¹⁸ reported that the conjugation of newly formed benzoic acid with glucuronic acid and with glycine is 2:1 after the feeding of phenylvaleric acid, phenyl- α,β -pentenic acid ($\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}:\text{CH}\cdot\text{COOH}$) or phenyl- β - γ -pentenic acid ($\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}:\text{CH}\cdot\text{CH}_2\cdot\text{COOH}$). The identity of the proportion of glucuronic acid and glycine used for the detoxication of benzoic acid and phenylacetic acid, irrespective of whether these aromatic acids were fed as such or whether they originated by oxidation of the aliphatic side chain,^{17,18} offers especially strong confirmation of the β -oxidation hypothesis.

Attention should likewise be called to the studies of Raper and Wayne,¹⁹ who fed phenylpropionic, phenylbutyric, phenylvaleric, and phenylcaproic acids to dogs, and noted that the end-products formed were in harmony with those one would expect according to the β -oxidation theory. However, when phenylnonanoic acid ($\text{C}_6\text{H}_5\cdot(\text{CH}_2)_8\cdot\text{COOH}$) or phenyldecanoic acid ($\text{C}_6\text{H}_5\cdot(\text{CH}_2)_9\cdot\text{COOH}$) was fed, the amount of benzoic and of phenylacetic acids formed, respectively, was less than that calculated from the phenyl residues involved. It is therefore suggested that, in addition to β -oxidation, some other type of breakdown obtains in the case of long-chain aliphatic acids.

However, the results of Quick¹⁸ throw some doubt on the hypothesis that the β -hydroxy and β -keto acids are normal intermediates in fatty acid oxidation. Since phenyl- β -hydroxypropionic acid ($\text{C}_6\text{H}_5\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{COOH}$) is excreted largely unchanged by dogs, and only 25% is oxidized to benzoic acid, there is some question as to whether or not it is a normal intermediary product in the oxidation of phenylpropionic acid. Moreover, the proportion of newly-synthesized benzoic acid formed after the feeding of phenylpropionic acid which is detoxicated with glucuronic acid and with glycine was found to be 1:1, in sharp contrast to the 3:1 ratio of these components when benzoic acid itself was administered.

Acetophenone ($\text{C}_6\text{H}_5\cdot\text{CO}\cdot\text{CH}_3$), the ketone formed when β -ketopropionic acid is heated, is partially changed to benzoic acid and partially reduced to phenylmethylecarbinol ($\text{C}_6\text{H}_5\cdot\text{CHOH}\cdot\text{CH}_3$). In this case, the benzoate is largely conjugated with glycine. According to Quick,¹⁸ this phenomenon obtains because of the slow rate of production of benzoic acid. These experiments confirm the earlier results of Thierfelder and Daiber.²⁰

¹⁹ H. S. Raper and E. J. Wayne, *Biochem. J.*, **22**, 188-197 (1928).

²⁰ H. Thierfelder and K. Daiber, *Z. physiol. Chem.*, **130**, 380-396 (1923).

Phenylmethylcarbinol is reported to be excreted as the glucuronate. On the basis of these results, Quick¹⁸ states that "Therefore neither phenyl- β -hydroxypropionic acid nor acetophenone appears to be a normal intermediate in the catabolism of phenylpropionic acid."

It was reported by Quick¹⁸ that mandelic acid ($\text{C}_6\text{H}_5 \cdot \text{CHOH} \cdot \text{COOH}$) is neither oxidized nor conjugated by the dog, but is excreted unchanged. This latter finding is in harmony with the results of Knoop,⁴ although the latter worker had not investigated whether or not the end-products of oxidation of mandelic acid were excreted as glucuronates.

The formation of ketone bodies from the fatty acids having an even number of carbon atoms is another proof of the β -oxidation theory. Thus, when butyric acid was fed to fasting rats as the sodium salt, β -hydroxybutyrate ($\text{CH}_3 \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{COOH}$) and acetoacetate ($\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH}$) were excreted in the urine, exemplifying an oxidation on the β -carbon atom.²¹

The Relationship between Butyric Acid and the Oxytetranoic Acids

The formation of acetone, which occurs concomitantly, results from the decarboxylation of acetoacetic acid, as follows:

The Transformation of Acetoacetic Acid to Acetone

Moreover, an amount of the ketone bodies identical with that obtained following an isomolecular dose of butyrate originated when caproic acid^{21,22} or sorbic acid ($\text{CH}_3 \cdot \text{CH} : \text{CH} \cdot \text{CH} : \text{CH} \cdot \text{COOH}$)²² was given to fasting rats; this must likewise indicate a removal of two carbons. Moreover, all even-chain acids from C_4 to C_{18} were shown to give rise to ketone bodies when fed either as their sodium salts²¹ or as their ethyl esters.²³

Had only one carbon atom at a time been removed from the even-numbered fatty acids, instead of two carbons, an acid with an uneven number of carbon atoms would have resulted. There is considerable evidence that the fate of the odd-carbon acids is entirely different from that of the even-carbon acids. In the first place, whereas all even-carbon

²¹ J. S. Butts, C. H. Cutler, L. Hallman, and H. J. Deuel, Jr., *J. Biol. Chem.*, **109**, 597-613 (1935).

²² H. J. Deuel, Jr., C. E. Calbert, L. Anisfeld, H. McKeehan, and H. D. Blunden, *Food Research*, **19**, 13-19 (1954).

²³ H. J. Deuel, Jr., L. F. Hallman, J. S. Butts, and S. Murray, *J. Biol. Chem.*, **116**, 621-639 (1936).

acids containing four or more carbons produce an exogenous ketonuria when fed to fasting rats, the odd-carbon acids give rise to none, or to limited quantities of ketone bodies. Thus, Deuel and co-workers²³ reported no ketonuria after the administration of ethyl propionate or the ethyl esters of valeric, heptanoic, or nonanoic (pelargonic) acids. However, a slight ketonuria was noted after the sodium salts of valeric or heptanoic acids were fed.²¹ MacKay and co-workers²⁴ reported that the odd-chain fatty acids having more than three carbons do give rise to limited amounts of acetone bodies in rabbits, as evidenced by an increased level of blood ketones. The formation of the ketone bodies is attributed to the condensation of the acetic acid molecules which are formed by rupture of two-carbon fragments from odd-chain acids containing five or more carbons.

Another marked difference in the behavior of odd- and even-carbon acids is their relation to carbohydrate formation. No glycogen production could be observed when the ethyl esters of butyric, caproic, caprylic, capric, lauric, myristic, or oleic acid were fed to rats,²⁵ nor when the sodium salts of diacetic, butyric, caproic, or caprylic acid were administered.²⁶ On the other hand, there is ample evidence that the odd-chain fatty acids give rise to glycogen. Ringer²⁷ reported, in 1912, that propionic acid resulted in "extra" sugar in the phlorhizinized dog; moreover, it was later shown that the administration of valeric and heptanoic acids likewise resulted in glucose formation, to the extent of three carbons in each molecule.²⁸ An increase in blood sugar in rabbits was noted by Pollak²⁹ after the intravenous injection of the salts of propionic and valeric acids, but not following the administration of salts of acetic, butyric, acetoacetic, or caproic acids. In addition, Deuel and collaborators reported that propionic, valeric, heptanoic, and nonanoic acid gave rise to definite amounts of liver glycogen when fed to fasting rats as their sodium salts,²⁶ or as their ethyl esters.²⁵ MacKay *et al.*²⁴ likewise found that propionic acid produced glycogen when fed to fasting rabbits. Atchley,³⁰ employing the newly-developed technic involving countercurrent distribution, was able to separate propionic acid, rather than butyric acid, when valeric acid was

²⁴ E. M. MacKay, A. N. Wick, and C. P. Barnum, *J. Biol. Chem.*, **136**, 503-507 (1940).

²⁵ J. S. Butts, H. Blunden, W. Goodwin, and H. J. Deuel, Jr., *J. Biol. Chem.*, **117**, 131-133 (1937).

²⁶ H. J. Deuel, Jr., J. S. Butts, L. F. Hallman, and C. H. Cutler, *J. Biol. Chem.*, **112**, 15-23 (1935-1936).

²⁷ A. I. Ringer, *J. Biol. Chem.*, **12**, 511-515 (1912).

²⁸ A. I. Ringer, *J. Biol. Chem.*, **14**, 43-52 (1913).

²⁹ L. Pollak, *Biochem. Z.*, **127**, 120-136 (1922).

³⁰ W. A. Atchley, *J. Biol. Chem.*, **176**, 123-131 (1948).

oxidized by the kidney fatty acid-oxidizing system. When isocaproate was oxidized, isobutyric acid could be separated from the reaction mixture. These demonstrations constitute the most convincing proof of the β -oxidation theory yet obtained, since they involve the actual separation and identification of the reaction product of the oxidation of an odd-chain acid and a branched-chain acid.

Finally, Deuel *et al.*³¹ noted that glycogen synthesis is of a considerably higher order when the triglycerides of fatty acids with an odd number of carbons are fed than when the triglycerides of the even-chain acids of corresponding length are given. One can only conclude that the behavior of the odd-chain and that of the even-chain fatty acids differ so much as to preclude a change of one to the other during their catabolism. Therefore, the metabolism of both the even-chain and the odd-chain fatty acids must involve a removal of fragments of two carbons, or, at least, of an even number of carbon atoms, at a time.

In addition to the formation of ketone bodies from fatty acids as a result of metabolism in the intact animal, the many experiments with isolated tissues support the β -oxidation theory. Thus, Jowett and Quastel^{32,33} reported that an extensive ketone body formation occurred when liver slices were incubated with butyric, caproic, or caprylic acids; on the other hand, only a slight ketone body synthesis followed incubation with valeric, heptanoic, or nonanoic acids. Muñoz and Leloir^{34,35} likewise published papers dealing with the quantitative aspects of ketone body production by liver slices when different fatty acids were added to the substrate. Similar variations in ketone body production by the liver slices when even-chain or odd-chain fatty acids were employed as substrates have been reported by Witter, Cottone, and Stotz.³⁶

Added support for the β -oxidation hypothesis is to be obtained from the proof that the main end-product, acetic acid, is capable of oxidation in the tissues. Thus, Pardee, Heidelberger, and Potter³⁷ reported that acetate- 1-C^{14} was oxidized by slices and homogenates of rat tissues *in vitro*. Oxalo-

³¹ H. J. Deuel, Jr., J. S. Butts, H. Blunden, C. H. Cutler, and L. Knott, *J. Biol. Chem.*, **117**, 119–129 (1937).

³² M. Jowett and J. H. Quastel, *Biochem. J.*, **29**, 2159–2180 (1935).

³³ M. Jowett and J. H. Quastel, *Biochem. J.*, **29**, 2181–2191 (1935).

³⁴ L. F. Leloir and J. M. Muñoz, *Biochem. J.*, **33**, 734–746 (1939).

³⁵ J. M. Muñoz and L. F. Leloir, *J. Biol. Chem.*, **147**, 355–362 (1943); **153**, 53–59 (1944).

³⁶ R. F. Witter, M. A. Cottone, and E. Stotz, *J. Biol. Chem.*, **207**, 671–678 (1954).

³⁷ A. B. Pardee, C. Heidelberger, and V. R. Potter, *J. Biol. Chem.*, **186**, 625–635 (1950).

acetate stimulated the oxidation, and it is suggested that the oxidation of acetate proceeded through the citrate cycle.

b. Multiple Alternate Oxidation Theory. According to this hypothesis, fatty acids are simultaneously oxidized at alternate carbon atoms, in contradistinction to the stepwise procedure suggested in the original β -oxidation theory. Under these circumstances, capric acid would be immediately oxidized to a polyketonic acid containing ten carbon atoms before any rupture of the fatty acid chain could take place. Subsequent splitting of the polyketone might then be in such locations as to yield one or more molecules of acetoacetate. The oxidation and breakdown of capric acid might be pictured as follows:

Polyketone of capric acid

Possible Oxidation of Capric Acid by Multiple Alternate Oxidation

Thus, capric acid might yield two ketone body molecules plus one acetate if the rupture obtained at *A* and *B* simultaneously. Likewise, it might also yield five acetate molecules if a splitting occurred at all possible weak points in the molecule.

Hurtley³⁸ first proposed the scheme of multiple oxidation. Jowett and Quastel^{32,33,39} later suggested the multiple alternate oxidation theory to explain the fact that caprylic and capric acids yielded more than one ketone body per molecule, as contrasted with the result when butyric or caproic acid was employed as the substrate. Leloir and Muñoz³⁴ likewise confirmed the greater ketone body production of long-chain acids by liver slices.

Butts and associates²¹ noted that the administration of caprylic acid gave rise to twice the amount of ketone bodies in the urine of the fasting rats as did an isomolecular amount of butyric or caproic acids. It was suggested that this might be attributed to a δ -oxidation. However, in a later report,²³ after it was found that other long-chain acids were responsible for increasing ketone body production in fasting rats in proportion to the length of the fatty acid chain, it was suggested that, although butyric and

³⁸ W. H. Hurtley, *Quart. J. Med.*, **9**, 301-408 (1915-1916).

³⁹ M. Jowett and J. H. Quastel, *Biochem. J.*, **29**, 2143-2158 (1935).

caproic acids break down by β -oxidation, the even-chain carbon acids containing eight to fourteen carbon atoms are probably also broken down by δ and ζ -oxidation by a procedure of "multiple alternate oxidation." The data obtained from *in vivo* tests thus support the *in vitro* experiments of Jowett and Quastel.

The results of oxygen consumption tests on perfused livers and on liver slices have offered support for the hypothesis that long-chain fatty acids yield more ketone bodies per molecule than do the short-chain acids. Thus, Blixenkroner-Møller⁴⁰ reported that the ratio of oxygen consumption of the perfused livers of diabetic cats to ketone body production was such as to indicate that four ketone body molecules were formed from each fatty acid molecule oxidized, rather than that a more complete oxidation occurred, with the production of two carbon residues such as in acetic acid. Likewise, Stadie⁴¹ reported that the ratio of O_2 consumption to ketone production in liver slices of the diabetic cat was such as to preclude successive β -oxidation as the explanation for the fatty acid oxidation in liver. These calculations are summarized in Table 1.

The quantitative aspects of acetoacetate formation from long-chain fatty acids have recently been supported by an *in vitro* system. Thus, Witter, Cottone, and Stotz³⁶ reported that, when fatty acids ranging in length from C_2 to C_{17} were oxidized by particulate preparations from rat liver and by mitochondria, all acids were vigorously oxidized except acetic and propionic. The fatty acids with an even number of carbon atoms were changed quantitatively to ketone bodies. In the case of the odd-chain acids, the yield of acetoacetate was slightly in excess of one mole of acetoacetate for each mole oxidized.

Stadie⁴¹ suggests that the principal experimental fact which argues against the successive β -oxidation theory, and which might thus be considered to support the multiple alternate oxidation theory, is the failure to detect acetic acid⁴²⁻⁴⁴ or any of the hypothetical intermediate acids having less than twelve carbon atoms, in the tissues or depot fats.⁴⁵ Thus, Stadie *et al.*,⁴⁴ employing a procedure which was capable of detecting extremely small amounts of acetic acid, were unable to detect traces of this compound in liver slices producing large quantities of ketone bodies.

⁴⁰ N. Blixenkroner-Møller, *Z. physiol. Chem.*, **252**, 117-136 (1938).

⁴¹ W. C. Stadie, *Harvey Lectures*, **37**, 129-167 (1942).

⁴² G. Embden and L. Michaud, *Beitr. chem. Physiol. Pathol.*, **11**, 332-347 (1908).

⁴³ E. Toennissen and E. Brinkman, *Z. physiol. Chem.*, **252**, 169-195 (1938).

⁴⁴ W. C. Stadie, J. A. Zapp, Jr., and F. D. W. Lukens, *J. Biol. Chem.*, **137**, 75-87 (1941).

⁴⁵ D. L. Cramer and J. B. Brown, *J. Biol. Chem.*, **151**, 427-438 (1943).

TABLE 1
THE OXIDATION METABOLISM OF LIVER SLICES
OF SIX DEPANCREATIZED CATS^a

Category	Mean oxygen uptake (a), micromoles/ g./hr.	Ketone formation (b), mg./g.	Ratio of a/b
Oxygen uptake:			
Observed	87.5 ± 4.0	—	—
Deamination of amino acids ^b			
8.2 ± 1.2			
Carbon dioxide formation			
28.0 ± 5.0			
Total corrections	36.2		
Corrected oxygen uptake	51.3 ± 6.5	—	—
Ketone formation observed		46.5 ± 4.0	—
Oxygen uptake: ketone formation			
Observed (using corrected oxygen uptake)	—	—	1.10 ± 0.12
Theoretical for multiple alternate oxidation ^c	—	—	1.25
Theoretical for successive β -oxidation ^c	—	—	6.5

^a Adapted from W. C. Stadie, *Physiol. Revs.*, 25, 395-441 (1945), p. 402.

^b Calculated from urea and ammonia formation.

^c Calculated for palmitic acid.

However, these criticisms have been largely met by studies³⁰ which demonstrated that the extent of acetate formation, as registered by the sensitive lanthanum method,⁴⁶ varied with the intensity of fat oxidation. Leloir and Muñoz³⁴ also proved, by the use of this technic, that acetic acid was present when octanoic acid was degraded by liver slices. Moreover, Atchley³⁰ demonstrated the presence of propionic acid after valeric acid had been oxidized by the kidney, as well as of isobutyric acid, following the oxidation of isocaproic acid.

Although the multiple alternate oxidation theory is an attractive one which explains the fact that a greater ketone body formation results from long-chain acids than from short-chain acids, it lacks experimental confirmation. Thus, the presence of either mono- or polyketonic acids has not been established. Although an α, γ -diketo acid, acetoacetic acid ($\text{CH}_3\text{-}$

⁴⁶ D. Krüger and E. Tschirch, *Ber.*, 63, 826-836 (1930).

$\text{CO}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH}$), exhibits a marked metabolic activity *in vitro*,^{47,48} the α,γ -diketo homologue of octanoic acid was found by Lehninger⁴⁹ to be poorly absorbed from the intestine of the rat. As much as 67% could be recovered from the gastrointestinal tract twelve hours after the diketo acid was fed to rats; this poor absorption was attributed to the low diffusibility. It was later shown, however, that broken cell preparations of rat liver were able to oxidize this compound at a slow rate.⁴⁹

Moreover, although the β -oxidation theory in its original form fails to explain the greater ketone body formation from long-chain acids than from short-chain ones, the β -oxidation-acetic acid condensation theory of MacKay *et al.*⁵⁰ provides as satisfactory an explanation of this phenomenon as does the multiple alternate oxidation theory. The criticism of Stadie,⁴¹ that the presence of acetic acid in the liver has not been proved, is important; however, it may not be valid, if the formation is instantaneous, and if any acetate formed is immediately oxidized or converted to ketone bodies. The data accumulated by means of isotopic acids, and of $\text{CH}_3\text{-CO}\cdot\text{CoA}$, which have been discussed in relation to the synthesis of fatty acids, and which will be elaborated in the following section, would seem to be sufficiently substantiated to dispel any doubt that two-carbon intermediates are formed in the tissues. Crandall and Gurin⁵¹ interpreted the fact that β -labeled octanoate gives rise to acetoacetate with a predominance of the isotopic carbon in the carboxyl group as evidence that multiple alternate oxidation does not constitute a significant pathway in the oxidation of octanoate.

c. β -Oxidation-Acetic Acid Condensation Theory. According to this theory, the breakdown of fatty acids proceeds by β -oxidation, but the acetic acid molecules set free are able to condense, to yield acetoacetate molecules. Support for this theory comes from several types of experiment. Thus, as early as 1913, Friedmann⁵² reported that the perfusion of liver with acetic acid resulted in the formation of ketone bodies. MacKay and collaborators⁵⁰ noted that the administration of acetic acid to a phlorrhizine-treated dog increased the level of ketonuria. Moreover, when this acid was fed to fasting rats, a definite increase in ketone body excretion over and above that produced by control rats receiving a similar quantity of sodium

⁴⁷ H. A. Krebs and W. A. Johnson, *Biochem. J.*, **31**, 772-779 (1932).

⁴⁸ A. L. Lehninger, *J. Biol. Chem.*, **148**, 393-404 (1943).

⁴⁹ A. L. Lehninger, *J. Biol. Chem.*, **153**, 561-570 (1944).

⁵⁰ E. M. MacKay, R. H. Barnes, H. O. Carne, and A. N. Wick, *J. Biol. Chem.*, **135**, 157-163 (1940).

⁵¹ D. I. Crandall and S. Gurin, *J. Biol. Chem.*, **181**, 829-843 (1949).

⁵² E. Friedmann, *Biochem. Z.*, **55**, 436-442 (1913).

molecules of ketone bodies, as compared with 1.0 molecules for butyric acid. Although Witter *et al.*⁵⁸ reported this relationship for caproic acid and sorbic acid ($\text{CH}_3\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$) when these acids were incubated with liver slices, Deuel and co-workers have found that a 1:1 ratio obtains *in vivo* in the exogenous ketonuria of rats after the feeding of caproic acid²¹⁻²³ or of sorbic acid.²² This might suggest that the direct formation of acetoacetate was the most important change occurring *in vivo*, but that the synthesis of acetoacetate may obtain in tissues in which the end-products cannot be removed and in which the substrate remains in the tissue for a long enough period to allow an equilibrium to be reached.

Although the β -oxidation-acetic acid condensation theory does satisfactorily explain how the long-chain acids may have a greater ketogenic activity than the short-chain acids, as well as how acetic acid gives rise to ketone bodies, it does not necessarily completely invalidate the multiple alternate oxidation theory. It is possible that the formation of a polyketone is the first step in the oxidation of a long-chain fatty acid; this is followed by an explosion to yield a number of acetate fragments simultaneously. On the other hand, the theory of Knoop and of Dakin, which suggests that the oxidation of fatty acids occurs in a stepwise fashion on the β -carbon, is not completely ruled out.

The oxidation and resynthesis of fatty acid may be occurring simultaneously. This somewhat complicates the explanation of the mechanism of oxidation. In studies carried out on C^{14} -labeled palmitic acid in which the isotope had been introduced into either carboxyl position, the fifth carbon or the eleventh carbon, Chaikoff and co-workers⁵⁹ found that C^{14} was recovered only in the carboxyl and carbonyl carbons of acetoacetate. This would suggest that the oxidation of long-chain fatty acids involves the progressive cleavage of the chain into 2C units. The following sequence of results is described:

1. Oxidation of the β -carbon of the fatty acid of C_n length.
2. Cleavage of the molecule between the α - and β -carbon, yielding a C_{n-2} residue and a 2C unit which immediately enters into the 2C metabolic pool in the liver.
3. Random condensation of 2C units from the pool to give rise to acetoacetate.
4. Oxidation of the fifth carbon of the original C_n chain, now the β -carbon of the C_{n-2} fragment. Two separate processes (*A* and *B*) can now

⁵⁸ R. F. Witter, E. H. Newcomb, and E. Stotz, *J. Biol. Chem.*, **185**, 537-548 (1950).

⁵⁹ I. L. Chaikoff, D. S. Goldman, G. W. Brown, Jr., W. G. Dauben, and M. Gee, *J. Biol. Chem.*, **190**, 229-240 (1951).

occur. They take place simultaneously. *A.* Some β -oxidized C_{n-2} fatty acid molecules are cleaved between their α - and β -carbons (C_4 and C_5 of original C_n molecule) yielding a C_{n-4} residue and C2 fragments which enter the same metabolic pool referred to in step (2). *B.* An orientation occurs between the C2 fragments in the liver pool and the C_{n-2} fragments in such a way that, when α,β cleavage of the C_{n-2} residue occurs, simultaneous condensation with the oriented C2 units occurs. The acetoacetate would be predominantly labeled in the carboxyl group.

In a later study, Goldman *et al.*⁶⁰ studied the decarboxylation of the acetoacetate formed during the oxidation of carboxyl-labeled palmitic and octanoic acids by rat liver slices. In the presence of sufficient non-isotopic acetoacetate carrier, the thermal and catalytic procedures yielded essentially equivalent values for the specific activity of the carboxyl carbon.

The asymmetric labeling of acetoacetate was explained by Beinert and Stansley^{61,62} by the enzymatic acyl interchange between CoA and β -keto acids. The acetoacetate cleavage enzyme, *E*, catalyzes the condensation of acetoacetate from two molecules of AcCoA. If AcCoA is labeled, the acetoacetate formed is symmetrically labeled. However, in the presence of added unlabeled acetoacetate, Ac^*CoA gives rise to acetoacetate labeled preferentially in the carboxyl half ($Ac \cdot Ac^*O^-$). The total amount of label incorporated under these conditions is fifty times that obtained in the absence of added acetoacetate. Both of these effects can be duplicated with higher β -keto acids.

The production of asymmetrically labeled acetoacetate is related to the rate of the following reactions:

Under certain conditions, $EAcCoA$ can react with $Ac \cdot CoA$ to form $EAc \cdot \cdot Ac^*CoA$; this results from a positional difference between the C2 units, rather than from a variation in chemical reactivity. This accounts for the fact that asymmetrically labeled acetoacetate may arise during fatty acid oxidation.

Brown and Chaikoff⁶³ postulated that two types of C_2 derivatives arise

⁶⁰ D. S. Goldman, G. W. Brown, Jr., H. R. Matheson, and I. L. Chaikoff, *J. Biol. Chem.*, **195**, 415-422 (1952).

⁶¹ H. Beinert and P. G. Stansley, *Federation Proc.*, **12**, 176 (1953).

⁶² H. Beinert and P. G. Stansley, *J. Biol. Chem.*, **204**, 67-76 (1953).

⁶³ G. W. Brown, Jr., and I. L. Chaikoff, *Biochim. et Biophys. Acta*, **11**, 37-51 (1953).

in the oxidation of short-chain fatty acids of the even series, namely $\text{CH}_3\text{--CO--}$ and $\text{--CH}_2\text{CO--}$. Two types of acetoacetate will be formed, one by the condensation of the first with the second type of C_2 compound, while the second arises exclusively from the second type. It is believed that, when acetoacetate is synthesized by slices or by washed homogenates of rat liver, the activity of 40 to 50% of the terminal two-carbon groups is identical with that of the two-carbon fragments from the rest of the molecule. The occurrence of asymmetric labeling of acetoacetate is related to the proportion of the two types of two-carbon compounds from which the acetoacetate is derived.

d. ω -Oxidation Theory. In addition to oxidation on the β -carbon, the fatty acids of medium chain length have been shown in some cases to be first oxidized on the ω -carbon, *i.e.*, on the terminal carbon furthest from the carboxyl group. As a result of this type of oxidation, dicarboxylic acids are produced which apparently are then broken down by β -oxidation, from either or both ends. Verkade, van der Lee *et al.*^{64,65} were the first investigators to recognize this type of oxidation. It was demonstrated that, in man, the ingestion of glycerides of capric acid and of undecanoic acid was followed by the excretion of dicarboxylic acids. Nonanoic acid⁶⁵ and caprylic acid had weak diacidogenic properties; lauric acid glycerides gave rise only to traces of dicarboxylic acids or to none at all, while tridecanoic acid was devoid of diacidogenic effects.⁶⁶ In the case of the dog, the metabolic pattern for the C_7 to C_{11} acids differed somewhat. According to Verkade,⁶⁶ this discrepancy may be related to the fact that, in contradistinction to man, it is difficult to produce ketosis in the dog, and that the lack of diacidogenesis is "possibly related and to be expected with the carnivora in general."

It has been observed that ω -oxidation does not occur in the case of straight-chain fatty acids having more than twelve carbon atoms. In contradistinction to this finding is the demonstration by Weitzel⁶⁷ of this type of oxidation, as evidenced by the presence of dicarboxylic acids in the urine of dogs after the feeding of α -substituted myristic or stearic acid in which the side chain has more than one carbon. As far as is known, this is the only example of ω -oxidation of C_{14} or C_{18} fatty acids.

The possible course of oxidation of the terminal methyl group which

⁶⁴ P. E. Verkade and J. van der Lee, *Biochem. J.*, **28**, 31-40 (1934).

⁶⁵ P. E. Verkade, M. Elzas, J. van der Lee, H. H. de Wolff, A. Verkade-Sandbergen, and D. Van der Sande, *Proc. Acad. Sci. Amsterdam, Sect. Sci.*, **35**, 251-266 (1932); P. E. Verkade and J. van der Lee, *Ibid.*, **36**, 314-323 (1933).

⁶⁶ P. E. Verkade, *Chemistry & Industry*, **57**, 704-711 (1938).

⁶⁷ G. Weitzel, *Z. physiol. Chem.*, **287**, 254-296 (1951).

occurs during the formation of dicarboxylic acids has been suggested by Stadie² as follows:

When tricaprin is metabolized in man by ω -oxidation, the hydrolysis of the triglyceride occurs first, followed by the oxidation of the free fatty acid, capric acid, to the dicarboxylic acid with the same number of carbons. In this case, sebacic acid is formed. The newly-formed dicarboxylic acid is then subjected to β -oxidation from one or from both ends, yielding a series of dicarboxylic acids which have two, four, or six carbons less than does the original acid.⁶⁸ It is possible that the simplest dicarboxylic acid, oxalic acid ($\text{HOOC}\cdot\text{COOH}$), which is normally present in the urine of man to the extent of 20 to 40 mg. per day,⁶⁹ may owe its origin to a reaction of this nature. A similar suggestion is made that food fats are the "parent substances of the C_4 system," which includes succinic acid ($\text{HOOC}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$). In the experiments of Verkade and van der Lee,⁶⁸ the dicarboxylic acids, sebacic, suberic, and adipic, were isolated from the urine after tricaprin had been ingested. A probable scheme of oxidation is illustrated below:

The Oxidation of Tricaprin in Man as an Example of ω -Oxidation of Fatty Acids

⁶⁸ P. E. Verkade and J. van der Lee, *Z. physiol. Chem.*, **227**, 213-222 (1934).

⁶⁹ P. Müller, *Verhandl. Ver. schweiz. Physiol.*, **11**, 8-9 (1937); *Chem. Abstr.*, **32**, 6308 (1938); *Physiol. Abst.*, **22**, 349 (1937).

After the administration of the triglyceride of nonanoic acid, azelaic acid ($\text{HOOC} \cdot (\text{CH}_2)_7 \cdot \text{COOH}$) and pimelic acid ($\text{HOOC} \cdot (\text{CH}_2)_5 \cdot \text{COOH}$) were identified in the urine. However, Verkade and van der Lee⁷⁰ were unable to demonstrate the excretion of dicarboxylic acids after the ingestion of triglycerides of the higher fatty acids.

The occurrence of a diaciduria is believed by Verkade⁶⁶ to be related to carbohydrate metabolism. Whereas the concomitant metabolism of carbohydrate is known to suppress ketonuria, Verkade and co-workers^{65,71} found that the excretion of dicarboxylic acids by man, after the ingestion of triundecylin, is augmented by the ingestion of carbohydrate. It is suggested that, during fasting, a diaciduria does not occur; this is considered to be a "pathological fact,"⁷¹ inasmuch as in the absence of carbohydrate utilization "the classical way of degradation of the saturated fatty acids (unilateral β -oxidation) is promoted at the expense of the way of degradation by ω -oxidation, with subsequent bilateral oxidation." More information is needed before one can understand the relationship of diaciduria to carbohydrate metabolism.^{72,73}

Flaschenträger and co-workers⁷²⁻⁷⁴ confirmed the fact that the C_3 to C_{11} acids give rise to dicarboxylic acids. However, it was recorded that the proportion of fatty acids undergoing ω -oxidation is very small (usually 0.1-1.0%), although in one instance the quantity excreted amounted to 5.8% of that ingested.⁷⁴ However, Flaschenträger *et al.* are of the opinion that the importance of ω -oxidation is minimal. Emmrich and Nebe⁷⁵ noted that the feeding of fatty acids having an uneven number of carbon atoms to man and to dogs resulted in the excretion of small amounts of pimelic acid, while the administration of hydrogenated coconut oil was followed by the excretion of sebacic acid. Moreover, Bernhard⁷⁶ concluded that ω -oxidation is not a step in the normal catabolism of fatty acids. This opinion is based upon the fact that, although succinic acid is metabolized when fed to dogs or rats, adipic (C_6), suberic (C_8), sebacic (C_{10}), and higher fatty acids (identified by labeling with deuterium) are not metabolized to any appreciable extent.

⁷⁰ P. E. Verkade and J. van der Lee, *Z. physiol. Chem.*, **225**, 230-244 (1934).

⁷¹ P. E. Verkade, J. van der Lee, and M. Elzas, *Biochim. et Biophys. Acta*, **2**, 38-56 (1948).

⁷² B. Flaschenträger and K. Bernhard, *Helv. Chim. Acta*, **18**, 962-972 (1935).

⁷³ B. Flaschenträger, K. Bernhard, C. Löwenberg, and M. Schläpfer, *Z. physiol. Chem.*, **225**, 157-167 (1934).

⁷⁴ B. Flaschenträger and K. Bernhard, *Z. physiol. Chem.*, **238**, 221-232 (1936).

⁷⁵ R. Emmrich and E. Nebe, *Z. physiol. Chem.*, **266**, 174-182 (1940).

⁷⁶ K. Bernhard, *Helv. Chim. Acta*, **24**, 1412-1425 (1941).

However, considerable evidence has been adduced to indicate that diaciduria occurs when fatty acids having side chains are metabolized. Kuhn, Köhler, and Köhler⁷⁷ and Kuhn and Löw⁷⁸ reported that a side chain in the α - or the β -position resulted in the formation of dicarboxylic acids. Asano and Yamakawa⁷⁹ found that the yield of dicarboxylic acids was increased by a factor of ten when side chains were present; their work indicated that a side chain at the α -position is as effective as one at the β -position in facilitating ω -oxidation. Yamakawa⁸⁰ also reported that branched-chain fatty acids having sixteen or seventeen carbons first underwent ω -oxidation, and that this was followed by β -oxidation, with the liberation of two-carbon fragments. After 2-phenylstearic acid ($\text{CH}_3 \cdot (\text{CH}_2)_{15} \cdot \text{CH}(\text{C}_6\text{H}_5) \cdot \text{COOH}$) was injected into rabbits, Tamemasa⁸¹ demonstrated the presence of α -phenyladipic acid, $\text{HOOC} \cdot (\text{CH}_2)_3 \cdot \text{CH}(\text{C}_6\text{H}_5) \cdot \text{COOH}$, which presumably originated by ω -oxidation and six β -oxidations of the 2-phenylstearic acid. In another series of tests in which ethyl-2-phenylstearic acid was injected into rabbits, Tamemasa⁸² confirmed the fact that 2-phenyladipic acid was excreted. However, a small amount of α -phenylglutaric acid, $\text{HOOC} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{C}_6\text{H}_5) \cdot \text{COOH}$, was likewise identified. It was postulated that this might arise by dehydrogenation of the stearic acid molecule at C_9 - C_{10} , followed by oxidative cleavage to α -phenylazelaic acid. The 2-phenylglutaric acid could then originate as a result of 2 β -oxidations.

Flaschenträger *et al.*⁷³ believe that, when β -oxidation is blocked by substitution in either the α - or the β -position, ω -oxidation occurs as an emergency mechanism for fatty acid oxidation. These workers are of the opinion that this method of disposal of the fatty acid may be called into play when no other pathway of oxidation is available, or when normal processes are over-taxed by the administration of excessively large doses of normal fats. Carter⁸³ suggests that the emergency theory of Flaschenträger *et al.*⁷³ explains the striking cessation of oxidation which is known to occur above lauric acid. Fatty acids having less than twelve carbon atoms cannot be stored in the animal body, and hence must be oxidized as they

⁷⁷ R. Kuhn, F. Köhler, and L. Köhler, *Z. physiol. Chem.*, **242**, 171-197 (1936); **247**, 197-219 (1937).

⁷⁸ R. Kuhn and I. Löw, *Z. physiol. Chem.*, **259**, 182-193 (1939).

⁷⁹ M. Asano and T. Yamakawa, *J. Biochem. (Japan)*, **37**, 329-338 (1950).

⁸⁰ T. Yamakawa, *J. Biochem. (Japan)*, **37**, 343-353 (1950).

⁸¹ O. Tamemasa, *J. Pharm. Soc. Japan*, **71**, 1015-1018 (1951); *Chem. Abst.*, **46**, 2149 (1952).

⁸² O. Tamemasa, *J. Pharm. Soc. Japan*, **71**, 1019-1024 (1951); *Chem. Abst.*, **46**, 2149 (1952).

⁸³ H. E. Carter, *Biol. Symposia*, **5**, 47-63 (1941).

are absorbed. When they are administered in large doses, the normal processes of β -oxidation are exceeded, and ω -oxidation comes into play as a supplementary mechanism to rid the body of the fatty acids which cannot be stored. Since lauric acid and higher acids can be partially deposited in the tissues, two pathways of disposal are possible (β -oxidation or storage), and there is less requirement for ω -oxidation. This interpretation has been supported by the results of Stevens⁸⁴ in Carter's group. It was found that β -phenyl acids from C_5 to C_7 were excreted unchanged, for the most part, but that those from C_8 to C_{12} were excreted only to the extent of about 20% as dienoic acids. The diacidogenic property of lauric acid was markedly increased by substitution of the phenyl group in the β -position. This prevents storage, and thus necessitates increased emergency oxidation in the form of ω -oxidation.

e. Miscellaneous Types of Oxidation. In addition to β - and ω -oxidation, certain other types of oxidation have been suggested to explain the catabolism of fatty acids in certain specific cases. Thus, Clutterbuck and Raper⁸⁵ showed that γ - and δ -oxidation, as well as β -oxidation, could occur *in vitro* when the ammonium salts of the longer-chain fatty acids were oxidized with H_2O_2 . There was no indication that a similar pattern might obtain *in vivo*. In later work, these investigators⁸⁶ were unable to observe any excretion of phenylsuccinic acid, $HOOC \cdot CH(C_6H_5) \cdot CH_2 \cdot COOH$, after the subcutaneous administration of phenylhexanoic acid, $HOOC \cdot CH(C_6H_5) \cdot (CH_2)_4 \cdot COOH$, to dogs. The former compound should have originated had γ -oxidation taken place.

f. Desaturation Theory as a Method of Fatty Acid Oxidation. A number of years ago, Leathes and collaborators⁸⁷⁻⁸⁹ advanced the hypothesis that the first step in the oxidation of fatty acids involves their desaturation in the liver. These unsaturated fatty acids were shown to occur not only in the phospholipid fraction but also in the neutral fat. Two main types of dehydrogenation of fatty acids take place in the animal body; namely, α, β -dehydrogenation and 9,10-dehydrogenation. The relationship between dehydrogenation and oxidation of fatty acids has been reviewed by Breusch.¹

⁸⁴ C. M. Stevens, Thesis, Univ. Illinois, 1941; cited by H. E. Carter, *Biol. Symposia*, 5, 47-63 (1941), p. 60.

⁸⁵ P. W. Clutterbuck and H. S. Raper, *Biochem. J.*, 19, 385-396 (1925).

⁸⁶ P. W. Clutterbuck and H. S. Raper, *Biochem. J.*, 19, 911-914 (1925).

⁸⁷ J. B. Leathes and L. Meyer-Wedell, *J. Physiol.*, 38, xxxvii-xl (1909).

⁸⁸ E. L. Kennaway and J. B. Leathes, *Lancet*, 176, 95-98 (1909, I); *Proc. Roy. Soc. Med.*, 2, Part III, *Pathol. Sect.*, 136-144 (1909).

⁸⁹ J. B. Leathes and H. S. Raper, *The Fats*, 2nd ed., Longmans Green, New York, 1925.

(a) α, β -Dehydrogenation. Quagliariello^{90,91} advanced the hypothesis that α, β -dehydrogenation accompanies or precedes the β -oxidation of fatty acids. Thus, a typical fatty acid would be dehydrogenated as follows:¹

This reaction would be analogous to the conversion of succinic acid to fumaric acid.

However, although the dehydrogenation of succinic acid occurs readily in tissues, the corresponding reaction to the α, β -unsaturated acid,⁹² aconitic acid, does not occur in the case of tricarballic acid, although Quastel⁹³ was able to demonstrate some dehydrogenation of tricarballic acid by *Bacillus (Escherichia) coli*.

a'. Detection of α, β -Unsaturated Acids: The occurrence of α, β -unsaturated acids can be proved by virtue of the fact that they react with di-(*p*-dimethylaminophenyl)-ureide to form characteristic yellow ureides;⁹⁴ on the other hand, saturated fatty acids form colorless ureides. This reaction has been employed successfully by Breusch and Ulusoy⁹⁵ for the determination of α, β -unsaturated fatty acids in fats. Even oily fatty acids can be precipitated as crystalline ureides from an ether solution. The reaction is as follows:

The Reaction Involved in the Separation of α, β -unsaturated fatty Acids from a Fatty Acid Mixture with di-(*p*-dimethylaminophenyl)-Ureide, According to the Method of Zetzsche *et al.*⁹⁴

⁹⁰ G. Quagliariello, *Atti accad. nazl. Lincei, Classe sci. fis. mat. e nat.* [6], 16, 387-389, 552-554 (1932).

⁹¹ G. Quagliariello, *Angew. Chem.*, 47, 370 (1934).

b'. Reactions in Which α,β -Dehydrogenation Occurs: A large number of instances may be cited in which α,β -desaturation has been unequivocally proved.

(a') In the Oxidation of β -Methylphenyl Fatty Acids.—Carter *et al.*⁹⁶ have shown that the animal cannot demethylate phenyl- β -methyl fatty acids, and that they are excreted in part as α,β -unsaturated fatty acids. Thus, β -methylphenylbutyric acid is catabolized as follows:

In contradistinction to the dehydrogenation of the β -methyl- γ -phenylbutyric acid, Kuhn and Livada⁹⁷ failed to demonstrate a corresponding reaction in the case of the next lower homologue. Thus, when β -methylphenylpropionic acid was given to rabbits, 30 to 35% was excreted in the urine in free form, while 20 to 25% was coupled with glycine. The metabolism was similar to that of β -methyleinnamic acid in rabbits and dogs, although a larger proportion was excreted in the free form when the unsaturated acid was given.

(b') In the Oxidation of Phenyl Fatty Acids.—Desaturation at the α,β -position can likewise be observed in the case of unsubstituted phenyl fatty acids whose molecule does not contain a side chain methyl. Thus, Mazza⁹⁸ isolated phenylcrotonic acid after incubation of phenylbutyric acid with surviving liver.

(c') In the Removal of Water from β -Hydroxy-Acids.—Snapper and Grünbaum⁹⁹ showed that β -phenyl- β -hydroxypropionic acid is converted to cinnamic acid by the removal of a molecule of water when the kidney is perfused with the hydroxy acid.

⁹² F. L. Breusch, *Science*, **97**, 490–492 (1943).

⁹³ J. H. Quastel, *Biochem. J.*, **20**, 168–194 (1926).

⁹⁴ F. Zetzsche, E. Lüscher, H. E. Meyer, H. Overbeck, and H. Lindlar, *Ber.*, **71**, 1088–1093, 1516–1521, 2095–2102 (1938).

⁹⁵ F. L. Breusch and E. Ulusoy, *Arch. Biochem.*, **11**, 489–498 (1946).

⁹⁶ H. E. Carter, E. Osman, H. Levine, and S. Gamm, *J. Biol. Chem.*, **128**, xiii (1939).

⁹⁷ R. Kuhn and K. Livada, *Z. physiol. Chem.*, **220**, 235–246 (1933).

⁹⁸ F. P. Mazza, *Arch. sci. biol. (Italy)*, **21**, 320–331 (1935).

⁹⁹ I. Snapper and A. Grünbaum, *Chinese J. Physiol.*, **15**, 301–308 (1940).

The Desaturation of β -Phenyl- β -Hydroxypropionic Acid by Removal of One Molecule of Water⁹⁹

Although similar dehydrogenations have not been reported in the non-phenylated hydroxy fatty acids, the reaction is believed to occur in these latter acids as well. The formation of α -imino acids from the corresponding α -amino acids is a common example of α,β -dehydrogenation in another group of compounds.

(d') In the Metabolism of α,β -Dideutero-Acids.—The data of Morehouse¹⁰⁰ also support the hypothesis that α,β -desaturation occurs. Thus, when α,β -dideuterobutyric acid was fed to rats, the β -hydroxybutyrate excreted contained only 4% of the original deuterium. However, when Morehouse¹⁰⁰ fed β,γ -dideuterobutyrate, she was able to obtain five times the amount of deuterium from the β -hydroxybutyrate in the urine that was present when the α,β -acid was fed. Although this experiment may be interpreted as indication of a preliminary α,β -dehydrogenation, Breusch¹ suggests that β -ketobutyric acid might be the first product formed. The enol of this form might readily exchange its deuterium in the α -position with protons of ordinary water.¹ Deuterium-free β -hydroxybutyric acid would be formed as the second step, as indicated below:

The reaction with β,γ -dideuterobutyric acid is pictured as follows:¹

Although the data of Morehouse¹⁰⁰ are not a definite proof of α,β -dehydrogenation, they do demonstrate that the γ -position is attacked very much less readily than is the α - or β -position; this is in line with the β -oxidation theory.

c'. The Behavior of α,β -Unsaturated Acids: Crotonic acid, $\text{CH}_3 \cdot \text{CH} : \text{CH} \cdot \text{COOH}$, the simplest even-carbon member of the α,β -unsaturated acids, is metabolized in a manner similar to that of the corresponding saturated acid, namely butyric acid. Thus, Blunden¹⁰¹ reported that the quantity of

¹⁰⁰ M. G. Morehouse, *J. Biol. Chem.*, 129, 769-779 (1939).

¹⁰¹ H. D. Blunden, *The Intermediary Metabolism of Butyric Acid as Determined from the Physiological Behavior of the Various Four-Carbon Acids*, Dissertation, Univ. Southern California, 1938.

ketone bodies and the qualitative distribution between the acetoacetate and β -hydroxybutyrate fractions in the urine of fasting rats were identical, irrespective of whether butyric acid or an isomolecular amount of crotonic acid was fed as their sodium salts. Crotonic acid was shown to yield a slightly greater amount of ketone bodies than does butyric acid, with liver slices,¹⁰² or when oxidized by the cyclophorase liver system of Grafflin and Green.¹⁰³ It has been reported that α,β -hexenoic acid ($\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}:\text{CH}\cdot\text{COOH}$)¹⁴ and sorbic acid ($\text{CH}_3\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$)²² produce the same amounts of ketone bodies as does caproic acid when fed to fasting animals. In addition, Cohen¹⁰² demonstrated that sorbic acid produces ketone bodies with liver slices, while Grafflin and Green¹⁰³ found that sorbic acid is metabolized by the kidney cyclophorase system, and that *trans*- Δ,α,β -hexenoic acid and sorbic acid are metabolized by rabbit liver preparations in a manner similar to the effect of caproic acid. The metabolism of α,β -pentenoic acid ($\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}:\text{CH}\cdot\text{COOH}$), *cis*- and *trans*- α,β -hexenoic acid and sorbic acid were all found to give rise to considerable amounts of acetoacetate.

Jowett and Quastel³⁹ are of the opinion that crotonic acid is not an intermediate of butyric acid oxidation, although it is also convertible to ketone bodies. On the other hand, Cohen¹⁰² suggested that a definite chemical grouping is required for oxidation by the so-called β -oxidase system.

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The skeleton group has the basic structure, $-\text{CH}:\text{CHC}-$, which would fit in well with the theory that dehydrogenation precedes oxidation. Lipmann and Perlmann¹⁰⁴ suggested that an intermediary desaturation may be associated with a phosphorylation, which would result in oxidation according to the following scheme:

Suggested Mechanism by Which Unsaturated Linkages Function in the Oxidation of Fatty Acids¹⁰⁴

Reaction (2) occurs by virtue of the energy-rich phosphate bond which is formed in reaction (1). While it is uncertain to what degree the unsaturated fatty acids play a role in the oxidation of fatty acids, it is certain that the

¹⁰² P. P. Cohen, *J. Biol. Chem.*, 119, 333-346 (1937).

¹⁰³ A. L. Grafflin and D. E. Green, *J. Biol. Chem.*, 176, 95-115 (1948).

¹⁰⁴ F. Lipmann and G. E. Perlmann, *Arch. Biochem.*, 1, 41-50 (1942).

unsaturated fatty acids may be oxidized to the ketone bodies in a manner similar to that of the corresponding saturated acids.

(b) β,γ -Dehydrogenation. Kleinzeller¹⁰⁵ demonstrated that the pathway of metabolism of the simplest β,γ -unsaturated acid, vinylacetic acid ($\text{CH}_2\text{:}-\text{CH}\cdot\text{CH}_2\cdot\text{COOH}$) may be similar to that of butyric acid. Acetoacetate was also shown to arise when vinylacetic acid was oxidized by the liver cyclophorase system.^{103,105} Presumably, this may mean that a shift of the double bond from the β,γ - to the α,β -position occurs as a preliminary to oxidation.

(c) 9,10-Dehydrogenation. There is considerable evidence that the animal possesses the ability to bring about a dehydrogenation at the 9,10-position. Thus, the classical studies of Schoenheimer and Rittenberg,¹⁰⁶ and those of Stetten and Schoenheimer,¹⁰⁷ although not defining the site of dehydrogenation, were generally accepted as being concerned with the formation of double bonds in the 9,10-positions. Lang and Adickes¹⁰⁸ report that oleic acid originates when a fatty acid dehydrase acts on stearic acid, but that no α,β -octadecenoic acid is formed. The dehydrogenase system employed by Champougny and LeBreton¹⁰⁹ likewise produces desaturation at the 9:10 position in the case of octadecanoic acid.

a'. Fatty Acids Effective as Substrates. In practically all cases, monoethenoid acids which occur physiologically have the double bond in the 9,10-position, irrespective of whether they contain only ten carbons or more. This is also true for the poly-unsaturated acids, linoleic and linolenic, but not for arachidonic acid, which is 5,8,11,14-eicosatetraenoic acid.

The fatty acids occurring naturally include 9,10-decenoic acid, $\text{CH}_2\text{:CH}\cdot(\text{CH}_2)_7\cdot\text{COOH}$, first detected in butterfat by Smedley¹¹⁰ and isolated by Bosworth and Brown,¹¹¹ as well as a series of higher homologues demonstrated in butterfat by Hilditch and Longenecker,¹¹² including:

9,10-dodecenoic acid (0.9%), $\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}\text{:CH}\cdot(\text{CH}_2)_7\cdot\text{COOH}$;
 9,10-tetradecenoic acid (1.7%), $\text{CH}_3\cdot(\text{CH}_2)_3\cdot\text{CH}\text{:CH}\cdot(\text{CH}_2)_7\cdot\text{COOH}$;
 9,10-hexadecenoic or palmitoleic acid (3.7%), $\text{CH}_3\cdot(\text{CH}_2)_5\cdot\text{CH}\text{:CH}\cdot(\text{CH}_2)_7\cdot\text{COOH}$;
 9,10-decenoic acid, $\text{CH}_2\text{:CH}(\text{CH}_2)_7\cdot\text{COOH}$,

was present to the extent of 0.4%.

¹⁰⁵ A. Kleinzeller, *Biochem. J.*, **37**, 678-682 (1943).

¹⁰⁶ R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **113**, 505-510 (1936).

¹⁰⁷ De W. Stetten, Jr., and R. Schoenheimer, *J. Biol. Chem.*, **133**, 329-345 (1940).

¹⁰⁸ K. Lang and F. Adickes, *Z. physiol. Chem.*, **262**, 123-127 (1939-1940).

¹⁰⁹ J. Champougny and E. LeBreton, *Compt. rend. soc. biol.*, **141**, 450-453 (1947)

¹¹⁰ I. P. Smedley, *Biochem. J.*, **6**, 451-461 (1912).

¹¹¹ A. W. Bosworth and J. B. Brown, *J. Biol. Chem.*, **103**, 115-134 (1933).

¹¹² T. P. Hilditch and H. E. Longenecker, *J. Biol. Chem.*, **122**, 497-506 (1938).

According to Appel, Böhm, Keil, and Schiller,¹¹³ even the unsaturated acids having thirteen, fifteen, or seventeen carbons, which occur in the fat depots after the feeding of synthetic fats with an odd number of carbon atoms, have their first double bond at the 9,10-position. Although some of the plant oils contain the double bond in another position, Millican and Brown¹¹⁴ report that a majority have their first double bond in this 9,10-location. Closely allied hydroxy-compounds with hydroxyl groups in the 9,10-position include aleuretic acid, $\text{CH}_2\text{OH} \cdot (\text{CH}_2)_5 \cdot \text{CHOH} \cdot \text{CHOH} \cdot (\text{CH}_2)_7 \cdot \text{COOH}$, found in shellac,¹¹⁵⁻¹¹⁷ and 9,10-dihydroxystearic acid, present in castor oil.¹¹⁸⁻¹¹⁹

b'. 9,10-Dehydrogenation as a Normal Pathway of Metabolism: Verkade and van der Lee^{66,120,121} observed that appreciable amounts of sebatic acid were excreted by healthy men after triundecylin had been administered. It is suggested that an intermediate unsaturated compound, namely 10-undecenoic acid ($\text{CH}_2 \cdot \text{CH} \cdot (\text{CH}_2)_8 \cdot \text{COOH}$), is formed presumably from undecanoic acid, which splits at the double bond to yield sebatic acid. In fact, when triundecylenin (containing 10:11 unsaturated undecenoic acid), was fed, sebatic acid was actually obtained in the urine. It was therefore suggested that the double bond may be a point of attack for the splitting of the fatty acids and the subsequent formation of dicarboxylic acids.

On the other hand, if oxidation of the fatty acids were to first involve dehydrogenation at the 9,10-position, with the resultant splitting of the chain, one might expect that oleic acid would split to yield azelaic and nonanoic acids as follows:

However, when triolein was fed in large amounts to man in the form of olive oil, Verkade and van der Lee^{120,121} were unable to isolate azelaic acid from

¹¹³ H. Appel, H. Böhm, W. Keil, and G. Schiller, *Z. physiol. Chem.*, **274**, 186-205 (1942); **282**, 220-244 (1947).

¹¹⁴ R. C. Millican and J. B. Brown, *J. Biol. Chem.*, **154**, 437-450 (1934).

¹¹⁵ W. Nagel, *Ber.*, **60B**, 605-609 (1927).

¹¹⁶ H. Raudnitz, H. Schindler, and F. Petru, *Ber.*, **68**, 1675-1676 (1935).

¹¹⁷ A. Tschirch, *Schweiz. Apoth. Z.*, **60**, 609-611 (1922).

¹¹⁸ A. Eibner and E. Münzing, *Chem. Umschau*, **32**, 166-176 (1925); *Chem. Abst.*, **19**, 3027 (1925).

¹¹⁹ G. King, *J. Chem. Soc.*, **1942**, 387-391.

¹²⁰ P. E. Verkade and J. van der Lee, *Proc. Acad. Sci. Amsterdam, Sect. Sci.*, **37**, No. 9, 590-597 (1934).

¹²¹ P. E. Verkade and J. van der Lee, *Z. physiol. Chem.*, **230**, 207-215 (1934).

the urine. Moreover, the formation of nonanoic acid probably does not occur, inasmuch as it would be expected to form azelaic acid as the result of ω -oxidation, and this has not been observed. Verkade and van der Lee^{120,121} reported similar negative results with erucic acid, insofar, as the non-appearance of nonanoic acid is concerned. In addition, Deuel *et al.*²³ proved that ethyl oleate gives rise to the same proportion of ketone bodies as does stearic acid. This would preclude the splitting of this acid at the 9,10-bond. Bernhard and Gloor¹²² likewise postulate that man and dog cannot oxidize oleic acid at the double bond, thus facilitating the formation of azelaic acid. It was found that when healthy subjects were given labeled D-azelaic acid, the latter was excreted in unchanged form, without variation in the original isotope content. This would not be the case if the dicarboxylic acid were an intermediate in oleic acid oxidation. Moreover, oleyl alcohol was oxidized to the acid by the dog, rather than changed to azelaic acid. It was likewise shown that, when β -oxidation was inhibited by blocking the carboxyl group (formation of diethyl amide), ω -oxidation resulted, rather than a 9,10-splitting. In the case of N-butylamide oleic acid and anilid oleic acid, no metabolic products whatsoever could be isolated from the urine. On the other hand, 9,10-dihydroxystearic acid and 9,10-diketostearic acid, when fed to dogs, were found to be oxidized to azelaic acid.

Breusch¹ is of the opinion that dehydrogenation in the 9,10-position is probably not involved in the enzymic degradation of fatty acids. This dehydrogenation is regarded as a mechanism for the control of the physical consistency of the body fats. It is suggested that dehydrogenation may have the function of rendering the fatty acids more liquid and diffusible, and therefore more readily absorbable; sodium oleate is diffused with less difficulty than is sodium stearate. The presence of unsaturated fatty acids renders fats more digestible in the presence of high concentrations of dietary calcium than in the case of more highly saturated fats.¹²³

c'. Oxidation of 9,10-Unsaturated Fatty Acids with Triple Bonds: In contradistinction to the fact that the doubly-bonded fatty acids are not split at their unsaturated linkages, compounds possessing triple bonds are readily decomposed at the site of their unsaturation. Thus, Bernhard and Gloor¹²⁴ reported that stearolic acid ($\text{CH}_3\cdot(\text{CH}_2)_7\text{CH}:\text{C}\cdot(\text{CH}_2)_7\text{COOH}$), stearoyl alcohol ($\text{CH}_3\cdot(\text{CH}_2)_7\text{C}:\text{C}\cdot(\text{CH}_2)_7\text{CH}_2\text{OH}$), behenolic acid ($\text{CH}_3\cdot(\text{CH}_2)_7\text{C}:\text{C}\cdot(\text{CH}_2)_{11}\text{COOH}$), and undecynoic acid ($\text{CH}_3\text{C}:\text{C}\cdot(\text{CH}_2)_7\text{--}$

¹²² K. Bernhard and U. Gloor, *Helv. Chim. Acta*, **35**, 608-616 (1952).

¹²³ A. L. S. Cheng, M. G. Morehouse, and H. J. Deuel, Jr., *J. Nutrition*, **37**, 237-250 (1949).

¹²⁴ K. Bernhard and U. Gloor, *Helv. Chim. Acta*, **36**, 296-299 (1953).

COOH) labeled with deuterium, gave rise to labeled azelaic acid in the case of dogs. This dicarboxylic acid could be isolated from the urine, proving that a biologic splitting occurred at the triple bond of the fatty acid.

g. Hydrogenation of Unsaturated Fatty Acids. The liver has the capacity to alter fats by saturating them, as well as by bringing about the reverse change. Probably the same enzymes are concerned with the hydrogenation as are necessary to effect the dehydrogenation.

(a) *Hydrogenation of α,β -Unsaturated Compounds.* Linneweh¹²⁵ was the first to observe that γ -crotonobetaine could be saturated to yield the corresponding butyrobetaine.

Kuhn, Köhler, and Köhler⁷⁷ reported that the hydrogenation of the ω,β -double bond of geranic acid occurs together with ω -oxidation when this compound is metabolized.

Illustration of the Metabolism of Geranic Acid (I), with the Formation of Hildebrandt Acid (II) by ω -Oxidation Alone, or of Dihydro-Hildebrandt Acid (III) by ω -Oxidation Together with α,β -Hydrogenation⁷⁷

Fischer¹²⁶ observed that hydrogenation is brought about by yeast acting on unsaturated aldehydes and alcohols, but only when the double bond is in the α,β -position.¹²⁷ Fischer and Bielig¹²⁷ demonstrated that α,β -saturation may be effected in animals, but only under certain conditions. Thus, when cinnamyl alcohol, $\text{C}_6\text{H}_5\cdot\text{CH}:\text{CH}\cdot\text{CH}_2\text{OH}$, was fed to dogs, the following changes were observed: α,β -hydrogenation alone resulting in the formation of β -phenylpropanol, $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\text{OH}$ and, on the addition of water, in the formation of β -hydroxy- β -phenylpropanol, $\text{C}_6\text{H}_5\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{CH}_2\text{OH}$. These end-products were present to the extent of only 3%; β -hydroxy- β -phenylpropanol might have arisen from β -oxidation of β -

¹²⁵ W. Linneweh, *Z. physiol. Chem.*, 181, 54-57 (1929).

¹²⁶ F. G. Fischer, *Angew. Chem.*, 53, 461-471 (1940).

¹²⁷ F. G. Fischer and H. J. Bielig, *Z. physiol. Chem.*, 266, 73-98 (1940).

phenylpropanol, but this appears unlikely. The greatest proportion (60%) of the cinnamyl alcohol was oxidized only at the alcohol group, to form α,β -dehydrophenylpropionic acid, $\text{C}_6\text{H}_5\cdot\text{CH}:\text{CH}\cdot\text{COOH}$, without α,β -hydrogenation or with the addition of hydrogen to the double bonds to form β -phenylpropionic acid, $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$.

However, when an ethyl group was present in the cinnamyl alcohol, no α,β -saturation occurred, but only the oxidation of the alcohol group to the unsaturated acid.

Fischer and Bielig¹²⁷ likewise demonstrated that the feeding of ketones containing α,β -unsaturated bonds, such as $\text{C}_6\text{H}_5\cdot\text{CH}:\text{CH}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CH}_3$, may result only in the production of a saturated ketone, $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CH}_3$, or in the formation of a saturated secondary alcohol, $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{CH}_3$. It was likewise observed that a certain amount of oxidation occurred on the benzene ring, since $p\text{-HO}\cdot\text{C}_6\text{H}_4\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CH}_3$ was obtained.

(b) *Hydrogenation of 9,10-Unsaturated Compounds.* A number of workers¹²⁸⁻¹³² have demonstrated that unsaturated dietary fats may be partially hydrogenated before they are deposited in the animal body. Since most of these fats have fatty acids with unsaturation in the 9,10-position, it is evident that the hydrogenation involves the 9,10-linkage. Hilditch and Pedelty¹³³ reported that oleoglycerides may be transformed to stearglycerides by enzymic hydrogenation. Zeller and Maschek¹³⁴ isolated an enzyme from pumpkin seedlings which has the capacity to bring about the saturation of unsaturated acids.

(2) *Enzyme Systems Involved in Fat Oxidation*

Several oxidation systems for fatty acids have been widely investigated, and considerable information is available as to the mechanism of their action. One of these systems, which acts on saturated and unsaturated fatty acids, is the so-called fatty acid oxidase system. The second enzyme sys-

¹²⁸ T. P. Hilditch, *The Chemical Constitution of Natural Fats*, 2nd ed., Wiley, New York, 1947.

¹²⁹ D. Rittenberg and R. Schoenheimer, *J. Biol. Chem.*, **117**, 485-490 (1937).

¹³⁰ J. A. Lovern, *Biochem. J.*, **30**, 2023-2026 (1936).

¹³¹ A. Banks and T. P. Hilditch, *Biochem. J.*, **26**, 298-308 (1932).

¹³² T. P. Hilditch and H. E. Longenecker, *Biochem. J.*, **31**, 1805-1819 (1937).

¹³³ T. P. Hilditch and W. H. Pedelty, *Biochem. J.*, **34**, 971-979 (1940).

¹³⁴ A. Zeller and F. Maschek, *Biochem. Z.*, **312**, 354-369 (1942).

tem, which requires a fatty acid with at least one double bond as a substrate, and which catalyzes the reaction at a greatly augmented rate with a diethenoid acid, is called lipoxidase. In addition to the oxidase systems, the closely related dehydrogenases are likewise important in the oxidation reaction. Phillips and Williams¹³⁵ reported the presence of an oxidative system in chicken fat.

a. Fatty Acid Oxidase Systems. (*a*) *Fatty Acid Oxidase of Lehninger.* Although the oxidation of fatty acids by the liver tissue of the intact animal, and even by isolated liver slices, has been reported many times, Muñoz and Leloir³⁵ were the first to demonstrate the oxidation of fatty acids by cell-free preparations of the liver. These workers found that, when butyrate was incubated with particulate matter from guinea pig liver, to which Mg^{++} , cytochrome-*c*, orthophosphate, adenylic acid and an intermediate from the Krebs tricarboxylic acid cycle were added, an oxidation with molecular oxygen occurred. The enzyme system was extremely labile, and in only a few cases could oxidation of higher acids, such as octanoic acid, be demonstrated. The ketone bodies were the principal oxidation products.

In 1944, Lehninger^{136,137} first demonstrated independently that saturated fatty acids from C_4 to C_{18} could be oxidized by homogenates of rat liver supplemented with cytochrome-*c*, in calcium-free saline. This preparation proved to be much more stable than that of Muñoz and Leloir.³⁵ Shortly after the discovery of the oxidation system, Lehninger¹³⁸ demonstrated that the activity resided in an easily-sedimented particulate fraction of the homogenate. After separation and washing, this fraction oxidized fatty acids without significant endogenous oxygen uptake, provided that Mg^{++} , ATP, and orthophosphate were present. This fraction brought about a practically complete oxidation of fatty acids to ketone bodies, but the system did not have the capacity to oxidize the latter compounds.¹³⁹ Lehninger^{139,140} made the important observation that, if oxaloacetate, fumarate, or malonate was added to the incubation mixture, the yield of acetoacetate was decreased, and some of the fatty acid carbon became incorporated into citrate, α -ketoglutarate, and succinate. On the basis of proof obtained in studies with isotopically-labeled fatty acids that fatty acids are broken down to two-carbon fragments,¹⁴¹ it was suggested that the ac-

¹³⁵ H. J. Phillips and I. L. Williams, *Science*, **117**, 658-659 (1953).

¹³⁶ A. L. Lehninger, *J. Biol. Chem.*, **154**, 309-310 (1944).

¹³⁷ A. L. Lehninger, *J. Biol. Chem.*, **157**, 363-381 (1945).

¹³⁸ A. L. Lehninger, *J. Biol. Chem.*, **161**, 437-451 (1945).

¹³⁹ A. L. Lehninger, *J. Biol. Chem.*, **164**, 291-306 (1946).

¹⁴⁰ A. L. Lehninger, *J. Biol. Chem.*, **161**, 413-414 (1945).

¹⁴¹ S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **155**, 143-151 (1949).

tive intermediate in acetoacetate formation and in citrate synthesis is a two-carbon compound.¹³⁹ Lehninger³ later postulated that the two-carbon unit in question is acetyl-CoA as isolated by Lynen *et al.*¹⁴² In outlining the requirements of the fatty acid oxidase system from mitochondria, Lehninger¹⁴³ recorded, in 1952, that Mg^{++} , ATP, an optimal concentration of electrolytes or of non-electrolytes such as sucrose, and a priming metabolite, possibly a Krebs citric acid cycle intermediate, which is necessary for producing oxidative phosphorylation, are the essential components. Lehninger suggests that an asymmetric acetoacetate may be formed from the fatty acid, probably due to the formation of more than one molecular species of active acetyl from the fatty acid chain. Lehninger³ reviewed the subject of enzymic oxidation of fatty acids through 1951, and Lynen¹⁴⁴ summarized the role of CoA in fatty acid oxidation.

a'. Conditions Affecting Stability: Although the system described by Lehninger was considerably more stable than that of Muñoz and Leloir³⁵ it was destroyed when attempts were made to purify it further. It was stable for some hours at 0°C., but decomposed within twenty minutes when incubated in the absence of substrate and ATP. The optimum pH was found to be 7.4. Lehninger³ noted that oxidation of fatty acids by this enzyme was strongly inhibited by fluoride, calcium ions, 2,4-dinitrophenol, methylene blue, arsenate, arsenite, as well as by certain phenyl-substituted fatty acids. High concentrations of fatty acids, especially of the long-chain members, were shown to inhibit the activity of the enzyme, presumably by their surface effects.^{3,138} Optimum concentrations of acids in the substrate were as follows: butyrate, 0.01 *M*; octanoate, 0.001 *M*; and palmitate, 0.00025 *M*.

b'. The Nature of the Particulate Matter: Potter¹⁴⁵ was of the opinion that the activity of the fatty acid oxidase system was dependent upon the presence of unbroken cells in the particulate preparations, especially because of the sensitivity of the system to changes of tonicity.¹⁴⁶ However, Lehninger³ did report that the oxidase system could withstand exposure of the particles to distilled water for short periods, but it was necessary to restore approximate isotonicity to obtain oxidation. Moreover, no intact liver cells could be observed microscopically.

In later work, the liver homogenates were fractionated according to the

¹⁴² F. Lynen, E. Reichert, and L. Rueff, *Ann.*, 574, 1-32 (1951).

¹⁴³ A. L. Lehninger, *Biochem. J.*, 51, xv-xvi (1952).

¹⁴⁴ F. Lynen, *Harvey Lectures*, 48, 210-244 (1952-1953).

¹⁴⁵ V. R. Potter, *J. Biol. Chem.*, 163, 437-446 (1946).

¹⁴⁶ A. L. Lehninger and E. P. Kennedy, *J. Biol. Chem.*, 173, 753-771 (1948).

new ultracentrifugal technic¹⁴⁷ into cell nuclei, mitochondria, microsomes, and soluble cytoplasm. In these tests, Kennedy and Lehninger^{148,149} proved that the fatty acid oxidase system was localized in the mitochondrial fraction; the activity of the saline-washed preparations used earlier was attributed to their content of agglutinated mitochondria. The activity of mitochondria in fatty acid oxidation was confirmed by Schneider.¹⁵⁰ It is now known that isolated mitochondria can catalyze all reactions of the Krebs cycle, and also all necessary phosphorylations. Lehninger³ was unable to dissociate oxidase activity from native mitochondrial structure.

c'. The Requirement for a "Primer" or "Sparker." Even in the early work of Muñoz and Leloir³⁵ in 1943-1944, it was evident that some "priming" or "sparking" material was necessary to permit the oxidation to proceed. At first, Lehninger^{137,138} was of the opinion that it was necessary only to generate ATP from adenylic acid by phosphorylations which were coupled with the oxidations in the Krebs cycle. However, Lehninger³ proved that a small quantity of "primer" was required in addition to ATP. Cross *et al.*¹⁵¹ suggested that the function of the "primer" is to cause oxidative phosphorylation, since substances which bring about the reverse reaction inhibit fatty acid oxidation. Kennedy and Lehninger¹⁵² later reported that fatty acid oxidation in suspensions of rat liver mitochondria could be activated not only by intermediates of the Krebs cycle but also by the oxidation of relatively high concentrations of the reduced diphosphopyridine nucleotide (DPNH₂), in which coupled oxidative phosphorylation is known to occur. The oxidation of fatty acids ceased when DPNH₂ was completely oxidized, but it could be prolonged if high concentrations of adenine nucleotides were also present. When DPNH₂ was used as the priming agent, octanoate was quantitatively oxidized to two acetoacetate molecules, while palmitate was converted to four acetoacetate molecules. When succinate was employed as the activating agent, less acetoacetate was formed, and part of it was completely oxidized *via* the Krebs cycle.

In the more recent work of Judah and Rees,¹⁵³ it was demonstrated that variations occur in the activation agent in the case of liver mitochondria

¹⁴⁷ G. H. Hogeboom, W. C. Schneider, and G. E. Pallade, *J. Biol. Chem.*, **172**, 619-635 (1948).

¹⁴⁸ E. P. Kennedy and A. L. Lehninger, *J. Biol. Chem.*, **172**, 847-848 (1948).

¹⁴⁹ E. P. Kennedy and A. L. Lehninger, *J. Biol. Chem.*, **179**, 957-972 (1949).

¹⁵⁰ W. C. Schneider, *J. Biol. Chem.*, **176**, 259-266 (1948).

¹⁵¹ R. J. Cross, J. V. Taggart, G. A. Covo, and D. E. Green, *J. Biol. Chem.*, **177**, 655-678 (1949).

¹⁵² E. P. Kennedy and A. L. Lehninger, *J. Biol. Chem.*, **190**, 361-368 (1951).

¹⁵³ J. D. Judah and K. R. Rees, *Biochem. J.*, **55**, 664-668 (1953).

from different animals. In the rat, activation of the octanoate oxidation by liver mitochondria was brought about by ATP or by a system generating ATP. However, when kidney and liver mitochondria obtained from the rabbit were used, the oxidation of octanoate could not be sparked by ATP unless the ω -oxidation of an intermediate of the citric acid cycle occurred concomitantly. The action of 2,4-dinitrophenol (DNP) in inhibiting fatty acid oxidation was shown to be due to its action in preventing ATP synthesis. The action could be overcome by using an excess of ATP. Appel and Kiese¹⁵⁴ confirmed the fact that the sparking effect of the citric acid cycle is necessary to enable the Lehninger enzyme system¹³⁷ to function in the anaerobic decomposition of caprylic acid. These workers demonstrated the decomposition of one out of ten micromoles of the acid when ten to twenty micromoles of methylene blue were also added to the system along with the enzyme.

d'. Oxidative Phosphorylation and Fatty Acid Oxidation: Oxidation of fatty acids has been shown to be accompanied by an oxidative phosphorylation. Johnson and Lardy¹⁵⁵ reported that the oxidation of acetate, butyrate, caprylate, and crotonate by washed residue of rabbit kidney homogenate in the absence of fluoride is accompanied by a net uptake of orthophosphate. P:O ratios of 1.0 to 1.7 were noted. In contradistinction to these results, the P:O ratios were approximately 3.0 for pyruvate, acetoacetate, and β -hydroxybutyrate, in the presence of fluoride, and 1.0 to 1.5 in its absence. Fluoride inhibits fatty acid oxidation at some step prior to the formation of the β -keto-acid and subsequent to the oxidation of the unsaturated acid. Gillette and Kalnitsky¹⁵⁶ suggest that fluoroacetate inhibits oxidation at the point of primary oxidation of the fatty acid to the 2-C intermediate. It is believed that this is accomplished by removing Mg^{++} , since it was shown that Mg^{++} and sodium fluoroacetate can form a stable complex. The formation of fluorocitrate is likewise suggested as a possible cause of inhibition. This type of inhibition can be overcome by the addition of an excess of Mg^{++} .

(b) *Cyclophorase Preparations.* Green and co-workers¹⁵⁷ referred to their preparations of the well-washed, easily sedimented particulate matter of liver and kidney, which is insoluble in approximately isotonic KCl solutions, as "cyclophorase." Their material was shown to bring about a

¹⁵⁴ W. Appel and M. Kiese, *Biochem. Z.*, **322**, 156-164 (1951).

¹⁵⁵ R. B. Johnson and H. A. Lardy, *J. Biol. Chem.*, **184**, 235-242 (1950).

¹⁵⁶ J. R. Gillette and G. Kalnitsky, *J. Biol. Chem.*, **187**, 679-686 (1950).

¹⁵⁷ D. E. Green, W. F. Loomis, and V. H. Auerbach, *J. Biol. Chem.*, **172**, 389-403 (1948).

quantitative conversion of pyruvate to CO_2 and H_2O *via* the tricarboxylic acid cycle. In a subsequent study, Grafflin and Green¹⁰³ reported the complete oxidation of a series of saturated straight-chain acids, of some β -hydroxy- and β -keto-acids, and also of some unsaturated acids, by cyclophorase, in the presence of intermediates of the tricarboxylic acid cycle. On the other hand, certain α - and β -substituted acids were not attacked; γ -phenyl acids were found to be inhibitors of fatty acid oxidation, although they were slowly oxidized by the cyclophorase system.

Lehninger³ is of the opinion that the differences between his oxidase system and the cyclophorase system are only superficial, and are related to the different conditions under which the studies were carried out. One variation is the yield of acetoacetate, which is much lower in the cyclophorase preparation than in that of Lehninger. This results from the larger proportion of fatty acid which is completely oxidized in the former case as the result of the presence of a greater amount of tricarboxylic acid intermediates. In addition, cyclophorase is more sensitive to malonate than is the Lehninger preparation. Lehninger³ states that this sensitivity is related not only to the proportion of fatty acid passing through the tricarboxylic acid cycle, but also to the "sparking" phenomenon. The older the preparation, the more "sparker" is required, and the greater the capacity of malonate to effect an inhibition. Knox and associates¹⁵⁸ and Grafflin and Green¹⁰³ reported that the "sparking" phenomenon could be brought about anaerobically by use of ferricyanide as a hydrogen acceptor.

(c) *Fatty Acid Oxidase of Drysdale and Lardy.* Drysdale and Lardy¹⁵⁹ described an enzyme system which oxidized fatty acids in the presence of an artificial electron acceptor. The enzyme system was prepared by the extraction of an acetone-dried powder of rat liver mitochondria with water. The extract consists of soluble proteins which are not sedimented when centrifuged at $120,000 \times g$. for three hours. ATP is required for activation; one mole of ATP permits the oxidation of one mole of fatty acid. CoA is likewise needed, in addition to ATP. When fatty acids labeled with C^{14} in the carboxyl group were oxidized with this enzyme system, the resulting acetoacetate was found to be labeled on both the carboxyl and the carbonyl groups. This fact indicates that the oxidase functions by splitting off two-carbon fragments from the fatty acids; they are then recombined to yield acetoacetate. When oxaloacetate is present, the fatty acids are quantitatively converted to citrate.

¹⁵⁸ W. E. Knox, B. N. Noyce, and V. H. Auerbach, *J. Biol. Chem.*, **176**, 117-122 (1948).

¹⁵⁹ G. R. Drysdale and H. A. Lardy, *J. Biol. Chem.*, **202**, 119-136 (1953).

(d) *Fatty Acid Oxidase of Kalnitsky and Barron.* Kalnitsky and Barron¹⁶⁰ prepared a fatty acid oxidase system from rabbit kidney which strongly oxidized a number of fatty acids, including acetic, butyric, β -hydroxybutyric, crotonic, vinylacetic, α -ketovaleric, methylethylacetic ($\text{CH}_3\text{-CH-(C}_2\text{H}_5\text{)-COOH}$), caproic and α -ketocaproic acids. On the other hand, formic, propionic, and α -hydroxybutyric and α -ketobutyric acids were oxidized slowly. The oxidase lost its potency in five hours, except toward caproic and α -ketocaproic acids. Fluoroacetate and fluorobutyrate inhibited the oxidation of most of the acids.

(e) *Fatty Acid Oxidation According to Green and Mii.* Green and Mii,¹⁶¹ and others,¹⁶²⁻¹⁷⁰ have outlined the present theories (1954) of fatty acid oxidation in animal tissues, in terms of the enzymes required and the reactions catalyzed, as described below:

a'. *Activation Enzymes.* These catalyze the formation of acyl-CoA from the free fatty acid, with the concomitant change of ATP to AMP (adenosine monophosphate), and the formation of pyrophosphate. Mahler, Wakil, and Bock¹⁷¹ prepared a highly purified form of the agent which activates fatty acids (FAAE), from beef liver. This agent catalyzes the formation of acyl CoA from a wide variety of fatty acids in the presence of CoA and ATP. At least three different enzymes act on the acyl CoA compounds with various specificity ranges covering the gamut from C_4 to C_{18} .

(a') *Butyryl Coenzyme A Dehydrogenase.*—Green, Mii, and Mahler¹⁶² isolated and purified the enzyme which mediates the first step in the oxidation of the lower fatty acids; this acts at a maximal rate on butyryl CoA, and hence the name butyryl CoA dehydrogenase is proposed for the protein. Mahler¹⁶³ reported that this enzyme is a cuproflavoprotein containing 1.2% (by weight) of riboflavin, and 0.345% of copper. The copper:flavin ratio was found to equal 2. The flavin part of the prosthetic group has been shown to be identical with flavin-adenine-dinucleotide (FAD). Mahler¹⁶³ prepared a copper-free enzyme by dialysis against cyanide which was still

¹⁶⁰ G. Kalnitsky and E. S. G. Barron, *Arch. Biochem.*, **19**, 75-87 (1948).

¹⁶¹ D. E. Green and S. Mii, *Federation Proc.*, **12**, 211 (1953).

¹⁶² D. E. Green, S. Mii, and H. R. Mahler, *J. Biol. Chem.*, **206**, 1-12 (1954).

¹⁶³ H. R. Mahler, *J. Biol. Chem.*, **206**, 13-26 (1954).

¹⁶⁴ S. J. Wakil and H. R. Mahler, *J. Biol. Chem.*, **207**, 125-132 (1954).

¹⁶⁵ S. J. Wakil, D. E. Green, S. Mii, and H. R. Mahler, *J. Biol. Chem.*, **207**, 631-638 (1954).

¹⁶⁶ H. Beinert, *J. Biol. Chem.*, **205**, 575-584 (1953).

¹⁶⁷ S. Wakil and H. R. Mahler, *Federation Proc.*, **12**, 285 (1953).

¹⁶⁸ D. S. Goldman, *J. Biol. Chem.*, **208**, 345-357 (1954).

¹⁶⁹ P. Hele, *J. Biol. Chem.*, **206**, 671-676 (1954).

¹⁷⁰ A. Millerd and J. Bonner, *Arch. Biochem. Biophys.*, **49**, 343-355 (1954).

¹⁷¹ H. R. Mahler, S. J. Wakil, and R. M. Bock, *J. Biol. Chem.*, **204**, 453-468 (1953).

actively reduced by butyryl-CoA, although the activity was enhanced by the presence of the Cu ion.

(b') Unsaturated Fatty Acyl-Coenzyme A Hydrase.—Wakil and Mahler¹⁶⁴ were the first to isolate and to purify a hydrating enzyme from beef liver mitochondria which had a specific action on unsaturated acyl derivatives of CoA. The preparation was shown to catalyze the hydration of α,β -, and also of β,γ -unsaturated acyl-CoA derivatives. The enzyme is specific for the *trans* isomer; at equilibrium, the ratio of β -hydroxy to total unsaturated acyl-CoA derivative equals 1.4 ± 0.2 . The hydrase is activated with reduced glutathione (GSH) and cysteine; it is inhibited by *p*-chloromercuribenzoate, iodoacetamide, and iodosobenzoate.

(c') β -Hydroxyacyl-Coenzyme A Dehydrogenase.—Wakil and collaborators¹⁶⁵ likewise isolated and partially purified a second enzyme from beef liver mitochondria which catalyzes the oxidation of β -hydroxyacyl-CoA derivatives in the presence of diphosphopyridine nucleotide (DPN), which serves as an electron acceptor. The enzyme was found to act on all β -hydroxyacyl-CoA derivatives from C₄ to C₁₂; it was optically specific for the product of the unsaturated acyl-CoA hydrase. *i.e.*, *d*- β -hydroxyacyl-CoA. The optimal pH was found to be 10.0. At this pH the reaction proceeded completely from left to right, while at a lower value the equilibrium could be shifted in favor of the β -keto-acyl-CoA formation by the use of the Mg ion as a complexing agent.

b'. Fatty Acid Oxidasas: These enzymes catalyze the oxidation of acyl-CoA to β -ketoacyl-CoA, with DPN as the primary external electron acceptor.¹⁶¹ Beinert¹⁶⁶ was able to prepare acetoacetyl-CoA ($\text{CH}_3\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CoA}$), β -ketoheptanoyl-CoA ($\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CoA}$), and β -keto-octanoyl-CoA ($\text{CH}_3\cdot(\text{CH}_2)_4\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CoA}$) by enzymatic dehydrogenation of the corresponding β -hydroxy derivatives of CoA. The compounds were identified by determination of the nature of the β -keto acid after alkaline hydrolysis, by enzymatic reactions, and by their spectral properties. The compounds were found to be of the same order of stability as other known acyl derivatives of CoA. They were readily susceptible to enzymatic cleavage in the presence of reduced CoA.

According to Wakil and Mahler,¹⁶⁷ the sparking or activation of fatty acids and their oxidized derivatives before oxidation by fatty acid oxidase systems involves the formation of S-acylated CoA derivatives.

c'. Cleavage Enzymes: These bring about the change of β -ketoacyl-CoA to acetyl-CoA and acyl-CoA. The latter product contains two less carbons than does its parent keto compound.¹⁶¹ Goldman¹⁶⁸ recently reported the fifteen-fold purification of the cleavage enzyme (CE) obtained

from beef liver mitochondria. It is believed that the enzyme acts by the formation of $E\text{Ac}^-$ (acetate enzyme) as an intermediary complex.

As soon as acetoacetyl-CoA is formed, the reaction may be terminated in either of the following ways:¹⁶¹

1. By enzymatic deacylation of acetoacetyl-CoA to acetoacetate.
2. By cleavage of acetoacetyl-CoA to two molecules of acetyl-CoA, followed by their condensation with oxaloacetate.

Thus, according to reaction 1, acetoacetate will be the end-product of fatty acid oxidation while, according to reaction 2, citrate is formed, and the acid may become completely oxidized to CO_2 and H_2O .

(f) *Acetate-Activating Enzyme*. Hele¹⁶⁹ prepared an acetate-activating enzyme (AAE) from beef heart mitochondria. The partially purified enzyme was believed to be associated with a single protein. AAE was found to be very unstable under the conditions of electrophoresis; at higher purity levels, it was found to undergo inactivation after dialysis. Beinert and associates¹⁷² also reported the isolation of an enzyme system from pig and rabbit heart which catalyzes the reversible conversion of acetate and CoA to acetyl CoA, and the simultaneous change of ATP to AMP and inorganic pyrophosphate. Millerd and Bonner¹⁷⁰ described a similar AAE system from the plant kingdom, in this case from spinach. This system may be associated with mitochondria; it has been demonstrated in all plant tissues which have been investigated. The AAE from plants was found to be active with a variety of normal and branched-chain fatty acids, in addition to acetate. Acetoacetate was also formed by the plant acetoacetate cleavage enzyme.

(g) *Aldehyde Dehydrogenase Enzyme*. The association of the oxidation of the fatty acid aldehydes with that of the fatty acids can be predicated by the demonstration that the appropriate enzyme system occurs in nature. Burton and Stadtman¹⁷³ prepared a purified aldehyde dehydrogenase from the anaerobe, *Clostridium kluyveri* which catalyzes the DPN-linked oxidation of acetaldehyde in the presence of CoA to acetyl CoA. This aldehyde dehydrogenase was also shown to effect the oxidation of propionaldehyde, butyraldehyde, and glycolaldehyde to the corresponding acyl CoA derivative.

According to Harting and Velick,¹⁷⁴ glyceraldehyde-3-phosphate de-

¹⁷² H. Beinert, D. E. Green, P. Hele, H. Hift, R. W. von Korff, and C. V. Ramakrishnan, *J. Biol. Chem.*, **203**, 35-45 (1953).

¹⁷³ R. M. Burton and E. R. Stadtman, *J. Biol. Chem.*, **202**, 873-890 (1953).

¹⁷⁴ J. Harting and S. F. Velick, *J. Biol. Chem.*, **207**, 857-865 (1954).

hydrogenase is an enzyme which catalyzes the reversible oxidative phosphorylation of acetaldehyde to acetylphosphate. Inorganic phosphate and diphosphopyridine nucleotide (DPN) participate in the reaction. Aldehyde is also oxidized in the absence of phosphate or arsenate. The same enzyme is capable of producing oxidative phosphorylation of propionaldehyde and of butyraldehyde.

(h) *β -Hydroxybutyrate Dehydrogenase System.* Lehninger and Greville¹⁷⁵ have shown that *L*- β -hydroxybutyrate causes the reduction of DPN, presumably by the already known¹⁷⁶ *L*-specific DPN-linked β -hydroxybutyric dehydrogenase which does not attack the *D*-isomer.¹⁷⁵ The *D*-isomer also causes reduction of DPN, but only if the extracts are supplemented with ATP, CoA, and Mg. Under these conditions, the *D*- β -hydroxybutyrate was shown to be capable of forming a CoA derivative at the expense of ATP. It is suggested that a dehydrogenase, specific for the *D*-isomer, reversibly catalyzes the reduction of *D*- β -hydroxybutyrate CoA, with the formation of acetoacetyl CoA.

(i) *General Considerations in Fatty Acid Oxidase Systems.* The most important fact is the universality of action of the oxidase systems. Thus, Kennedy and Lehninger reported that all of the saturated straight-chain acids from butyrate to stearate were oxidized by isolated mitochondria.¹⁷⁷ Cyclophorase was found to be inactive with acids having more than thirteen carbons.³ This is probably to be ascribed to the technical difficulties of preparing suitable emulsions of these higher fatty acids at a pH of 7.4.

The fatty acid oxidases also oxidize the unsaturated acids. Thus, Kennedy and Lehninger¹⁷⁷ showed that oleic, linoleic, linolenic, and vaccenic acids are all readily oxidized; priming is required in all cases. Autooxidation, however, is only a negligible factor. It is remarkable that even the unnatural *trans* isomer of oleic acid, elaidic acid, is as rapidly oxidized as is oleic acid.¹⁷⁷ Grafflin and Green¹⁰³ reported that both *cis* and *trans* forms of crotonic acid and β , γ -unsaturated acids are oxidized by cyclophorase.

In order that the oxidase system may function, it is necessary that the substrate have a free carboxyl group. Thus, long-chain fatty alcohols, aldehydes, amides, and *N*-acylglycine derivatives are not oxidized. Although fatty acid esters and phospholipids are oxidized, it seems probable that oxidation occurs only after hydrolysis of the ester linkage, since fat oxidase systems contain esterases. However, when the substrate contains two car-

¹⁷⁵ A. L. Lehninger and G. D. Greville, *Biochim. et Biophys. Acta*, **12**, 188-202 (1953).

¹⁷⁶ D. E. Green, J. G. Dewar, and L. F. Leloir, *Biochem. J.*, **31**, 934-949 (1937).

¹⁷⁷ E. P. Kennedy and A. L. Lehninger, *J. Biol. Chem.*, **185**, 275-285 (1950).

boxyl groups, as in the case of the dicarboxylic acids, adipic, glutaric, pimelic, and azelaic, the fatty acid oxidase has been found to be inert.¹⁰³

In addition to liver preparations, which yield a potent fatty acid oxidase,¹³⁸⁻¹⁴⁰ in the presence of ATP,¹⁰³ kidney tissue and heart muscle¹⁷⁸ serve as satisfactory sources of the enzyme. In the latter two tissues, the oxidation proceeds *via* the tricarboxylic acid cycle, and acetoacetate does not accumulate.^{3,178} The presence of fatty acid oxidase in slices of lung, spleen, brain, heart, and skeletal muscle has been demonstrated by Geyer *et al.*¹⁷⁹ Lung and spleen are especially active.³ Fatty acid oxidase preparations of the liver mitochondria in isotonic sucrose were reported to be relatively stable to freezing and storage at a low temperature.³⁶

b. Lipoxidase. (a) *Introduction.* Lipoxidase is an enzyme active primarily in the oxidation of the polyethenoid acids. For a number of years, it was erroneously considered to be a carotene oxidase. Thus, Haas and Bohn¹⁸⁰ demonstrated that pigments could be bleached by an enzyme present in soybeans. The enzyme was shown to destroy vitamin A activity completely.¹⁸¹ Moreover, a similar phenomenon was brought about by the enzymes in alfalfa,^{182,183} which were later found to be lipoxidases.^{184,185}

André and Hou,¹⁸⁶ in 1932, were the first to discover the fat-oxidizing enzyme in soybeans (*Glycine soja*), and to propose the name "lipoxidase." Apparently this is the same enzyme which had previously been referred to as "carotene oxidase." Four years later, Craig¹⁸⁷ reported a similar enzyme system in white lupine (*Lupinus albus* L.). It was shown by Sumner and Dounce¹⁸⁸ that the production of peroxides occurred concomitantly with the action of carotene oxidase. Somewhat later, it was reported^{189,190} that carotene oxidase bleaches carotene only if fat is present. Although Tauber¹⁹⁰ believed that carotene was oxidized by the peroxide formed by the oxidation of unsaturated acids ("unsaturated fat oxidase"), Sumner¹⁹¹

¹⁷⁸ A. Lehninger, *J. Biol. Chem.*, **165**, 131-145 (1946).

¹⁷⁹ R. P. Geyer, L. W. Matthews, and F. J. Stare, *J. Biol. Chem.*, **180**, 1037-1045 (1950).

¹⁸⁰ L. W. Haas and R. M. Bohn, *U. S. Patents Nos.* 1,957,333 to 1,957,337 (May 1, 1934).

¹⁸¹ C. N. Frey, A. S. Schultz, and R. F. Light, *Ind. Eng. Chem.*, **28**, 1254 (1936).

¹⁸² S. M. Hauge and W. Aitkenhead, *J. Biol. Chem.*, **93**, 657-665 (1931).

¹⁸³ S. M. Hauge, *J. Biol. Chem.*, **108**, 331-336 (1935).

¹⁸⁴ H. L. Mitchell and S. M. Hauge, *J. Biol. Chem.*, **163**, 7-14 (1946).

¹⁸⁵ H. L. Mitchell and H. H. King, *J. Biol. Chem.*, **166**, 477-480 (1946).

¹⁸⁶ E. André and K. Hou, *Compt. rend.*, **194**, 645-647 (1932); **195**, 172-174 (1932).

¹⁸⁷ F. N. Craig, *J. Biol. Chem.*, **114**, 727-746 (1936).

¹⁸⁸ J. B. Sumner and A. L. Dounce, *Enzymologia*, **7**, 130-132 (1939).

¹⁸⁹ J. B. Sumner and R. J. Sumner, *J. Biol. Chem.*, **134**, 531-533 (1940).

¹⁹⁰ H. Tauber, *J. Am. Chem. Soc.*, **62**, 2251 (1940).

¹⁹¹ R. J. Sumner, *J. Biol. Chem.*, **146**, 215-218 (1942).

states that the oxygen which reacts with carotene is derived from some intermediate which is produced during the peroxidation of the unsaturated fat. The early work on lipoxidase has been reviewed by Süllman¹⁹² and by Jezeski,¹⁹³ a comprehensive summary is that of Bergström and Holman.¹⁹⁴

(b) *Distribution of Lipoxidase.* Lipoxidase is widely distributed in the plant kingdom. Thus, as has already been reported, lipoxidase occurs in soybeans,¹⁸⁶ white lupine,¹⁸⁷ alfalfa,^{182,183} and potatoes;¹⁹⁵ in addition, lipoxidase has been detected in the seeds of a variety of legumes,¹⁹⁶ in radishes,¹⁹⁵ in soybean meal preparations,¹⁹⁷ and in a number of species of beans and peas.¹⁹⁸ Süllman¹⁹⁹ likewise reported the presence of this enzyme in a number of plant tissues. According to Van Fleet^{200,201} the lipoxidase content is high at the seedling stage in plants grown on an alkaline medium. Lipoxidase is activated in areas in the plant which are neutral or alkaline, where water loss has occurred, and below injured surfaces. The lipoxidase content has been shown to decrease in germinating soybeans by the second day after planting, simultaneously with a reduction in the linoleate and linolenate contents.

In contradistinction to the results on plant tissues, the unsaturated fat oxidases are not so widely distributed in animal tissues. It is also uncertain whether or not the enzyme is similar to lipoxidase. Although Banks²⁰² described a heat-labile system in herring muscle which stimulated rancidity in fat, the active substance was later shown to involve hemin (hematin) proteins;^{203,204} the action was quite different from that of lipoxidase. A rancidity-provoking enzymic action in pig muscle²⁰⁵ was later proved to be due to its hemoglobin and myoglobin content.²⁰⁶ An unsaturated fat oxidase has been reported in rat liver and in rat gastric mucosa,²⁰⁷ as well as in

¹⁹² H. Süllman, *Fermentforschung*, 17, 610-631 (1945).

¹⁹³ J. J. Jezeski, *Trans. Am. Assoc. Cereal Chemists*, 5, 37-49 (1947); *Chem. Abst.*, 41, 4933 (1947).

¹⁹⁴ S. Bergström and R. T. Holman, *Advances in Enzymol.*, 8, 425-457 (1948).

¹⁹⁵ V. A. Kirsanova, *Biokhimiya*, 3, 191-200 (1938); *Chem. Abst.*, 33, 8640 (1939); *Chimie & industrie*, 41, 984 (1939).

¹⁹⁶ H. H. Strain, *J. Am. Chem. Soc.*, 63, 3542 (1941).

¹⁹⁷ R. J. Sumner and D. K. Tressler, *Ind. Eng. Chem.*, 35, 921 (1943).

¹⁹⁸ R. Reiser and G. S. Fraps, *J. Assoc. Offic. Agr. Chemists*, 26, 186-194 (1943).

¹⁹⁹ H. Süllman, *Experientia*, 1, 323-324 (1945).

²⁰⁰ D. S. Van Fleet, *Am. J. Botany*, 29, 747-755 (1942).

²⁰¹ D. S. Van Fleet, *J. Am. Chem. Soc.*, 65, 740 (1943).

²⁰² A. Banks, *J. Soc. Chem. Ind.*, 56, 13-15T (1937).

²⁰³ A. Banks, *J. Soc. Chem. Ind.*, 63, 8-13 (1944).

²⁰⁴ A. Banks, Personal communication (1947); cited by S. Bergström and R. T. Holman, *Advances in Enzymol.*, 8, 425-457 (1948), p. 443.

²⁰⁵ C. H. Lea, *J. Soc. Chem. Ind.*, 56, 376-380T (1937).

²⁰⁶ B. M. Watts and D.-H. Peng, *J. Biol. Chem.*, 170, 441-453 (1947).

²⁰⁷ E. L. Hove, *Science*, 98, 433-434 (1943).

the striated muscle of the rabbit.²⁰⁸ Brocklesby and Rogers²⁰⁹ described a substance in salmon liver and in pilchard oil which is very effective in destroying vitamin A. Carter²¹⁰ found an enzyme in dogfish and halibut liver oils which greatly reduced the vitamin A content. This might also be considered to be an unsaturated fatty acid oxidase. However, none of these enzyme systems has been well characterized or prepared in reasonably pure form. It is not certain whether or not any of these systems is identical with soybean lipoxidase.

(c) *Acids Effective as Substrates.* According to a number of investigators,^{196, 211, 212} only those acids which have an unsaturated linkage in the 9:10 position are attacked by lipoxidase. However, since many of these tests were made on impure substrates, the results are questionable. Holman and Burr²¹³ reported that crude extracts of lipoxidase attacked linoleic acid, ethyl linoleate, linolenic acid, ethyl linolenate, and methyl arachidonate; it was later demonstrated that these essential acids were all oxidized at the same rate.²¹⁴

Sumner²¹² had suggested that the optimal molecular configuration for activity was $\text{—CH:CH}\cdot\text{CH}_2\cdot\text{CH:CH}\cdot(\text{CH}_2)_7\cdot\text{COOH}$, and that the double bond nearer the carboxyl should probably have a *cis* configuration. This hypothesis was based upon the fact that oleic acid, but not erucic acid (in which no double bond occurs in the 9,10 position), was active. However, since arachidonic acid has no double bond at position 9, it is evident that this hypothesis must be modified. Bergström and Holman¹⁹⁴ have indicated that the configuration necessary for activity is the following: $\text{—CH:CH}\cdot\text{CH}_2\cdot\text{CH:CH}\text{—}$, with both double bonds *cis*. This is in line with the findings of Holman and Elmer²¹⁴ that only the *cis* isomers of linoleic and linolenic acids serve as active substrates. Apparently, acids with conjugated systems, as for instance, elaeostearic acid, are not attacked by lipoxidase.^{213, 215} Süllman²¹⁶ reported that oxidation of linoleic acid by a lipoxi-

²⁰⁸ H. Süllman, Personal communication (1947); cited by S. Bergström and R. T. Holman, *Advances in Enzymol.*, 8, 425–457 (1948), p. 443.

²⁰⁹ H. N. Brocklesby, *Fisheries Research Board, Can., Bull. No. 59*, 1–431 (1941); with N. I. Rogers, *Ibid.*, *Progress Repts., Pacific Coast Stas.*, 50, 4–8 (1941); *Chem. Abst.*, 36, 2371 (1942).

²¹⁰ N. M. Carter, *Fisheries Research Board, Can., Ann. Rept. 1941, Append. V; Rept. Pacific Fisheries Exptl. Sta. (Prince Rupert, B.C.)*, 1941, 31–35.

²¹¹ H. A. Spöehr, J. H. C. Smith, H. C. Strain, and W. H. Milner, *Carnegie Inst. Wash. Yearbook*, 39, 147–154 (1940).

²¹² R. J. Sumner, *J. Biol. Chem.*, 146, 211–213 (1942).

²¹³ R. T. Holman and G. O. Burr, *Arch. Biochem.*, 7, 47–54 (1945).

²¹⁴ R. T. Holman and O. C. Elmer, *J. Am. Oil Chemists' Soc.*, 24, 127–129 (1947).

²¹⁵ H. Süllman, *Helv. Chim. Acta*, 27, 789–793 (1944).

²¹⁶ H. Süllman, *Helv. Chim. Acta*, 24, 1360–1380 (1941).

dase prepared from soybean used one mole of oxygen, while oxidation of linolenic acid under similar conditions required two molecules of O_2 . In contradistinction to these results, only 0.1 mole of O_2 was utilized per mole of oleic or ricinoleic acid.

(d) *Isolation of Pure Lipoxidase.* Sällman²¹⁷ prepared an acetone powder of soybean lipoxidase. Cosby and Sumner²¹⁸ effected a 1:60 concentration of the enzyme, while Balls, Axelrod, and Kies²¹⁹ were able to prepare an aqueous extract of soybean meal with a specific activity 115 times that of a 2.5% water extract of the meal. Theorell, Bergström, and Åkeson²²⁰ reported the preparation of an electrophoretically-homogeneous enzyme which had an activity of 130 units per mg.; later, an improved method of preparation was reported.^{194,221} Theorell, Holman, and Åkeson^{222,223} have now prepared a crystalline lipoxidase from soybean flour, by precipitation with ammonium sulfates, which is electrophoretically homogeneous at pH 6.0, and which is homogeneous to sedimentation and diffusion patterns. The following properties are reported:²²³ sedimentation constant, 5.62×10^{-13} ; diffusion constant, 5.59×10^{-7} ; partial specific volume, 0.750; and molecular weight, 102,000. The isoelectric point is at a pH of approximately 5.4. The absorption spectrum is that of a common protein with a moderately high absorption at 280 m μ . Lipoxidase is a colorless protein, devoid of iron and containing no detectable prosthetic group. It is soluble in dilute salt solution, but insoluble in water.¹⁹⁴ The activity of the enzyme was found to be such that 330 moles of linoleic acid were oxidized per mole per second under the conditions of the test.²²³ Holman and associates²²⁴ reported that crystalline lipoxidase contains 938 amino acid residues per molecule; the authors accounted for 94% of the composition of the molecule.

(e) *Properties of Lipoxidase.* The optimum pH for lipoxidase activity has been reported as 9.4, with continued effectiveness at a high rate at a pH²²⁵ up to 11.7. However, Smith²²⁶ stated that the optimum pH of soy-

²¹⁷ H. Sällman, *Helv. Chim. Acta*, **26**, 2253-2263 (1943).

²¹⁸ E. L. Cosby and J. B. Sumner, *Arch. Biochem.*, **8**, 259-264 (1945).

²¹⁹ A. K. Balls, B. Axelrod, and M. W. Kies, *J. Biol. Chem.*, **149**, 491-504 (1943).

²²⁰ H. Theorell, S. Bergström, and Å. Åkeson, *Arkiv. Kemi, Mineral., Geol.*, **19 A**, No. 6, 1-9 (1944).

²²¹ H. Theorell, S. Bergström, and Å. Åkeson, *Pharm. Acta Helv.*, **21**, 318-324 (1946); also cited by S. Bergström and R. T. Holman, *Advances in Enzymol.*, **8**, 425-457 (1948), p. 445.

²²² H. Theorell, R. T. Holman, and Å. Åkeson, *Arch. Biochem.*, **14**, 250-252 (1947).

²²³ H. Theorell, R. T. Holman, and Å. Åkeson, *Acta Chem. Scand.*, **1**, 571-576 (1947).

²²⁴ R. T. Holman, F. Panzer, B. S. Schweigert, and S. R. Ames, *Arch. Biochem.*, **26**, 199-204 (1950).

²²⁵ R. T. Holman, *Arch. Biochem.*, **15**, 403-413 (1947).

²²⁶ G. N. Smith, *Arch. Biochem.*, **19**, 133-143 (1948).

bean lipoxidase for the oxidation of methyl linoleate at 25°C. was 6.5. Bergström and Holman¹⁹⁴ recorded the optimum temperature as 30°C. The activity of the enzyme declines precipitously above this temperature; the temperature-accentuated inactivation is caused by contact with either the substrate or the reaction products. Tappel *et al.*²²⁷ found that the activity of lipoxidase is reduced by freezing to less than 1% of that found in the liquid system at the same temperature. The lipoxidase-catalyzed oxidation of linoleate was found to have the relatively low activation energy of 4300 cal. per mole.²²⁷

Holman²²⁵ reported that pure lipoxidase is not inhibited by pyrophosphate, fluoride, cyanide, azide, mercury ions, *p*-chloromercuribenzoate or diethylthiocarbamic acid, even when these are present in high concentrations. This indicates that neither heavy metals nor sulfhydryl groups are concerned with the functional activity of the enzyme. Although Hummel and Mattill²²⁸ have suggested that lipoxidase is an aerobic dehydrogenase, Balls and Kies²²⁹ are of the opinion that the fact that lipoxidase is not inhibited by a specific oxidase inhibitor, sodium cyanide (NaCN), is not sufficient reason for classifying it in this category.

The action of antioxidants on lipoxidase offers another interesting approach to the problem of linoleate oxidation. Thus, although Bergström and Holman¹⁹⁴ note that some inhibition of lipoxidase activity is caused by α -naphthol and by α -tocopherol, they suggest that it is due to antioxidant activity rather than to true enzymic inhibition. Tappel and associates²²⁷ found that two well-known antioxidants were effective inhibitors of lipoxidase, namely nordihydroguaiaretic acid and propyl gallate. Moreover, the effective concentrations were sufficiently low to render the use of these antioxidants practical for food protection. On the other hand, it was shown that α -tocopherol was rapidly oxidized by the lipoxidase system. Bergström and Holman¹⁹⁴ note that some substrate competition is exhibited, in decreasing order, by elaidolinolenic acid, 10,12-linoleic acid, oleic acid, and octanoic acid.

Although Balls *et al.*²¹⁹ and Theorell and co-workers²²⁰ believed that lipoxidase is activated by a polypeptide present in soybeans²³⁰ which could augment the enzyme activity^{230,231} by as much as 300%, the activator is not

²²⁷ A. I. Tappel, W. O. Lundberg, and P. D. Boyer, *Arch. Biochem. Biophys.*, **42**, 293-304 (1953).

²²⁸ J. P. Hummel and H. A. Mattill, *Proc. Soc. Exptl. Biol. Med.*, **55**, 31-33 (1944).

²²⁹ A. K. Balls and M. W. Kies, *J. Biol. Chem.*, **153**, 337-338 (1944).

²³⁰ M. W. Kies, *J. Biol. Chem.*, **170**, 121-132 (1947).

²³¹ M. W. Kies, *Federation Proc.*, **6**, 267 (1947).

required or effective for the lipoxidase preparation of Smith and Sumner,²³² nor has it been found to be necessary to activate crystalline lipoxidase.¹⁹⁴

(f) *The Mechanism of Action of Lipoxidase.* It has been suggested that the course of oxidation catalyzed by lipoxidase is similar to that occurring in the autoxidation of fats;^{233,234} hydroperoxides are formed, which are largely conjugated. Holman²²⁵ and Bergström and Holman²³⁵ later showed that, with a low substrate concentration, and at 0°C., linoleic acid is oxidized to yield totally conjugated peroxides. When the temperature is increased, side reactions or subsequent oxidation or decomposition of the peroxides occurs. The postulated pathway of oxidation is given below:

The Oxidation of Linoleic Acid with Lipoxidase as Proposed by Bergström and Holman^{194,255}

The oxidation of linoleic acid proceeds in the form of a chain reaction, as a result of the transfer of a hydrogen from the methylene group on C₁₁ of

²³² G. N. Smith and J. B. Sumner, *Arch. Biochem.*, **19**, 89-94 (1948).

²³³ S. Bergström, *Arkiv. Kemi, Mineral., Geol.*, **21 A**, No. 15, 1-8 (1945).

²³⁴ R. T. Holman, *Arch. Biochem.*, **10**, 519-529 (1946).

²³⁵ S. Bergström and R. T. Holman, *Nature*, **161**, 55 (1948).

linoleic acid to V or VI, whereby these latter compounds are changed to VII and VIII, respectively. Thus, linoleic acid is changed to II, which can then be further oxidized by either of the above pathways. It has been suggested that the function of lipoxidase may be to initiate the chain reactions by removing the hydrogen atom from the linoleic acid methylene group.

However, Tappel, Boyer, and Lundberg²³⁶ noted several differences in the decomposition of linoleate brought about by autoxidation and by catalysis with lipoxidase, respectively. For example, the extinction coefficient at 232.5 $m\mu$ for the peroxides formed in the presence of lipoxidase was higher than that which resulted from the autoxidation of linoleate. Antioxidants inhibited the oxidation of linoleate as the result of a direct effect upon lipoxidase and because of a preferential oxidation of the antioxidant. To replace the assumption that the oxidation of linoleate proceeds as a chain reaction, Tappel *et al.*²³⁶ suggest the formation of a biradical from linoleate and oxygen on the enzyme surface. The biradical may accept electrons from antioxidants, or may react to give the conjugate linoleate peroxide.

On the other hand, Khan²³⁷ noted that the infrared absorption spectra of the peroxide concentrate (reduced) from methyl linoleate autoxidized below 0°C. with dispersed oxygen, and the peroxide concentrate (reduced) from linoleic acid oxidized at 0°C. in the presence of lipoxidase with dispersed oxygen, are identical at the same wave lengths, proving the presence of the two major isomers, *cis-trans* conjugated and *trans-trans* conjugated. Thus, although lipoxidase catalysis may have proceeded through the controlled mechanisms proposed by Tappel *et al.*,²³⁶ it has also stabilized the free radicals at the 9- and 13-positions of the linoleate system, producing the conjugated isomers.²³⁷ The free radicals initiated at the 11-position are able to change over to the more stable forms.

a'. Coupled Reactions: There is considerable evidence that a coupled oxidation occurs in a number of easily oxidized compounds and linoleic acid under the action of lipoxidase. Substances which have been found to exert this effect include various carotenoids,^{196,211} chlorophyll,¹⁹⁶ hemin,²³⁸ ascorbic acid¹⁹⁶ (although slowly) and certain dyes.¹⁹⁶

Holman²³⁹ reported that, in the coupled oxidation of carotene and linoleic acid with pure lipoxidase, the amount of carotene destroyed and the amount of diene conjugated are proportional to the enzyme concentration and to the

²³⁶ A. L. Tappel, P. D. Boyer, and W. O. Lundberg, *J. Biol. Chem.*, 199, 267-281 (1952).

²³⁷ N. A. Khan, *Arch. Biochem. Biophys.*, 44, 247-249 (1953).

²³⁸ M. W. Kies, *Federation Proc.*, 5, 141 (1946).

²³⁹ R. T. Holman, *Federation Proc.*, 7, 160-161 (1948).

time interval. It is therefore obvious that the destruction of carotene is a satisfactory index of the degree of oxidation, as well as of the activity of the enzyme. It has also been reported that, in the case of both carotene and bixin, the amount of destruction of the carotenoid is proportional to its concentration.¹⁹⁴

Sumner and Smith²⁴⁰ report that bixin has certain advantages over carotene as an index of the activity of lipoxidase, and that an induced or coupled reaction exists between bixin and methyl linoleate as a result of lipoxidase activity.²⁴¹ In a more complete study of the bixin-linoleate destruction by lipoxidase, Kunkel²⁴² reported that the rate of diene conjugation of linoleate was approximately linear with time when the system was aerated. However, the presence of bixin was shown to inhibit diene conjugation at first; however, as the bixin was destroyed the rate of diene conjugation increased above that in the control series.

Holman²⁴³ observed that, during the germination of soybeans, carotenoids began to increase on the fifth day, and chlorophyll on the seventh day. The fat content began to decrease after the third day, while the iodine number was found to drop after five days. Lipoxidase activity declined sharply after the second day. It is suggested that the function of lipoxidase may be to initiate the oxidation of linoleic and linolenic acids in the germinating seeds, after which the breakdown continues autocatalytically.

c. Dehydrogenase Systems. Two different dehydrogenase systems have been described. One of these acts to produce α,β -unsaturated compounds, while the second one is concerned only with 9,10-dehydrogenation.

(a) α,β -Dehydrogenases. Although little information has been obtained as to the specific dehydrogenases which act in the α,β -position, probably due to difficulties in analytical methods as a result of steric hindrance, several such preparations have been reported.

Muñoz and Leloir³⁵ described a dehydrogenase from guinea pig liver which is apparently distinct from the dehydrogenase of Lang. The one from guinea pig liver is very labile, and it catalyzes dehydrogenation only of the lower fatty acids (3 to 8 carbons). For maximum activity, fumarate, cytochrome-*c*, inorganic phosphate, adenylic acid, and Mg^{++} or Mn^{++} are required. In addition to the saturated acids in the category listed, crotonic, isocrotonic, and β -hydroxybutyric acids may also serve as substrates. Fluoride, iodoacetate, arsenate, and malonate completely inhibit the action of the enzyme.³⁵

²⁴⁰ J. B. Sumner and G. N. Smith, *Arch. Biochem.*, **14**, 87-92 (1947).

²⁴¹ G. N. Smith and J. B. Sumner, *Arch. Biochem.*, **17**, 75-80 (1948).

²⁴² H. O. Kunkel, *Arch. Biochem.*, **30**, 306-316 (1951).

²⁴³ R. T. Holman, *Arch. Biochem.*, **17**, 459-466 (1948).

Fatty acid dehydrogenases were localized in the mitochondria of rat liver by Witter and collaborators.²⁴⁴ The substrates in decreasing order of activity included oleate, hexanoate, palmitate, 2-hexenoate, β -hydroxyhexanoate, β -hydroxybutyrate, and β -ketohexanoate. This dehydrogenase is believed to carry the reaction beyond the stage of the α,β -unsaturated acid. Green and co-workers¹⁷⁶ reported that β -hydroxybutyric dehydrogenase is inhibited by 0.03 *M*-iodoacetate and by arsenite. This would suggest that —SH groups are required for activity. Singer and Barron²⁴⁵ confirmed the fact that an —SH group is required in the activating protein for β -hydroxybutyric dehydrogenase.

After reviewing all of the available evidence of α,β -unsaturation, Breusch¹ states that "there also exists an enzyme system which, though perhaps less important and quite unrelated to the enzyme system which brings about β -oxidation of fatty acids, catalyzes α,β -dehydrogenation or hydrogenation of fatty acids. The occurrence of α,β -hydrogenation has been proved beyond doubt; this proof is still outstanding for α,β -dehydrogenation."

(b) *9,10-Dehydrogenases*. a'. Preparation and Activation: Lang alone²⁴⁶ and with co-workers^{247,248} first prepared an active fatty acid dehydrogenase, from the liver and the muscle of rats, in which the activity increases with the length of the carbon chain of the substrate. It is specific for the higher fatty acids. Adenylic acid was shown to be a co-dehydrogenase in muscle. Inosinic acid and ATP were equally effective as coenzymes. These results have been confirmed by a number of workers.^{109,249} This enzyme is apparently similar to that isolated from ox liver by Annau and co-workers.²⁵⁰ Both are activated by adenylic acid and by hypoxanthine;²⁵¹ however, since hypoxanthine is a more effective activator than is adenylate, Burton²⁵¹ suggests that the latter may exert its action through the hypoxanthine formed from it.

Champougny and LeBreton^{109,252} reported that a phosphorylation of the fatty acids is necessary before the palmitic acid $\rightarrow$ palmitoleic acid or the stearic acid $\rightarrow$ oleic acid transformations can occur. Jacob and Pascaud²⁵³ confirmed this finding, but believe that phosphorylation is catalyzed, not by

²⁴⁴ R. F. Witter, M. Cottone, and W. J. Pories, *Federation Proc.*, **12**, 291–292 (1953).

²⁴⁵ T. P. Singer and E. S. G. Barron, *J. Biol. Chem.*, **157**, 241–253 (1945).

²⁴⁶ O. St. A. K. Lang, *Z. physiol. Chem.*, **261**, 240–248 (1939).

²⁴⁷ K. Lang and H. Mayer, *Z. physiol. Chem.*, **261**, 249–252 (1939).

²⁴⁸ K. Lang and H. Mayer, *Z. physiol. Chem.*, **262**, 120–122 (1939).

²⁴⁹ T. Fontaine, *Bull. soc. chim. biol.*, **25**, 286–292 (1943).

²⁵⁰ E. Annau, A. Eperjessy, and O. Felszeghy, *Z. physiol. Chem.*, **277**, 58–65 (1942).

²⁵¹ K. Burton, *Nature*, **161**, 606 (1948).

²⁵² J. Champougny and E. LeBreton, *Compt. rend. soc. biol.*, **141**, 45–48 (1947).

²⁵³ A. Jacob and M. Pascaud, *Compt. rend. soc. biol.*, **143**, 1476–1478 (1949).

a phosphomonoesterase, but rather by a phosphorylase requiring adenosine or adenylic acid as a coenzyme. It had been shown earlier that vitamins B, nicotinamide, pyridoxine, and pantothenic acid act as coenzymes.²⁵⁴

b'. The Distribution of 9,10-Dehydrogenase: In addition to the liver and muscle as sources of dehydrogenases,^{247,248} the small intestine (rats),²⁵⁵ bile, pancreas,²⁵⁶ and kidneys,²⁵⁷ have also been reported to be sites of origin. Annau *et al.* noted their presence in the liver, but they found that only lecithin, palmitic acid, and stearic acid could be dehydrogenated.

Shapiro and Wertheimer,²⁵⁸ Quagliariello alone⁹⁰ and with Scoz²⁵⁹ called attention to the presence of a fatty acid dehydrogenase in adipose tissue itself. The enzyme requires the presence of a coenzyme, adenylic acid, and of inorganic phosphate. It has an optimum pH of 8.0. The highest activity is found in relation to natural, long-chain fatty acids; its effectiveness decreases with diminishing chain length. In addition to fatty acids, this enzyme attacks phospholipids and succinic acid, but not neutral fat. The enzyme is also present in liver, heart, muscle, and testes. The action of the dehydrogenase is not decreased by monoiodoacetate, sodium fluoride, phlorhizin, maleic acid, pyrophosphate, or benzoate at fairly high concentrations, but it is inhibited by oxygen.

(3) Pathways of Fatty Acid Oxidation

Crandall and co-workers²⁶⁰ suggested the existence of alternative pathways for fat oxidation, one by means of acetone body production in the liver, and a second one which does not involve ketogenesis. It is assumed that ketogenesis is a mechanism which supplies the tissues with substances which can be used as a partial replacement for glucose in metabolism, and which therefore conserves the carbohydrate reserve and decreases the need for gluconeogenesis. Barnes and collaborators²⁶¹ have independently arrived at essentially the same conclusion by studying the ketone body utilization in animals in a state of ketosis produced by pancreatic diabetes or by phlorhizin.

However, in view of the more recent demonstration by the use of iso-

²⁵⁴ J. Champougny and E. LeBreton, *Compt. rend. soc. biol.*, 141, 43-45 (1947).

²⁵⁵ H. D. Cremer, *Z. physiol. Chem.*, 263, 240-242 (1940).

²⁵⁶ H. Tangl and N. Berend, *Biochem. Z.*, 232, 181-188 (1931).

²⁵⁷ F. P. Mazza, *Boll. soc. ital. biol. sper.*, 9, 298-299 (1934).

²⁵⁸ B. Shapiro and E. Wertheimer, *Biochem. J.*, 37, 102-104 (1943).

²⁵⁹ G. Quagliariello and G. Scoz, *Arch. sci. biol. (Italy)*, 17, 513-529; 530-545 (1932).

²⁶⁰ L. A. Crandall, Jr., H. B. Ivy, and G. I. Ehni, *Am. J. Physiol.*, 131, 10-17 (1940); *J. Biol. Chem.*, 138, 123-128 (1941).

²⁶¹ R. H. Barnes, D. R. Drury, P. O. Greeley, and A. N. Wick, *Am. J. Physiol.*, 130, 144-150 (1940).

topes that acetate is the primary degradation product, and that such acetate may combine with oxaloacetate to be oxidized *via* the citric acid cycle, one may postulate that the oxidation of fat in the peripheral tissues in the absence of ketosis occurs because the acetate produced by oxidation is readily removed and oxidized to CO_2 and water. On the other hand, in tissues like the liver (particularly during fasting), there is less ability to oxidize acetate *via* the citric acid cycle. The consequent accumulation of the acetate results in the formation of acetone bodies.

(4) *The Metabolism of the Ketone Bodies*

a. Introduction. The most fruitful source of information concerning the intermediary metabolism of fatty acids has been afforded by their close relationship to the ketone bodies. This applies not only to the vast amount of work reported prior to the employment of the isotopic technics, but also to the investigations which have been carried out subsequently with isotopes.

The accumulation of the ketone bodies in the blood in abnormally large amounts is referred to by the terms *ketosis* or *ketonemia*, while the presence of these substances in the urine is designated by the terms *ketonuria* or *acetonuria*. The *ketone bodies*, or *acetone bodies*, include the three substances, acetoacetic (or diacetic) acid, β -hydroxybutyric acid, and acetone which always occur in association. It is generally considered that acetoacetate is the primary product. It is in equilibrium with β -hydroxybutyrate, while acetone can be formed from it by decarboxylation. These relationships are indicated below:

The Metabolic Interrelations of the Ketone Bodies

It is generally believed that ketone bodies are compounds normally occurring in the animal body. Under conditions in which carbohydrates are present in the diet in normal amounts, only traces of these substances are found in the blood, tissue fluids, and urine. However, when carbohydrate is unavailable, as in fasting, when it cannot be utilized, as in diabetes, or when the body is called upon to metabolize excessive amounts of fat, a marked ketosis, with a concomitant ketonuria, may ensue.

Substances which are convertible to the ketone bodies are referred to as

ketogenic, while substances which tend to prevent the accumulation of the ketone bodies in the tissues are called *antiketogenic* by one school, and *ketolytic*²⁶² by another group. The difference in terminology refers to the mechanism by which it is believed that these substances prevent the accumulation of ketone bodies.

It is generally accepted that, under normal conditions, fatty acids are oxidized to CO_2 and H_2O , thus setting free the maximum amount of energy that is bound up in the molecule. In the course of degradation of the fatty acids, the ketone bodies are intermediates. It has long been the accepted opinion that, in order to complete the oxidation of the fatty acids to CO_2 and H_2O , carbohydrate must be simultaneously oxidized to effect the complete breakdown of the ketone bodies. Naunyn²⁶³ elaborated the theory of "secondary oxidation," and Rosenfeld²⁶⁴ stated, many years ago, that "fats burn only in the flame of carbohydrate." While this aphorism has been somewhat in disrepute in recent years, the fact remains that ketonuria develops in the absence of carbohydrates or carbohydrate precursors, while it disappears when such substances are metabolized. The demonstration of the interrelation between the metabolism of ketone bodies and the Krebs cycle has revived the acceptance of the Naunyn-Rosenfeld statement. The historical aspects of ketosis were reviewed by Magnus-Levy,²⁶⁵ Shaffer,²⁶⁶ and Stadie,² while the more recent metabolic work has been summarized in the papers of MacKay²⁶⁷ and of others. One of the best critical reviews to be found is the monograph of Peters and Van Slyke.²⁶⁸

(a) *Historical Development.* Acetone was the first of the ketone bodies to be recognized in biological material. Thus Petters,²⁶⁹ in 1857, discovered acetone in the urine of diabetics. He considered it to be an intermediate of glucose. Acetoacetic acid was the second ketone body to be identified. Gerhardt²⁷⁰ described the presence of this compound in diabetic urine eight years after the discovery of acetone.

²⁶² According to G. Lusk, *Elements of the Science of Nutrition*, 4th ed., Saunders, Philadelphia, 1928, page 665, the term "ketolytic," in place of "antiketogenic," was introduced at the suggestion of Professor S. R. Benedict.

²⁶³ B. Naunyn, cited by P. A. Shaffer, *Medicine*, 2, 375-404 (1923).

²⁶⁴ G. Rosenfeld, *Berl. klin. Wochschr.*, 43, 978-981 (1906).

²⁶⁵ A. Magnus-Levy, *Ergeb. inn. Med. u. Kinderheilk.*, 1, 352-419 (1908).

²⁶⁶ P. A. Shaffer, *Medicine*, 2, 375-404 (1923).

²⁶⁷ E. M. MacKay, *J. Clin. Endocrinol.*, 3, 101-110 (1943).

²⁶⁸ J. P. Peters and D. D. Van Slyke, *Quantitative Clinical Chemistry*, 2nd ed., vol. I, Williams & Wilkins, Baltimore, 1946, pp. 489, 494.

²⁶⁹ W. Petters, *Prager Vierteljahresschr. prakt. Heilk.*; cited by F. Hirschfeld, *Z. Klin. Med.*, 28, 176-209 (1895), p. 176.

²⁷⁰ C. Gerhardt, *Wien. med. Presse*, 6, No. 28, 673 (1865); also cited by F. Hirschfeld, *Z. klin. Med.*, 28, 176-209 (1895), p. 176.

Neither acetone nor acetoacetic acid contains an asymmetric carbon atom; however, β -hydroxybutyrate does contain a carbon atom which renders it optically active. Had it not been for the latter fact, the isolation of this third ketone body might have been delayed later than 1884, the year in which Külz²⁷¹ discovered it. Since a marked levorotation of the urine was noted after the fermentation of the sugar, it was certain that some other abnormal substance, in addition to glucose, was present in the urine. Külz succeeded in isolating and characterizing β -hydroxybutyrate (pseudo-oxybutyric acid). Simultaneously with the work of Külz, Stadelmann²⁷² reported the presence of an acid in the urine of diabetics which was incorrectly described as crotonic acid. Minkowski,²⁷³ continuing the investigations of Stadelmann, came to the conclusion that the acid in question was not crotonic acid but rather β -hydroxybutyric acid.

(b) *The Relationship of Ketonuria to the Fatty Acid:Glucose Ratio.* The first recognition that ketonuria occurred when insufficient carbohydrate was being oxidized was that of Hirschfeld²⁷⁴ in 1895. However, Stadelmann²⁷⁵ had pointed out several years earlier that a relationship existed between the formation of β -hydroxybutyric acid and the occurrence of coma. Magnus-Levy²⁶⁵ showed that, when an 11.7 g. dose of β -hydroxybutyric acid was given to a normal dog, it was completely oxidized and did not appear in the urine; on the other hand, when the acid was administered to a phlorhizinized dog, it was largely excreted in the urine. Neubauer²⁷⁶ found that, when either acetoacetic acid or β -hydroxybutyric acid was given to a diabetic patient, both compounds were excreted in the urine. This indicated that the two acids were in equilibrium; in cases of marked ketosis, the β -hydroxybutyrate comprised 60 to 80% of the total ketone bodies.

Shaffer²⁷⁷ was the first to suggest a quantitative relationship between the amounts of sugar, protein, and fat metabolized and the quantity of ketone bodies produced. The amount of *ketogenic* matter metabolized, calculated in millimoles, was determined by the use of the following constants:

²⁷¹ E. Külz, *Z. Biol.*, 20, n.s. 2, 165-178 (1884).

²⁷² E. Stadelmann, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, 17, 419-444 (1883).

²⁷³ O. Minkowski, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, 18, 35-48 (1884).

²⁷⁴ F. Hirschfeld, *Z. klin. Med.*, 28, 176-209 (1895).

²⁷⁵ E. Stadelmann, *Über den Einfluss der Alkalien auf den menschlichen Stoffwechsel. Experimentelle-klinische Untersuchungen*, Stuttgart, 1890; cited by G. Lusk, *Elements of the Science of Nutrition*, 4th ed., Saunders, Philadelphia, 1928, p. 658.

²⁷⁶ O. Neubauer, *Verhandl. deut. Kongr. inn. Med.*, 27, 566-574 (1910).

²⁷⁷ P. A. Shaffer, *J. Biol. Chem.*, 47, 449-473 (1921); 49, 143-162 (1921); 54, 399-441 (1922).

$$\text{One g. fat} = \frac{1000}{874} \times 3 = 3.43 \text{ millimoles fatty acid}$$

$$\text{One g. protein N (from leucine, tyrosine)} = 15 \text{ millimoles fatty acid}$$

In the case of fat, the figure of 3.43 is assumed on the basis of a single ketone body per fatty acid residue for triolein (mol. wt. = 874). In determining the ketogenic figure for protein, the value of 15 millimoles per g. of urine nitrogen is an estimated value by the use of which close checks between predicted and actual ketonuria can be demonstrated.

On the other hand, the antiketogenic values were calculated in millimoles as follows:^{266, 267}

$$\text{One g. glucose} = \frac{1000}{180} = 5.56 \text{ millimoles glucose}$$

$$\text{One g. fat} = \frac{1000}{874} \times \frac{1}{2} = 0.57 \text{ millimole glucose}$$

$$\text{One g. urinary N} = 3.6 \text{ g. glucose}$$

$$3.6 \text{ g. glucose} = \frac{3600}{180} = 20 \text{ millimoles glucose}$$

The estimation of the antiketogenic substance from glucose in millimoles, is based upon the molecular weight of 180 for this monosaccharide. In the case of fat, the glucose equivalent is calculated on the basis that each fat molecule gives rise to one-half of a glucose molecule from the glycerol moiety. The calculation of the millimoles of glucose per g. of urinary nitrogen is based upon the theoretical relationship of urinary glucose to urinary nitrogen of 3.60:1 which obtains in a fasting diabetic subject.

Shaffer²⁶⁶ assumed that one molecule of glucose is able to cause the complete oxidation of two molecules of fatty acid, and that all fatty acids in excess of this value are excreted as ketone bodies in the urine. In a large number of experiments cited from the literature, in which the amount of the several foodstuffs metabolized was reported, the expected ketonuria was calculated. Remarkable agreement between calculated and determined values was recorded in a number of instances.

Woodyatt²⁷⁸ also employed calculations similar to those of Shaffer to estimate ketonuria. However, instead of being based upon millimoles, these values were estimated on the basis of actual grams of the several

²⁷⁸ R. T. Woodyatt, *Arch. Internal Med.*, 28, 125-141 (1921).

foodstuffs metabolized. Total glucose and total fatty acids were calculated as follows:

$$\begin{aligned}\text{Glucose} &= C + 0.58 P + 0.1 F \\ \text{Total fatty acids} &= 0.46 P + 0.9 F \\ C &= \text{carbohydrate} \\ F &= \text{fat} \\ P &= \text{protein}\end{aligned}$$

The glucose arising from protein is calculated on the basis of the accepted hypothesis that protein gives rise to 58% glucose. In the case of fat, the glycerol which is converted to glucose is 10% of the total weight of this foodstuff metabolized. Protein is believed to give rise to 46% fatty acids, while 90% of the fat molecule is known to be made up of fatty acids. Woodyatt stated that one g. of carbohydrate must be available to effect the complete oxidation of 1.5 g. of fatty acid, giving a fatty acid: glucose ratio (F.A.:G) of 1.5:1 as the threshold of ketosis. Since the molecular weight of glucose is 180 and the average value for the molecular weight of palmitic and oleic acids is 270, this would entail a F.A./G. ratio on the molecular basis of 1:1, which is only one-half that cited by Shaffer.²⁶⁶

Although these calculations were widely employed for the determination of satisfactory diets to prevent ketosis, and there has been considerable discussion as to which formula and method of calculation is correct, less emphasis has been placed on this subject since the advent of the insulin treatment of diabetes. Moreover, it should be pointed out that, on a theoretical basis, the calculations are not correct, in line with our present knowledge. In the first place, it is now known that several ketone molecules are formed for each long-chain fatty acid molecule.^{21,23,32-34,39,41,44} Secondly, no account is taken of the well-known fact that a difference in susceptibility to ketonuria exists between men and women.²⁷⁹

b. Sources of Ketone Bodies in the Animal. (a) *Formation from Even-Chain Fatty Acids.* The principal sources of the ketone bodies in the animal organism are the even-chain fatty acids. The proof of the above statement is based upon experimental feeding of fatty acids to diabetic or fasting rats or to dogs, upon liver perfusion tests, chiefly with dogs, and upon experiments with tissue slices.

Butts and collaborators²¹ demonstrated that butyric, caproic, and caprylic acids, when fed as their sodium salts, were excreted as acetone bodies when administered to fasting rats; on the other hand, practically no

²⁷⁹ H. J. Deuel, Jr., and M. Gulick, *J. Biol. Chem.*, **96**, 25-34 (1932).

ketone bodies could be noted when isomolecular quantities of the odd-carbon acids, valeric and heptanoic acids, were given. In later studies from this group,²³ in which ethyl esters were fed to rats, it was found that all even-chain saturated acids from C₄ to C₁₈ were ketogenic. Moreover, ethyl oleate, likewise, was shown to be converted to the ketone bodies. Butyric acid was found to produce a ketonuria in fasting rats which was quantitatively identical with that induced by an isomolecular amount of sodium acetoacetate,²¹ or by that of ethyl acetoacetate.²³ Gottschalk²⁸⁰ reported an experiment in which it was found that a marked ketonuria resulted from the feeding of butyric acid to a man on a carbohydrate-free diet. Deuel *et al.*²³ were unable to demonstrate any ketogenic action when the ethyl esters of any of the odd-carbon acids from C₃ to C₁₁ were fed to fasting rats.

Embsden and co-workers^{7,8} demonstrated that ketone bodies originated in the perfusing fluid when surviving livers of dogs were perfused with blood containing caproic, caprylic, or capric acids; on the other hand, no ketosis developed when odd-carbon fatty acids were added to the perfusate. These results were partially confirmed by Dakin,¹⁴ who observed ketonemia after caproic acid or after a number of intermediates had been added to Ringer solution used to perfuse a surviving liver.

The results with tissue slices have confirmed the *in vivo* tests. Thus, Jowett and Quastel³² reported a marked ketone body formation by liver slices which were incubated with the even-chain fatty acids. The values for ketone body formation expressed as Q_{ac} (equivalent cu. mm. as gas per mg. dry weight per hour) were as follows: control, 0.20; C₂, 0.42; C₄, 2.55; C₆, 2.62; C₈, 1.80; and C₁₀, 1.39. The corresponding values for the odd-chain fatty acids were as follows: control, 0.20; C₃, 0.12; C₅, 0.51; C₇, 0.88; and C₉, 0.72. Cohen¹⁰² reported the occurrence of ketogenesis by liver slices incubated with butyrate or caproate, but not when propionate or valerate was employed. Acetate gave slightly positive results. Leloir and Muñoz³⁴ likewise demonstrated the synthesis of ketones as a result of incubation of liver slices with the salts of even-chain fatty acids.

Cheldelin and Beinert²⁸¹ reported that cyclophorase produces large amounts of ketone bodies from the even-numbered fatty acids, and only a small quantity from odd-numbered acids. Thus, the acetoacetate formed approaches 1 mole for the C₆ acid, and exceeds this figure for octanoic acid (1.5 mole). In the case of the lower odd-numbered fatty acids, the acetoacetate content is very small; in the case of the C₉ and C₁₁ acids, 0.5 mole of acetoacetate is formed per mole of fatty acid oxidized.

²⁸⁰ A. Gottschalk, *Klin. Wochschr.*, **11**, 978-980 (1932).

²⁸¹ V. H. Cheldelin and H. Beinert, *Biochim. et Biophys. Acta*, **9**, 661-673 (1952).

Unsaturated fatty acids have also been found to be ketogenic. These include erotonic acid ($\text{CH}_3\text{CH}:\text{CH}\cdot\text{COOH}$), as demonstrated by Blunden¹⁰¹ with fasting rats, and by Jowett and Quastel³² with liver slices, vinylacetic acid ($\text{CH}_2:\text{CH}\cdot\text{CH}_2\cdot\text{COOH}$), as proved by Kleinzeller,¹⁰⁵ who employed liver slices from fasted pigeons and rats, and sorbic acid ($\text{CH}_3\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$), as proved by Cohen¹⁰² and by Witter *et al.*⁵⁸ with liver slices, and by Deuel *et al.*²² *in vivo* in fasting rats.

(b) *Formation from Acetic Acid.* There seems to be no doubt that two acetic acids or two molecules closely related to acetate can condense to yield acetoacetate by the following scheme:

The formation of ketone bodies from acetate is supported by the demonstration of an increased ketonuria when acetate is fed to phlorhizinized dogs,⁵⁰ and by the finding of Loeb²⁸² and of Friedmann⁵² that abundant ketone formation resulted from the perfusion of surviving liver with acetate. Moreover, Swendseid, Barnes *et al.*⁵³ showed by the use of carboxyl-labeled acetic acid that ketone bodies may form by the condensation of acetate molecules. In many additional experiments^{51,141} longer-chain fatty acids labeled only in the carboxyl group have been shown to give rise to acetoacetate having an isotopic carbonyl carbon; this could result only from its synthesis from acetic acid molecules. Finally, a number of reports have demonstrated that ketogenesis occurs in liver slices in which acetate is the substrate.^{32,102} All of these investigations point to the fact that acetate may serve as a building stone for acetoacetate, although it is not so effective as butyric acid or longer even-chain fatty acids. This has been discussed fully in the section on the synthesis of fatty acids (see pages 11 to 26), and in the discussion of the β -oxidation-acetic acid condensation theory (see pages 83-87). However, there are negative reports in regard to this condensation as, for example, that of Schultz and Smith.²⁸³ These workers were unable to demonstrate any significant changes in the blood ketones in fasted, non-fasted, or phlorhizinized goats following the oral or intravenous administration of acetate, although butyric acid produced a rapid and immediate rise in blood ketones, and caproic, caprylic, and capric acids caused an increase in blood ketones after one hour.

(c) *Formation from Butyric Acid.* Although the majority of the experimental evidence favors the idea that butyric acid is converted solely to

²⁸² A. Loeb, *Biochem. Z.*, **47**, 118-126 (1912).

²⁸³ L. H. Schultz and V. R. Smith, *J. Dairy Sci.*, **32**, 817-822 (1949).

ketone bodies, several reports have been published which indicate that the administration of butyrate is followed by an increase in glycogen stores in the liver. Thus, Blixenkron-Møller²⁸⁴ reported that an increase in liver glycogen occurred in cat liver perfused with butyric acid; it was suggested that butyric acid was changed to succinic acid as a result of ω -oxidation. Succinic acid is believed to be a glucose former.^{285, 286} However, Deuel *et al.*²⁸⁷ questioned this interpretation, since succinic acid does not exert a ketolytic effect. On the other hand, MacKay *et al.*²⁸⁸ reported that succinic acid exerts an antiketogenic effect if it is fed to rats as the free acid, and not as the sodium salt; under the latter condition, an alkali excess remains which can itself produce a ketonuria. Stöhr²⁸⁹ and Dziewiatkowski and Lewis²⁹⁰ reported that, when unfasted rats were given butyrate,²⁹⁰ or when sodium acetate was administered with glucose to fasted rats,²⁸⁹ a higher figure for liver glycogen obtained than was the case in control rats. Finally, Buchanan *et al.*²⁹¹ found that isotopic carbon appeared in liver glycogen in excess of that expected from bicarbonate, when rats were fed butyrate with radioactive carbon in the carboxyl group.

Many experimental reports show results exactly opposite to those of the experiments described above. In a large number of tests on fasting rats under a variety of conditions, the administration of butyric acid either as the sodium salt or as the ethyl ester did not give rise to any demonstrable amount of glycogen, whereas the feeding of odd-chain acids under similar conditions resulted in the formation of well-defined stores of glycogen.^{25, 26, 31} In addition, the feeding of butyrate to phlorhizinized dogs leads to no increase in urinary glucose.²⁸ Moreover, the excretion of acetone bodies in the urine of rats was identical in amount with that obtained in animals receiving equimolecular amounts of diacetic acid.²¹ Finally, Deuel *et al.*²⁹² showed that no extra glycogen accumulated in the livers of rats fed glucose four hours earlier, irrespective of whether or not sodium butyrate

²⁸⁴ N. Blixenkron-Møller, *Z. physiol. Chem.*, **252**, 137-150 (1938).

²⁸⁵ A. I. Ringer, E. M. Frankel, and L. Jonas, *J. Biol. Chem.*, **14**, 539-550 (1913).

²⁸⁶ A. Korányi and A. Szent-Györgyi, *Orvosi Hetilap*, **81**, 615-618 (1937); *Chem. Abst.*, **31**, 6335 (1937).

²⁸⁷ H. J. Deuel, Jr., S. Murray, and L. Hallman, *Proc. Soc. Exptl. Biol. Med.*, **37**, 413-414 (1937).

²⁸⁸ E. M. MacKay, J. W. Sherrill, and R. H. Barnes, *J. Clin. Invest.*, **18**, 301-305 (1939).

²⁸⁹ R. Stöhr, *Z. physiol. Chem.*, **217**, 141-152 (1933).

²⁹⁰ D. D. Dziewiatkowski and H. B. Lewis, *J. Biol. Chem.*, **153**, 49-52 (1944).

²⁹¹ J. M. Buchanan, A. B. Hastings, and F. B. Nesbett, *J. Biol. Chem.*, **150**, 413-425 (1943).

²⁹² H. J. Deuel, Jr., C. Johnston, M. G. Morehouse, H. S. Rollman, and R. J. Winzler, *J. Biol. Chem.*, **157**, 135-140 (1945).

had been given. Also, the feeding of butyrate to unfasted rats did not lead to significant deviation from the control levels.

It should be pointed out that sodium butyrate is widely employed as a ketogenic agent to produce an exogenous ketonuria. If it were converted to an appreciable extent to glucose, this would result in a marked reduction in the level of ketonuria. Although Buchanan *et al.*²⁹¹ did obtain some radiocarbon in the glycogen fraction after feeding isotopic butyrate, the glycogen deposited comprised less than 5% of the ingested butyrate, and amounted to only about 3 mg. Obviously, the formation of glucose does not represent a major pathway of transformation of butyrate.

(d) *Formation from Odd-Chain Fatty Acids.* The question as to whether or not odd-chain fatty acids are ketogenic is disputable. In tests in which the sodium salts of valeric and heptanoic acids were ingested²¹ as well as in studies in which the ethyl esters of propionic, valeric, heptanoic, nonanoic, or undecanoic acids²³ were fed to fasting rats, only a slight ketonuria occurred if, in fact, any increase in ketone body excretion over the control level could be noted. On the other hand, glycogen synthesis was observed when the sodium salts of the C₃, C₅, C₇, and C₉ acids were administered,²⁶ and also when the ethyl esters of C₃, C₅, C₇, C₉, and C₁₁ acids were given.²⁵ In contradistinction to this finding, the sodium salts of the even-chain acids or their ethyl esters gave rise to an intense ketonuria when fed to fasting rats, and no glycogen formation could be observed. Krainick²⁹³ injected solutions of the various fatty acids into human subjects. Although he was able to demonstrate typical curves showing increased blood levels of ketone bodies in the case of C₄, C₆, C₈, and C₁₁ acids, no increases were obtained with the odd-chain acids from C₃ to C₁₁, except a minimal rise in the case of C₇. Embden and Marx,⁸ likewise, were unable to detect any ketone bodies in the fluid used in the perfusion of surviving livers of dogs after odd-numbered fatty acids had been added to it.

On the other hand, MacKay and collaborators²⁴ reported that a ketonemia obtained when the ethyl or glycerol esters of valeric, heptanoic, and undecanoic acid were fed to rats; no quantitative data were available, but approximately the same levels of blood ketones were found as in the case of even-numbered acids. Jowett and Quastel³² also reported ketogenesis when odd-numbered fatty acids were incubated with liver slices. However, in every case, the even-numbered fatty acids produced more ketone bodies than did the odd-chain acids in ratios varying between 2:1 and 5:1. Califano²⁹⁴ reported that liver slices produced only small amounts of ketone bodies when incubated with odd-chain fatty acids.

²⁹³ H. G. Krainick, *Klin. Wochschr.*, 19, 803-808 (1940).

²⁹⁴ I. Califano, *Biochem. Z.*, 289, 354-364 (1937).

The mechanism of oxidation of the odd fatty acids is not known, but it is presumed that the β -oxidation-acetic acid condensation theory also applies. Under these conditions, one might postulate that heptanoic acid would give rise to two acetic acid molecules (which could condense to yield acetoacetate) and one molecule of propionic acid (which could be changed to glucose).

The Oxidation of Heptanoic Acid to Yield Acetoacetic Acid and Glucose

Propionic acid is a strong glycogen former and does not give rise to any ketone bodies. As the length of the odd-chain acid is increased, a larger proportion of the molecule is available for acetoacetate formation from the acetic acid set free, and a smaller proportion is convertible to propionic acid. One would therefore expect that the higher odd fatty acids might exhibit some degree of ketonuria. However, the excretion of ketone bodies is suppressed to some extent by the presence of sugar being formed concomitantly from the propionic acid moiety produced.

The above mechanism for the metabolism of *n*-valeric acid has been confirmed by Siegel and Lorber,²⁹⁵ who made use of doubly-labeled valeric acid. When carboxyl- C^{13} , γ - C^{14} -*n*-valeric acid was fed to fasted rats along with glucose, about three times as much of the C^{14} was recovered in the liver glycogen as of the C^{13} . Moreover, C^{13} was found only on carbons 3 and 4 of glucose prepared from this glycogen, while C^{14} was present on carbons 1, 6, and 2, 5, predominantly and equally. According to these workers, their findings correspond to an *in vivo* metabolic pathway for *n*-valerate involving β -oxidation to "acetate" and "propionate."

On the basis of these principles, Kahn²⁹⁶ prepared an artificial triglyceride composed of the odd-carbon acid, margaric acid ($\text{CH}_3 \cdot (\text{CH}_2)_{15} \cdot \text{COOH}$), which was called "intarvin" by Keefer *et al.*,²⁹⁷ who reported that this substance was less ketogenic than were ordinary fats. However, their results

²⁹⁵ I. Siegel and V. Lorber, *J. Biol. Chem.*, 189, 571-576 (1951).

²⁹⁶ M. Kahn, *Am. J. Med. Sci.*, 166, 826-833 (1923).

²⁹⁷ C. S. Keefer, W. A. Perlzweig, and W. S. McCann, *Bull. Johns Hopkins Hosp.*, 35, 265-270 (1924).

are not entirely conclusive. Uhlmann²⁹⁸ also tentatively recommended the treatment of diabetics with fats composed of an odd number of carbons. Synthetic fats made in Germany from petroleum, during World War II, contained a considerable proportion of fatty acids having an uneven number of carbons. Flössner²⁹⁹ and Kabelitz³⁰⁰ both reported that a lower degree of ketonuria occurs in diabetics when synthetic fat is used than when natural fats are ingested, although the latter author states that the metabolism of unstabilized diabetics was severely upset by the excess of synthetic fat. Divergent conclusions have been reported by a number of other workers.³⁰¹⁻³⁰³

(e) *Formation from Branched-Chain Acids.* In addition to isovaleric acid, which is believed to be an intermediate of leucine, and α -ketovaleric acid, which arises from valine, a number of other branched-chain fatty acids have been investigated, to determine their ketogenic activity. These results are summarized in Table 2.

In the oxidation of fatty acids, β -oxidation will occur unless the molecule is altered by the introduction of a new group. The amino group, when introduced into the α -position, which is the normal one in the physiologic amino acids, will have the net effect of shortening the acid by one carbon insofar as oxidation is concerned.¹⁰² Thus, an α -amino acid with an even number of carbons will suffer the fate of the odd-carbon fatty acid with one less carbon; on the other hand, the α -amino acid containing an odd number of carbons will behave, on oxidation, like the even-chain fatty acid with one less carbon. The introduction of methyl or ethyl groups into the fatty acids tends to block β -oxidation. Thus, Wick³⁰⁴ was unable to induce ketosis in the rabbit with α -methylbutyric acid, α -ethylbutyric acid, or with α -methyl-valeric acid. Carter⁸³ reviewed the subject of the oxidation of branched-chain acids.

(f) *Formation from Amino Acids.* Although most of the amino acids are glucose-formers, there is convincing evidence that leucine and possibly several other amino acids can give rise to ketone bodies.

a'. Leucine: Embden, Salomon, and Schmidt⁷ reported that, when leucine was added to the perfusing fluid passed through a surviving liver, acetone bodies were formed in large amounts. The natural isomer was

²⁹⁸ R. Uhlmann, *Deut. Arch. klin. Med.*, **161**, 165-173 (1928).

²⁹⁹ O. Flössner, *Ernährung*, **8**, 89-93 (1943).

³⁰⁰ G. Kabelitz, *Klin. Wochschr.*, **23**, 13-16 (1944).

³⁰¹ H. Lundin, *J. Metabolic Research*, **4**, 151-176 (1923).

³⁰² F. S. Modern, *J. Metabolic Research*, **4**, 177-188 (1923).

³⁰³ D. M. Lyon, W. Robson, and A. C. White, *Brit. Med. J.*, **1925**, **I**, 207-210.

³⁰⁴ A. N. Wick, *J. Biol. Chem.*, **141**, 897-903 (1941).

TABLE 2

THE KETOGENIC ACTIVITY OF BRANCHED FATTY ACIDS

Name	Branched fatty acid tested	Formula	Ketogenic activity reported by				
			Baer-Blum ^a	Embsden et al. ^b	Lang-Adickes ^c	Wick ^d	Others
Isobutyric		(CH ₃) ₂ :CH·COOH	0	0	0	0	—
α-Methylbutyric		CH ₃ ·CH ₂ ·CH(CH ₃)·COOH	+	+	0	0	+ ^e
α-Ethylbutyric		CH ₃ ·CH ₂ ·CH(C ₂ H ₅)·COOH	+	—	—	0	—
β-Methylbutyric (isovaleric)		CH ₃ ·CH(CH ₃)·CH ₂ ·COOH	+	+	+	+	+ ^{f,g}
α-Methylvaleric		CH ₃ ·CH ₂ ·CH ₂ ·CH(CH ₃)·COOH	0	—	—	0	—
α-Ethylvaleric		CH ₃ ·CH ₂ ·CH ₂ ·CH(C ₂ H ₅)·COOH	—	—	—	0	—
β-Methylvaleric		CH ₃ ·CH ₂ ·CH(CH ₃)·CH ₂ ·COOH	—	+	+	+	—
γ-Methylvaleric		CH ₃ ·CH(CH ₃)·CH ₂ ·CH ₂ ·COOH	—	0	+	+	—
α-Methylcaproic		CH ₃ ·CH ₂ ·CH ₂ ·CH ₂ ·CH(CH ₃)·COOH	—	—	0	+	—
α-Ethylcaproic		CH ₃ ·CH ₂ ·CH ₂ ·CH ₂ ·CH(C ₂ H ₅)·COOH	—	—	0	0	—

+, ketone body formation; 0, no ketone body formation; —, no data.

^a Administration to diabetics, J. Baer and L. Blum, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **55**, 89–115 (1906); **56**, 92–100 (1907).

^b Liver perfusion, G. Embsden, H. Salomon, and F. Schmidt, *Beitr. chem. Physiol. u. Pathol.*, **8**, 129–155 (1906).

^c Incubation of liver slices, K. O. St. A. Lang and F. Adickes, *Z. physiol. Chem.*, **263**, 227–234 (1940).

^d Ketonemia after injection in rabbits, A. N. Wick, *J. Biol. Chem.*, **141**, 897–903 (1941).

^e Liver perfusion, J. Wirth, *Biocem. Z.*, **27**, 20–26 (1910). Liver slices, M. J. Coon and N. S. B. Abrahamsen, *J. Biol. Chem.*, **195**, 805–812 (1952).

^f Incubation of liver slices, P. P. Cohen, *J. Biol. Chem.*, **119**, 333–346 (1937).

^g Labeled isovaleric acid incubated with liver slices, I. Zabin and K. Bloch, *J. Biol. Chem.*, **185**, 117–129 (1950).

found to be much more ketogenic than the unnatural one.³⁰⁵ It was shown almost simultaneously by Baer and Blum³⁰⁶ that a greatly increased excretion of β -hydroxybutyric acid followed the administration of 33.7 g. of the amino acid to a diabetic patient. Butts and co-workers³⁰⁷ confirmed the fact that leucine gives rise to ketone bodies when fed to normal fasting rats; moreover, no glycogen was formed in rats after the administration of this amino acid. This confirms earlier reports³⁰⁸⁻³¹⁰ that leucine is not a glucose-former. Finally, Edson³¹¹ noted that acetone bodies were formed by liver slices when leucine was added to the substrate. This finding was confirmed by Cohen.¹⁰²

The pathway of metabolism of leucine, as pictured by Lusk,³¹² is given below:

The Conversion of Leucine to β -Hydroxybutyric Acid³¹²

A number of reports also indicate that isovaleric acid, one of the intermediates postulated in the leucine $\rightarrow$ β -hydroxy-butyrate reaction, is ketogenic. Thus, Cohen¹⁰² and Lang and Adickes³¹³ found that liver slices converted isovaleric acid into ketones. Zabin and Bloch,³¹⁴ employing labeled isovaleric acid, which was incubated with liver slices, showed that the isovaleric acid was broken at the β -carbon to yield a 2-carbon and a 3-carbon fragment. The isopropyl moiety was believed to be the more important source of the ketone bodies. In contradistinction to the

³⁰⁵ G. Embden, *Beitr. chem. Physiol. Pathol.*, **11**, 348-355 (1908).

³⁰⁶ J. Baer and L. Blum, *Naunyn-Schmiedeberg's Arch. exp'tl. Pathol. Pharmacol.*, **55**, 89-115 (1906).

³⁰⁷ J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, **120**, 289-295 (1937).

³⁰⁸ O. Simon, *Z. physiol. Chem.*, **35**, 315-323 (1902).

³⁰⁹ R. H. Wilson and H. B. Lewis, *J. Biol. Chem.*, **85**, 559-569 (1929-1930).

³¹⁰ J. T. Halsey, *Am. J. Physiol.*, **10**, 229-235 (1903-1904).

³¹¹ N. L. Edson, *Biochem. J.*, **29**, 2498-2505 (1935).

³¹² G. Lusk, *Science of Nutrition*, 4th ed., Saunders, Philadelphia, 1928, pp. 235, 640, 681.

³¹³ K. O. St. A. Lang and F. Adickes, *Z. physiol. Chem.*, **263**, 227-234 (1940).

³¹⁴ I. Zabin and K. Bloch, *J. Biol. Chem.*, **185**, 117-129 (1950).

above hypotheses, Coon and Gurin³¹⁵ have proposed an alternate pathway to explain the formation of acetoacetate from leucine. It is suggested that only the α and β carbons of leucine split off to form the C_2 intermediate which is capable of condensing to yield acetoacetate. The conclusions were reached on the basis of the results of *in vitro* studies with liver slices, employing leucine labeled with C^{14} in the β -position, and of the results obtained in *in vivo* studies with a phlorhizinized rat fed this labeled leucine. The following metabolic pathway is proposed:

The Conversion of Leucine to Acetoacetate as Proposed by Coon and Gurin³¹⁵

Coon and Gurin³¹⁵ are of the opinion that the isopropyl residue is not completely oxidized to CO_2 in the liver, and that it does not, to any appreciable extent, furnish a C_2 fragment similar to that arising from the α - and β -carbons of leucine. The earlier results of Bloch,³¹⁶ in which it was shown that only 0.5 mole of acetoacetate arose from one mole of leucine, would appear to support this new concept of the intermediary metabolism of leucine. Plaut and Lardy³¹⁷ suggested the possibility that the conversion of the isopropyl group of isovaleric acid to acetoacetate may be the result of a stoichiometric fixation of CO_2 . It was also demonstrated that CO_2 could be incorporated into the carboxyl group of acetoacetate formed from crotonate, butyrate, nonylate, and pyruvate by rat liver slices. This incorporation did not occur in the acetoacetate formed from *dl*- β -hydroxybutyrate and valerate.

b'. Norleucine: There is a lack of agreement on the question of whether or not norleucine, $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$, is a ketogenic amino acid. Although Edson³¹¹ found that norleucine was converted to

³¹⁵ M. J. Coon and S. Gurin, *J. Biol. Chem.*, **180**, 1159-1167 (1949).

³¹⁶ K. Bloch, *J. Biol. Chem.*, **155**, 255-263 (1944).

³¹⁷ G. W. E. Plaut and H. A. Lardy, *J. Biol. Chem.*, **186**, 705-716 (1950).

ketone bodies by liver slices, Cohen¹⁰² did not confirm this result. Moreover, in *in vivo* tests, Butts *et al.*³⁰⁷ were unable to demonstrate any ketonuria resulting from the feeding of norleucine to fasting rats. In fact, the latter workers demonstrated that norleucine possesses marked ketolytic properties, and is a source of glycogen in the rat. The glycogenic properties of this amino acid confirm the earlier results of Greenwald,³¹⁸ who demonstrated that "extra sugar" resulted after the feeding of the racemic as well as the D- and L- forms of norleucine to phlorhizinized dogs. The weight of evidence would seem to indicate that norleucine is a glycogenic and not a ketogenic acid.

c'. Isoleucine: Isoleucine, $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$, has properties intermediate between those of leucine and of norleucine. Although Dakin³¹⁹ was unable to demonstrate the formation of either "extra glucose" or acetone bodies when isoleucine was given to a phlorhizinized dog, Wirth³²⁰ did report the occurrence of ketonemia when blood containing isoleucine was perfused through a surviving liver. On the other hand, Butts, Blunden, and Dunn³⁰⁷ noted that the feeding of DL-isoleucine resulted in the production of a small amount of glycogen while, under certain other conditions, isoleucine served as a ketogenic acid. It was suggested that, following deamination of the amino acid and the subsequent decarboxylation, the ultimate formation of glycogen or ketone bodies was dependent upon whether a demethylation or a deethylation occurred on the β -carbon atom.

d'. Valine: The early studies of Embden *et al.*,⁷ in which liver perfusion was employed, yielded no evidence that valine, $(\text{CH}_3)_2\text{CH} \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$, gives rise to ketone bodies. Moreover, Dakin³²¹ was unable to demonstrate any sugar formation or ketone synthesis after the feeding of valine to phlorhizinized dogs. Chase and Lewis³²² did not observe any increase in liver glycogen three hours after the administration of valine. Edson³¹¹ was the only observer to report that valine was ketogenic; he tested this property of valine by incubating it with liver slices. Cohen¹⁰² failed to confirm the above finding, but reported instead that valine possesses an antiketogenic effect.

Later *in vivo* studies have apparently established the fact that valine is a glucose-former. Rose and co-workers³²³ demonstrated, by the use of the

³¹⁸ I. Greenwald, *J. Biol. Chem.*, **25**, 81-86 (1916).

³¹⁹ H. D. Dakin, *Oxidations and Reductions in the Animal Body*, 2nd ed., Longmans, Green, London, 1923.

³²⁰ J. Wirth, *Biochem. Z.*, **27**, 20-26 (1910).

³²¹ H. D. Dakin, *J. Biol. Chem.*, **14**, 321-333 (1913).

³²² B. W. Chase and H. B. Lewis, *J. Biol. Chem.*, **106**, 315-321 (1934).

³²³ W. C. Rose, J. E. Johnson, and W. J. Haines, *J. Biol. Chem.*, **145**, 679-684 (1942).

phlorhizin technic on dogs, that three of the five carbons of L-valine, D-valine, and DL-valine can be converted to glucose. In addition, Butts and Sinnhuber³²⁴ showed that glycogen is deposited in the livers of rats to a small extent following the administration of DL-valine. As a further confirmation of the glucose-forming properties of valine, these latter workers reported that it exerts a ketolytic effect when tested in rats with either exogenous or endogenous ketonuria.³²⁴

Rose and co-workers³²³ postulated that valine is metabolized as illustrated below:

The Intermediate Reactions in the Transformation of Valine to Glucose³²³

In further confirmation of the glucose-forming action of valine, Rose *et al.*³²³ proved that α -ketoisovalerate, $(\text{CH}_3)_2\text{CH}\cdot\text{CO}\cdot\text{COOH}$, is converted to glucose by the phlorhizinized dog to the same extent as is valine, *i.e.*, three of its five carbon atoms are changed. Moreover, Ringer, Frankel, and Jonas³²⁵ reported that the second postulated intermediate in the valine $\rightarrow$ glucose reaction, namely isobutyric acid, $(\text{CH}_3)_2\text{CH}\cdot\text{COOH}$, is transformed to glucose to the extent of 3 carbons.

e'. Isovaline: Although there are no data on the ketogenic activity of DL-isovaline, $\text{CH}_3\cdot\text{CH}_2\cdot\text{C}(\text{CH}_3)(\text{NH}_2)\cdot\text{COOH}$, Butts and Sinnhuber³²⁴ reported that it is devoid of glycogenic activity.

f'. Tyrosine and Phenylalanine: Baer and Blum³²⁶ reported that both tyrosine, $\text{HO}\cdot\text{C}_6\text{H}_4\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, and phenylalanine, $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, are ketogenic under certain conditions. Moreover, Embden and co-workers⁷ found that not only were tyrosine and phenylalanine converted to ketone bodies when perfused through a surviving liver, but homogentisic acid, $(\text{HO})_2\cdot\text{C}_6\text{H}_3\cdot\text{CH}_2\cdot\text{COOH}$, also, followed the same course of metabolism. Muconic acid, $\text{HOOC}\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$, which presumably is an intermediate in the phenylalanine $\rightarrow$ β -hydroxybutyrate reaction, likewise yields ketone bodies when perfused through a

³²⁴ J. S. Butts and R. O. Sinnhuber, *J. Biol. Chem.*, **139**, 963-969 (1941).

³²⁵ A. I. Ringer, E. M. Frankel, and L. Jonas, *J. Biol. Chem.*, **14**, 525-538 (1913).

³²⁶ J. Baer and L. Blum, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **56**, 92-100 (1907).

liver.³²⁷ Neither phenylalanine³²¹ nor tyrosine^{321,328} yields sugar when fed to phlorhizinized dogs.

On the other hand, Butts and associates³²⁹ were unable to demonstrate any ketonuria when either DL-phenylalanine or DL-tyrosine was fed to fast-ing rats. The amino acids appeared to have different metabolic pathways. Thus, phenylalanine gave rise to glycogen, and reduced an exogenous ketonuria, while tyrosine exerted no such effect. Moreover, homogentisic acid was excreted after the administration of phenylalanine, but not after that of tyrosine.³²⁹

Further proof that tyrosine is capable of conversion to acetoacetic acid has been recorded by Weinhouse and Millington.^{330,331} It was suggested that the data indicate that the α - and β -carbons of tyrosine become the carboxyl and α -carbon of acetoacetate, respectively. Two of the carbons in the benzene ring become the β - and γ -carbons in acetoacetate. It is interesting that Wakeman and Dakin,³³² many years before isotopic techniques were available, suggested that two of the four carbons of acetoacetate are probably derived from the phenyl ring, and two from the side-chain. In view of the conflicting evidence presented by several investigators, it is probably better at the present time to reserve judgment as to whether or not phenylalanine and tyrosine are ketogenic.

g'. Miscellaneous Amino Acids: Edson³¹¹ reported that α -aminobutyric acid, $\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, is slightly ketogenic, while Cohen¹⁰² found that this amino acid is toxic for liver slices. α -Aminovaleric acid, $\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, was shown, by the liver slice technic, to be ketogenic, while α -aminocaproic acid, $\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, gave negative results.¹⁰² Cohen,¹⁰² using the liver slice technic, demonstrated that, of the branched-chain α -amino acids, α -aminoisobutyric acid, $(\text{CH}_3)_2\cdot\text{C}(\text{NH}_2)\cdot\text{COOH}$, is toxic, α -aminoisovaleric acid, $(\text{CH}_3)_2\cdot\text{CH}\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, is not ketogenic, while α -aminoisocaproic acid, $(\text{CH}_3)_2\cdot\text{CH}\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, is ketogenic. The α -hydroxy- and α -keto acids of the normal and branched α -amino acids behaved in a manner similar to that of their parent amino acids.¹⁰²

(g) *Formation from Pyruvate.* There have been a number of reports in the literature to the effect that acetic acid is formed in the animal body from pyruvate (see discussion in Chapter V). Since acetoacetate can readily

³²⁷ M. Hensel and R. Riesser, *Z. physiol. Chem.*, **88**, 38-43 (1913).

³²⁸ A. J. Ringer and G. Lusk, *Z. physiol. Chem.*, **66**, 106-119 (1910).

³²⁹ J. S. Butts, M. S. Dunn, and L. F. Hallman, *J. Biol. Chem.*, **123**, 711-718 (1938).

³³⁰ S. Weinhouse and R. H. Millington, *J. Biol. Chem.*, **175**, 995-996 (1948).

³³¹ S. Weinhouse and R. H. Millington, *J. Biol. Chem.*, **181**, 645-653 (1949).

³³² A. J. Wakeman and H. D. Dakin, *J. Biol. Chem.*, **9**, 139-150 (1911).

be formed from acetate, it would appear that pyruvate can be considered as one of the parent substances of the ketone bodies.

Moreover, there is direct evidence that acetoacetate can accumulate in amounts up to 28% of that of the pyruvate oxidized^{333,335} when liver slices break down pyruvate in the presence of malonate³³³ or of ammonium chloride.³³⁴ Moreover, pyruvate is quantitatively converted to acetoacetate by the washed-cell liver preparation of Lehninger,^{3,146} When fumarate was added to the washed-cell preparations, acetoacetate formation was suppressed¹⁴⁰ in favor of a greater production of di- and tricarboxylic acids. Since the *in vitro* conditions which affect the relative yields of acetoacetate and the polycarboxylic acids were the same for octanoic acid and for pyruvic acid, it was believed that an acetyl-like C₂ compound was common to both fats and carbohydrates.

In a later study of the parallel behavior of octanoate and pyruvate with Lehninger's preparation, Crandall and Gurin⁵¹ found that only the α - and β -carbons of pyruvate become incorporated into the acetoacetate molecule. However, octanoate differed from pyruvate in the production of asymmetrically-labeled acetoacetate from carboxyl-labeled octanoic acid. When the octanoate was labeled on the carboxyl or β -carbon, acetoacetate was produced, with more C¹³ in the carboxyl than in the carbonyl group.³³⁵ On the other hand, when ζ -labeled octanoate was incubated with liver homogenate, the acetoacetate formed was predominantly labeled in the carbonyl group.³³⁶ It is suggested that the fatty acids, on oxidation, give rise to two species of C₂ fragments in the formation of acetoacetate; one of these, which is not readily acetylated, is derived from the ζ - and ω -carbons of octanoate, while the other type originates from the first six carbons of octanoate. The terminal fragment has a tendency to acetylate rather than to be acetylated. One possibility which is suggested is that pyruvate and acetate form two C units which are identical with the two C units derived from the first six carbons of octanoate.³³⁶ Shreeve³³⁷ has likewise reported that propionate is as effective as pyruvate as a precursor of acetyl groups in the intact rat.

However, the conversion of pyruvate to acetoacetate in the normal animal appears highly improbable. It will be observed that the only conditions in which this change has been observed are in *in vitro* tests where an abnormal metabolite is present which causes an alteration in the usual

³³³ E. Annau, *Z. physiol. Chem.*, **224**, 141-149 (1934).

³³⁴ N. L. Edson, *Biochem. J.*, **29**, 2082-2094 (1935).

³³⁵ E. A. Evans, Jr., *Biochem. J.*, **34**, 829-837 (1940).

³³⁶ D. I. Crandall, R. O. Brady, and S. Gurin, *J. Biol. Chem.*, **181**, 845-852 (1949).

³³⁷ W. W. Shreeve, *J. Biol. Chem.*, **195**, 1-10 (1952).

course of metabolism. Moreover, Weinhouse and Millington³³⁸ concluded that, in contrast to its action in homogenates of liver, pyruvate does not significantly increase acetoacetate production in liver slices. It is also suggested that pyruvate is not ketogenic in the intact normal liver cell. Bloch³³⁹ likewise points out that, in spite of the evidence that pyruvic acid is a source of ketone bodies, this reaction is contrary to results obtained with intact animals. Pyruvate has the ability to yield glycogen and to suppress ketonuria in the fasting rat,³⁴⁰ whereas fatty acids provoke a ketonuria. Under such conditions the catabolites of fat and pyruvate do not follow a single pathway. The derivation of acetoacetate from pyruvate may be a favored reaction under special conditions *in vitro*; however, if it occurs at all, it is quantitatively insignificant in the pyruvate metabolism of the intact animal. Bloch concludes that "The hypothesis that a C₂ fragment arises in the oxidation of both carbohydrate and fat would seem to be untenable unless it is assumed that the C₂ fragments are not identical."

In a number of reactions the behavior of pyruvate differs from that of fatty acids. Thus, whereas acetate serves as an excellent source of the acetyl group for both aromatic amines (such as sulfanilamide) and for α -amino acids (such as phenylaminobutyric acid), pyruvate, when administered as tagged alanine, can acetylate α -amino acids only.³⁴¹ Another difference is that pyruvate (alanine) does not serve as a building stone for cholesterol either *in vivo*³⁴¹ or *in vitro*.³⁴² An additional variation between the physiologic behavior of acetate and that of lactate (which is in equilibrium with pyruvate) is that acetate may serve as the source of carbons 2 and 8 in the purine nucleus, while lactate cannot be incorporated into the uric acid molecule. Thus, if pyruvic acid or lactic acid were converted to the same C₂ fragment which originates from acetate, they should mediate identical reactions in the acetylation of aromatic amines and in the synthesis of cholesterol and of uric acid. Since this does not occur, one must conclude that no important part of pyruvate metabolism proceeds by way of the acetyl group.

(h) *Formation from Dicarboxylic Acids.* On the basis of studies with liver slices, Edson³⁴³ concluded that the dicarboxylic acids are not ketogenic.

³³⁸ S. Weinhouse and R. H. Millington, *J. Biol. Chem.*, **193**, 1-10 (1951).

³³⁹ K. Bloch, *Physiol. Revs.*, **27**, 574-620 (1947).

³⁴⁰ I. Shapiro, *J. Biol. Chem.*, **108**, 373-387 (1935).

³⁴¹ K. Bloch and D. Rittenberg, *J. Biol. Chem.*, **159**, 45-58 (1945).

³⁴² D. Rittenberg, E. Borek, and K. Bloch, *Federation Proc.*, **5**, 151 (1946).

³⁴³ N. L. Edson, *Biochem. J.*, **30**, 1855-1861 (1936).

However, oxalic acid, when present in concentrations too low to inhibit respiration in liver slices, appeared to have some ketogenic action. The explanation for this effect is not immediately apparent.

c. Conditions Resulting in Ketonuria. Many circumstances will result in the production of ketosis. It is generally believed that animals especially susceptible to one type of ketonuria exhibit a high degree of susceptibility to other types of ketonuria as well.

(a) *Fasting.* The most common type of ketonuria is produced by inanition. The rate of development and the intensity of this type of ketosis vary with species. Thus, Crandall³⁴⁴ found that ketonemia developed in men after a fast lasting thirty-nine hours, while two to three days elapsed before it was exhibited in dogs. In the case of man, the maximum ketonemia was reached after three or four days; from then on the level of ketone bodies in the blood continued practically at a constant level, although the rate was sometimes slightly lower than the maximum obtained early in the fast. The average ketonuria values of normal male subjects for the first four days, as reported by Deuel and Gulick,²⁷⁹ expressed in g. per sq. meter of body surface, were as follows: 0 day, 0.02; first day, 0.05; second day, 0.86; third day, 1.90; and fourth day, 2.66. The highest amounts excreted during this period by a male subject, expressed in total ketone bodies for the control day and the succeeding four fast days, were the following: 0.04, 0.28, 5.01, 8.06, and 8.94 g. In the case of the normal female subjects, the average values expressed in g./sq. meter of body surface were as follows: 0 day, 0.02; first day, 0.54; second day, 4.25; third day, 8.47; and fourth day, 6.56. The maximum excretion by a woman expressed in total ketone bodies for the control day and for the succeeding three fast days were the following: 0.03, 0.05, 4.02, and 20.67 g.

In the case of prolonged fasts, there is likewise evidence that the ketonuria continues until the fasts are discontinued. Thus, in the experiment on the professional faster, Succi, studied by Luciani,³⁴⁵ the values for urinary β -hydroxybutyric acid (expressed in g.) were recorded by Brugsch,³⁴⁶ from the twenty-third to the thirtieth day, respectively, as follows: 9.24, 8.43, 9.85, 5.28, 11.62, 6.99, 9.15, and 13.60 (Av. 9.34). The excretion of acetone in g. over the same period was the following: 0.569, 0.410, 0.463, 0.569, 0.525, 0.339, 0.242, and 0.115 (Av. 0.404). Similar data are avail-

³⁴⁴ L. A. Crandall, Jr., *J. Biol. Chem.*, 138, 123-128 (1941).

³⁴⁵ L. Luciani, *Das Hungern*, Trans. M. C. Fraenkel, Hamburg & Leipzig, 1890; cited by G. Lusk, *Science of Nutrition*, 4th ed., Saunders, Philadelphia and New York, 1928, p. 91.

³⁴⁶ T. Brugsch, *Z. exptl. Pathol. u. Therap.*, 1, 419-430 (1905); cited by G. Lusk, *Science of Nutrition*, 4th ed., Saunders, Philadelphia and New York, 1928, p. 94.

able in the case of the professional faster, Levanzin, who was studied by Benedict;³⁴⁷ the latter subject excreted 5.0 g. of β -hydroxybutyrate on the twenty-first day, and 4.5 g. on the thirty-first fast day.

(b) *The Ingestion of a Protein-Fat Diet.* Ketonuria occurs in human subjects maintained on a protein-fat diet largely devoid of carbohydrate. McClellan and DuBois³⁴⁸ and Tolstoi³⁴⁹ reported that ketonuria occurred continuously in the case of two Arctic explorers who were on a protein-fat diet over the course of a year. Deuel *et al.*³⁵⁰ showed that the level of ketonuria was maintained on a similar diet for as long as twenty-seven days. The acetone-body excretion of one subject in g. for the days on the protein-fat diet were as follows: 1, 0.14; 2, 1.01; 3, 1.10; 4, 2.50; 5, 2.71; 6, 4.89; 7, 6.41; 8, 5.17; 9, 8.23; 10, 6.21; 11, 5.30; 12, 6.31; 13, 7.05; 14, 6.62; 15, 5.26; 16, 5.73 (25 g. glucose taken on this day); 17, 4.45; 18, 5.91; and 19, 5.70. The maximum values correspond almost exactly to those for the same subject during a complete fast, which were as follows, expressed in g.: 0, 0.64; 1, 0.30; 2, 5.17; 3, 7.28, 4, 8.95, 5, 6.64; 6, 9.52; and 7, 7.60. However, the maximum level of ketonuria was reached more slowly on the protein-fat regimen than during starvation.

(c) *Diabetes Mellitus.* Prior to the advent of insulin, marked ketosis was an invariable concomitant of diabetes mellitus. Frequently, the extreme acidosis so engendered was partially responsible for the fatal outcome of this disease. For example, the ketonuria (β -hydroxybutyrate) of a patient reported by Mosenthal and Lewis³⁵¹ for a period shortly before death was as follows (expressed in g.): Day 1, 64.9; Day 2, 66.0; Day 3, 50.6; Day 4, 64.9; Day 5, 78.0; Day 6, 111.2; Day 7, 106.6; and Day 8, 73.0. The patient died on the second day after the last determination of ketonuria. The subject refused food during this period, receiving largely alcoholic beverages. One factor which may have contributed to the high ketonuria was the fact that NaHCO_3 was administered at a high level.

In another case of severe diabetes mellitus in the pre-insulin era, which was studied by Geyelin and DuBois,³⁵² the high ketonuria decreased with clinical improvement. Thus, the subject, Cyril K., presented β -hydroxybutyrate values on thirteen days as follows: 43.7, 34.6, 60.0, 53.0, 57.9, 55.2, 70.8, 75.1, 87.4, 58.5, 56.8, and 41.2 g. As the symptoms cleared up, the ketonuria decreased to 26.2 g. on the fourteenth day, and to 10.95 g. on

³⁴⁷ F. G. Benedict, *A Study of Prolonged Fasting*, Carnegie Inst., Washington, Publ. No. 203, 5-416, 1915, p. 283.

³⁴⁸ W. S. McClellan and E. F. DuBois, *J. Biol. Chem.*, 87, 651-668 (1930).

³⁴⁹ E. Tolstoi, *J. Biol. Chem.*, 83, 753-758 (1929).

³⁵⁰ H. J. Deuel, Jr., M. Gulick, and J. S. Butts, *J. Biol. Chem.*, 98, 333-351 (1932).

³⁵¹ H. O. Mosenthal and D. S. Lewis, *Bull. Johns Hopkins Hosp.*, 28, 187-191 (1917).

the fifteenth day. By the twenty-first day, a value of 0.24 g. was obtained.

The deleterious effect of large doses of sodium bicarbonate on ketone body excretion probably accounts for the high level of acetonuria in a subject investigated by Weiland.³⁵³ This patient, who received green vegetables, 200 g. of meat, and 120 g. of sodium bicarbonate daily, had a combined acetone and β -hydroxybutyrate excretion over the four days of observation of 105.2, 80.2, 121.0, and 103.8 g.

(d) *Pancreatic Diabetes and Alloxan Diabetes.* Allard³⁵⁴ found that depancreatized dogs developed a considerable acidosis, together with excretion of acetone bodies; they died in coma.

Alloxan, a substance which can be readily synthesized in the laboratory from uric acid, produces an experimental type of diabetes³⁵⁵⁻³⁵⁹ which is closely related to that following pancreatectomy. Dunn and co-workers^{360,361} demonstrated that this drug causes a specific degeneration of the islands of Langerhans of the pancreas, without evident injury to other organs or glands, except for an early tubular nephritis.³⁶⁰ According to Banerjee and Bhattacharya,³⁶² acetonuria reaches a maximum one week after the injection of alloxan into rabbits. Acetonuria is not related to the level of glycosuria. The diabetogenic action of alloxan may be prevented by the injection of glutathione, which accelerates the destruction of alloxan in the blood;³⁶³ moreover, animals such as the guinea pig whose blood has a high glutathione content are more resistant to alloxan than are other animals.³⁶⁴⁻³⁶⁶ In 1948, Lukens³⁶⁷ reviewed the subject of alloxan diabetes.

(e) *Phlorhizin Diabetes.* It is well known that, during fasting in phlorhizin glycosuria, carbohydrates are not oxidized, and that an acidotic condition resembling diabetes mellitus obtains. However, when carbohydrate

³⁵² H. R. Geyelin and E. F. DuBois, *J. Am. Med. Assoc.*, **66**, 1532-1534 (1916).

³⁵³ W. Weiland, *Z. exptl. Pathol. Therap.*, **12**, 116-151 (1913).

³⁵⁴ E. Allard, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **59**, 391-396 (1908).

³⁵⁵ C. C. Bailey and O. T. Bailey, *J. Am. Med. Assoc.*, **122**, 1165-1166 (1943).

³⁵⁶ J. S. Dunn and N. G. B. McLetchie, *Lancet*, **1943**, *II*, 384-386.

³⁵⁷ M. G. Goldner and G. Gomori, *Endocrinology*, **33**, 297-308 (1943).

³⁵⁸ R. Carrasco-Formiguera, *J. Lab. Clin. Med.*, **29**, 510-517 (1944).

³⁵⁹ J. A. Ruben and K. Y. Yardumian, *Science*, **103**, 220-221 (1946).

³⁶⁰ J. S. Dunn, H. L. Sheehan, and N. G. B. McLetchie, *Lancet*, **1943**, *I*, 484-487.

³⁶¹ J. S. Dunn, J. Kirkpatrick, N. G. B. McLetchie, and S. V. Telfer, *J. Pathol. Bacteriol* **55**, 245-257 (1943).

³⁶² S. Banerjee and G. Bhattacharya, *J. Biol. Chem.*, **178**, 145-149 (1949).

³⁶³ A. Lazarow, *Proc. Soc. Exptl. Biol. Med.*, **61**, 441-447 (1946).

³⁶⁴ E. S. West and D. M. Highet, *Proc. Soc. Exptl. Biol. Med.*, **68**, 60-62 (1948).

³⁶⁵ M. G. Goldner, *Bull. N. Y. Acad. Med.*, **21**, 44-45 (1945).

³⁶⁶ M. Griffiths, *Australian J. Exptl. Biol. Med. Sci.*, **26**, 339-346 (1948).

³⁶⁷ F. D. W. Lukens, *Physiol. Revs.*, **28**, 304-330 (1948).

is given to the phlorhizinized dog in a sufficiently large dose to charge the tissues with carbohydrate temporarily, a portion of this foodstuff is oxidized, and the diabetic symptoms disappear.³⁶⁸ Wierzuchowski^{369,370} was the first to observe that the dramatic improvement in physical condition noted when glucose was given to a phlorhizinized dog was related not only to its increased blood sugar level but also to the disappearance of the acidosis. Acetonuria had vanished within four hours after the administration of glucose, and the CO₂-combining power of the blood returned to normal within this period.

Goldfarb *et al.*³⁷¹ reported that rats, fasted and phlorhizinized over periods of one to four days, excreted ketone bodies varying in amount between 277 and 1760 mg. It was concluded that the production of the ketone bodies closely approximated the theoretical yield based upon corrected factors for the ketogenic and antiketogenic values of the foodstuffs oxidized. In a later report, Goldfarb³⁷² arrived at similar conclusions, following an investigation of various species of the monkey family.

(f) *Alkalosis, Acidosis, and Ketosis.* The ketone bodies are readily manufactured in the body whenever it is necessary to combat alkalosis. Davies and associates³⁷³ were the first to note that, when alkalosis due to CO₂ deficit produced by hyperventilation was present, acetonuria occurred. This observation was confirmed by a number of workers.³⁷⁴⁻³⁷⁶

Several investigators have reported on the ketogenic effect of an alkalosis produced by the ingestion of an excess of alkali. Booher and Killian³⁷⁵ noted that abnormally large amounts of ketone bodies were produced in human subjects as the result of an excessive administration of alkali or because of the loss of HCl by vomiting. Butts and associates³⁷⁷ found that an increased ketonuria over and above the minimal value found during fasting in rats resulted from the administration of sodium bicarbonate. The effect of alkali administration was much more pronounced in the female than it was in the male animals. The studies of MacKay and co-workers,³⁷⁸

³⁶⁸ H. J. Deuel, Jr., H. E. C. Wilson, and A. T. Milhorat, *J. Biol. Chem.*, **74**, 265-298 (1927).

³⁶⁹ M. Wierzuchowski, *J. Biol. Chem.*, **67**, xlii (1926).

³⁷⁰ M. Wierzuchowski, *J. Biol. Chem.*, **68**, 385-397 (1926).

³⁷¹ W. Goldfarb, S. B. Barker, and H. E. Himwich, *J. Biol. Chem.*, **105**, 283-286 (1934).

³⁷² W. Goldfarb, *J. Biol. Chem.*, **116**, 787-791 (1936).

³⁷³ H. W. Davies, J. B. S. Haldane, and E. L. Kennaway, *J. Physiol.*, **54**, 32-45 (1920).

³⁷⁴ H. Beumer and A. Soecknick, *Z. Kinderheilk.*, **37**, 236-241 (1924).

³⁷⁵ L. E. Booher and J. A. Killian, *Proc. Soc. Exptl. Biol. Med.*, **21**, 528-529 (1924).

³⁷⁶ J. B. S. Haldane, *Lancet*, **1924**, *I*, 537-538.

³⁷⁷ J. S. Butts, H. J. Deuel, Jr., and L. Hallman, *Proc. Soc. Exptl. Biol. Med.*, **32**, 898-899 (1934-1935).

³⁷⁸ E. M. MacKay, A. N. Wick, H. O. Carne, and C. P. Barnum, *J. Biol. Chem.*, **138**, 63-68 (1941).

demonstrated, in the case of the rat, that acidosis reduces ketonuria while alkalosis augments this condition. This is explained on the basis that acidosis produces an antiketogenic effect due to increased protein catabolism, which results in an increased glucose formation. Alkalosis, on the other hand, was believed to have the opposite effect on this process. In the case of man, Beumer and Soecknick³⁷⁴ and Porges and Lipschütz³⁷⁹ demonstrated that fasting ketonuria is exaggerated by the ingestion of alkali, while Adlersburg³⁸⁰ noted that ketosis of fasting was decreased by the ingestion of acid ammonium phosphate. In the light of the later experimental work, it seems probable that the excessively high levels of ketonuria reported in diabetic subjects in pre-insulin days may have been partly caused by the extremely high dosages of alkali employed. According to Lusk,³¹² dosages of sodium bicarbonate as high as 200 g. per day were in common use. This subject has been discussed further in the early section on diabetes mellitus (see page 122).

Although the administration of acid-producing salts such as NH_4Cl results in a decrease of ketonuria in the intact animal, just the opposite effect has been reported in isolated tissues. Recknagel and Potter,³⁸¹ using rat liver homogenates supplemented with ATP, confirmed the earlier reports of Annau³³³ obtained with liver mince, and of Edson³³⁴ with liver slices, which demonstrated that ammonium chloride exerts a ketogenic effect. It is suggested that the following steps are involved: (1) A preliminary vigorous reductive amination of α -ketoglutaric acid takes place; (2) because of the interruption in the Krebs cycle at the stage of the oxidation of α -ketoglutarate to succinate, the system fails to supply sufficient oxaloacetate for citric acid condensation; and (3) since the preferential pathway to citric acid is no longer available, the metabolism of ketone body precursors is shunted in the direction of acetoacetate formation.

(g) *Experimental Ketonuria in Rats.* The rat is an animal which, under ordinary conditions, produces a ketonuria of such low intensity as to render it entirely valueless for studies in this field. Thus, Levine and Smith³⁸² reported that the total ketonuria for a four-day period calculated for a 250 g. rat was 16.9 mg. The total ketone excretion for a group of nine rats with an average weight of 224 g. varied between 2.3 and 19.2 mg. Cori and Cori³⁸³ observed that rats excreted 6.2 mg. per 100 g. rat per day in

³⁷⁹ O. Porges and H. Lipschütz, *Naunyn-Schmiedeberg's Arch. expl. Pathol. Pharmacol.*, **97**, 379-385 (1923).

³⁸⁰ D. Adlersburg, *Biochem. Z.*, **143**, 527-532 (1923).

³⁸¹ R. O. Recknagel and V. R. Potter, *J. Biol. Chem.*, **191**, 263-275 (1951).

³⁸² H. Levine and A. H. Smith, *J. Biol. Chem.*, **75**, 1-22 (1927).

³⁸³ G. T. Cori and C. F. Cori, *J. Biol. Chem.*, **72**, 615-625 (1927).

the summer, while the average was only 1.9 mg. per day in the winter. In the experiments of Butts and Deuel,³⁸⁴ no sexual variation in fasting ketonuria was noted in the rat. The average ketonuria for the male rats (based upon a 250 g. animal for four days), during two periods, amounted to 5.5 and 7.5 mg., while the comparative results on females were 4.8 and 7.0 mg. Apparently, the sex variation in ketonuria does not obtain for the fasting ketonuria in the rat, as it does for starvation ketosis in human subjects.

a'. Exogenous Ketonuria: Butts and Deuel³⁸⁴ reported that a physiologic ketonuria can be produced in rats if they are given orally, during fasting, a solution of acetoacetate which has been adjusted to a pH of 7.40. This ketonuria is reproducible in different animals, and the experiment can, in most cases, be continued for five or even seven days without fatality. The most satisfactory dosage of sodium acetoacetate was found to be 150 mg. (calculated as acetone) per 100 sq. cm. of body surface per day, given in divided doses in the morning and in the evening. Surface area can be calculated by the formula of Lee,³⁸⁵ based upon the weight on the first day of the test. Under usual conditions, no food is given during the ketonuria test, but substances which are to be tested as sources of ketolytic material are administered in appropriate dosages at the same time that the ketogenic material is given. It is most important that a supply of water be continuously available, as the animals must have a sufficient amount because of the diuretic effect of the ketones. Urine collections can be made in flasks connected with funnels above which round cages are suspended, as described by Levine and Smith.³⁸⁶ To prevent contamination of the urine with feces, a cone made of wire netting is inserted in the neck of the funnel. Urine is collected under paraffin oil to prevent the volatilization of acetone.

Although acetoacetate was administered in the early experiments to produce the ketosis,^{21,340,384,387} it was later found that butyric acid was equally effective, and it has since been used in place of acetoacetate. Not only is butyric acid cheaper, more uniform, and much more easily prepared for use than is acetoacetate, but solutions of sodium butyrate are more stable than are those of sodium acetoacetate. In the later tests by the Deuel group and the Butts group and others, in which exogenous ketonuria was employed to study the ketolytic action of various metabolites, sodium butyrate was exclusively employed as the ketogenic agent. Because of the

³⁸⁴ J. S. Butts and H. J. Deuel, Jr., *J. Biol. Chem.*, **100**, 415-428 (1933).

³⁸⁵ M. O. Lee, *Am. J. Physiol.*, **89**, 24-33 (1929).

³⁸⁶ H. Levine and A. H. Smith, *J. Lab. Clin. Med.*, **11**, 168-172 (1925).

higher degree of ketonuria exhibited by female rats as compared with male rats,³⁸⁴ animals of the former sex have usually been preferred when it is desirable to produce a high level of ketonuria in the control group. The output of ketone bodies is sufficiently high to make a collection of urine and a determination of ketone bodies possible on a daily basis. From a single animal, one may obtain several confirmatory results over the four or five days during which the experiment is continued. For more exact quantitative comparisons of the ketolytic action of different metabolites, it is preferable to compare levels of acetonuria of a control group receiving only the butyrate with that of another group receiving butyrate plus the metabolite under study.

There are a number of indications that the ketonuria produced by the administration of sodium butyrate is physiologic, and compares with fasting ketonuria in man. In the first place, the sex difference in susceptibility to ketonuria, first reported by Deuel and Gulick²⁷⁹ in human subjects, has been repeatedly observed in rats since this phenomenon was first reported by Butts and Deuel.³⁸⁴ Moreover, the greater ketolytic effect exhibited by galactose over that by glucose, first demonstrated by Deuel, Gulick, and Butts³⁵⁰ in man, has also been shown to obtain in rats.³⁸⁷ Finally, the fact that no ketonuria occurs when the ketogenic acid is administered to normally-fed rats,³⁸⁸ as well as the fact that the level of ketone body excretion is reduced in proportion to the amount of ketolytic material fed, are indications of the physiologic nature of the ketosis. Because this type of ketonuria is produced as a result of ketogenic material fed simultaneously to the experimental animal, it has been referred to as "exogenous ketonuria."³⁸⁹

b'. Endogenous Ketonuria: When rats which have developed fatty livers as a result of being placed on a low protein, choline-free diet are fasted, a ketonuria of considerable magnitude occurs, in spite of the fact that no exogenous ketogenic material is being given.³⁹⁰ This phenomenon is in contradistinction to the situation in normal rats, in which no appreciable ketonuria obtains during fasting unless ketogenic acids are fed simultaneously. The type of ketonuria resulting when rats with fatty livers are fasted is known as "endogenous ketonuria."³⁸⁹ Endogenous ketonuria is also to be considered a physiologic type of ketonuria. Thus, female

³⁸⁷ J. S. Butts, *J. Biol. Chem.*, **105**, 87-96 (1934).

³⁸⁸ J. S. Butts, *The Comparative Ketolytic Action of Glucose, Galactose, and Lactose, When Administered to Rats Suffering from an Artificial Ketosis*, Thesis, Univ. Southern California, Dept. Biochem. Nutrition, 1933.

³⁸⁹ H. J. Deuel, Jr., L. F. Hallman, and S. Murray, *J. Biol. Chem.*, **124**, 385-393 (1938).

³⁹⁰ H. J. Deuel, Jr., L. F. Hallman, and S. Murray, *J. Biol. Chem.*, **119**, 257-268 (1937).

rats exhibit a higher level of ketonuria than do the males. This response is identical with that of human subjects during fasting, and with that of rats subjected to an exogenous ketonuria. Moreover, ketolytic substances such as glucose tend to suppress the endogenous ketonuria, as would be expected if it is assumed to be a physiologic condition.

One might expect the endogenous type of ketonuria to be preferable to the exogenous type for experimental studies, since the rate of production of ketone bodies is constant in the first case, while it must be highest during a few hours after the ketogenic acid is fed in the latter type of ketonuria. However, substances which themselves are not ketolytic but become active only after transformation to glucose or to some similar precursor may have a less pronounced ketolytic effect when tested against an endogenous ketonuria than when the test animals have an exogenous ketonuria. In all probability, this may be explained by the fact that the fatty liver is unable to bring about the essential changes in the metabolite which can be readily effected by a liver containing a normal proportion of fat. For a further discussion of this point, the reader is referred to *The Lipids*, Volume II, Chapter VI.

Janes and Brady³⁹¹ noted that, when excessive doses of niacin were given to rats fasted for periods up to five days, a marked ketonuria occurred. It is stated that the ketogenic properties of niacin are not associated with its acid or vasodilatory properties. It is possible that a choline deficiency of sufficient magnitude may develop to bring about the ketonuria, as is the case in the fatty livers. Niacin is detoxicated by methylation, and, according to some reports, its administration in excessive amounts may result in the development of fatty livers (see *The Lipids*, Volume II, Chapter VI).

d. Factors Altering the Extent of Ketonuria. The susceptibility of an animal to ketosis is a function of species, age, sex, nutritional condition, hormonal control, and many related factors.

(a) *Species as a Factor in Ketonuria.* Man^{279,344} and monkey^{392,393} have been generally considered to be the species most susceptible to a fasting ketonuria. The level of ketonuria in the adult dog, which is classed as a carnivore, is extremely low.^{344,394} It has been stated that only a minimum ketonuria develops during fasting in steers, goats,³⁹² rabbits,³⁹⁵ and rats.³⁸²⁻³⁸⁴

³⁹¹ R. G. Janes and J. Brady, *Am. J. Physiol.*, 159, 547-550 (1949).

³⁹² I. A. Mirsky, N. Nelson, I. Grayman, and S. Elgart, *Endocrinology*, 31, 264-270 (1942).

³⁹³ T. E. Friedemann, *J. Biol. Chem.*, 105, 335-341 (1934).

³⁹⁴ F. M. Allen, *J. Metabolic Research*, 4, 199-222 (1923).

³⁹⁵ L. Kallós-Deffner, *Z. ges. expit. Med.*, 92, 389-393 (1933).

Although, in general, such species variations are valid, there are many conditions in which ketonuria becomes an important problem in the latter group of animals. The most complete review on the subject of ketosis in domestic animals is that of Sampson.³⁹⁶

a'. Ketosis in Cattle: It is becoming apparent that, among the domestic animals, the ruminants are especially susceptible to ketosis. Information now available indicates that cattle, sheep, and goats readily develop ketonuria. Nothing is known at present about the susceptibility of the camel or of members of the deer family to ketosis but, according to Sampson,³⁹⁶ it will be no surprise if future studies reveal both experimental and spontaneous ketonuria in these ruminants, especially under circumstances which produce this condition in cows and ewes. Practically all reports of ketonuria in ruminants are concerned with the development of this condition in the female, where it is associated with lactation or with pregnancy, no reports of spontaneous ketosis in male ruminants are known to the author.

The nervous form of ketosis or acetonemia in cows is a disease which has been recognized for over a century, but the causative factor has only recently been understood. According to Udall,³⁹⁷ Landel described this condition as early as 1849, at which time he classified it under the name of "mania puerperalis." Other early reports of this disease in cows are cited in the textbook of Fleming³⁹⁸ published in 1879. The occurrence of acetonemia in cattle as the most prominent symptom of this disease was first recognized by Sjollema and van der Zande³⁹⁹ in Holland in 1923. Five years later, Hupka⁴⁰⁰ suggested that it was, in all probability, a common cattle disease in Germany. In the United States, acetonemia in cattle was first described by Truax⁴⁰¹ in Iowa (1930), and by Hayes⁴⁰² in Texas (1931). Since that time, the incidence of this condition has been noted in many locations in the United States and in a number of European countries. For a more complete discussion of the distribution of acetonemia in cattle, the reader is referred to the report by Sampson.³⁹⁶

³⁹⁶ J. Sampson, "Ketosis in Domestic Animals," *Univ. Illinois, Agr. Expt. Sta., Bull. No. 524*, 407-470 (1947).

³⁹⁷ D. H. Udall, *The Practice of Veterinary Medicine*, 4th ed., Udall, Ithaca, N. Y., 1943; cited by J. Sampson "Ketosis in Domestic Animals," *Univ. Illinois, Agr. Expt. Sta., Bull. No. 524*, 1947, p. 411.

³⁹⁸ G. Fleming, *A Textbook of Veterinary Obstetrics*, Cogswell, New York, 1879, pp. 687-689; cited by J. Sampson, *Univ. Illinois, Agr. Expt. Sta., Bull. No. 524*, 1947, p. 411.

³⁹⁹ B. Sjollema and J. E. van der Zande, *J. Metabolic Research*, 4, 525-533 (1923).

⁴⁰⁰ E. Hupka, *Deut. tierärztl. Wochschr.*, 36, Special No., 98-101 (1928); also cited by J. Sampson, *Univ. Illinois, Agr. Expt. Sta., Bull. No. 524*, 1947, pp. 417, 419.

⁴⁰¹ E. R. Truax, *Fort Dodge Biochem. Rev.*, 1, 5-7 (1930); cited by J. Sampson, *Univ. Illinois, Agr. Expt. Sta., Bull. No. 524*, 1947, p. 416.

⁴⁰² W. F. Hayes, *North Am. Veterinarian*, 12, No. 3, 31-33 (March 1931).

There appears to be general agreement that spontaneous or clinical ketosis of cows and also of ewes is associated with hypoglycemia. Thus, it has been reported that an inverse relationship exists between the levels of total ketone bodies and glucose in the blood of ruminants suffering from ketosis.⁴⁰³⁻⁴⁰⁵ The average ketonemia and blood sugar of nine healthy cows were found to be 2.21 mg. % (0 to 7.07) and 48.2 mg. % (36.2 to 59.9), respectively, as contrasted with mean values of 55.8 mg. % (22.0 to 176.1) and 28.5 mg. % (18.1 to 44.1), respectively, in the case of nine cows suffering from ketosis.³⁹⁶ The symptoms of the ketosis resulting from hypoglycemia are believed to be due to the hypoglycemia itself, while the acetonemia is attributed to the failure of carbohydrate oxidation. According to Kruse (cited by Hupka)^{400,406} typical symptoms of acetonemia in cows were produced in healthy animals by the administration of large doses of insulin. However, clinical ketosis has likewise been reported in cows having a fairly normal blood sugar level.⁴⁰⁶⁻⁴⁰⁸ Recovery of normal appetite and milk flow does not occur until ketonemia has been dissipated.⁴⁰⁹ Moreover, Shaw⁴¹⁰ was unable to prevent the incidence of ketosis in one herd of high-producing Holstein cattle by feeding molasses for several weeks prior to parturition. It was, however, recognized that a close relationship exists between the amount of liver glycogen and the development of ketosis. Although most workers have reported that clinical ketosis in dairy cattle usually occurs from one to six weeks after parturition,^{399,408,411} Knodt *et al.*⁴¹² state that factors such as gestation, parturition, stage of lactation, and sudden cessation of milking do not ordinarily exert much influence on the concentrations of acetone bodies in the blood and urine.

Acetonemia in cattle has been related to vitamin B₁ deficiency,⁴¹³ but this appears questionable, inasmuch as the mature ruminant is able to

⁴⁰³ J. Sampson and C. E. Hayden, *Cornell Vet.*, 26, 183-199 (1936).

⁴⁰⁴ J. W. Groenewald, H. Graf, and R. Clark, *Onderstepoort J. Vet. Sci. Animal Ind.*, 17, 225-244 (1941); J. W. Groenewald, H. Graf, P. M. Becker, J. R. Malan, and R. Clark, *Ibid.*, 245-296 (1941).

⁴⁰⁵ M. H. Roepke, *J. Am. Vet. Med. Assoc.*, 100, 411-415 (1942).

⁴⁰⁶ Kruse, *Beitrag zur Aetiologie der Gebärpause*, Inaug. Diss., Hannover, 1927; cited by E. Hupka, *Deut. tierärztl. Wochschr.*, 36, Special No., 98-101 (1928), p. 101.

⁴⁰⁷ J. Sampson, A. C. Gonzaga, and C. E. Hayden, *Cornell Vet.*, 23, 184-207 (1933).

⁴⁰⁸ G. F. Boddie, *Vet. Record*, 15, 1539-1546 (1935); *Chem. Abst.*, 30, 6054 (1936).

⁴⁰⁹ J. Sampson and L. E. Boley, *Am. J. Vet. Research*, 2, 327-332 (1941).

⁴¹⁰ J. C. Shaw, *J. Dairy Sci.*, 26, 1079-1090 (1943).

⁴¹¹ C. W. Duncan, C. F. Huffman, and H. A. Tobin, *J. Am. Vet. Med. Assoc.*, 95, 690-700 (1939).

⁴¹² C. B. Knodt, J. C. Shaw, and G. C. White, *J. Dairy Sci.*, 25, 851-860 (1942).

⁴¹³ B. Carlström, K. Myrbäck, N. Holmin, and A. Larsson, *Skand. Vet. Tidskr.*, 29, 403-444, 589-597 (1939); *Abst. Vet. Bull.*, 11, 470-472 (1941); *Acta Med. Scand.*, 102, 175-213 (1939).

synthesize this vitamin by the action of microorganisms in the rumen or other parts of the digestive tract.^{414,415} Patton⁴¹⁶⁻⁴¹⁸ is of the opinion that ketonemia in cattle is related to a deficiency of vitamin A, but the fact that acetoneuria has been observed in cattle fed liberal amounts of green alfalfa, and even in cows on green pasture, seems to preclude that possibility.^{408,409,419,420} Moreover, Shaw *et al.*⁴²¹ reported normal values for plasma carotene (325 $\mu\text{g. \%}$) and vitamin A (23.2 $\mu\text{g. \%}$) in nine cows with ketosis in which the blood glucose averaged 27.6 mg. $\%$ and the blood acetone bodies presented a mean of 37.8 mg. $\%$. Likewise, vitamin A therapy has proved of little value in the treatment of acetoneuria in cattle.⁴²¹⁻⁴²³ The clinical ketonemia of cattle has also been attributed to dysfunction of the pituitary gland;⁴²⁴ however, the data do not appear to be conclusive.

According to Knodt *et al.*⁴²⁵ the average mean content of total acetone bodies in 126 analyses of blood of normal cattle was 2.99 mg. $\%$ (0.69 to 5.54), while the mean value for 58 analyses of cows with ketosis was found to be 33.6 mg. $\%$ (2.40 to 89.3).⁴²⁶ Urinary acetone bodies averaged 10 mg. $\%$ (86 analyses varying between 2.50 and 70.0), while the figure for cows with ketosis averaged 253.8 mg. $\%$ (53 analyses varying between 10.0 and 1568). According to Sjollem and van der Zande,³⁹⁹ urinary acetone bodies are present to the extent of 10 to 13 g. per kg. urine (1000 to 1300 mg. $\%$) in acetoneuria, whereas the normal level of urinary ketonuria is 0.1 to 0.7 g. per kg. urine (10 to 70 mg. $\%$). However, other workers^{413,414} state that the urine of normal cows usually contains 0.3 g. of ketone bodies per liter (30 mg. $\%$), or less.

Saarinén and Shaw⁴²⁷ noted that plasma phospholipids and the cholesterol

⁴¹⁴ H. H. Dukes, *The Physiology of Domestic Animals*, 6th ed., Comstock, Ithaca, N. Y., 1947, p. 527.

⁴¹⁵ L. A. Maynard, *Animal Nutrition*, 2nd ed., McGraw-Hill, New York & London, 1947, p. 208.

⁴¹⁶ J. W. Patton, *Vet. Med.*, 39, 150-153 (1944).

⁴¹⁷ J. W. Patton, *Vet. Med.*, 39, 271-278 (1944).

⁴¹⁸ J. W. Patton, *Vet. Med.*, 40, 163-167 (1945).

⁴¹⁹ J. C. Shaw, R. C. Powell, Jr., and G. C. White, *J. Am. Vet. Med. Assoc.*, 100, 473-478 (1942).

⁴²⁰ S. J. Roberts, *North Am. Veterinarian*, 27, No. 5, 284-287 (May, 1946).

⁴²¹ J. C. Shaw, L. D. Matterson, M. E. Surgenor, and C. A. Hourigan, *J. Am. Vet. Med. Assoc.*, 106, 285-291 (1945).

⁴²² C. E. Hayden, M. G. Fincher, S. J. Roberts, W. J. Gibbons, and A. G. Danks, *Cornell Vet.*, 36, 71-84 (1946).

⁴²³ J. M. Hendershot, *J. Am. Vet. Med. Assoc.*, 108, 74-75 (1946).

⁴²⁴ M. G. Fincher and C. E. Hayden, *Cornell Vet.*, 30, 197-217 (1940).

⁴²⁵ C. B. Knodt, J. C. Shaw, and G. C. White, *J. Dairy Sci.*, 25, 837-849 (1942).

⁴²⁶ J. Sampson and C. E. Hayden, *J. Am. Vet. Med. Assoc.*, 86, 13-23 (1935).

⁴²⁷ P. Saarinén and J. C. Shaw, *J. Dairy Sci.*, 33, 496-507 (1950).

ester fractions were low, particularly in the later stages of ketonuria. Neutral fats were relatively low in the early stages of spontaneous ketosis, while they were normal or high in the latter stages. When cows were given 70 to 80% of their caloric requirement, the plasma lipids were found to resemble those in the early stages of ketosis,⁴²⁸ When they received only 35% of their total nutritional requirement, the postpartal plasma lipid values were similar to those of cows in advanced stages of ketosis.⁴²⁸ The predisposing factor in the development of spontaneous ketosis cannot be traced to fatty livers.⁴²⁹

The milk obtained from cows affected with ketosis contains increased ketone bodies. Blood values for ketone bodies as high as 300 to 450 mg. % were recorded by Sjollem and van der Zande,³⁹⁹ while the corresponding figures of 7.4 to 30.9 mg. % have been reported in the milk of cows with acetonemia.³⁹⁶ In contradistinction to these values, the milk of normal cows contains only small amounts of ketone bodies,^{409,411,412}

b'. Ketosis in Sheep: As in the case of cattle, ketosis in sheep is largely confined to the female. However, in contradistinction to cows, in which ketosis occurs as a post-parturient complication, in ewes it occurs previous to parturition.³⁹⁶ Although acetonemia in sheep has been referred to by at least nineteen names, the term, "pregnancy disease," is the most common designation. According to Groenewald and associates,⁴⁰⁴ the first reference to pregnancy disease ("domsiekte," or "stupid sickness") in ewes was that of Steele in 1890, who called it "parturient fever in sheep." Henning (also cited by Groenewald *et al.*)⁴⁰⁴ stated that the disease had been known in the Cape Colony earlier than 1882. Apparently, the occurrence of pregnancy disease in ewes is world-wide.^{404,430} It probably occurs throughout the United States wherever sheep are raised.³⁹⁶

The general consensus of opinion of a number of workers^{404,430-434} is that pregnancy disease develops in ewes which are underfed during pregnancy, and especially during the last one-third of the gestation period, when the

⁴²⁸ P. Saarinen and J. C. Shaw, *J. Dairy Sci.*, **33**, 508-514 (1950).

⁴²⁹ P. Saarinen and J. C. Shaw, *J. Dairy Sci.*, **33**, 515-525 (1950).

⁴³⁰ L. M. Roderick and G. S. Harshfield, *N. Dakota Agr. Expt. Sta., Tech. Bull. No. 261*, 3-31 (Oct., 1932).

⁴³¹ C. S. M. Hopkirk, *Australian Vet. J.*, **10**, 54-64 (1934); cited by J. W. Groenewald, H. Graf, and R. Clark, *Onderstepoort J. Vet. Sci. Animal Ind.*, **17**, 225-244 (1941), p. 233.

⁴³² L. M. Roderick, G. S. Harshfield, and M. C. Hawn, *J. Am. Vet. Med. Assoc.*, **90** [n.s. 43], 41-50 (1937).

⁴³³ A. H. H. Fraser, W. Godden, L. C. Snook, and W. Thomson, *J. Physiol.*, **94**, 346-357 (1938).

⁴³⁴ A. H. H. Fraser, W. Godden, L. C. Snook, and W. Thomson, *J. Physiol.*, **97**, 120-127 (1939).

glycogen requirement is greatest.⁴³² During pregnancy disease in the ewe, the hypoglycemia and acetonemia are similar to those occurring in ketosis in the cow.⁴⁰³ Table 3 shows the values for blood sugar and blood ketones of normal pregnant ewes, of ewes in which an experimental ketosis was produced during later pregnancy either by fasting or by feeding rations low in caloric value, and of animals which developed a spontaneous ketosis.

TABLE 3

TOTAL KETONE BODIES AND SUGAR IN THE BLOOD OF HEALTHY EWES DURING ADVANCED PREGNANCY, AND IN EWES AFFECTED WITH EXPERIMENTAL OR CLINICAL KETOSIS (PREGNANCY DISEASE)^a

Category	Number of ewes	Blood ketone bodies, mg. %	Blood sugar, mg. %
Normal	9	2.04 (0.5-5.2)	43.3 (38.4-52.0)
Experimental ketosis	8	25.4 (17.0-40.9)	26.1 (14.7-53.1)
Spontaneous ketosis	9	41.4 (15.3-96.2)	27.7 (17.0-50.8)

^a Adapted from J. Sampson, *Ketosis in Experimental Animals*, Univ. Illinois Agr. Exptl. Sta., Bull. No. 524, 407-470 (1947), p. 442.

Data similar to those recorded in Table 3 have been reported by other workers.^{407,430,435,436} The levels of ketonuria observed in ewes and cows are similar. Thus, Sampson *et al.*⁴⁰⁷ reported a value of 736 mg. % of total ketone bodies in mild pregnancy disease in the ewe, while the urine of ewes affected with severe ketosis may contain as much as 1400 mg. per liter.³⁹⁶

The cause of ketosis in ewes is believed to be traceable to the lack of sufficient carbohydrate in the diet. Thus, in treatment of this condition, the administration of large doses of carbohydrate is recommended.^{396,404} Although it has been suggested that pregnancy disease in sheep may be the result of a deficiency in vitamin A,⁴³⁷ this hypothesis is open to question. In the first place, Barron,⁴³⁸ and Underwood and co-workers,⁴³⁹ demonstrated that the level of vitamin A in the livers of ewes having pregnancy disease is practically the same as that in the livers of normal ewes. Values are high in both cases. Moreover, in view of the negative results obtained

⁴³⁵ J. Sampson and L. E. Boley, *Cornell Vet.*, 30, 392-401 (1940).

⁴³⁶ H. S. Cameron and H. Goss, *J. Am. Med. Assoc.*, 96, 165-170 (1940).

⁴³⁷ J. O. Foss and D. F. Eveleth, *Vet. Med.*, 39, 313-315 (1944).

⁴³⁸ N. S. Barron, *Vet. Record*, 54, 29-39 (1942); *Chem. Abst.*, 38, 1770 (1944).

⁴³⁹ E. J. Underwood, D. H. Curnow, and F. L. Shier, *Australian Vet. J.*, 19, 164-173 (1943); *Vet. Record*, 56, 222 (1944).

in the treatment of acetonemia in cows with vitamin A,⁴²¹⁻⁴²³ a similar therapy in the case of ewes would seem to be of doubtful value. Pugh⁴⁴⁰ reported that ketonemia in sheep is accompanied by a marked decrease in the rate at which injected acetate is metabolized.

c'. Ketosis in Goats: The goat apparently develops ketonemia in a manner similar to that of sheep. Here again, spontaneous acetonemia has been noted only in females. Forbes⁴⁴¹ reported the following values for blood acetone bodies in goats on low-calorie diets, expressed in mg. %: late pregnancy, 25; early lactation, 18; the eleventh week of lactation, 4; and early pregnancy, 3.7. Normal values were found to range from 2 to 4 mg. %. Low blood-sugar values (Av. 28 mg. %) were also noted during the period of maximum ketonemia, while the maximum blood-sugar value (46 mg. %) occurred in conjunction with a minimum blood acetone value. Typical clinical ketosis was observed in a herd of goats in which twelve fatalities resulted among the pregnant does.⁴⁴² Ketonuria values as high as 450 mg. % were recorded.

d'. Ketosis in Hogs: The occurrence of ketosis in swine was not recognized until 1940, when Hull and Nolan⁴⁴³ described three cases which had been so diagnosed in the Department of Animal Pathology of the Kentucky Agricultural Experiment Station. The occurrence of spontaneous ketonemia in swine was confirmed by Sampson, Hanawalt, and Graham.⁴⁴⁴ On the other hand, young pigs were shown by Woods⁴⁴⁵ to have a "low liability to ketosis." This was likewise reported by Hanawalt and Sampson,⁴⁴⁶ who failed to demonstrate any significant ketonemia or ketonuria in a pig fasted for as long as twenty-eight days.

Although the blood and urine of healthy pregnant and lactating sows contain only traces of acetone bodies,³⁹⁶ it is believed that, when their intake of readily available carbohydrate or potential carbohydrate is insufficient, ketosis develops.⁴⁴⁴ This would suggest that the etiology of ketonemia in cows and ewes is identical with that in the sow. Sampson,³⁹⁶ and Sampson, Hanawalt, and Graham⁴⁴⁴ recorded the following data during late pregnancy on healthy sows (a) and on those having ketosis (b): ketone bodies, (a), trace (4), and (b), 60.2 mg. % (36.1 to 95.2); blood sugar, (a), 62.1 mg. % (40.7 to 80.2) and (b), 63.6 (54.2 to 79.1). Although there is evidence that

⁴⁴⁰ P. S. Pugh, *Nature*, 170, 978-979 (1952).

⁴⁴¹ R. M. Forbes, *Cornell Vet.*, 33, 27-47 (1943).

⁴⁴² A. T. Gilyard and R. T. Gilyard, *Cornell Vet.*, 25, 201-202 (1935).

⁴⁴³ F. E. Hull and A. F. Nolan, *J. Am. Vet. Med. Assoc.*, 97, 162-164 (1940).

⁴⁴⁴ J. Sampson, V. M. Hanawalt, and R. Graham, *Cornell Vet.*, 33, 355-359 (1943).

⁴⁴⁵ E. B. Woods, *Am. J. Physiol.*, 79, 321-340 (1926-1927).

⁴⁴⁶ V. M. Hanawalt and J. Sampson, *Am. J. Vet. Research*, 8, 73-81 (1946).

blood ketone levels may be as high or higher in sows than in cows and ewes affected with severe ketonemia, the blood-sugar values examined in the small number of tests failed to show low values for the animals which exhibited the ketonemia.

e'. Ketosis in Fowls: Mirsky and co-workers⁴⁴⁷ reported that fasting produced a rapid accumulation of ketone bodies in the blood of normal ducks; values as high as 45 mg. % were noted.³⁹⁶ Fasting likewise produced an appreciable ketonemia in well-nourished hens.⁴⁴⁸ However, following pancreatectomy, only a temporary diabetes was found to occur in the chicken,⁴⁴⁹ and a mild form in the duck.^{447,450}

f'. Ketosis in Miscellaneous Animals: According to Cervello and Girgenti,⁴⁵¹ a fasting ketonuria is observed in the dog. When the pancreas is removed, in this species, a typical diabetes accompanied by ketosis occurs.⁴⁵²

As has been discussed earlier, the dog is extremely resistant to ketosis during fasting;^{344,394} however, puppies, in contrast to adult dogs, develop a marked fasting ketosis.³⁹⁴ Ketosis follows the injection of phlorhizin into the dog,⁴⁵³ as well as that of alloxan.³⁶⁰ The injection of anterior pituitary extract resulted in experimental diabetes, and its complications.^{454,455} However, spontaneous ketosis is rare in the dog.⁴⁵⁶

Although little is known of the condition of spontaneous ketonemia in the cat, Long and Lukens⁴⁵⁷ noted that severe diabetes resulted from pancreatectomy. However, Barker⁴⁵⁸ was unable to demonstrate augmented ketonuria, commensurate with the larger amounts of fat burned, in depan-

⁴⁴⁷ I. A. Mirsky, N. Nelson, I. Grayman, and M. Korenberg, *Am. J. Physiol.*, **135**, 223-229 (1941).

⁴⁴⁸ J. Sampson and H. H. Dukes, unpublished data cited by J. Sampson, *Ketosis in Domestic Animals*, Univ. Illinois, Agr. Expt. Sta., Bull. No. 524, 1947, p. 461.

⁴⁴⁹ T. Koppányi, A. C. Ivy, A. L. Tatum, and F. T. Jung, *Am. J. Physiol.*, **78**, 666-674 (1926).

⁴⁵⁰ R. Sprague and A. C. Ivy, *Am. J. Physiol.*, **115**, 389-394 (1936).

⁴⁵¹ C. Cervello and F. Girgenti, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **75**, 153-167 (1914).

⁴⁵² R. G. Sprague, *Diabetes Mellitus*, in F. Tice, *Practice of Medicine*, vol. 9, W. F. Prior, Hagerstown, 1922, 69-134 (pp. 70, 71).

⁴⁵³ F. M. Allen and M. B. Wishart, *J. Metabolic Research*, **4**, 223-254 (1923).

⁴⁵⁴ H. M. Evans, K. Meyer, M. E. Simpson, and F. L. Reichert, *Proc. Soc. Exptl. Biol. Med.*, **29**, 857-858 (1932).

⁴⁵⁵ F. G. Young, *Lancet*, 1937, **II**, 372-374.

⁴⁵⁶ E. Huttyra, J. Marek, and R. Manninger, *Pathology and Therapeutics of the Diseases of Domestic Animals*, 4th ed., vol. III, A. Eger, Chicago, 1938; cited by J. Sampson, *Ketosis in Domestic Animals*, Univ. Illinois, Agr. Expt. Sta., Bull. No. 524, 407-470 (1947), p. 461.

⁴⁵⁷ C. N. H. Long and F. D. W. Lukens, *J. Exptl. Med.*, **63**, 465-490 (1936).

⁴⁵⁸ S. B. Barker, *Proc. Soc. Exptl. Biol. Med.*, **34**, 893-897 (1936).

creatized dogs with markedly increased fat metabolism caused by exercise or by the administration of dinitrophenol.

(b) *Age as a Factor in Ketonuria.* It is generally considered that very young animals are more susceptible to ketosis than are older animals. Moreover, the severity of the ketosis is greater in the young. Peters and Van Slyke²⁶⁸ are of the opinion that this phenomenon may be traced to the greater rapidity with which the glycogen stores are expended in the young than in older individuals.^{459,460}

On the other hand, Knodt and associates⁴⁶¹ reported a progressive rise in the blood acetone values from birth to maturity. Their data are summarized in Table 4.

TABLE 4
COMPARISON OF BLOOD AND URINARY ACETONE BODIES
OF VARIOUS AGE GROUPS OF CATTLE^a

Category	Number of samples	Mg. % of acetone bodies		
		Total acetone bodies	Acetone + acetoacetate	β -Hydroxybutyrate
Blood				
Birth	8	1.28 $\pm$ 0.75	0.71 $\pm$ 0.17	0.57 $\pm$ 0.84
Birth to 10 months	65	1.49 $\pm$ 0.66	0.81 $\pm$ 0.11	0.68 $\pm$ 0.59
9-26 months	100	2.04 $\pm$ 0.73	0.98 $\pm$ 0.45	1.06 $\pm$ 0.60
Mature	427	2.66 $\pm$ 1.23	1.08 $\pm$ 0.66	1.58 $\pm$ 1.00
Urine				
Birth	1	1.17	0.93	0.24
Birth to 10 months	65	6.42 $\pm$ 4.88	2.94 $\pm$ 1.97	3.48 $\pm$ 3.56
9-26 months	100	14.30 $\pm$ 5.67	5.31 $\pm$ 2.48	8.99 $\pm$ 4.55
Mature	427	11.81 $\pm$ 6.56	4.52 $\pm$ 2.85	7.29 $\pm$ 4.78

^a Adapted from C. B. Knodt, J. C. Shaw, and G. C. White, *J. Dairy Sci.*, 25, 861-867 (1942).

Although no information is available as to the effect of age on the resulting ketonuria in the rat, some data on comparative liver glycogen are suggestive. Thus, it was shown⁴⁶² that the level of glycogen in the livers of unfasted rats is highest at thirty-nine to forty days, under which circumstances it exceeds 8%. From this value, it was found to decrease gradually

⁴⁵⁹ I. A. Mirsky and W. E. Nelson, *Am. J. Diseases Children*, 67, 100-105 (1944).

⁴⁶⁰ I. A. Mirsky, M. Korenberg, N. Nelson, and W. E. Nelson, *Endocrinology*, 28, 358-367 (1941).

⁴⁶¹ C. B. Knodt, J. C. Shaw, and G. C. White, *J. Dairy Sci.*, 25, 861-867 (1942).

⁴⁶² H. J. Deuel, Jr., J. S. Butts, L. F. Hallman, S. Murray, and H. D. Blunden, *J. Biol. Chem.*, 119, 617-620 (1937).

to 4% in males (and to lower values in females). These new levels are maintained until the rats are seventeen to twenty-four months of age. Since the level of ketonuria is usually inversely related to the carbohydrate stores, one would expect a lower susceptibility to ketonuria in very young rats.

(c) *Sex as a Factor in Ketonuria.* The most important physiologic factor which influences the level of ketonemia and ketonuria is undoubtedly sex. There is ample evidence that the pattern of fat metabolism is quite different for the female than it is for the male. Thus, it was noted earlier that the storage fats in the female are greater in amount than in the male. Moreover, the situation is reversed in the case of glycogen, which appears to be stored at a higher level in the male rat as contrasted with the female (see *The Lipids*, Vol. II, Chapter VI).

The principal organ in which sex differences in fat storage can be observed is the liver. In most cases of unfasted animals, and in practically all instances in which fasted rats are tested, it can be shown that liver glycogen values are higher in the male, and liver lipids higher in the female, than in the livers of the opposite sex. However, the higher level of liver lipids in the female rats, as contrasted with the male animals, is confined to the neutral fat, as the average cholesterol and lecithin appear to be higher in the livers of males than in this organ in the females.

The sex difference in fat metabolism is exhibited most strikingly in relation to ketosis. In the previous section on the effect of species on ketonemia and ketonuria, it was pointed out that spontaneous ketonemia in cattle, sheep, goats, and hogs occurs only in the female. Sampson³⁹⁶ states that so far as he is aware "evidence has not been presented to show that significant ketonemia occurs in steers, bulls, wethers, and rams, no matter what the disease with which they are affected." He states further: "A significant spontaneous ketosis of the ovine and bovine species in this country is limited almost exclusively to females during pregnancy and lactation . . ."

In the case of rats, although there is no sex difference in the slight ketonuria occurring during fasting, the female excretes a much larger proportion of ketone bodies after the feeding of ketogenic acids than does the male, in the condition described as exogenous ketonuria.^{384,389} The same situation obtains in rats with fatty livers (endogenous ketonuria), in the spontaneous ketonuria which results on fasting.³⁹⁰ Beach and associates⁴⁶³ noted that a similar sex difference obtains in the ketonuria occurring after

⁴⁶³ E. F. Beach, P. J. Bradshaw, and N. R. Blatherwick, *Am. J. Physiol.*, 166, 364-373 (1951).

the production of diabetes with alloxan. Emerson⁴⁶⁴ likewise found that a greater ketonuria develops in female rats on exposure to ether anesthesia than results in male animals. The high degree of ketonuria produced in the female rat by feeding ketogenic acids was found to be abolished by ovariectomy. Thus, the level of exogenous ketonuria in spayed female rats was about half that observed in normal males.⁴⁶⁵ Female guinea pigs also exhibit a considerably greater acetoneuria when tested by exogenous ketonuria than do the male animals.³⁸⁴ However, Chamberlin *et al.*⁴⁶⁶ reported

Fig. 1. The urinary excretion of ketone bodies by normal men and women during fasting. The broken lines represent maximum and minimum figures for 5 women, and the solid lines maximum and minimum values for 5 men. The results are expressed in grams (as acetone) per sq. meter body surface.^{268,279}

that the degree of ketonuria occurring as a result of an exclusively beef heart diet, or following fasting, in cats, was actually higher in males than in females. However, the levels of output of ketone bodies recorded were extremely low, being approximately only 0.7 mg. per 100 sq. cm. in the case of the male animals, and 0.3 mg. per 100 sq. cm. in the females. It would be interesting to determine what sex differences would obtain if an exogenous

⁴⁶⁴ G. A. Emerson, *J. Pharmacol. Exptl. Therap.*, **55**, 90-96 (1935).

⁴⁶⁵ C. F. Grunewald, C. H. Cutler, and H. J. Deuel, Jr., *J. Biol. Chem.*, **105**, 35-43 (1934).

⁴⁶⁶ E. Chamberlin, W. H. Furgason, and V. E. Hall, *J. Biol. Chem.*, **121**, 599-606 (1937).

ketonuria were produced. One interesting fact which was recorded was the absence of any increase in the level of ketonuria on fasting.

Man has been shown to exhibit the greatest sex difference in starvation ketosis. Thus, Deuel and Gulick²⁷⁹ found that the average ketone body excretion, when based upon surface area, was approximately four times as great in women as in men. The sex difference in the excretion of ketone bodies over a four-day period is illustrated in Figure 1.

(d) *Type of Food Ingested as a Factor in Ketonuria.* It has long been known that the type of food ingested affects the extent of ketonuria during the period when any particular regimen is being consumed, and subsequently during a period of fasting. Thus, a protein-fat diet largely free from carbohydrate caused a persistent ketonuria over a period of a year in the case of two Arctic explorers studied by McClellan and DuBois.³⁴⁸ When extra fat is added to a diet lacking in carbohydrate, the extent of ketonuria is exaggerated.^{348, 467, 468} The increased ketonuria occurs concomitantly with a decreased nitrogen excretion. On the other hand, a reduction of fat in the ketogenic diet was shown by Salomonsen⁴⁶⁸ to increase the protein loss as indicated by an increased urinary nitrogen.

The extent of ketosis occurring during the period of alimentation is also influenced by the caloric intake. Thus, it has been repeatedly demonstrated that the onset of acetolemia in cattle, and in pregnancy disease in ewes, almost invariably occurs when the level of food intake is considerably below the required amount. The reader is referred to the earlier section on species as a factor in ketonuria for a further exposition of this point (see page 146).

Finally, Deuel *et al.*³⁹⁰ reported that, following a low-protein, low-choline diet such as to induce the development of fatty livers, rats excreted a high level of ketone bodies on subsequent fasting. It is believed that ketonuria occurs during fasting following this deficient diet because of the stimulation of fat breakdown due to the high level of this foodstuff in the liver. Some justification for this viewpoint was furnished by the fact that the rate of decrease of liver lipid in the female rats during fasting was greater than that in males; this finding was consistent with a higher endogenous ketonuria in female rats than was observed in males.

MacKay *et al.*⁴⁶⁹ challenged the viewpoint that the level of liver fat is the deciding factor in effecting the ketosis. Thus, they were unable to

⁴⁶⁷ G. Becker, *Acta Med. Scand.*, 63, 313-335 (1926).

⁴⁶⁸ L. Salomonsen, *Acta Paediat.*, 9, Suppl. I, 1-146 (1929-1930).

⁴⁶⁹ E. M. MacKay, H. O. Carne, A. N. Wick, and F. E. Visscher, *J. Biol. Chem.*, 141, 889-896 (1941).

find any relationship between the level of ketonuria and the liver fat content *per se*, or between the amount of lipotropic agents such as methionine or choline, or of a liver-fattening substance, for instance cystine, and the degree of fasting ketonuria in the rat. On the other hand, it is suggested that the rapidity of onset and the degree of ketosis are inversely related to the protein content of the preceding diet. The hypothesis is advanced that this effect is related to the amount of protein stored prior to the onset of the fasting period. The greater the amount of stored protein available in the tissues at the onset of the starvation regimen, the larger the potential reserve of antiketogenic material for catabolism during fasting. Such antiketogenic material assists the rats in maintaining their liver glycogen and blood sugar at higher levels. Tidwell and Treadwell⁴⁷⁰ also reported that the preceding protein intake by rats is a major dietary factor affecting the degree of the subsequent fasting ketonemia. In a later communication, these workers⁴⁷¹ stated that the reduction in ketonuria was not due to a metabolic lag or to an increased flushing out of metabolic end-products during the fast. Rather, it appeared to be related to the increased protein catabolism which occurred during the period of the fast. However, the administration of an amount of glucose equivalent to that formed by the increased rate of protein catabolism failed to produce the corresponding antiketogenic effect previously attributed to it. It is suggested that the greater fasting ketosis noted following a low-protein diet may be related to an increased rate of utilization of carbohydrate by these animals, as indicated by the rapid disappearance of liver glycogen. Apparently, more experimental work is needed before the relationship between fatty livers and fasting ketonuria is clarified.

(e) *Adaptation as a Factor in Ketonuria.* It has been generally considered by some nutritionists that adaptation to ketonuria may occur. Thus, Heinbecker⁴⁷²⁻⁴⁷⁴ reported that three successive groups of fasting Eskimos developed a lower degree of ketonuria than is usual in the case of white persons living in the temperate zones. He suggests that the mechanism for the oxidation of fat is modified, since the Eskimo is able to burn a considerable amount of fat to completion without the aid of carbohydrate. The levels of plasma lipids of Arctic Eskimos⁴⁷⁵ are similar to those considered normal for white individuals in the temperate zones. The question

⁴⁷⁰ H. C. Tidwell and C. R. Treadwell, *J. Biol. Chem.*, **162**, 155-161 (1946).

⁴⁷¹ H. C. Tidwell and C. R. Treadwell, *J. Biol. Chem.*, **164**, 313-319 (1946).

⁴⁷² P. Heinbecker, *J. Biol. Chem.*, **80**, 461-475 (1928).

⁴⁷³ P. Heinbecker, *J. Biol. Chem.*, **93**, 327-336 (1931).

⁴⁷⁴ P. Heinbecker, *J. Biol. Chem.*, **99**, 279-282 (1932-1933).

⁴⁷⁵ A. C. Corcoran and I. M. Rabinowitch, *Biochem. J.*, **31**, 343-348 (1937).

which arises is whether the Eskimos are inherently less susceptible to ketosis, or whether they have become adapted to a meat diet. In the year-long experiment carried out in the case of the white Arctic explorers, who consumed only a meat diet, McClellan and DuBois³⁴⁸ noted that an apparent adaptation to the diet obtained, inasmuch as the ketonuria tended to diminish after several months on the carbohydrate-free diet.

Samuels and co-workers⁴⁷⁶ demonstrated that the spontaneous and forced activity exhibited by fasting rats, as well as the duration of survival, are related to the prefast diet. Marked increase in survival time, and in the amount of work performed before exhaustion, were observed in the case of rats receiving fat diets as contrasted with those on carbohydrate or protein diets. Roberts and Samuels⁴⁷⁷ reported that the rats which had been given a high-fat diet exhibited a lower susceptibility to insulin injection than did animals previously fed a high-carbohydrate regimen. This resistance to insulin by the fat-fed group was found to be ascribable to the sparing action of the diet on liver glycogen.^{478, 479} Even in the case of hepatectomized rats, the animals previously conditioned to fat survived about twice as long after the operation as did animals previously conditioned to carbohydrate.⁴⁷⁹ Gilmore and Samuels⁴⁸⁰ carried out experiments with isolated tissues; they noted a lower rate of glucose utilization when the rats had received a high-fat diet than was the case when they had been fed on a high-carbohydrate regimen. Lundbaek and Stevenson⁴⁸¹ observed that the rate of glucose utilization *in vitro* by the diaphragm of fat-fed rats was only approximately one-half of that of carbohydrate-fed animals, when the tissues were immersed in glucose solution.

El Rawi and Geiger⁴⁸² demonstrated that adaptation to a fat diet develops in rats fed a diet containing 15% protein and the balance largely composed of fat, as contrasted with one containing chiefly carbohydrate. Not only were the fat-adapted rats able to maintain higher liver glycogen and blood-sugar levels during subsequent fasting, but they were likewise found to be more resistant to insulin. It would therefore seem probable that the body accommodates itself to different dietary regimens by the development of diverse enzyme systems for carrying out the catabolism

⁴⁷⁶ L. T. Samuels, R. C. Gilmore, and R. M. Reinecke, *J. Nutrition*, **36**, 639-651 (1948).

⁴⁷⁷ S. Roberts and L. T. Samuels, *Proc. Soc. Exptl. Biol. Med.*, **53**, 207-208 (1943).

⁴⁷⁸ L. T. Samuels, R. M. Reinecke, and H. A. Ball, *Proc. Soc. Exptl. Biol. Med.*, **49**, 456-458 (1942).

⁴⁷⁹ S. Roberts, L. T. Samuels, and R. M. Reinecke, *Am. J. Physiol.*, **140**, 639-644 (1944).

⁴⁸⁰ R. C. Gilmore, Jr., and L. T. Samuels, *J. Biol. Chem.*, **181**, 813-820 (1949).

⁴⁸¹ K. Lundbaek and J. A. F. Stevenson, *Federation Proc.*, **7**, 75 (1948).

⁴⁸² I. El Rawi and E. Geiger, unpublished results, 1953.

of the foodstuffs. Thus, during periods of stress, such as during fasting, variations in response become evident, because of the particular enzyme systems present in the animals as a result of their previous adaptation.

(f) *Hormones as Related to Ketonuria.* Many hormones influence the degree of ketonuria of the animal. The profound effect of sex on susceptibility to fasting ketonuria is undoubtedly based upon a hormonal relationship. The fact that the high level of ketonuria of the female is abolished after ovariectomy would seem to indicate that the hormone responsible for the ketonuria is produced in the ovary, either directly or due to external stimulation. The pancreatic hormone, insulin, is another substance which is indirectly related to ketonuria by virtue of its regulation of carbohydrate metabolism. Following pancreatectomy, ketonuria usually occurs concomitantly with the glycosuria.

A third hormone which appears to be directly concerned with ketosis is produced in the anterior lobe of the hypophysis; it is frequently referred to as the "ketogenic" or "diabetogenic" hormone, since the injection of this substance is usually followed by ketonuria. Anselmino and Hoffmann⁴⁸³ were the first to demonstrate, in 1931, that extracts of the anterior lobe of the pituitary provoked ketonemia when injected into rats. It was subsequently reported⁴⁸⁴ that such ketogenic extracts also produced fatty infiltration of the liver; it was likewise claimed that they regulated the level. Many observers⁴⁸⁵⁻⁴⁹² have confirmed the lipogenic effect of the extracts of the anterior lobe, as well as their effects on ketonuria. Since the ketonuria following injection of the extract of the anterior lobe of the pituitary is accompanied by a fatty infiltration of the liver, it is believed that carbohydrate combustion is retarded or completely abolished under these conditions, with the result that fat serves as the sole source of energy. The development of ketosis is therefore the result both of the increased level of fat oxidation and of the failure of carbohydrate to act ketolytically. This suggestion is in harmony with the observation that the ketogenic hormone provokes a ketonuria in fasted and possibly also to a lesser extent

⁴⁸³ K. J. Anselmino and F. Hoffmann, *Klin. Wochschr.*, 10, 2380-2383 (1931).

⁴⁸⁴ K. J. Anselmino, F. Hoffmann, and E. Rhoden, *Arch. ges. Physiol. (Pflüger's)*, 237, 515-516 (1936).

⁴⁸⁵ C. H. Best and J. Campbell, *J. Physiol.*, 86, 190-203 (1936).

⁴⁸⁶ P. T. Black, J. B. Collip, and D. L. Thomson, *J. Physiol.*, 82, 385-391 (1934).

⁴⁸⁷ V. G. Foglia and P. Mazzocco, *Compt. rend. soc. biol.*, 127, 150-152 (1938).

⁴⁸⁸ E. G. Fry, *Endocrinology*, 21, 283-291 (1937).

⁴⁸⁹ E. M. MacKay and R. H. Barnes, *Am. J. Physiol.*, 118, 525-527 (1937).

⁴⁹⁰ A. H. Neufeld and J. B. Collip, *Endocrinology*, 25, 768-774 (1939).

⁴⁹¹ J. H. Burn and H. W. Ling, *Quart. J. Pharm. and Pharmacol.*, 2, 1-16 (1929).

⁴⁹² J. S. Butts, C. H. Cutler, and H. J. Deuel, Jr., *J. Biol. Chem.*, 105, 45-58 (1934).

in fed rats,^{485,486,493} but that ketonuria is abolished when the carbohydrate metabolism is stimulated by the administration of glucose.⁴⁹² The hyperlipemic response to the pituitary hormone is not affected by the removal of the pancreas.^{489,494} The ketonuria is suppressed by adrenalectomy;⁴⁹⁵ fatty infiltration of the liver is likewise abolished by this procedure.⁴⁸⁹

On the other hand, hypophysectomy reverses the changes caused by the injection of the pituitary hormone. In the first place, it considerably reduces the incidence of hyperlipemia;⁴⁹⁶ after extirpation of the anterior lobe of the pituitary, the fatty infiltration of the liver following pancreatectomy is decreased. In fact, according to Issekutz and Verzár,⁴⁹⁷ fatty livers do not develop in hypophysectomized rats, even after the treatment of the animals with powerful hepatotoxic agents such as carbon tetrachloride and phosphorus. However, the effect of hypophysectomy in preventing fatty livers is completely reversed by the adrenocortical hormones. Oastler and Anderson⁴⁹⁸ believe that the absence of ketonuria in hypophysectomized animals is related to an increase in the renal threshold for ketone bodies following the operation. Bondy and Wilhelmi⁴⁹⁹ reported that the rate of formation of ketone bodies by rat liver slices from hypophysectomized rats was depressed below that of liver slices from normal rats. This reduced activity was accounted for entirely on the basis of the reduced activity of the thyroid gland; this effect was completely counteracted by thyroxine.

According to Lotspeich,⁵⁰⁰ liver slices from rats which had received growth hormone (also elaborated in the anterior lobe of the pituitary gland) produced significantly more acetoacetate than did liver slices obtained from rats previously fed calorically equivalent diets corresponding to those of the experimental animals. When insulin was also administered, an even greater production of acetoacetate was noted in the liver slices. Thus, it is suggested that, under these conditions, insulin is *hyperketogenic* rather than antiketogenic. The explanation of these findings is not immediately available.

The reports in the literature on the action of the adrenal hormones are

⁴⁹³ R. A. Shipley and C. N. H. Long, *Biochem. J.*, **32**, 2242-2256 (1938).

⁴⁹⁴ J. M. Muñoz, *Compt. rend. soc. biol.*, **127**, 156-157 (1938).

⁴⁹⁵ E. M. MacKay and R. H. Barnes, *Am. J. Physiol.*, **118**, 184-189 (1937).

⁴⁹⁶ I. L. Chaikoff, G. E. Gibbs, G. F. Holtom, and F. L. Reichert, *Am. J. Physiol.*, **116**, 543-550 (1936).

⁴⁹⁷ B. v. Issekutz, Jr., and F. Verzár, *Arch. ges. Physiol. (Pflüger's)*, **240**, 624-635 (1938).

⁴⁹⁸ E. G. Oastler and A. B. Anderson, *Biochem. J.*, **33**, 1094-1098 (1939).

⁴⁹⁹ P. K. Bondy and A. E. Wilhelmi, *J. Biol. Chem.*, **186**, 245-251 (1950).

⁵⁰⁰ W. D. Lotspeich, *Federation Proc.*, **12**, 91 (1953).

conflicting. Although Bennett *et al.*⁵⁰¹ noted that ACTH produced an increased ketonemia in a fasted normal dog, and that it also augmented the concentration of ketone bodies in the blood of the fasted rat, and the rate of their excretion in the urine,⁵⁰² Kinsell and associates⁵⁰³ obtained diametrically opposed results in tests of human subjects. Thus, it was found that the injection of either ACTH or cortisone into most fasting individuals resulted in a partial or complete suppression of the hyperketonemia and ketonuria induced by fasting. According to the above workers, these results may be interpreted to mean either that adrenal steroids increase the rate of utilization of ketones by peripheral tissues, that they decrease the rate of formation of ketone bodies from fatty acids, and at the same time increase the rate of direct utilization of fatty acids for energy purposes by peripheral tissues, or finally that certain adrenal steroids so change the metabolic pathway of fatty acids that mid-zone metabolites other than ketones originate. Hexose is suggested as a possible metabolite.

On the other hand, adrenalectomy reduces or abolishes the ketosis of fasting,⁴⁹⁵ that resulting from pancreatectomy⁵⁰⁴ or from phlorhizin,⁵⁰⁵ that occurring in pregnancy,⁵⁰⁶ as well as that resulting from the injection of the ketogenic hormone of the anterior pituitary, when determined by the degree of ketonuria.⁴⁹⁵ MacKay and Barnes⁵⁰⁷ interpreted the results of their tests, in which they found a lower exogenous ketonuria in adrenalectomized than in normal animals, as indicative of the fact that an increased level of ketolysis obtains following the removal of the adrenal glands. This is in line with the results of Bondy and Wilhelmi,⁴⁹⁹ which demonstrated in the case of rat liver slices that adrenalectomy did not reduce the rate of ketogenesis, although in the later periods it did prevent the normal increase in blood ketones. At this later stage, a decrease in the rate of ketogenesis by liver slices did occur.

Although Butts *et al.*⁴⁹² observed no increased ketonuria in the intact rat when antuitrin was given, Pittoni and Rossi⁵⁰⁸ reported an increased

⁵⁰¹ L. L. Bennett, J. F. Garcia, and C. H. Li, *Proc. Soc. Exptl. Biol. Med.*, **69**, 52-53 (1948).

⁵⁰² L. L. Bennett, R. E. Kreiss, C. H. Li, and H. M. Evans, *Am. J. Physiol.*, **152**, 210-215 (1948).

⁵⁰³ L. W. Kinsell, S. Margen, G. D. Michaels, R. Reiss, R. Frantz, and J. Carbone, *J. Clin. Invest.*, **30**, 1491-1502 (1951).

⁵⁰⁴ C. N. H. Long and F. D. Lukens, *Proc. Soc. Exptl. Biol. Med.*, **32**, 743-745 (1935).

⁵⁰⁵ G. Evans, *Am. J. Physiol.*, **114**, 297-308 (1936).

⁵⁰⁶ E. M. MacKay and R. H. Barnes, *Proc. Soc. Exptl. Biol. Med.*, **34**, 682-683 (1936).

⁵⁰⁷ E. M. MacKay and R. H. Barnes, *Am. J. Physiol.*, **122**, 101-104 (1938).

⁵⁰⁸ A. Pittoni and C. R. Rossi, *Bull. soc. chim. biol.*, **33**, 1025-1031 (1951).

production of acetoacetate in rats treated with anterior pituitary hormone, which resulted in a ketonemia. It is suggested by the latter workers that this may result from an increased ketone body formation in the liver due to a decrease in carbohydrate metabolism. Kinsell *et al.*⁵⁰⁹ noted that testosterone propionate exerted a beneficial effect on the ketonuria in a diabetic patient. It is suggested that this may have been due in part to a direct effect of testosterone upon fat or ketone metabolism.

e. The Mechanism of the Effect of Carbohydrate on Ketonuria. It has long been recognized that a close relationship obtains between the appearance of ketone bodies in the blood or urine and the failure to oxidize carbohydrate. One of the most familiar aphorisms is the statement of Rosenfeld²⁶⁴ that "fats burn in the flame of carbohydrates." This was modernized by Woodyatt⁵¹⁰ in 1916 to the following statement: "When the mixture of metabolites oxidizing in the body contains more than three molecules of higher fatty acid to one of glucose, then the body 'smokes' with acidosis compounds like an automobile with too much oil in the cylinders." The subject of the interrelationship of ketosis and carbohydrate oxidation has been reviewed by Deuel and Morehouse,⁵¹¹ and also by Stadie.²

As early as 1895, Hirschfeld²⁷⁴ recognized that the condition responsible for ketone body production is a failure in carbohydrate metabolism. A similar concept of the concurrent oxidation of carbohydrate and fat was held by Geelmuyden,^{512,513} and it was the basis for the theory of Shaffer²⁷⁷ that a quantitative ratio must exist between the antiketogenic and the ketogenic fractions if ketonuria is to be prevented. The original concept of Shaffer⁵¹⁴ was based upon *in vitro* experiments in which it was demonstrated that the presence of glucose was essential if acetoacetate was to be destroyed in alkaline solution with H_2O_2 . Ultimately, Shaffer²⁶⁶ suggested that two molecules of acetoacetate were destroyed for each molecule of glucose oxidized. It was suggested and demonstrated from calculations of published experiments that any excess acetoacetate over that accounted for on a ketogenic:antiketogenic ratio of 2:1 was excreted.^{266,277} On the other hand, Woodyatt²⁷⁸ suggested that 1 g. of

⁵⁰⁹ L. W. Kinsell, S. Margen, G. D. Michaels, and D. P. McCallie, *J. Clin. Invest.*, **30**, 1486-1490 (1951).

⁵¹⁰ R. T. Woodyatt, *J. Am. Med. Assoc.*, **66**, 1910-1913 (1916).

⁵¹¹ H. J. Deuel, Jr., and M. G. Morehouse, *Advances in Carbohydrate Chem.*, **2**, 119-160 (1946).

⁵¹² H. C. Geelmuyden, *Z. physiol. Chem.*, **41**, 128-152 (1904).

⁵¹³ H. C. Geelmuyden, *Z. physiol. Chem.*, **58**, 255-271 (1908-1909).

⁵¹⁴ P. A. Shaffer, *J. Biol. Chem.*, **47**, 433-448 (1921).

glucose must be available for the oxidation of 1.5 g. of fatty acid; this is a 1:1 ratio on a molecular basis. The appearance of a diabetic acidosis when the F.A.:G ratio was less than 2:1 was explained by Shaffer²⁷⁷ as due to the uneven distribution of metabolites. Details of these concepts have been presented elsewhere (see page 122).

Since the demonstration that more than one molecule of ketone bodies originates from each fatty acid oxidized, and especially since the demonstration of the possibility of synthesis of acetoacetate from acetic acid molecules, the theory that a quantitative relationship exists between the ketone bodies and the glucose oxidized has become less popular. However, the current concept that the ketone bodies must be oxidized *via* the tri-carboxylic acid cycle with oxaloacetic acid, $\text{HOOC}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH}$, as the catalytic agent, is, in reality, an exposition of the original theory of Shaffer. In fact, Medes and her associates⁵⁷ state that "there now exists a body of circumstantial evidence indicating that the metabolism of fatty acids, whether at the acetate or acetoacetate level, is coupled with a carbohydrate intermediate, which may be oxalacetate."

(a) *Theories to Explain the Mechanism of the Action of Carbohydrate on Ketosis.* The action of carbohydrates in preventing or abolishing ketosis has been explained on the basis of *ketolysis* and on that of *antiketogenesis*. According to the ketolysis theory, the oxidation of carbohydrate or of substances which are potential sources of carbohydrate causes the disappearance of ketosis and ketonuria by producing a concomitant destruction of ketone bodies. One explanation of this effect is that acetoacetate and the active carbohydrate intermediate are conjugated, and that the new compound is capable of being oxidized. Another suggestion is that the oxidation of acetoacetate results from a catalytic effect exerted by carbohydrate, although all evidence indicates that the oxidation of the ketone bodies is bound up with the metabolism of finite amounts of carbohydrate. The first of these theories has been supported by the experimental results of Deuel and associates.³⁸⁹

According to the antiketogenesis theory, carbohydrate is preferentially oxidized by the animal body; consequently, when carbohydrate is available for oxidation, the formation of ketone bodies is sufficiently suppressed so that the quantity produced by the liver does not exceed the capacity of the tissues to oxidize them. If this concept were correct, there would be no direct relationship between the oxidation of glucose and that of acetoacetate. Cohen¹⁰² suggested that carbohydrates reduce ketonuria because of antiketogenesis; this results from the fact that the carbohydrate intermediates which compete with those of fat for the oxidative enzyme surfaces

can prevent the fat from being oxidized. The review by MacKay²⁶⁷ on the subject supports this theoretical mechanism for the action of carbohydrate.

(b) *Experimental Evidence Concerning the Mechanism of the Action of Carbohydrate on Ketonuria.* a'. *In Vitro* Analogy of Coupled Reactions: The most cogent arguments for the ketolysis theory are in *in vitro* experiments of Shaffer.⁵¹⁴ In these tests, it was noted that only a slight oxidation of an alkaline solution of acetoacetate occurred in the test-tube when H_2O_2 was added, unless glucose was also present. Thus, only 8% of acetoacetate disappeared in sixteen hours when H_2O_2 was used alone, while, in the presence of a small amount of glucose, 74% of the ketogenic material was oxidized. It was found that, as a requirement for this reaction, glucose itself must be oxidized.⁵¹⁵ According to the latter workers, "*the rate of acetoacetate disappearance becomes equal to and parallels the rate of sugar oxidation; that is to say, the rate of keto acid consumption, or 'ketolysis,' is determined and limited by the rate of sugar oxidation.*" Shaffer and Friedemann⁵¹⁵ reported that glycolaldehyde, $\text{HOCH}_2\cdot\text{CHO}$, as well as glucose, produced a ketolytic effect; some evidence was adduced that a condensation product resulting from a Knoevenagel type of reaction had been formed between this compound and one or two acetoacetate molecules, whereby oxidation was accelerated.

Further support for the theory that a coupled reaction occurs is to be found in the experiments of West.^{516,517} Thus, a number of condensation products, in which the addition was on the α -carbon of the keto acid, were found to be much more labile than the parent substances. These condensation products were shown to have higher reducing potentials than that of acetoacetate, which would indicate a possible explanation for this phenomenon on an electronic basis.⁵¹⁶ West⁵¹⁷ later proved that a condensation product was formed between acetoacetic ester and glucose. It is stated that "*it is plausible to suppose that various oxidations may occur in the animal body in which one compound aids the oxidation of another by combination with it, forming a complex more easily oxidized by the body than one or both constituents.*"

Another type of evidence which lends support to the theory that coupled reactions can occur in the body is one described by Westerfeld, Stotz, and Berg.⁵¹⁸ These workers established the fact that the administration of pyruvate accelerates the rate of alcohol metabolism. It was suggested

⁵¹⁵ P. A. Shaffer and T. E. Friedemann, *J. Biol. Chem.*, **61**, 585-623 (1924).

⁵¹⁶ E. S. West, *J. Biol. Chem.*, **66**, 63-75 (1925).

⁵¹⁷ E. S. West, *J. Biol. Chem.*, **74**, 561-589 (1927).

⁵¹⁸ W. W. Westerfeld, E. Stotz, and R. L. Berg, *J. Biol. Chem.*, **144**, 657-665 (1942).

that the pyruvate effect might be due to a condensation of the pyruvate with acetaldehyde (arising from the oxidation of alcohol) to give acetoin ($\text{CH}_3\cdot\text{CO}\cdot\text{CHOH}\cdot\text{CH}_3$). It was also evident from the report of Leloir and Muñoz⁵¹⁹ that pyruvate could augment the oxidation of alcohol by liver slices. Dontcheff⁵²⁰ likewise observed, by study of the whole animal, that the feeding of large doses of carbohydrate to fasted rats stimulated alcohol oxidation. Carpenter and Lee,⁵²¹ in tests of human subjects, found that alcohol was metabolized more rapidly when given with glucose or fructose than when galactose was ingested.

In a later study, Westerfeld, Stotz, and Berg⁵²² presented evidence that the increased utilization of alcohol in the presence of pyruvate was, in fact, a coupled oxidation-reduction reaction between pyruvate and alcohol. Von Euler and Adler⁵²³ and Quibell⁵²⁴ had established the fact that this is a coenzyme-linked reaction in which both the oxidation of alcohol and the reduction of pyruvate⁵²⁵ are dependent upon the presence of diphosphopyridine nucleotide (coenzyme I).

Acetoin is the coupled reaction product arising when an animal carboxylase system acts upon pyruvate and alcohol or acetaldehyde. Berg and Westerfeld⁵²⁶ reported that pyruvate and propionaldehyde also react to give a 5-carbon ketol, by condensation of the 2-carbon fragment of decarboxylated pyruvate with 3-carbon propionaldehyde.

b'. The Tricarboxylic Acid Cycle and the Oxidation of Ketone Bodies: The present concept is that oxaloacetic acid is the active agent in combining with acetoacetate, thus bringing the latter into the tricarboxylic cycle (citric acid cycle), and so effecting the oxidation of ketone bodies. As early as 1891, Claisen and Hori⁵²⁷ described the condensation of oxaloacetic acid and acetic acid by the aldol method to yield citric acid. It is now accepted that the action of acetic and acetoacetic acid in the formation of citric acid resembles that of oxaloacetate, and that presumably they yield a similar active condensing agent. Although Breusch⁵²⁸ postulated the enzymatic synthesis of citric acid from oxaloacetic acid two years before

⁵¹⁹ L. F. Leloir and J. M. Muñoz, *Biochem. J.*, **32**, 299-307 (1938).

⁵²⁰ L. Dontcheff, *Compt. rend. soc. biol.*, **126**, 462-464, 465-467 (1937).

⁵²¹ T. M. Carpenter and R. C. Lee, *J. Pharmacol. Exptl. Therap.*, **60**, 254-263, 264-285, 286-295 (1937).

⁵²² W. W. Westerfeld, E. Stotz, and R. L. Berg, *J. Biol. Chem.*, **149**, 237-243 (1943).

⁵²³ H. von Euler and E. Adler, *Z. physiol. Chem.*, **226**, 195-212 (1934).

⁵²⁴ T. H. Quibell, *Z. physiol. Chem.*, **251**, 102-108 (1938).

⁵²⁵ D. E. Green and J. Brosteaux, *Biochem. J.*, **30**, 1489-1508 (1936).

⁵²⁶ R. L. Berg and W. W. Westerfeld, *J. Biol. Chem.*, **152**, 113-118 (1944).

⁵²⁷ L. Claisen and E. Hori, *Ber.*, **24**, 120-127 (1891).

⁵²⁸ F. L. Breusch, *Z. physiol. Chem.*, **250**, 262-280 (1937).

World War II, the further exposition of the theory occurred somewhat later. This investigator^{92,529,530} suggested that the oxidation of β -keto acids was accelerated by an enzyme, which he called "citrogenase"; this caused a condensation of oxaloacetic acid and of higher β -keto acids to form citric acid or other tricarboxylic acids in equilibrium with it. A corresponding enzyme was present, not only in the heart muscle,⁹² but likewise in the kidney, where it was shown to occur in the insoluble particles from homogenized cortex.^{531,532} Martius⁵³³ also confirmed the condensation of oxaloacetic and β -keto acids. However, neither Weil-Malherbe⁵³⁴ nor Krebs and Eggleston⁵³⁵ were able to confirm the occurrence of citrogenase, the enzyme involved in citric acid synthesis. Thunberg⁵³⁶ reviewed the formation, occurrence, and function of citric acid.

Buchanan and co-workers⁵³⁷ reported that intermediates of the Krebs cycle, when added to a homogenate of guinea pig kidney cortex, stimulated the disappearance of acetoacetate. It was concluded that the mechanism is similar to that suggested by Wood *et al.*⁵³⁸ for the oxidation of pyruvate. *Cis*-aconitic acid was assumed to be the condensation product of oxaloacetic acid and of a 2-carbon intermediate common to the metabolism of pyruvate and of acetoacetate. It is now believed that the acetate involved in the condensation with oxaloacetate is an active form which can readily be synthesized from acetoacetate.

There has been considerable confusion as to whether or not acetic acid and acetoacetate follow the same course of metabolism, and whether they are both metabolized in conjunction with the tricarboxylic cycle. Quastel and Wheatley⁵³⁹ found that malonate, which inhibits the succinoxidase system,^{540,541} slows down the oxidation of both fatty acids and ketone bodies. In fact, they recognized that the oxidation of these substances was related to the tricarboxylic acid cycle. Edson and Leloir⁵⁴¹ confirmed

⁵²⁹ F. L. Breusch, *Enzymologia*, **11**, 169-173 (1944).

⁵³⁰ F. L. Breusch and H. Keskin, *Enzymologia*, **11**, 243-252 (1944).

⁵³¹ H. Wieland and C. Rosenthal, *Ann.*, **554**, 241-260 (1943).

⁵³² F. E. Hunter and L. F. Leloir, *J. Biol. Chem.*, **159**, 295-310 (1945).

⁵³³ C. Martius, *Z. physiol. Chem.*, **279**, 96-104 (1943).

⁵³⁴ H. Weil-Malherbe, *Nature*, **153**, 435-436 (1944).

⁵³⁵ H. A. Krebs and L. V. Eggleston, *Nature*, **154**, 209-210 (1944).

⁵³⁶ T. Thunberg, *Physiol. Revs.*, **33**, 1-12 (1953).

⁵³⁷ J. M. Buchanan, W. Sakami, S. Gurin, and D. W. Wilson, *J. Biol. Chem.*, **157**, 747-748 (1945).

⁵³⁸ H. G. Wood, C. H. Werkman, A. Hemingway, and A. O. Nier, *J. Biol. Chem.*, **139**, 483-484 (1941).

⁵³⁹ J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **29**, 2773-2786 (1935).

⁵⁴⁰ R. P. Geyer, L. W. Matthews, and F. J. Stare, *J. Biol. Chem.*, **182**, 101-108 (1950).

⁵⁴¹ N. L. Edson and L. F. Leloir, *Biochem. J.*, **30**, 2319-2332 (1936).

these findings, and suggested that pyruvate and acetate combine, resulting in the formation of α -ketoglutarate. Wieland and Rosenthal¹⁵³¹ confirmed these findings. Buchanan and associates⁵⁴² reported that, when acetate or acetoacetate was incubated with kidney homogenates, C^{13} was found in the α -ketoglutarate, succinate, and fumarate. The quantity of C^{13} obtained accounted for a large portion of the isotopic C which disappeared from the acetate or acetoacetate. Since C^{13} is in the α -carbon of ketoglutarate, it is believed that citrate is excluded as an important intermediate. Evidence is adduced to indicate that no "extra" carbohydrate is formed during the metabolism of fatty acids.

Both the carboxyl and the carbonyl carbons of acetoacetate have been shown by Weinhouse, Medes, and Floyd^{543,544} to enter the tricarboxylic acid cycle. Acetoacetate, marked with C^{13} in either of the above positions, was shown to give rise to citrate containing C^{13} when it was incubated with oxaloacetate along with homogenates of kidney, muscle, or brain. It is therefore certain that acetoacetate breaks down to two C_2 fragments before reacting with oxaloacetate.

Some confusion was due to the fact that the disappearance of acetoacetate reported in the earlier studies of Krebs and Eggleston⁵⁴⁵ with suspensions of sheep heart homogenate under anaerobic and aerobic conditions, on the addition of fumarate, L-malate, oxaloacetate, or α -ketoglutarate, was shown in their later results⁵⁴⁶ to have been due merely to reduction of the acetoacetate to β -hydroxybutyrate rather than to its incorporation into the tricarboxylic acid cycle. When the tests were carried out under aerobic conditions with minced sheep heart or kidney cortex, and using the same metabolites as above, definite proof was obtained that acetoacetate contributes to the formation of acids of the tricarboxylic acid cycle. Oxaloacetate and fumarate were both shown to increase the rate of oxidation of acetoacetate.

Although the evidence would seem to be sufficient to prove that oxaloacetate reacts with acetoacetate or with acetate to form citrate, the nature of the compound which is common to these two substances is not known. One would naturally expect acetate itself to be the compound involved in both cases. However, in the tests of Buchanan *et al.*,⁵⁴⁷ when acetoacetate

⁵⁴² J. M. Buchanan, W. Sakami, S. Gurin, and D. W. Wilson, *J. Biol. Chem.*, **159**, 695-709 (1945).

⁵⁴³ S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **166**, 691-703 (1946).

⁵⁴⁴ N. F. Floyd, G. Medes, and S. Weinhouse, *J. Biol. Chem.*, **171**, 633-638 (1947).

⁵⁴⁵ H. A. Krebs and L. V. Eggleston, *Biochem. J.*, **39**, 408-419 (1945).

⁵⁴⁶ H. A. Krebs and L. V. Eggleston, *Biochem. J.*, **42**, 294-305 (1948).

⁵⁴⁷ J. M. Buchanan, W. Sakami, S. Gurin, and D. W. Wilson, *J. Biol. Chem.*, **169**, 403-410 (1947).

containing C^{13} in both carboxyl and carbonyl positions, and ordinary acetate, were incubated with kidney extracts containing α -ketoglutarate, the acetate isolated at the conclusion of the tests contained much less C^{13} than did α -ketoglutarate. This was interpreted to mean that acetoacetate did not pass through the stage of acetate while being incorporated into the tricarboxylic acid cycle. In later studies by Buchanan and co-workers,⁵⁴⁸ it was found that acetoacetate *per se* behaved differently from the acetoacetate originating when octanoate was oxidized. Acetoacetate was found to be resistant to splitting into 2-carbon fragments and recombining at random. Since it is known, however, that all 4 carbons of acetoacetate are convertible into citrate,⁵³¹ this would appear to necessitate the preliminary formation of two 2-carbon fragments. This intermediate is not the ordinary acetate; therefore, it is believed that it may be an activated form of acetate (see page 15).

The citric acid cycle is operative in effecting the oxidation not only of acetoacetate but also of the longer aliphatic fatty acids. Thus, Lehninger¹⁴⁰ found that the 2-carbon fragments formed on oxidation of octanoic acid with liver slices containing ATP are condensed to citric acid (if oxaloacetate is present), or to acetoacetic acid. He states that neither butyric acid, crotonic acid, vinylacetic acid, DL- β -hydroxybutyric acid, aldol, nor acetylpyruvate is an intermediate.

According to Stern and Ochoa,⁵⁴⁹ the formation of citric acid from oxaloacetate and acetate involves a 2-stage reaction which is as follows:

(1) Formation of the active acetate derivative. This results from the action of an acetate-activating enzyme system which includes CoA and ATP. The active acetate is believed to be acetylphosphate or a compound closely related to it.

(2) Condensation of active acetate and oxaloacetate to form citrate through the action of a condensing enzyme. This apparently involves an addition reaction rather than a condensation between the molecules, with the elimination of a small molecule. The reaction proceeds as follows:

⁵⁴⁸ J. M. Buchanan, W. Sakami, and S. Gurin, *J. Biol. Chem.*, **169**, 411-418 (1947).

⁵⁴⁹ J. R. Stern and S. Ochoa, *Federation Proc.*, **9**, 234-235 (1950).

The Condensation of Oxaloacetate and of Activated Acetate to Form Citric Acid⁵⁵⁰

The condensing enzyme is present in a number of animal tissues, including skeletal muscle, liver, kidney, brain, heart, as well as in yeast and several bacterial systems, *Escherichia coli* (Escherich's carbohydrate-fermenting intestinal bacillus) and *Azotobacter agile* (aerobic bacterium from water and soil, which fixes free nitrogen). The enzyme which activates acetate apparently has wide distribution in animal tissues, as well as in *E. coli* and *A. agile*.⁵⁵⁰ A comprehensive chart indicating how acetoacetate and acetic acid enter the citric acid cycle is given in Fig. 2.

By means of this oxidation of one acetic acid molecule, six molecules of H₂O are formed, and four molecules of H₂O are used; two molecules of CO₂ are formed. The net result is given in the following equation:

Stern and Ochoa⁵⁵¹ demonstrated that acetate or acetoacetate condenses with oxaloacetate to give rise to citric acid rather than to *cis*-aconitic acid or to isocitric acid. This proof was rendered possible by the preparation from pigeon liver of an enzyme system relatively free from the *cis*-aconitase enzyme system. Novelli and Lipmann,^{552,553} as well as Stern and Ochoa,⁵⁵¹ demonstrated that ATP, Mg⁺⁺ ions, and CoA were necessary for the formation of citrate from acetate and oxaloacetate. Since, in this

⁵⁵⁰ E. S. West and W. R. Todd, *Textbook of Biochemistry*, 2nd ed., Macmillan, New York, 1955.

⁵⁵¹ J. R. Stern and S. Ochoa, *J. Biol. Chem.*, **179**, 491-492 (1949).

⁵⁵² G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **171**, 833-834 (1947).

⁵⁵³ G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **182**, 213-228 (1950).

Fig. 2. The oxidation of acetone bodies by incorporation in the tricarboxylic cycle in the peripheral tissues.⁵⁵⁰

case, acetate formed citrate more readily than acetoacetate, it was believed that acetate must be formed from acetoacetate before condensation with oxaloacetate takes place.

Because of the importance of CoA in effecting the incorporation of the C₂ unit into the tricarboxylic acid cycle,⁵⁵³ one would expect that this condensation would be depressed in pantothenic acid deficiency. Thus, Olson *et al.*⁵⁵⁴ found that the condensation of acetate proceeds at a markedly subnormal rate in homogenized heart ventricle from pantothenic acid-deficient ducklings. Cheldelin and co-workers⁵⁵⁵ reported that the oxidation of caproate and butyrate by rats deficient in pantothenic acid was less than one-half of that of control animals. Moreover, pantothenic acid deficiency symptoms were found to be accentuated on a high-fat diet.⁵⁵⁶

Beatty and West⁵⁵⁷ have shown that most of the substances related to the tricarboxylic acid cycle are ketolytic. Thus, oxaloacetic acid and its precursors (succinic, malic, α -ketoglutaric, aspartic, glutamic, and *cis*-aconitic acids and alanine) were all able to reduce an exogenous ketonuria produced by the administration of sodium butyrate. These results are regarded as additional evidence supporting the operation of the Krebs cycle in the animal and favoring the theory of fatty acid oxidation *via* the tricarboxylic cycle. Ketosis results from a lack of oxaloacetic acid.

c'. The Specific Effect of Glucose and of Glucose Precursors on Ketonuria: If antiketogenesis is the correct explanation for the depressing effect of carbohydrate on ketonuria, then any substance capable of oxidation in the body should cause the disappearance of the ketone bodies, irrespective of whether or not the substance in question gives rise to carbohydrate.

However, the ability of substances to reduce either exogenous or endogenous ketonuria is limited to those compounds, which are capable of forming liver glycogen or of producing "extra sugar" when administered to phlorhizinized or depancreatized dogs. Thus, in addition to D-glucose, which has repeatedly been used as a standard for comparison in such studies,^{22,307,324,329,340,350,558-566} D-galactose,^{350,387,566} D-fructose,³⁵⁰ lactose,³⁸⁷

⁵⁵⁴ R. E. Olson, E. G. Hirsch, H. Richards, and F. J. Stare, *Arch. Biochem.*, **22**, 480-482 (1949).

⁵⁵⁵ V. H. Cheldelin, A. P. Nygaard, C. M. Hale, and T. E. King, *J. Am. Chem. Soc.*, **73**, 5004-5005 (1951).

⁵⁵⁶ W. D. Lotspeich, *Proc. Soc. Exptl. Biol. Med.*, **73**, 85-87 (1950).

⁵⁵⁷ C. H. Beatty and E. S. West, *J. Biol. Chem.*, **190**, 603-610 (1951).

⁵⁵⁸ H. J. Deuel, Jr., L. F. Hallman, S. Murray, and J. H. Hilliard, *J. Biol. Chem.*, **125**, 79-84 (1938).

⁵⁵⁹ C. Johnston and H. J. Deuel, Jr., *J. Biol. Chem.*, **149**, 117-124 (1943).

⁵⁶⁰ C. E. Vaniman and H. J. Deuel, Jr., *J. Biol. Chem.*, **152**, 565-570 (1944).

D-mannose,⁵⁵⁸ and cellobiose⁵⁶⁰ also exhibit the same ketolytic properties as does glucose, or are even more effective. In addition to the above-mentioned sugars, the hexitols,⁵⁵⁹ a series of glucose intermediates³⁴⁸ including L-lactic acid, pyruvic acid, and glycerol, as well as the glucose-forming amino acids^{307, 318, 319, 323, 329, 561-565, 567} produce similar responses in phlorhizinized animals when tested for "extra sugar" formation, for the production of liver glycogen, and for their ability to reduce ketonuria.³²⁴ However, the ketolytic effect of sorbitol was only 50% that of glucose in tests of ketonuria, and only 25% in endogenous ketonuria.⁵⁵⁹ Sharp and Berg⁵⁶⁸ found that DL-lysine monohydrochloride fed to fasted rats produced no greater glycogen deposition than was observed in fasted controls, and that the average total excretion of acetone bodies was slightly greater than in the corresponding control groups which had received no lysine. Cahill,⁵⁶⁹ in his chapter on *Metabolism of Proteins and Amino Acids*, included a table showing the comparative carbohydrate-forming activity of various amino acids when evaluated by these three methods.

The results obtained with the hexitol, sorbitol, present some interesting features. When this polyhydric alcohol is fed to rats having an exogenous ketonuria, it exerts approximately 50% of the ketolytic effect which is produced by an equivalent amount of glucose. On the other hand, when it is fed to fasting rats in which an endogenous ketonuria exists, it is only approximately 25% as effective as glucose.⁵⁵⁹ Johnston and Deuel⁵⁵⁹ believe that the latter result may be attributed to the fact that the functional activity of the fatty livers in the rats is presumably sufficiently reduced to slow down the conversion of sorbitol to glucose in this organ. This is the first essential step in the metabolism of sorbitol if this substance is to exert its depressing action on ketonuria.

Edson⁵⁷⁰ reported that, when sorbitol was tested for its ability to reduce the spontaneous ketogenesis in the liver slices obtained from fasted rats, sorbitol was more effective than glucose or fructose. L-Iditol was likewise

⁵⁶¹ J. S. Butts, M. S. Dunn, and L. F. Hallman, *J. Biol. Chem.*, **112**, 263-274 (1935-1936).

⁵⁶² J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, **119**, 247-255 (1937).

⁵⁶³ J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, **124**, 709-714 (1938).

⁵⁶⁴ J. S. Butts and R. O. Sinnhuber, *J. Biol. Chem.*, **140**, 597-602 (1941).

⁵⁶⁵ L. F. Remmert and J. S. Butts, *J. Biol. Chem.*, **144**, 41-46 (1942).

⁵⁶⁶ H. J. Deuel, Jr., E. M. MacKay, P. W. Jewel, M. Gulick, and C. F. Grunewald, *J. Biol. Chem.*, **101**, 301-322 (1933).

⁵⁶⁷ W. K. Hall, J. R. Doty, and A. G. Eaton, *Am. J. Physiol.*, **131**, 252-255 (1940).

⁵⁶⁸ G. O. Sharp and C. P. Berg, *J. Biol. Chem.*, **141**, 739-745 (1941).

⁵⁶⁹ W. M. Cahill, *Metabolism of Proteins and Amino Acids*, in M. Sahyem, *Outline of the Amino Acids and Proteins*, Reinhold, New York, 1948, 179-196 (p. 189).

⁵⁷⁰ N. L. Edson, *Biochem. J.*, **30**, 1862-1869 (1936).

shown to decrease spontaneous ketogenesis in liver slices.⁵⁷¹ Rat liver and kidney, and most mammalian livers, were shown to possess a dehydrogenase which catalyzes a reversible oxidation of sorbitol to D-fructose, and of L-idoitol to L-sorbose; coenzyme I is required for its activity.⁵⁷² When sorbitol is oxidized by liver slices, glucose is the main product. However, Blakley⁵⁷² later demonstrated that sorbitol is unable to alter the spontaneous ketonuria in liver homogenates. It was also found that the oxidation of octanoate with acetoacetate formation in washed mitochondria suspensions is unaffected by the simultaneous dehydrogenation of sorbitol. Thus, under conditions in which a competition might exist for coenzyme I or ATP, sorbitol dehydrogenation was without effect on fatty acid oxidation.

Thus, it is evident that glucose, other related sugars, glucose intermediates (including some amino acids), and also the hexitols, are invariably able to reduce ketonuria. The ability to reduce exogenous ketonuria is believed to be a property inherent in glucose-forming compounds.

d'. The Negative Effect of Non-Glucose Precursors on Ketonuria: In contradistinction to the consistent ketolytic effect observed in the case of all the glucose-forming intermediates discussed above, there is no instance in which a ketolysis can be demonstrated with non-glycogenic compounds. Thus, Shapiro³⁴⁰ reported that DL-lactic acid, acetaldehyde, ethylene glycol, and ethyl alcohol had no effect on ketonuria; furthermore, these substances were not effective as sources of sugar, as judged by liver glycogen studies. Similarly leucine,³⁰⁷ and cystine⁵⁶³ are neither glycogenic nor ketolytic.

Shapiro³⁴⁰ noted that, in a certain proportion of tests with ethyl alcohol and ethylene glycol, a reduction in ketonuria obtained. However, this effect was found only in cases in which a concomitant increase in protein metabolism occurred. In no single instance was a reduction of the ketonuria observed when a simultaneous rise in protein metabolism was not present.

The negative ketolytic effect of cystine calls for comment. Dakin³²¹ obtained "extra sugar" when cysteine was fed as the sodium salt to phlorrhizinized dogs. One might naturally expect that cystine would respond in a manner similar to that of cysteine. However, Butts *et al.*⁵⁶³ were unable to demonstrate any deposition of liver glycogen, or a lowering in the level of experimental ketonuria, after the administration of L-cystine to rats in the form of a suspension. It was shown on the basis of the

⁵⁷¹ R. L. Blakley, *Biochem. J.*, 49, 257-271 (1951).

⁵⁷² R. L. Blakley, *Biochem. J.*, 52, 269-279 (1952).

increased urinary excretion of inorganic sulfate, that a major portion of the amino acid had been absorbed and metabolized. The discrepancy between the dog and the rat experiments may be due to a species difference, to a variation in the metabolism of cystine and cysteine, or, as seems more probable, to a conversion of cysteine to serine in the Dakin experiments, due to the action of the alkali used to dissolve the amino acid.

The investigation of the role of alcohol in ketotic rats has been extended by Deuel, Hallman, and Murray.³⁸⁹ In the latter tests, a comparison was made of the ketonuria produced experimentally by the exogenous or endogenous method, when isodynamic quantities of D-glucose or ethyl alcohol were fed to rats. If the antiketogenesis theory is correct, any fat-sparing substance should reduce the ketonuria as effectively as an equivalent amount of glucose or of glucose precursors. According to the review of Carpenter,⁵⁷³ there is preponderant evidence not only that alcohol can be used as a general source of energy, but also that it can serve specifically as a source of energy for muscle contraction.

In the rat experiments in which sodium butyrate was fed to produce the exogenous ketonuria,³⁸⁹ the average ketonuria per 100 gm. rat per day was 118.6 mg. in the control group, 26.3 mg. in the group fed 100 mg. D-glucose in addition to the butyrate, and 108.9 mg. in the case of the rats receiving the butyrate and also an amount of ethyl alcohol isodynamic with 100 mg. of glucose. In the tests in which endogenous ketonuria was employed, the average ketonuria was 57.9 mg./100 g. daily in the control group, 8.0 mg./100 g. daily in the group receiving 100 mg. of glucose each day, and 53 mg./100 g. daily in the alcohol-fed group. The amount of carbohydrate required to abolish the ketonuria almost completely is equivalent isodynamically to only a fraction of the fat broken down. The authors calculated that the fat utilized in the above tests amounted to 2464 mg. (control group), 2407 mg. (D-glucose-fed group) and 2392 mg. (alcohol-fed group). Thus, the ketone body excretion of the control group was decreased 93% by an amount of glucose dynamically equivalent to only 2.3% of the total fat catabolized. Similar conclusions were reached in the case of fasting ketonuria in man when D-galactose was fed.³⁸⁹ Thus, it was demonstrated that ketonuria was practically abolished by an amount of glucose too small to alter the fat metabolism to any significant degree. These experiments strongly support the ketolysis theory.

e'. The Effect of Glucose on the Exogenous Ketonuria of Rats: The experiments cited in the previous section on endogenous ketonuria indicate that ketone bodies disappear from the urine as the result of the adminis-

⁵⁷³ T. M. Carpenter, *J. Nutrition*, 6, 205-224 (1933).

tration of an amount of glucose sufficient to replace only an insignificant amount of the fat being catabolized. It is difficult to present decisive evidence that this carbohydrate effect is being exerted only on that portion of the fat being metabolized in the liver which is the precursor of the ketone bodies. However, in the case of exogenous ketonuria, one is not faced with any similar difficulty in determining the amount of ketogenic material being metabolized. Thus, when a given amount of sodium acetoacetate, sodium butyrate, or sodium caproate is fed, it cannot be stored or withheld, but it must be metabolized, resulting in the production of acetone bodies or, in case ketolytic material is available, converted to CO_2 and H_2O . If the metabolism of acetoacetic acid or of other ketogenic acids were not influenced by D-glucose, or if it were used in preference to D-glucose, then its elimination should not be changed, after sugar feeding, from that recorded in fasting. On the other hand, if D-glucose is preferentially oxidized, then the proportion of ketone bodies should be increased when glucose is administered. Just the opposite effect has been consistently observed.

There is considerable evidence that acetoacetate, β -hydroxybutyrate, butyrate, and caproate cannot be stored in the animal body. Thus, the injection of β -hydroxybutyrate into fasting or fed nephrectomized rats or dogs is followed by its rapid disappearance from the blood and the tissues.⁵⁷⁴⁻⁵⁷⁷ Butyrate⁵⁷⁸ or caproate⁵⁷⁹ is given as the triglyceride, it is not stored in detectable amounts. The fact that D_2O is rapidly excreted in the urine after the feeding of deuteriotributyryl⁵⁸⁰ would seem to indicate that it is rapidly metabolized and not directly built into long-chains acids. It is thus evident, in the tests with exogenous ketonuria, that ketogenic acids must be metabolized whether or not glucose is available. When glucose is present, these acids are no longer eliminated as ketone bodies, but are presumably broken down to CO_2 and H_2O . If glucose effects this change, the most logical explanation is that it does so by ketolysis.

The proponents of the antiketogenesis theory accept the fact that, in exogenous ketonuria, the administration of glucose is followed by the disappearance of the ketonuria. However, these results are interpreted

⁵⁷⁴ I. L. Chaikoff and S. Soskin, *Am. J. Physiol.*, **87**, 58-72 (1928).

⁵⁷⁵ I. A. Mirsky and R. H. Broh-Kahn, *Am. J. Physiol.*, **119**, 734-739 (1937).

⁵⁷⁶ I. A. Mirsky, N. Nelson, and I. Grayman, *J. Biol. Chem.*, **130**, 179-186 (1939).

⁵⁷⁷ H. J. Deuel, Jr., L. F. Hallman, P. O. Greeley, J. S. Butts, and N. Halliday, *J. Biol. Chem.*, **133**, 173-182 (1940).

⁵⁷⁸ H. C. Eckstein, *J. Biol. Chem.*, **81**, 613-628 (1929).

⁵⁷⁹ H. C. Eckstein, *J. Biol. Chem.*, **84**, 353-357 (1929).

⁵⁸⁰ M. G. Morehouse, *J. Biol. Chem.*, **155**, 33-38 (1944).

as indicating that glucose inhibits the endogenous fasting ketonuria, thus acting antiketogenically.²⁶⁷ The administered ketogenic acids can then replace the endogenous ketone bodies and be entirely oxidized by the peripheral tissues, without a resulting ketonuria. However, Deuel and Morehouse⁵¹¹ pointed out that this interpretation is not consistent with the isodynamic law, even if one assumes 100% transformation of fat to ketone bodies.

f'. The Effect of Glucose on the Rate of Disappearance of Ketone Bodies in the Intact Animal: Other experimental evidence important in determining whether the ketolytic or the antiketogenic theory best explains the inhibitory action of glucose on ketone body formation involved studies of the effect of glucose on the rate of disappearance of the ketone bodies. Chaikoff and Soskin⁵⁷⁴ were unable to demonstrate any alteration in the rate of utilization of acetoacetate when injected into eviscerated, nephrectomized dogs, irrespective of whether or not glucose was present. Utilization values in both cases varied between 120 and 130 mg./kg./hr.⁵¹¹ On the other hand, the rate of utilization of acetoacetate was only 8.5 mg./kg./hr. in the case of a depancreatized, nephrectomized dog, as contrasted with a figure of 210 mg./kg./hr. in a diabetic eviscerated dog.⁵¹¹ The variation in this case was explained by the fact that the ketone bodies were added to the blood in the first instance by the liver. Mirsky and co-workers were unable to demonstrate any alteration in the rate of disappearance of injected acetoacetate as a result of the administration of glucose to nephrectomized rabbits⁵⁷⁵ or of injected β -hydroxybutyrate after it was given to nephrectomized rats.⁵⁷⁶ However, Deuel and associates⁵⁷⁷ found that β -hydroxybutyrate was oxidized to an amount statistically greater in glucose-fed rats than in fasted nephrectomized rats. Thus, in the 75-minute interval after the injection of β -hydroxybutyrate, 22.5 ± 1.5 mg. and 12.6 ± 1.2 mg. per 100 sq. cm. were oxidized by the glucose-fed animals and by the fasted rats, respectively. The corresponding values for the 150-minute interval were 23.2 ± 0.4 mg. and 15.4 ± 1.3 mg. per 100 sq. cm., respectively. In the tests by Deuel *et al.*,⁵⁷⁷ especially purified β -hydroxybutyrate was used, and the depressing effect of ether and of trauma on carbohydrate metabolism was prevented by carrying out the nephrectomies on the day prior to the experiment.

g'. The Effect of Glucose on the Rate of Disappearance of Ketone Bodies in Liver Slices *in vitro*: Since the liver is able to synthesize ketone bodies as well as to utilize them, the ketone body balance in liver under any set of conditions will be the sum of the two effects. A number of workers have recognized that the liver is the chief organ of ketogene-

sis.^{32, 33, 581-589} On the other hand, liver slices from rats are able to destroy ketone bodies under anaerobic and aerobic conditions.^{584, 590} Malonic acid acts as an agent inhibiting this reaction.

The rate of spontaneous ketogenesis is accelerated in liver slices obtained from fasted rats, and is depressed in slices from well-fed animals when the substrate contains no ketone bodies or when butyrate is present.⁵⁹¹⁻⁵⁹³ Moreover, Cohen and Stark⁵⁹³ reported that, when an acetoacetate substrate was employed, the rate of ketone body disappearance was greater in preparations of liver from well-fed rats, rabbits, and guinea pigs than in slices from the livers of fasted animals of the same species. Quastel and Wheatley⁵⁹⁴ reported that glycogen (but not D-glucose) reduced the Q_{O_2} and ketone body production of liver slices from fasted rats which were immersed in a substrate containing butyrate, crotonate, or caproate. In one series of tests, it was found that the addition of propionate to a substrate containing butyrate caused a marked depression in acetoacetate utilization, although these workers were unable to duplicate the results a year later.⁵⁹⁵ The positive results were attributed to a substrate competition between propionate and butyrate,⁵⁹⁴ although the authors suggest that the results might likewise be attributed to ketolysis. However, on the basis of a reduced Q_{O_2} , they concluded that antiketogenesis rather than ketolysis best explained the lowered ketone body formation in liver slices with a glycogen substrate.⁵⁹⁴ Cohen and Stark⁵⁹³ attributed similar results to ketolysis.

The experiments of Bobbitt and Deuel⁵⁹¹ likewise indicate that ketolysis is the theory which best explains the action of carbohydrate on ketone body disappearance. It was demonstrated in these tests not only that the

⁵⁸¹ G. Embden and F. Kalberlah, *Beitr. chem. Physiol. Pathol.*, **8**, 121-128 (1906).

⁵⁸² G. Embden and H. Engel, *Beitr. chem. Physiol. Pathol.*, **11**, 232-326 (1908); G. Embden and L. Lattes, *Ibid.*, **11**, 327-331 (1908).

⁵⁸³ M. Almagia and G. Embden, *Beitr. chem. Physiol. Pathol.*, **6**, 59-62 (1905).

⁵⁸⁴ I. Snapper and A. Grünbaum, *Biochem. Z.*, **181**, 410-417, 418-424 (1927); **185**, 223-228 (1927).

⁵⁸⁵ I. Snapper, A. Grünbaum, and J. Neuberger, *Biochem. Z.*, **167**, 100-106 (1926).

⁵⁸⁶ H. E. Himwich, W. Goldfarb, and A. Weller, *J. Biol. Chem.*, **93**, 337-342 (1931).

⁵⁸⁷ W. Goldfarb and H. E. Himwich, *J. Biol. Chem.*, **101**, 441-448 (1933).

⁵⁸⁸ H. C. Harrison and C. N. H. Long, *J. Biol. Chem.*, **133**, 209-218 (1940).

⁵⁸⁹ I. A. Mirsky, *Am. J. Physiol.*, **115**, 424-428 (1936).

⁵⁹⁰ I. E. Stark and P. P. Cohen, *J. Biol. Chem.*, **123**, cxv (1938).

⁵⁹¹ B. G. Bobbitt and H. J. Deuel, Jr., *J. Biol. Chem.*, **143**, 1-9 (1942).

⁵⁹² S. Weinhouse, R. H. Millington, and B. Friedman, *J. Biol. Chem.*, **181**, 489-498 (1949).

⁵⁹³ P. P. Cohen and I. P. Stark, *J. Biol. Chem.*, **126**, 97-107 (1938).

⁵⁹⁴ J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **27**, 1753-1762 (1933).

⁵⁹⁵ J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **28**, 1014-1027 (1934).

ketone bodies present in the liver slices and substrate were reduced when the samples were obtained from well-fed rats, as contrasted with samples from fasted rats, but also that the consumption of butyrate from the substrate in the former case was at a considerably higher level than that in the liver slices from fasted rats. Thus, in the presence of carbohydrates, livers utilized significantly greater amounts of ketogenic acids in the substrate, and smaller quantities of ketone bodies remained unoxidized at the end of the incubation than could be noted in experiments in which liver slices were obtained from starved animals. Weinhouse *et al.*⁵⁹² also reported that the presence of carbohydrate did not cause inhibition of the oxidation of C₂ to C₈ fatty acids by liver slices, but that, in many cases, it actually resulted in an increased rate of fatty acid oxidation. In the absence of oxaloacetate, an accumulation of acetyl groups leads to their self-condensation, with the formation of acetoacetate. These authors⁵⁹² suggest that this is the explanation for the findings of Bobbitt and Deuel⁵⁹¹ that glycogen added to liver slices increased the rate of butyrate utilization but decreased the rate of ketogenesis.

Although Edson⁵⁷⁰ postulates that carbohydrates and their derivatives are antiketogenic and compete with carbohydrates for oxygen in the liver, his results are inferential, since they are not based upon a direct determination of the decrease in fatty acids. However, the conclusions of Bobbitt and Deuel⁵⁹¹ upon which they base their opinion that ketolysis best explains the action of carbohydrate on ketosis, are arrived at from experiments involving the determination not only of the ketone bodies present but also of the fatty acid disappearance during the course of an experiment.

(c) *Résumé of the Mechanism of Action of Carbohydrate on Ketonuria.* The most logical concept of the action of carbohydrate in depressing ketosis is that expressed by Rosenfeld²⁶⁴ many years ago, and cited earlier, namely that "fats burn in the flame of carbohydrate." Ketone bodies are broken down to active acetate, and this combines with oxaloacetate, and so enters the tricarboxylic cycle. When carbohydrate is present, so that the tricarboxylic acid cycle is functioning at a high level, adequate supplies of oxaloacetate are available to bring about the removal of all the ketone bodies formed. On the other hand, when the carbohydrate metabolism is at a minimum level, and hence the supply of oxaloacetate arising from carbohydrate is limited, there is insufficient oxaloacetate present to combine with all the available active acetate. The reaction of ketone bodies → active acetate becomes bogged down, and greater amounts of ketone bodies are retained in the tissues, in addition to those excreted.

Thus, most evidence indicates that the action of carbohydrate in the

suppression of ketosis is ketolytic. The explanation, based upon the incorporation of ketone bodies *via* active acetate into the tricarboxylic acid cycle, is practically identical with the original concept of Shaffer.²⁶⁶ The only variation is that Shaffer believed that the coupled reaction was between acetoacetate and a triose, while the current concept is that the coupling occurs between active acetate (formed from ketone bodies) and oxaloacetate.

f. The Comparative Ketolytic Effect of the Sugars. Although it would be expected that the ketolytic effect of sugars and of glucose intermediates would be in proportion to the D-glucose derived from the compound in question, galactose presents an interesting exception. Galactose is a sugar more difficult to metabolize than is glucose, since the galactosemia following galactose feeding is greater than the corresponding hyperglycemia after equivalent amounts of glucose have been given.⁵⁹⁶ Moreover, a galactosuria follows even moderate doses of galactose in the normal animal, while much larger amounts of glucose will not produce any detectable glycosuria.

In spite of the apparently difficult metabolism of galactose, it has repeatedly been demonstrated to have a greater ketolytic activity than has glucose. Thus, when given to fasting men or women or to men presenting a ketonuria produced by the ingestion of a protein-fat diet,³⁵⁰ the ketonuria was reduced to lower values, and the minimum level was attained at a longer period after the ingestion of this sugar than was the case when glucose was taken. In a typical experiment on a fasting man, the minimum ketonuria was obtained twenty hours after glucose and twenty-five hours after galactose had been taken, when 72 g. of the sugars were ingested; the resumption of the preprandial level of ketonuria occurred forty-four hours after the administration of glucose, but not until sixty-eight hours after that of galactose. In this experiment, the values for ketone body excretion during the period of maximum ketolysis were 0.260 g./8 hr. in the glucose test, and 0.201 g./8 hr. in the galactose experiment.³⁵⁰ Butts³⁸⁷ reported a similar phenomenon in rats, in which an exogenous ketonuria was produced by the administration of sodium acetoacetate twice daily. Moreover, the latter investigator demonstrated that the nitrogen-sparing action of glucose and galactose was in line with the ability of the animal to reduce ketonuria. As a possible explanation for these findings, Deuel *et al.*⁵⁶⁶ noted that the liver glycogen of dogs and of rats was higher six hours after the ingestion of glucose than following

⁵⁹⁶ M. Wierzechowski, W. Pieskow, and E. Owsiany, *Biochem. Z.*, 230, 146-172 (1931).

that of galactose; however, a higher level of glycogen was maintained in the liver for a longer period (twelve to seventy-two hours) after galactose feeding than when glucose was the sugar administered. This variation in retention of liver glycogen offers an excellent explanation for the prolonged inhibition of ketonuria following the ingestion of galactose.

It is not known whether or not the more prolonged retention of glycogen after galactose feeding, as compared with that after glucose, is related to a difference in composition or structure of the glycogen laid down. Galactogen, or "animal sinistrin," which is a polysaccharide composed of galactose molecules obtained from the protein gland and eggs of *Helix pomatia* (Roman land-snail) and also found in mussels, fish, and mammals, including man, has been described by several workers⁵⁹⁷⁻⁵⁹⁹ since its original discovery by Hammersten.⁶⁰⁰ However, it is not believed that this is the explanation, since Jewel⁶⁰¹ found that the glycogen arising after galactose feeding in dogs yielded sugars on hydrolysis which are completely fermentable with yeast. On the other hand, Bell⁶⁰² reported that the glycogen formed following galactose feeding is composed of 18 glucose units, as contrasted with the glycogen containing 12 glucose units which is normally formed.

In addition to galactose, there is some evidence that fructose and sucrose exhibit a more pronounced effect on ketonuria than does glucose.³⁵⁰ In rat experiments, lactose was shown to have an action intermediate between the high ketolytic effect exerted by galactose and the lower one which results from glucose.³⁸⁷

g. The Metabolism of Acetone. Whenever ketosis occurs, definite amounts of acetone ($\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_3$) appear in the blood, and usually in the liver. It has generally been assumed that, under such conditions, β -hydroxybutyric acid, acetoacetic acid, and acetone are all present. Whereas the reaction between β -hydroxybutyrate and acetoacetate has been recognized to be reversible, until recently the general opinion has been that the change of acetoacetate to acetone represented an irreversible reaction. However, in view of the experiments of Plaut and Lardy³¹⁷ with labeled acetone one must accept the fact that the reaction, $\text{acetoacetate} \rightleftharpoons \text{acetone}$

⁵⁹⁷ E. Baldwin and D. J. Bell, *J. Chem. Soc.*, 1938, 1461-1465; 1941, 125-132.

⁵⁹⁸ F. May, *Z. Biol.*, 92, 319-324, 325-330 (1932).

⁵⁹⁹ F. May, *Z. Biol.*, 95, 277-297, 401-430, 606-613, 614-634 (1934).

⁶⁰⁰ O. Hammersten, *Arch. ges. Physiol. (Pflüger's)*, 36, 373-456 (1885).

⁶⁰¹ P. W. Jewel, *A Chemical Study of the Glycogens Formed After the Administration of Glucose, Fructose, and Galactose*, Thesis, Dept. Biochem. Nutrition, Univ. Southern California, 1932.

⁶⁰² D. J. Bell, *Biochem. J.*, 30, 1612-1616 (1936).

+ CO₂, is to some extent reversible. Under certain conditions, the fixation of a small amount of carbon dioxide can occur in the acetone molecule, with the resultant formation of acetoacetate. Coon⁶⁰³ also proved that a similar fixation of carbon can take place to a large extent on the isopropyl alcohol fragment formed on the breakdown of leucine or isovaleric acid. This author indicates that a similar reaction may occur with acetone, but that this reaction is only a minor one.

Price and Rittenberg⁶⁰⁴ observed that acetone can be largely oxidized to CO₂ and exhaled in the respiratory air within twenty-four hours after the feeding of 1,3-C¹⁴-acetone to rats. When the C¹⁴-methyl-labeled acetone was given in small doses (0.1 to 0.6 mg./100 g. body weight) to rats by stomach tube, less than 10% was found to be exhaled as such. At least half of the ketone was oxidized and the C¹⁴O₂ excreted within twenty-four hours. Oxidation of acetone was likewise found to occur in alloxan-diabetic rats.⁶⁰⁴ Polonovski and Valdiguié⁶⁰⁵ suggest that the oxidation of acetone may occur *via* the tricarboxylic acid cycle.

A number of workers have noted that labeled carbon atoms of acetone may appear in cholesterol^{604,606,607} as well as in fatty acids.^{604,607} It is generally believed that the acetyl group is formed as an intermediate in these syntheses.^{604,605,607} According to Sakami, working alone⁶⁰⁸ and with Lafaye,⁶⁰⁹ acetone is split to yield a one-carbon and a two-carbon fragment which correspond to formate and acetate. Since carbonyl-labeled acetone was found to be converted into glycogen, it is believed that a third product of oxidation may be the unsplit 3-carbon acid identical with or similar to pyruvic acid.⁶⁰⁹ Price and Rittenberg⁶⁰⁴ also reported that acetone contributes carbon atoms to urea, arginine, aspartic and glutamic acids, liver glycogen and also liver and carcass cholesterol and carcass fatty acids. Wood and co-workers^{610,611} have found that *Clostridium acetobutylicum* (anaerobe from corn, potato, molasses, and soil) and *Cl. butylicum* (anaerobe producing butylic acid) ferment butyl alcohol, and change acetone to isopropyl alcohol. It is therefore evident that acetone is not

⁶⁰³ M. J. Coon, *J. Biol. Chem.*, **187**, 71-82 (1950).

⁶⁰⁴ I. D. Price and D. Rittenberg, *J. Biol. Chem.*, **185**, 449-459 (1950).

⁶⁰⁵ M. Polonovski and P. Valdiguié, *Compt. rend.*, **224**, 1531-1533 (1947).

⁶⁰⁶ E. Borek and D. Rittenberg, *J. Biol. Chem.*, **179**, 843-845 (1949).

⁶⁰⁷ I. Zabin and K. Bloch, *J. Biol. Chem.*, **185**, 131-138 (1950).

⁶⁰⁸ W. Sakami, *J. Biol. Chem.*, **187**, 369-378 (1950).

⁶⁰⁹ W. Sakami and J. M. Lafaye, *J. Biol. Chem.*, **193**, 199-203 (1951).

⁶¹⁰ H. G. Wood, R. W. Brown, C. H. Werkman, and C. G. Stuckwisch, *J. Am. Chem. Soc.*, **66**, 1812-1818 (1944).

⁶¹¹ H. G. Wood, R. W. Brown, and C. H. Werkman, *Arch. Biochem.*, **6**, 1812-1818 (1944).

an inert metabolite but one which is actively concerned in many intermediary reactions.

(5) *The Metabolism of Polyunsaturated Acids*

a. Interconversions of Linoleic Acid. (a) *Conversion to Arachidonic Acid.* Although there are decisive data to the effect that the animal cannot synthesize the so-called essential fatty acids (EFA) *de novo*, nevertheless considerable evidence indicates that a certain degree of interconvertibility exists between several members of the group.

Circumstantial proof of this interconversion consists in the fact that one essential fatty acid may prevent the deficiency caused by the exclusion of others from the diet. Thus, Turpeinen⁶¹² and Smedley-MacLean and Nunn⁶¹³ are of the opinion that linoleic acid is a precursor of arachidonic acid. The higher biopotency of arachidonic acid as compared with linoleic acid (see Chapter XI) is considered to be due to the fact that linoleate is somewhat inefficiently converted to arachidonate.

The most convincing type of proof of the linoleate $\rightarrow$ arachidonate reaction is based upon the use of balance experiments; thus, when linoleate is fed, increased amounts of arachidonate are found in the tissues of the animals, as compared with the level in control animals which have received no supplementation with linoleate. In 1926, Ellis and Isbell^{614,615} presented evidence of the appearance of arachidonic acid in the pig following the ingestion of peanuts, which contain linoleic acid. Nunn and Smedley-MacLean⁶¹⁶ demonstrated that the fat-deficient rat was able to convert linoleate to arachidonate. Widmer and Holman⁶¹⁷ obtained similar results in the case of rats. Smedley-MacLean and Hume⁶¹⁸ suggested that linoleic acid is transformed to clupanodonic acid. Rieckehoff and co-workers⁶¹⁹ also reported that corn oil (which contains linoleate but no arachidonate) markedly increases the content of the tetraenoic acid (presumably largely or entirely arachidonic acid) in the liver, heart, kidney, and brain of fat-deficient rats.

In addition to the pig and rat tests, evidence for the conversion of linoleate to arachidonate has been adduced from tests on chickens. Thus,

⁶¹² O. Turpeinen, *J. Nutrition*, **15**, 351-366 (1938).

⁶¹³ I. Smedley-MacLean and L. C. A. Nunn, *Biochem. J.*, **34**, 884-902 (1940).

⁶¹⁴ N. R. Ellis and H. S. Isbell, *J. Biol. Chem.*, **69**, 219-238 (1926).

⁶¹⁵ N. R. Ellis and H. S. Isbell, *J. Biol. Chem.*, **69**, 239-248 (1926).

⁶¹⁶ L. C. A. Nunn and I. Smedley-MacLean, *Biochem. J.*, **32**, 2178-2184 (1938).

⁶¹⁷ C. Widmer, Jr., and R. T. Holman, *Arch. Biochem.*, **25**, 1-12 (1950).

⁶¹⁸ I. Smedley-MacLean and E. M. Hume, *Biochem. J.*, **35**, 996-1002 (1941).

⁶¹⁹ I. G. Rieckehoff, R. T. Holman, and G. O. Burr, *Arch. Biochem.*, **20**, 331-340 (1949).

Reiser⁶²⁰ reported an increase in tetraenoic acids in the tissues of growing chicks on a fat-free diet when cottonseed oil or ethyl linoleate was fed. The increase was minimal in the neutral fat fraction of the carcass, more pronounced in the organ neutral fat, and increasingly greater in the carcass and organ phospholipids. In other studies,^{621,622} it was found that the unsaturated acids reached their minimum value in egg yolks after the hens had been fed a fat-free diet. Phospholipids were stored more efficiently than was neutral fat.⁶²³ It was noted that tetraenoic acids made their appearance in the yolk after cottonseed oil or ethyl linoleate had been fed.⁶²³

The most convincing proof for the synthesis of arachidonate from linoleate has been adduced by the use of tagged molecules. The mechanism of the change of one unsaturated acid to another is largely a matter of conjecture. Reiser⁶²³ suggested that "fragments of the ingested acids containing the double bonds might combine to form the more highly unsaturated members of the series." Thomasson⁶²⁴ is in agreement with Greenberg,⁶²⁵ who suggests that two molecules of linoleate give rise to one of arachidonate. Sinclair⁶²⁶ is likewise convinced of the synthesis of arachidonate from linoleate, "despite the improbability of a change involving the addition of two carbon atoms and desaturation at the 5:6 and 8:9 positions." Experimental evidence for these several conjectures was adduced by Mead and co-workers⁶²⁷ who showed that, when carboxyl-labeled acetate was injected into intact weanling rats, it appeared as the carboxyl carbon of arachidonic acid, as determined from the analysis of the degraded octabromides prepared from the acids separated from the tissues.

On the basis of a survey of the literature, Holman⁶²⁸ concluded that arachidonic acid does not occur in plants or in vegetable oils. He suggested that the one positive report was probably based upon a typographical error, and that arachidic acid was actually referred to. However, ara-

⁶²⁰ R. Reiser, *J. Nutrition*, **42**, 325-336 (1950).

⁶²¹ R. Reiser, *J. Nutrition*, **40**, 429-440 (1950).

⁶²² R. Reiser, B. Gibson, M. J. Carr, and B. G. Lamp, *J. Nutrition*, **44**, 159-175 (1951).

⁶²³ R. Reiser, *Arch. Biochem. Biophys.*, **32**, 113-120 (1951).

⁶²⁴ H. J. Thomasson, *Intern. Rev. Vitamin Research*, **25**, 62-82 (1953).

⁶²⁵ S. M. Greenberg, *Essential Fatty Acids*, Dissertation, Dept. Biochem. Nutrition, Univ. Southern California, 1951.

⁶²⁶ H. M. Sinclair, *Essential Fatty Acids and Their Relation to Pyridoxine*, in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia No. 9, Cambridge Univ. Press, 80-99, 1952.

⁶²⁷ J. F. Mead, G. Steinberg, and D. R. Howton, *J. Biol. Chem.*, **205**, 683-689 (1953).

⁶²⁸ R. T. Holman, Personal communication, 1955.

chidonic acid is usually a component of animal tissues, and may substitute for linoleate in the nutrition of different animals. These findings are interpreted as *a priori* evidence of the linoleate $\rightarrow$ arachidonate conversion.

(b) *Transformation to Polyunsaturated Acids Other Than Arachidonic Acid.* Experimental evidence has been adduced that, in addition to tetraenoic acids, other polyunsaturated acids may originate from linoleate. Although the data are still fragmentary, there appears to be a species difference in the transformations which can be effected with linoleic acid.

In the case of the laying hen, Reiser *et al.*^{622,623} found that linoleate was changed to pentaenoic acid as well as to tetraenoic acid. However, no hexaenoic acids originate in this species. In contradistinction to these results, the Holman group^{617,619} reported that, in the case of the rat, pentaenoic and hexaenoic acids arise from linolenic acid, and arachidonic (tetraenoic acid) from linoleic acid. Although these workers failed to note the formation of pentaenoic or hexaenoic acid when selected tissues were used, the evidence for the synthesis of both of these types of acids was obtained by analysis of the whole animal.

b. Interconversions of Linolenic Acid. The metabolism of linolenic acid poses certain interesting variations as compared with that of linoleic acid. One of the earliest observations was that this trienoic acid was present in the tissues to only an exceedingly small extent, even when there was a considerable amount present in the diet. This led Bloor⁶²⁹ to postulate that linolenic acid is preferentially utilized and deposited. However, it now appears that the deposition of linolenic acid in the tissue fat is a function of species. Thus, on the one hand, Ellis and Isbell^{614,615} observed that only small amounts of trienoic acids were deposited in pig fat, even when the swine were fed diets high in soybeans, which are relatively rich in linolenate. However, Beadle *et al.*⁶³⁰ did note that as much as 11.4% of trienoic acid was present in the "yellow" fat of hogs. On the other hand, rats which had been fed on a diet containing linseed oil were found to have as much as 25.6% of linolenate in their fat depots. According to Brooker and Shorland,⁶³¹ linolenic acid may comprise as much as 20% of the depot fat of pasture-fed horses. In general, however, linolenic acid is practically absent from depot fats.

The linolenate content of fats is influenced in an interesting manner by dietary conditions. The most striking fact is that the proportion of

⁶²⁹ W. R. Bloor, *Biochemistry of the Fatty Acids*, Reinhold, New York, 1943.

⁶³⁰ B. W. Beadle, O. H. M. Wilder, and H. R. Kraybill, *J. Biol. Chem.*, **175**, 221-229 (1948).

⁶³¹ E. G. Brooker and F. B. Shorland, *Biochem. J.*, **46**, 80-85 (1950).

linolenate in the storage fat is *decreased* by feeding polyunsaturated acids, and is *increased* by the administration of fat-free diets. Thus, Reiser *et al.*⁶²² reported that, on usual diets, the neutral fat and phospholipid of the egg-yolk fat of hens contained only 0.18% and 0.00%, respectively, of trienoic acids. After fifty-two weeks on a fat-free diet, the values of the above fractions were found to have increased to 0.42% and 2.3%, respectively. In contradistinction to the augmentative effect of fasting, the administration of corn oil decreased the proportion of trienoic acids in heart fatty acids from 22.2% to 4.8%.⁶¹⁹ More recently, Klein and Johnson⁶³² observed that the trienoic acid content of mitochondria, "poorly sedimentable layer" and microsomes of the livers of senescent rats, and of rats on a fat-free diet, was increased over the control level in both cases. Smedley-MacLean⁶³³ reported an increase in trienoic acid in fat deficiency; it was suggested that this "trienoic acid fraction" may represent partially-hydrogenated arachidonate, resulting from the attempt on the part of the rat to make the most of its stores of arachidonate.

The metabolic fate of ingested linolenic acid apparently varies with species. Reiser and co-workers⁶²² reported that linolenic acid is converted into dienoic, tetraenoic, pentacnoic, and hexaenoic acids in the hen; these polyunsaturated acids are deposited in the egg-yolk fat in increased amounts after linolenate feeding. On the other hand Holman⁶³⁴ believes that linolenic acid is converted chiefly to hexaenoic acid. In another paper,⁶³⁵ he also reported its conversion to unsaturated acids having four, five, and six double bonds.

The data recorded above are difficult to reconcile with the information available on the nutritional value of linolenic acid. A number of workers^{636,637} have adduced evidence to show that linoleic and linolenic acids have different functions. While linoleic and arachidonic acid support growth and also alleviate skin symptoms, linolenic acid apparently supports growth to only a slight extent, unless "sparked" with linoleic acid, and has no effect upon the dermatitis.⁶³⁷ It has been further demonstrated that linolenic acid is only slightly active in protecting rats from

⁶²² P. D. Klein and R. M. Johnson, *Federation Proc.*, 12, 231 (1953).

⁶³³ I. Smedley-MacLean, *The Metabolism of Fat*, Methuen, London, 1943.

⁶³⁴ R. T. Holman, *Proc. Third Conference on Research, Amer. Meat Inst.*, Chicago, 1951, pp. 1-10.

⁶³⁵ R. T. Holman, *Proc. Soc. Exptl. Biol. Med.*, 76, 100-102 (1951).

⁶³⁶ G. O. Burr, *Federation Proc.*, 1, 224-233 (1942).

⁶³⁷ S. M. Greenberg, C. E. Calbert, E. E. Savage, and H. J. Deuel, Jr., *J. Nutrition*, 41, 473-486 (1950).

injury due to x-irradiation,⁶³⁸ unless it is supplemented by linoleate. Deuel *et al.*⁶³⁹ noted a similar relationship between linolenate and linoleate employed as supplements in the case of fasting rats and lactating rats on a fat-free diet. Holman^{634,635} is of the opinion that the slight amount of tetraenoic acid synthesized in the animal as a result of the administration of linolenate is probably an inactive isomer. The whole subject must be reassessed in view of the recent finding of Thomasson⁶²⁴ that γ -linolenic acid (6,9,12-octadecatrienoic acid) is the active essential trienoic acid, while ordinary linolenic acid is believed to possess only a slight biopotency.

c. Interconversions of Elaeostearic Acid. Elaeostearic acid, 9,11,13-octadecatrienoic acid, the conjugated counterpart of linolenic acid, apparently undergoes a change, with the loss of a double bond, when given to rats. This change is assumed by Miller and Burr,⁶⁴⁰ on the basis of the fact that the absorption maximum of elaeostearic acid at 270 $m\mu$ is lost and a new one appears at 235 $m\mu$ when this fatty acid is metabolized by rats. According to Reiser,⁶²³ a similar change of elaeostearic acid to a dienoic acid occurs in the case of the hen fed tung oil, which contains α -elaeostearic acid as its principal fatty acid component. In another series of tests, Reiser and co-workers⁶²² reported that not only dienoic acids were formed but several other polyunsaturated acids having three to six double bonds.

d. Interconversions of the More Highly Unsaturated Acids. Information regarding the presence and fate of polyunsaturated acids higher than arachidonic acid is fragmentary. Holman and Taylor⁶⁴¹ have reported, on the basis of spectrophotometric analyses, that hexaenoic acid is deposited in increased amounts in the heart and brain, and pentaenoic acid in the liver of rats after supplementation with ethyl arachidonate. However, these authors likewise point out that it is questionable whether the more highly unsaturated acids arise from arachidonic acid; it is suggested that the impurities in the tetraenoic acid preparation might have been sufficient to account for the newly-formed 5- and 6-double bonded acids. Reiser⁶²⁰ noted a significant increase in dienoic acid, but no augmentation in trienoic acid, in growing chicks fed cod-liver oil which was free from dienoic and trienoic acids but which contained more highly unsaturated acids.

⁶³⁸ A. L. S. Cheng, T. M. Graham, R. B. Alfin-Slater, and H. J. Deuel, Jr., *J. Nutrition*, **55**, 647-653 (1955).

⁶³⁹ H. J. Deuel, Jr., C. R. Martin, and R. B. Alfin-Slater, *J. Nutrition*, **57**, 297-302 (1955).

⁶⁴⁰ E. S. Miller and G. O. Burr, *Proc. Soc. Exptl. Biol. Med.*, **36**, 726-729 (1937).

⁶⁴¹ R. T. Holman and T. S. Taylor, *Arch. Biochem.*, **29**, 295-301 (1950).

According to Clément and May,⁶⁴² conjugated tetraenoic acid can be hydrogenated to conjugated trienoic acid by the rat.

Figure 3 represents some of the known interconversions of the several polyunsaturated acids. For a further treatment of the physiology and chemistry of the polyunsaturated acids, particularly those having biological activity against the fat-deficiency syndrome, the reader is referred to a recent review of Deuel and Reiser,⁶⁴³ as well as to Chapter VII of this volume.

e. The Metabolism of Polyunsaturated Acids as Related to the State of Oxidation. One of the chief properties of the polyunsaturated acids is the

Fig. 3. The possible biologic interconversions of polyunsaturated fatty acids (supplied in text).⁶⁴³

readiness with which they enter into auto-oxidation. Several investigators have studied the role of such oxidations in the normal metabolism of the polyunsaturated acids.

Dubouloz *et al.*⁶⁴⁴ reported that peroxides of fatty acid esters formed after the ingestion of ethyl oleate are gradually eliminated from the gastrointestinal tract, and that no determinable quantities are deposited in the tissues. Although small amounts of such compounds apparently do occur in adipose tissue, the digestive origin was not proved. The action of cold or of x-irradiation was later shown⁶⁴⁵ to result in the formation of lipid peroxides in the skin of the rat.

On the basis of *in vitro* studies, Kunkel⁶⁴⁶ reported that *d*- α -tocopherol

⁶⁴² G. Clément and P. May, *J. Physiol. (Paris)*, **45**, 79-83 (1953).

⁶⁴³ H. J. Deuel, Jr., and R. Reiser, *Vitamins and Hormones*, **13**, 29-70 (1955).

⁶⁴⁴ P. Dubouloz, J. Fondarai, and C. Lagarde, *Biochim. et Biophys. Acta*, **3**, 371-377 (1949).

⁶⁴⁵ P. Dubouloz and J. Dumas, *Compt. rend.*, **234**, 2575-2576 (1952).

⁶⁴⁶ H. O. Kunkel, *Arch. Biochem.*, **30**, 317-325 (1951).

exerts a greater antioxidant effect in the photo-oxidation of methyl linoleate than does *d*- γ -tocopherol. However, the carotenoid, bixin, enhances the antioxidant capability of γ -tocopherol more than it does that of α -tocopherol. Hence, the γ -isomer of tocopherol possesses a greater anti-oxygenic activity than does the α -form in the presence of bixin, although the contrary is the case in the pure system. The apparent diene conjugation of methyl linoleate per mole of absorbed oxygen was found to be less in the presence of γ -tocopherol than when α -tocopherol or no tocopherol was present in the system.⁶⁴⁶

In *in vivo* tests on deficient rats, Witten and Holman⁶⁴⁷ demonstrated that the metabolic conversions of linoleate and linolenate by fat-deficient rats are not influenced by the "oxidative or reductive status" of the animals; the variations were studied by comparing the results of control experiments in which linoleate or linolenate was fed with others in which tocopherol or benzoyl peroxide was added. In later investigations, these workers⁶⁴⁸ reported that pyridoxine is also required for the normal inter-conversions of linoleate and linolenate. When pyridoxine and linoleate were administered together to fat-deficient, pyridoxine-deficient rats, growth, fat synthesis, and arachidonate synthesis were stimulated to a much greater extent than when either supplement was given by itself. Similar data were recorded for linolenate, with the exception that dermal symptoms were not relieved. Hexaenoic acid synthesis was also augmented when the trienoic acid was fed.

A still further investigation of the behavior of linoleate as related to oxidation was carried out by Holman and Greenberg.⁶⁴⁹ Whereas all supplements containing linoleate with or without peroxide were able to cure fat-deficiency symptoms, to reduce water consumption and to stimulate arachidonate synthesis, completely negative results were obtained in all respects by the following products: linoleate peroxide, reduced linoleate peroxide, decomposed linoleate peroxide, and conjugated linoleate. In fact, dermal symptoms were actually aggravated. It was suggested that these substances, all of which have conjugated double bond systems, actually produce toxic effects on fat-deficient rats.

(6) *The Site of Oxidation of Fats in the Animal Body*

a. The Liver as the Site of Oxidation. The liver has long been recognized as the key organ in the oxidation of fatty acids. Thus, it is immedi-

⁶⁴⁷ P. W. Witten and R. T. Holman, *Arch. Biochem. Biophys.*, **37**, 90-98 (1952).

⁶⁴⁸ P. W. Witten and R. T. Holman, *Arch. Biochem. Biophys.*, **41**, 266-273 (1952).

⁶⁴⁹ R. T. Holman and S. I. Greenberg, *Arch. Biochem. Biophys.*, **49**, 49-57 (1954).

ately responsive to any qualitative or quantitative changes in fat metabolism.

(a) *The Nature of Liver Fats.* The liver fats differ from the ordinary ingested fat and from depot fats in having a higher phospholipid content and a greater degree of unsaturation.

a'. Phospholipids: The relatively high phospholipid content in the liver has led to the hypothesis that lecithin is involved in the intermediary metabolism of fat. It was suggested that fat is changed into lecithin in order to allow it to pass into the tissue cells, since this phospholipid represents a form of fat which is readily transportable.⁶⁵⁰ It was also assumed that lecithin is susceptible to oxidation.

In spite of the high phospholipid content in the liver, the proportion of this component present in the organ is normally fairly constant under a variety of conditions.⁶⁵¹⁻⁶⁵³ However, Artom⁶⁵⁴ reported that the phospholipid content of the liver was increased as much as 35% after the feeding of large doses of fat. On the basis of this effect, Artom⁶⁵⁴ considers that the phospholipids represent normal intermediates in fat metabolism.

Another fact which indicates the importance of phospholipids as intermediates in the oxidation of fat in the liver is their relatively high rate of turnover. Thus, Artom, Sarzana, and Segré⁶⁵⁵ reported that, of the various tissues examined, radioactive phosphate was most rapidly incorporated into the phospholipids of the small intestine and liver. This rate of utilization by the liver was much higher on a fat diet than it was on a carbohydrate regimen. Fries *et al.*⁶⁵⁶ confirmed the fact that the feeding of fat increased the rate of turnover of P³² in the liver and intestine. It would thus appear highly probable that phospholipid formation is intimately connected with the catabolism of fat by the liver.

b'. Unsaturation of Liver Lipids: Under ordinary conditions, the liver lipids contain a far larger proportion of unsaturated fatty acids than are found in the lipids of other tissues,⁶⁵⁷ with the exception of the brain.⁶⁵⁸ This high unsaturation is not confined to the fatty acids in the phospholipids, but also applies to those which comprise the neutral fat.^{658,657} In

⁶⁵⁰ O. Loew, *Biol. Zentr.*, 11, 269-281 (1891).

⁶⁵¹ A. Mayer and G. Schaeffer, *J. physiol. path. gén.*, 16, 325-336 (1914).

⁶⁵² W. R. Bloor, *J. Biol. Chem.*, 80, 443-454 (1928).

⁶⁵³ R. G. Sinclair, *J. Biol. Chem.*, 82, 117-136 (1929).

⁶⁵⁴ C. Artom, *Arch. fisiol.*, 32, 57-86 (1933).

⁶⁵⁵ C. Artom, G. Sarzana, and E. Segré, *Arch. intern. physiol.*, 47, 245-276 (1938).

⁶⁵⁶ B. A. Fries, S. Ruben, I. Perlman, and I. L. Chaikoff, *J. Biol. Chem.*, 123, 587-593 (1938).

⁶⁵⁷ W. F. Bloor and R. H. Snider, *J. Biol. Chem.*, 87, 399-413 (1930).

⁶⁵⁸ R. H. Snider and W. R. Bloor, *J. Biol. Chem.*, 99, 555-573 (1933).

fact, the more highly unsaturated neutral fat appears to be confined largely to the liver.⁶⁵⁷

There is some question as to whether the hepatic lipids have high iodine values because they are desaturated *in situ* or whether this results from a selection of the highly unsaturated fatty acids from those brought to the liver by the blood stream for retention by this organ. The concept of Leathes and his co-workers⁸⁷⁻⁸⁹ that the liver desaturates fat as the preliminary step in its oxidation has not been adequately proved. When cod-liver oil was fed, the liver fat contained fatty acids with a higher iodine number than was the case on the basal diet, both in the case of rats⁸⁷ and in the case of dogs.⁶⁵⁹ On the other hand, Sinclair⁶⁵³ found that, although the administration of cod-liver oil to cast resulted in increased unsaturation in the phospholipid fraction of the liver, the iodine values did not exceed that of the cod-liver oil fed. Leathes and Meyer-Wedell⁸⁷ interpreted their results as indicating a desaturation by the liver, while Joannovics and Pick⁶⁵⁹ considered that the liver selected the more highly unsaturated acids furnished in the diet and carried them to the liver for retention. Imrie⁶⁶⁰ is of the opinion that the liver is the only organ which can cause any considerable degree of desaturation of the fatty acids.

However, a number of facts are opposed to the concept that the liver has an especial capacity for desaturation of the fatty acids. Thus, Klenk and v. Schoenebeck⁶⁶¹ found that the high degree of unsaturation of liver lipids was not due to C₁₈ acids which had been desaturated, but rather to a C₂₂-tetraenoic acid (arachidonic acid). Since it is known that arachidonic acid cannot be synthesized in the animal body,⁶³⁶ it is evident that the increased unsaturation of the liver lipids is not due to *in vivo* synthesis. In fact, Burr⁶³⁶ proved that the liver is unable to synthesize a dienoic acid such as linoleic acid. Moreover, McMaster and Drury⁶⁶² reported that 90% of the liver could be removed without altering the R.Q. of fasting rabbits; this was interpreted to mean that the liver does not carry out any unique function in fat catabolism.

Finally, although Bloor⁶²⁹ is of the opinion that desaturation is a normal stage in the oxidation of the fatty acids, the dehydrogenation occurs from the carboxyl end of the fatty acid, and not in the middle. Although Schoenheimer and Rittenberg¹⁰⁶ proved, by the use of deuterium-labeled saturated fatty acids, that desaturation occurs in the animal, it is uncertain

⁶⁵⁹ G. Joannovics and E. P. Pick, *Wien. klin. Wochschr.*, **23**, 573-577 (1910).

⁶⁶⁰ C. G. Imrie, *J. Pathol. Bacteriol.*, **19**, 245-257 (1914).

⁶⁶¹ E. Klenk and O. v. Schoenebeck, *Z. physiol. Chem.*, **209**, 112-133 (1932).

⁶⁶² P. D. McMaster and D. R. Drury, *Proc. Soc. Exptl. Biol. Med.*, **25**, 151-153 (1927).

which organ is responsible for the dehydrogenation. Tangl and Berend⁶⁶³ suggested that desaturation occurs during absorption from the intestine.

c'. Saturation of Liver Lipids: Although the liver lipids are usually more highly unsaturated than are other body lipids, this is not invariably the case. Thus, Hilditch¹²⁸ indicated that saturation of unsaturated fats in preparation for storage may be as important a transformation as the reverse change. Rittenberg and Schoenheimer¹²⁹ demonstrated that hydrogenation of unsaturated fats may occur in the animal organism. Mottram⁶⁶⁴ reported that the liver lipids of the plaice had a lower iodine number than did those in the myotomes of the fish. Lovern⁶⁶⁵ also failed to demonstrate desaturation by the liver of the conger eel (*Conger vulgaris*) or of the porpoise (*Phocaena communis*). This author¹³⁰ reported saturation of the fats of the tunny (*Thunnus thynnus*); he noted that the content of stearic acid increased concomitantly with a decrease in the C₁₈-unsaturated acids. Saturation of fatty acids in the fat depots of the pig was also noted by Banks and Hilditch.¹³¹ Similar hydrogenation was found in the depot fat of the ox, by Hilditch and Longenecker.¹³² It is suggested that the body attempts to maintain an approximately constant content of saturated acids; this is made possible by the *in vivo* hydrogenation of the unsaturated C₁₈ acids.

(b) *Fat Transport to and from the Liver.* The liver accurately mirrors any change in the mobilization of fats. Thus, Hynd and Rotter⁶⁶⁶ reported that, when mice or rats were given carbohydrate-free diets, the livers of these animals became engorged with fat. When rats were fasted, the livers continued to present increasingly high levels of fat as long as stores of depot fat were available.⁶⁶⁷⁻⁶⁷⁰ However, in the case of carnivorous animals, such as the dog and cat, whose diet contains little exogenous carbohydrate, liver lipids do not increase to such a marked extent during fasting as they do in other animals.⁶⁶⁶

After fatty meals, the fatty acids in the liver lipids tend to approximate the composition of the ingested fat.⁶⁷¹ On the other hand, during fasting,

⁶⁶³ H. Tangl and N. Berend, *Biochem. Z.*, **220**, 234-238 (1930).

⁶⁶⁴ V. H. Mottram, *J. Physiol.*, **45**, 363-369 (1912).

⁶⁶⁵ J. A. Lovern, *Biochem. J.*, **28**, 394-402 (1934).

⁶⁶⁶ A. Hynd and D. L. Rotter, *Biochem. J.*, **24**, 1390-1399 (1930).

⁶⁶⁷ H. M. Barrett, C. H. Best, and J. H. Ridout, *J. Physiol.*, **93**, 367-381 (1938).

⁶⁶⁸ J. H. Dible, *J. Pathol. Bacteriol.*, **35**, 451-466 (1932).

⁶⁶⁹ H. C. Hodge, P. L. MacLachlan, W. R. Bloor, C. A. Stoneburg, M. C. Oleson, and R. Whitehead, *J. Biol. Chem.*, **139**, 897-915 (1941).

⁶⁷⁰ P. L. MacLachlan, H. C. Hodge, W. R. Bloor, E. A. Welch, F. L. Truax, and J. D. Taylor, *J. Biol. Chem.*, **143**, 473-490 (1942).

⁶⁷¹ R. G. Sinclair, *J. Biol. Chem.*, **111**, 515-526 (1935).

the composition of the liver fatty acids closely resembles that of the fatty acids in depot fat.^{667,669,670} When minimal quantities of fat are present in the diet, the liver is the first organ to show fat synthesis. Artom⁶⁵⁴ and Artom and Peretti⁶⁷² consider that the increase in phospholipids in the liver during the absorption of ingested neutral fats may be interpreted as proof of the participation of the liver in the intermediate metabolism and transport of the fatty acids. Bernhard and Schoenheimer⁶⁷³ demonstrated that, although the composition of liver lipids changes more promptly than do the fat depots, all storage tissues ultimately exhibit similar changes. Whereas a 50% turnover of liver fatty acids occurred within one day in the case of mice, a longer interval was required for a comparable change in the fat depots.⁶⁷³

(c) *Ketone Body Formation in the Liver.* Another indication that the liver occupies a unique position in fat oxidation is the fact that it is the principal organ which is capable of ketone body formation. Thus, after perfusion of various organs, Embden *et al.*^{7,581-583} and Snapper and co-workers^{584,585} reported that only the liver formed appreciable quantities of ketone bodies, while skeletal muscles, kidney, and lung did not bring about a corresponding synthesis. This exclusive ketogenic capacity on the part of the liver was found to be true not only in the normal organ but also in pathologic conditions following pancreatectomy and phlorhizin poisoning. In these cases the production of ketone bodies was considerably increased.⁵⁸² Moreover, Himwich and associates^{586,587} demonstrated that the organs of the dog, except the liver, removed ketone bodies from the blood, while an increased amount was present in the venous blood coming from the liver, as contrasted with that in its arterial supply. Mirsky⁶⁷⁴ showed that the liver is required for the production of ketosis by anterior pituitary extract. Although Jowett and Quastel^{32,33} did note that small amounts of ketone bodies were formed from butyric acid by slices of spleen, testes, and kidney, in the presence of added fatty acids, the quantities produced by liver slices were ten to forty times as great. Harrison and Long⁵⁸⁸ reported that, when ketosis was produced in the intact rat by fasting, by phlorhizin, or by the injection of anterior pituitary extract, a large amount of ketone bodies was present in the liver, while none appeared in the muscle until the level of ketone bodies in the plasma had reached approximately 70 mg. %. Another indication of the hepatic origin of the ketone bodies is evident from the

⁶⁷² C. Artom and G. Peretti, *Arch. intern. physiol.*, **36**, 351-370 (1933).

⁶⁷³ K. Bernhard and R. Schoenheimer, *J. Biol. Chem.*, **133**, 713-720 (1940).

⁶⁷⁴ I. A. Mirsky, *Am. J. Physiol.*, **116**, 110-111 (1936).

experimental data of Mirsky⁵⁵⁹ and of Mirsky and Broh-Kahn.⁵⁷⁵ These workers reported that ketosis could not be produced in eviscerated rabbits. Crandall *et al.*,²⁶⁰ in experiments with unanesthetized angiotomized dogs (London cannulas in the portal and hepatic veins), found that ketone bodies were produced in the liver in fasted animals but not in unfasted ones. The synthesis of ketone bodies in the liver has been demonstrated by liver perfusion and by incubation of liver slices *in vitro*.^{39,104,334,541,595,675} Stadie, Zapp, and Lukens⁶⁷⁵ likewise observed that the R. Q. of isolated liver which is oxidizing chiefly fat is as low as 0.30. If one excludes the conversion of fat to carbohydrate as an explanation for this value, it can best be explained as due to an incomplete oxidation of fatty acids associated with the production of ketone bodies.

(d) *Fat Turnover in the Liver.* Fatty acids have a short half-life, probably about one day, in the liver, as contrasted with a period of five to nine days in the body carcass, and ten to fifteen days in brain tissue.⁶⁷³ Hevesy and co-workers⁶⁷⁶ noted that, in spite of the usual half-life of fatty acids of only one day in the livers of mice, an actual decrease of concentration of 50% has been demonstrated within thirty to sixty minutes after the injection of C¹⁴-labeled acetic acid. This suggests that a rapidly renewable fatty acid fraction may be present in the liver. Beeckmans and Elliott⁶⁷⁷ believe that this effect may be ascribed to the rapid turnover of a minor portion of the fatty acid present. However, there is no evidence of such a rapidly metabolizing fraction in the brain or muscle fatty acids.⁶⁷⁶

b. Extrahepatic Oxidation of Fat. Considerable circumstantial evidence indicates that fat must be burned directly by the extrahepatic tissues. Thus, the total quantity of ketone bodies formed by the liver of the diabetic animal is not large enough to account for the calories obtained from fat.^{678,679} Moreover, it is not possible to remove the ketone bodies from the blood rapidly enough, nor to accelerate their oxidation sufficiently, to provide the entire caloric needs of the animal.^{680,681}

In addition, there is positive evidence of the use of fat by non-hepatic tissues. Thus, measurements of respiratory quotients in resting, depan-

⁶⁷⁵ W. C. Stadie, J. A. Zapp, Jr., and F. D. W. Lukens, *J. Biol. Chem.*, **132**, 423-443 (1940).

⁶⁷⁶ G. Hevesy, R. Ruyssen, and M. L. Beeckmans, *Experientia*, **7**, 144-146 (1951).

⁶⁷⁷ L. M. Beeckmans and G. de Elliott, *Nature*, **167**, 200-201 (1951).

⁶⁷⁸ W. C. Stadie, *J. Clin. Invest.*, **19**, 843-861 (1940).

⁶⁷⁹ W. C. Stadie, *Ann. Internal Med.*, **15**, 783-797 (1941).

⁶⁸⁰ N. Nelson, I. Grayman, and I. A. Mirsky, *J. Biol. Chem.*, **140**, 361-364 (1941).

⁶⁸¹ E. T. Waters, J. P. Fletcher, and I. A. Mirsky, *Am. J. Physiol.*, **122**, 542-546 (1938).

creatized dogs,⁶⁸² and after hepatectomy in rabbits⁶⁸³ have been interpreted as proving extrahepatic oxidation of fats. Typical figures for the utilization of fat were likewise noted in heart-lung preparations,^{684,685} provided carbohydrate was not present in the perfusing fluid. Roberts *et al.*⁴⁷⁹ found that eviscerated animals, previously maintained on carbohydrate-free diets, subsisted on fat, sparing blood glucose, and that no ketone bodies accumulated in the blood or tissues.

In subsequent experiments, in which the fats were labeled with isotopes, more extensive information was obtained as to the site of fat utilization. For example, Weinhouse, Millington, and Volk⁶⁸⁶ demonstrated that the oxidation of palmitic acid is brought about by a series of extrahepatic tissues, including homogenized pigeon breast muscle and heart, rat heart, and rat kidney. Rat skeletal muscle and brain were found to be inactive in fat oxidation. In later studies,⁶⁸⁷ it was found by the use of labeled palmitate that a metabolically active fatty acid fragment occurs which is not in immediate equilibrium with the tissue lipids. Lehninger¹⁷⁸ likewise demonstrated that palmitic and octanoic acids could be metabolized by heart muscle, and subsequently incorporated into succinate and ketoglutarate.

Geyer and co-workers^{688,689} reported differences in the oxidation pattern of C¹⁴-labeled octanoic acid in liver and in kidney slices. In the latter issue, much more of the carboxyl group was converted to CO₂, while the acetoacetate formation was considerably less in kidney slices than in liver slices. Moreover, the proportions of CO₂ and acetoacetate obtained from odd-carbon and even-carbon acids of varying chain length in kidney were almost identical, in contrast to the marked variations in behavior exhibited by these classes of fatty acids in liver slices. In the cyclophorase system of Grafflin and Green¹⁰³ derived from kidney and liver, the oxidation of the C₄ to C₁₂ acids goes to completion.

In an attempt to determine the extent of the extrahepatic oxi-

⁶⁸² H. E. Himwich, W. Goldfarb, N. Rakeiton, L. H. Nahum, and D. DuBois, *Am. J. Physiol.*, **110**, 352-356 (1934).

⁶⁸³ D. R. Drury and P. D. McMaster, *J. Exptl. Med.*, **49**, 765-778 (1929).

⁶⁸⁴ E. W. H. Cruickshank and H. W. Kosterlitz, *J. Physiol.*, **99**, 208-223 (1941).

⁶⁸⁵ M. B. Visscher, *Proc. Soc. Exptl. Biol. Med.*, **38**, 323-325 (1938).

⁶⁸⁶ S. Weinhouse, R. H. Millington, and M. E. Volk, *J. Biol. Chem.*, **185**, 191-200 (1950).

⁶⁸⁷ M. E. Volk, R. H. Millington, and S. Weinhouse, *J. Biol. Chem.*, **195**, 493-501 (1952).

⁶⁸⁸ R. P. Geyer and M. Cunningham, *J. Biol. Chem.*, **184**, 641-646 (1950).

⁶⁸⁹ R. P. Geyer, M. Cunningham, and J. Pendergast, *J. Biol. Chem.*, **185**, 461-468 (1950).

dation of fat, Goldman, Chaikoff *et al.*⁶⁹⁰ carried out tests on normal and on liverless dogs which were receiving emulsified, carboxyl-labeled tripalmitin intravenously. Only about 40% as much triglyceride was utilized by the hepatectomized dogs as by normal animals, in spite of the fact that the plasma fatty acid levels were higher in the hepatectomized dogs than in the normal controls.

On the basis of observations by Lehninger¹⁷⁸ and by Chernick, Masoro, and Chaikoff,⁶⁹¹ Goldman *et al.*⁶⁹⁰ postulate that the oxidation of higher fatty acids must be a normal occurrence in muscle. Direct evidence of this utilization has been presented by a number of workers. Wertheimer and Ben-Tor⁶⁹² demonstrated that the fatty acids of rat serum were broken down when diaphragm was incubated in it. The oxidation of the serum fatty acids was completely inhibited by glucose, pyruvic acid, and acetoacetate, but only partially by galactose, fructose, and acetate. Thus, a competition exists between glucose and fatty acid oxidation in muscle. Hansen and Rutter⁶⁹³ reported that octanoic acid stimulates the oxidation in rat diaphragm. The isolated diaphragms from animals previously maintained on a high-carbohydrate intake were found to remove more octanoate from the medium than did similar tissues from animals previously fed a fat diet, but both stimulated oxygen consumption. Geyer and associates⁶⁹⁴ also found that octanoic and pentanoic acids, as well as palmitic acid, were oxidized at a normal rate by eviscerated rats when calculations were based upon specific activity data. Merlini⁶⁹⁵ reported that the saturated fatty acids from C₁ to C₇ are oxidized by auricle, while Pignatosa⁶⁹⁶ noted an irregular oxidation of fatty acids and esters by the crystalline lens of scorbutic guinea pigs.

One may conclude from these data that fats can be utilized by tissues other than the liver. It is of interest to ascertain to what extent ketone bodies participate in this oxidation, both from the standpoint of synthesis in the tissues involved, and also as related to their oxidation in extrahepatic organs.

(a) *Extrahepatic Synthesis of Acetone Bodies.* Although the liver is the

⁶⁹⁰ D. S. Goldman, I. L. Chaikoff, W. O. Reinhardt, C. Entenman, and W. G. Dauben, *J. Biol. Chem.*, **184**, 719-726 (1950).

⁶⁹¹ S. S. Chernick, E. J. Masoro, and I. L. Chaikoff, *Proc. Soc. Exptl. Biol. Med.*, **73**, 348-352 (1950).

⁶⁹² E. Wertheimer and V. Ben-Tor, *Biochem. J.*, **50**, 573-576 (1952).

⁶⁹³ R. G. Hansen and W. J. Rutter, *J. Biol. Chem.*, **195**, 121-126 (1952).

⁶⁹⁴ R. P. Geyer, W. R. Waddell, J. Pendergast, and G. S. Yee, *J. Biol. Chem.*, **190**, 437-443 (1951).

⁶⁹⁵ D. Merlini, *J. physiol. pathol. gén.*, **39**, 499-504 (1949).

⁶⁹⁶ G. Pignatosa, *Boll. oculist.*, **23**, 243-256 (1944).

chief site of ketogenesis due to fatty acid degradation and by condensation of acetic acid, muscle and other non-hepatic tissues can form ketone bodies. Thus, Medes and her co-workers,⁶⁹⁷ employing isotopic acetate, reported that ketogenesis is not exclusively a function of the liver, but that it may take place in tissues, such as the kidney, which utilize acetate.

Pennington⁶⁹⁸ reported that the epithelium of the reticulum and of the omasum of the sheep were as active in transforming acetic and butyric acids to ketone bodies as was liver tissue. However, the epithelia of the abomasum, cecum, and kidney produced a relatively small amount of ketone bodies.

The epithelial tissue of sheep rumen is able to convert butyric acid *in vitro* to ketone bodies.^{698,699} Ketone bodies were also shown to originate from acetic acid, but not from propionic acid; moreover, the latter compound was found to exert an antiketogenic effect.⁶⁹⁸ In a later study of the reactions in the epithelial tissue of sheep rumen,⁷⁰⁰ ketone bodies were produced quantitatively after the first 1.5 hours, from pyruvate, instead of the latter compound being converted to lactate. Ketone bodies were also produced from lactate, but at a slower rate than from pyruvate. In contrast to these results, glucose was metabolized without the production of ketone bodies, by the sheep rumen epithelia. Actually, glucose in the presence of CO₂ suppressed the endogenous production of ketone bodies, although it did not decrease the amount of pyruvate metabolized. It is suggested that, under these conditions, glucose is metabolized by a pathway other than *via* pyruvic acid.⁷⁰⁰

Isolated tissues other than the liver and, to some extent, the kidney, do not yield ketone bodies^{594,675,701} in appreciable amounts. The relatively slight degree of ketosis produced by kidney slices is attributed to the rapid oxidation of acetoacetate by this tissue.⁵⁹⁴

Greenbaum and McLean⁷⁰² have suggested, on the basis of tests on rats, that the growth hormone of the anterior lobe of the pituitary gland controls the extrahepatic utilization of fat. Since the liver is unable to catabolize the total fat disappearing from the body, it was concluded that the additional fat oxidized was catabolized outside of the liver, the growth hormone being the stimulating agent. A definite ketonuria was shown to obtain in adult female rats as a result of this catabolism.

⁶⁹⁷ G. Medes, S. Weinhouse, and N. F. Floyd, *J. Biol. Chem.*, **157**, 751-752 (1945).

⁶⁹⁸ R. J. Pennington, *Biochem. J.*, **51**, 251-258 (1952).

⁶⁹⁹ R. J. Pennington, *Biochem. J.*, **49**, lix (1951).

⁷⁰⁰ R. J. Pennington and T. M. Sutherland, *Biochem. J.*, **54**, xxvii (1953).

⁷⁰¹ R. A. Shipley, *Am. J. Physiol.*, **141**, 662-668 (1944).

⁷⁰² A. L. Greenbaum and P. McLean, *Biochem. J.*, **54**, 413-424 (1953).

(b) *The Utilization of Ketone Bodies by Extrahepatic Tissues.* Muscles and other extrahepatic tissues can bring about an oxidation of the ketone bodies to carbon dioxide and water, thus obtaining the maximum energy yield. According to Stadie,² the rate at which ketone bodies can be utilized by the tissues of various animals is in the neighborhood of 3000 μ M per 100 gm. per day. Marriott,⁷⁰³ and Snapper and Grünbaum⁵⁸⁴ have shown that the capacity of the tissues to utilize the ketone bodies is high. Proof that these substances can be metabolized in tissues other than the liver was adduced by Chaikoff and Soskin,⁵⁷⁴ who observed that diacetic acid injected into the depancreatized dog disappeared even after hepatectomy. The latter workers demonstrated that the rate of utilization of ketone bodies is a function of their level in the blood. Thus, while a high rate of disappearance of ketone bodies by depancreatized, hepatectomized dogs, which had also been nephrectomized, was noted when the plasma level of ketone bodies was 100 mg. % and the blood ketone values rapidly dropped to 20 to 30 mg. % there was a tendency toward leveling off at this lower level. This would suggest that the rate of utilization of ketones is very high where high blood ketone levels exist, but that the rate is not of any great magnitude when the ketone levels are those ordinarily obtained in ketosis. The results of Dye and Chidsey,⁷⁰⁴ as well as those of Blixenkrone-Møller,⁷⁰⁵ both support this conclusion. Nelson and collaborators⁶⁸⁰ likewise reported a relationship between the concentration of β -hydroxybutyrate in the blood and its rate of oxidation. Mirsky⁵⁸⁹ and Mirsky and Broh-Kahn⁷⁰⁶ reported the utilization of ketone bodies in blood from eviscerated dogs and rabbits. Harrison and Long⁵⁸⁸ observed that β -hydroxybutyrate was removed from the blood by the muscles of rats which had been fasted, or given anterior pituitary extract or phlorhizin, at the same rate as by those of normal animals. Barnes *et al.*⁷⁰⁷ and Waters and co-workers⁶⁸¹ noted the disappearance of the ketone bodies in the perfusing solution of heart-lung preparations.

Since the rate of utilization of ketone bodies by the extrahepatic tissues appears to be little influenced by diabetic conditions, it is believed that combustion of carbohydrate is not a prerequisite for their oxidation. Thus, the utilization of ketone bodies was reported in depancreatized dogs by Chaikoff and Soskin,⁵⁷⁴ and in those of Blixenkrone-Møller,⁷⁰⁵ in phlorhizin-

⁷⁰³ W. McK. Marriott, *J. Biol. Chem.*, **18**, 241-262 (1914).

⁷⁰⁴ J. A. Dye and J. L. Chidsey, *Am. J. Physiol.*, **127**, 745-750 (1939).

⁷⁰⁵ N. Blixenkrone-Møller, *Z. physiol. Chem.*, **253**, 261-275 (1938).

⁷⁰⁶ I. A. Mirsky and R. H. Broh-Kahn, *Am. J. Physiol.*, **120**, 446-450 (1936).

⁷⁰⁷ R. H. Barnes, E. M. MacKay, G. K. Moe, and M. B. Visscher, *Am. J. Physiol.*, **123**, 272-279 (1938).

ized rabbits and dogs, as well as in diabetic dogs, rabbits, and a goat by Barnes *et al.*²⁶¹ Friedemann⁷⁰⁸ also reported that the rate of disappearance of acetoacetate proceeded equally rapidly in normal, in phlorhizinized, and in depancreatized dogs. Stadie and co-workers⁶⁷⁵ noted that, when slices of liver and muscle were incubated together, the ketones produced by the liver were oxidized by the muscle.

Although the presence of sugar does not appear to be necessary for the oxidation of the ketone bodies in the isolated tissues, it is a well-known fact that glucose spells the difference between the presence or the absence of ketonuria in the fasting intact animal given ketogenic materials. Mirsky, Nelson, and Grayman,⁵⁷⁶ however, were unable to demonstrate any increased rate of oxidation of injected β -hydroxybutyrate in nephrectomized rats if a proper correction was made for endogenous ketogenesis, irrespective of whether or not glucose was given. On the other hand, Deuel *et al.*,⁵⁷⁷ in tests patterned after those of Mirsky *et al.*,⁵⁷⁶ did note an increased rate of disappearance of β -hydroxybutyrate, in the case of rats, when glucose was given. Additional evidence is necessary to answer this question more fully. This relationship has been discussed more completely in an earlier section (see page 172).

The maximum proportion of the metabolism which can be assumed to be due to the oxidation of ketone bodies is considerably under 100%. Stadie^{678,679} calculated that, in the case of diabetic patients, the energy obtained from the oxidation of the ketone bodies cannot account for more than one-half to one-third of the total caloric needs from fat. Koehler, Windsor, and Hill⁷⁰⁹ reached the conclusion that acetoacetate does not have the capacity to supply all the energy for sustenance in man. Their conclusions were based upon the results of perfusion tests with acetoacetate in normal subjects. Wick and Drury⁷¹⁰ arrived at essentially similar conclusions as a result of experiments on rabbits.

(7) Factors Affecting Fat Oxidation

Although the hypothesis was formerly accepted by most biochemists that fat is oxidized only when the carbohydrate reserves become depleted, this concept cannot be accepted now, in view of the newer experimental results obtained by the use of isotopes; the data indicate that fat is being deposited not only when the available carbohydrate is high, but also during periods of fasting. The fat storage depots must be regarded as dynamic

⁷⁰⁸ T. E. Friedemann, *J. Biol. Chem.*, **116**, 133-161 (1936).

⁷⁰⁹ A. E. Koehler, E. Windsor, and E. Hill, *J. Biol. Chem.*, **140**, 811-825 (1941).

⁷¹⁰ A. N. Wick and D. R. Drury, *J. Biol. Chem.*, **138**, 129-134 (1941).

rather than static. There can be no doubt that, in spite of the continuous turnover of fats during periods of normal nutrition, there are certain conditions under which fat oxidation is depressed, and other situations when fat oxidation is accelerated.

Geyer and associates⁷¹¹ demonstrated that the rate of palmitic acid oxidation in liver slices, but not in kidney slices, of rats was accelerated by previous fasting. The addition of pyruvate to the medium decreased the rate of oxidation of palmitic acid by liver and kidney slices from both non-fasted and fasted rats. On the other hand, just the opposite condition to that reported in fasting has been shown to obtain in congenitally obese mice.⁷¹² Thus, Salcedo and Stetten⁷¹² reported that the excess fat deposition in the congenitally obese strain maintained at Cold Spring Harbor resulted from the retarded metabolism of the depot fatty acids, while the proportion of dietary fatty acids directly stored was normal. In the tests of Guggenheim and Mayer,⁷¹³ it was found that, when fasted obese and non-obese mice were injected with carboxyl-labeled sodium acetate, the non-obese mice excreted one-third more labeled CO₂ during the three hours following the injection than did the obese animals. After labeled acetate was fed for three days to these two groups of mice, the retention of isotopic carbon was more than twice as great in the strain of congenitally obese mice as in the normal animals. It is suggested that a partial block in acetate utilization obtains, with a resultant increased lipogenesis. Pyruvate oxidation is likewise depressed in the obese strain of mice.

The results of Muñoz and Leloir³⁵ were interpreted as indicative of variations in fat oxidation as related to the length of the fatty acid chains. Thus, it was reported that fatty acids up to eight carbons in length caused a rapid increase in O₂ uptake of a homogenate of guinea pig liver, while fatty acids of twelve to eighteen carbons either caused no augmentation of oxygen uptake or resulted in an inhibition of this utilization. Weinhouse *et al.*⁶⁸⁶ have also shown that, whereas palmitic acid is converted both to CO₂ and to acetoacetate by rat liver slices and homogenates, the lower fatty acids are transformed chiefly to ketone bodies. However, Weinhouse and co-workers⁶⁸⁶ conclude that the same enzyme systems are involved in the oxidation of both longer- and shorter-chain acids.

Various parts of the fatty acid molecule appear to be oxidized simultaneously. Weinman *et al.*⁷¹⁴ found that rats injected with tripalmitin in

⁷¹¹ R. P. Geyer, E. J. Bowie, and J. C. Bates, *J. Biol. Chem.*, **200**, 271-274 (1953).

⁷¹² J. Salcedo, Jr., and De W. Stetten, Jr., *J. Biol. Chem.*, **151**, 413-416 (1943).

⁷¹³ K. Guggenheim and J. Mayer, *J. Biol. Chem.*, **198**, 259-265 (1952).

⁷¹⁴ E. O. Weinman, I. L. Chaikoff, W. G. Dauben, M. Gee, and C. Entenman, *J. Biol. Chem.*, **184**, 735-744 (1950).

which the palmitic acid was labeled with C^{14} at the carboxyl group or at carbons 6 or 11, respectively, expired $C^{14}O_2$ at the same rate. This means that, once the process of breakdown of palmitic acid is initiated in the intact rat, the molecule is disrupted in such a manner that all its carbons are converted to CO_2 at about the same time. This precludes the existence of a major portion of the carbon from palmitic acid as a stable intermediate of oxidation;¹⁰⁷ the number of molecules which become stabilized as lauric or myristic acid are few as compared with those completely oxidized. Lerner and associates⁷¹⁵ reported that approximately 36 to 59% of the $C^{14}O_2$ originating from palmitic acid labeled with C^{14} at its sixth carbon was eliminated within twenty-four hours after its intravenous injection.

In studies on labeled stearic acid, Weinman and collaborators⁷¹⁶ found some differences in the rate of utilization of the carboxyl carbon from that of the sixth carbon. Thus, after the injection of tristearin-1- C^{14} into fasted rats, 40 to 44% of the $C^{14}O_2$ was eliminated during the first twenty-four hours, as contrasted with only 30 to 38% of the labeled carbon as $C^{14}O_2$ after the injection of tristearin-6- C^{14} . It was also found that some isotopically marked tripalmitin was present in the body fats, although the activity of the 18-carbon fraction was twelve times as great as that of the 16-carbon fraction. The authors, therefore, interpret the differences in the rate of excretion of $C^{14}O_2$ to mean that some of the tristearin-6- C^{14} becomes stabilized at the palmitic acid stage, thus lessening the proportion available for complete oxidation of the molecule. Although the same change will obviously occur in the tristearin-1- C^{14} , it has already eliminated the C^{14} from its molecule.

Ascorbic acid was shown by Rusch and Klein⁷¹⁷ to play a role in the oxidation of emulsions of liver phospholipids. Abramson⁷¹⁸ reported that the oxidation of unsaturated fatty acids, as measured by the thiobarbituric acid reaction, was highest in the brain, nerve, and testis of normal guinea pigs; on the other hand, in the scorbutic guinea pig, oxidation was decreased in brain, testis, medulla oblongata, adrenal, and kidney, whereas it was unchanged from the normal in the liver, spleen, and heart. This decreased oxidation rate of unsaturated fats in scorbutic tissues could be corrected by the addition of ascorbic acid *in vitro*. When vitamin C was added to the tissues of normal animals, the oxidation rate was decreased in the liver, in-

⁷¹⁵ S. R. Lerner, I. L. Chaikoff, C. Entenman, and W. G. Dauben, *Proc. Soc. Exptl. Biol. Med.*, **70**, 384-387 (1949).

⁷¹⁶ E. O. Weinman, I. L. Chaikoff, B. P. Stevens, and W. G. Dauben, *J. Biol. Chem.*, **191**, 523-529 (1951).

⁷¹⁷ H. P. Rusch and B. E. Klein, *Cancer Research*, **1**, 465-472 (1941).

⁷¹⁸ H. Abramson, *J. Biol. Chem.*, **178**, 179-183 (1949).

creased in some tissues, and unchanged in others. 2,4-Dinitrophenol (DNP) has been shown to enhance the respiration of liver slices metabolizing octanoate and pyruvate; a greater proportion of acetoacetate was found than was noted in control tissues.⁷¹⁹ Richter⁷²⁰ suggested that galactose may have a specific effect upon the utilization of fat, since rats on a diet of margarine plus galactose survived longer than did animals which received only glucose with the fat.

Choline also plays a role in determining the rate of fat oxidation. Thus, Artom^{721,722} found that labeled palmitate or stearate was oxidized to a lesser extent, as determined by the production of $C^{14}O_2$, by liver slices of rats previously maintained on a choline-deficient diet than by slices of livers of rats previously on a stock diet, or on a low-protein regimen which was supplemented with choline. A high level of fat oxidation occurred in liver slices from choline-deficient animals which had been given injections of choline shortly before death. Since the *in vitro* addition of choline did not effect a similar stimulation in the rate of fat oxidation, it is suggested that the enhancement of fatty acid oxidation is not due to free choline but to some substance or substances formed from choline *in vivo*.

The rate at which octanoic acid-1- C^{14} is oxidized *in vitro* has been shown to be related to the inorganic salts in the medium. Geyer and co-workers⁷²³ reported that the rate of metabolism of this acid in rat liver slices was greatly increased when potassium or lithium salts were added to the medium at the expense of the sodium salts. The increase was reflected principally in the increased acetoacetate production. In the case of rat kidney slices, potassium stimulated the metabolism of octanoate, but lithium did not.

Vestling and co-workers⁷²⁴ demonstrated that leukemic liver systems are incapable of normal fatty acid oxidation. It is believed that the presence of malignant leucocytes in the liver leads to a failure in at least one important enzyme system.

It is likewise obvious that the rate of fat oxidation, particularly in the case of fasting animals or of those on a limited supply of calories, is regulated by the expenditure of energy. Under these conditions, fat calories

⁷¹⁹ P. Fantl, G. J. Lincoln, and J. F. Nelson, *Biochem. J.*, **48**, 96-99 (1951).

⁷²⁰ C. P. Richter, *Science*, **108**, 449-450 (1948).

⁷²¹ C. Artom, *Federation Proc.*, **12**, 171-172 (1953).

⁷²² C. Artom, *J. Biol. Chem.*, **205**, 101-111 (1953).

⁷²³ R. P. Geyer, M. F. Meadows, L. D. Marshall, and M. S. Gongaware, *J. Biol. Chem.*, **205**, 81-85 (1953).

⁷²⁴ C. S. Vestling, S. Kaufman, R. E. Maxwell, and H. Quastler, *J. Biol. Chem.*, **165**, 385-386 (1946).

will be the source of energy for work; they are largely utilized for the extra calories produced for specific dynamic action, and they will serve to furnish the means for the increased caloric expenditure occasioned by exposure to low environmental temperatures. Likewise, the increased basal metabolism resulting from an abnormal production of thyroxine, or from the administration of thyroid, causes an increased fat oxidation if carbohydrate is not available in a sufficient amount to supply the needed energy. Harel-Ceddaha⁷²⁵ has shown that the oxidation of fatty acids required for the chemical temperature regulation of warm-blooded animals submitted to the action of cold is catalyzed by epinephrine. This hormone was found to increase the oxidation of octanoic acid by liver homogenates.

(8) *The Inhibition of Fatty Acid Oxidation*

The oxidation of fatty acids can be inhibited by compounds which compete for the enzymes with the normal oxidation products. Malonic acid, $\text{HOOC}\cdot\text{CH}_2\cdot\text{COOH}$, is the best known of these inhibiting agents. Geyer, Cunningham, and Pendergast⁷²⁶ reported that the effect of all inhibitors, except maleic and malonic acids, on carboxyl-labeled octanoic acid, consists in a decrease in both the CO_2 and the acetoacetate formation by rat liver slices. Although acetoacetate formation was inhibited to a greater extent than was CO_2 formation, the ratio of labeling in the carbonyl and carboxyl groups in the acetoacetate remained normal. On the other hand, malonic acid caused a decrease in the formation of CO_2 from octanoate, but an increase in radioactivity of both the carbonyl and the carboxyl groups of acetoacetate.

(9) *The Oxidation and Metabolism of Parenterally Administered Fats*

Although the administration of glucose and of amino acids by the intravenous route has been a routine procedure over a number of years, the successful parenteral administration of fats has only recently been achieved. The importance of developing a satisfactory procedure for the administration of fat by pathways other than the oral one has been recognized, since the use of this foodstuff is necessary if caloric needs must be met by exclusively intravenous feeding. The administration of fat intravenously has the advantage of providing not only a readily concentrated source of calories but also one which possesses only slight osmotic properties. The

⁷²⁵ L. Harel-Ceddaha, *Compt. rend.*, **236**, 2114–2116 (1953).

⁷²⁶ R. P. Geyer, M. Cunningham, and J. Pendergast, *J. Biol. Chem.*, **188**, 185–191 (1951).

great research activity in the field of parenteral fat administration is evident from the fact that Johnson⁷²⁷ had compiled a bibliography of 170 items up to September 1951; about one-half of these are for the period subsequent to 1940.

a. Subcutaneous Administration of Fat. Von Leube,⁷²⁸ in 1895, first called attention to the possible nutritional value of fats given subcutaneously. He noted the rapid disappearance of very large injections of camphorated oil from the site of administration. The results of daily subcutaneous injections of butterfat into an emaciated dog, over a period of six weeks, indicated that fats given hypodermically were of distinct value in nutrition. Although Winternitz⁷²⁹ and Henderson and Crofutt⁷³⁰ discounted the effectiveness of administering fat subcutaneously, and suggested that the absorption is too slow to be practical, Mills⁷³¹ demonstrated the absorption of a variety of fats and oils when administered to cats by the subcutaneous route. He suggested the application of such injections in the treatment of wasting diseases, and in cachectic conditions associated with imperfect metabolic processes, and especially in tuberculosis, in which the intolerance to fats is almost symptomatic.

b. Intravenous Administration of Fat. According to Holt *et al.*,⁷³² only a few instances of intravenous fat administration had been recorded prior to 1935, but these had in some cases been successful. Murlin and Riche⁷³³ followed the respiratory quotient in two dogs after fat had been given intravenously. Since the changes in R. Q. were comparable to those resulting after fat was given orally,⁷³⁴ it was concluded that intravenously-injected fat was normally metabolized. The results of Holt *et al.*⁷³² demonstrated that lecithin-stabilized fats were satisfactorily utilized by normal infants.

(a) *Cause of Toxicity of Fat Preparations for Intravenous Use.* A number of the earlier products used for parenteral fat feeding proved to be toxic. One of the conditions which may render a preparation unsatisfactory for intravenous use is the presence of too large fat globules. Thus, in the preparation of Dunham and Brunschwig,⁷³⁵ one-third of the dogs died during the injections of emulsified olive oil or lard oil in which the size of the

⁷²⁷ E. Johnson, *Intravenous Injection of Fats and Oils for Nutritive Purposes*. Bibliography, Army Med. Library (Reference Div.), Washington, D. C., Dec. 12, 1951.

⁷²⁸ W. Von Leube, *Sitzungsber. physik.-med. Ges. Würzburg*, 1895, No. 1, 5-11.

⁷²⁹ H. Winternitz, *Z. klin. Med.*, 50, 80-101 (1903).

⁷³⁰ Y. Henderson and E. F. Crofutt, *Am. J. Physiol.*, 14, 193-202 (1905).

⁷³¹ L. H. Mills, *Arch. Internal Med.*, 7, 694-719 (1911).

⁷³² L. E. Holt, Jr., H. C. Tidwell, and T. McNair Scott, *J. Pediat.*, 6, 151-160 (1935).

⁷³³ J. R. Murlin and J. A. Riche, *Proc. Soc. Exptl. Biol. Med.*, 13, 7-8 (1915).

⁷³⁴ J. R. Murlin and G. Lusk, *J. Biol. Chem.*, 22, 15-41 (1915).

⁷³⁵ L. J. Dunham and A. Brunschwig, *Arch. Surg.*, 48, 395-405 (1944).

fat particles was from a fraction to 2 μ , or even as large as 15 to 30 μ in diameter. Symptoms of toxicity include vomiting, excessive salivation, pulmonary embolism and infarction, thrombophlebitis and hemolysis.⁷³⁶ Anemia has also been reported.^{735,736} To ensure that the fat preparations are harmless, it is also necessary that the stabilizers be relatively non-toxic.⁷³⁶

Fat preparations have frequently produced febrile reactions after being introduced intravenously. Shafiroff and co-workers⁷³⁷ reported frequent febrile responses in patients receiving intravenous fat, probably due to the sudden plethora of fat. Lambert, Miller, and Frost⁷³⁸ observed a febrile response to the intravenous injection of fat in rabbits and occasionally in dogs. This fat preparation, however, was found to contain no pyrogens resulting from bacterial contamination. These workers suggest that the increase in body temperature under these conditions is to be considered a "thermogenic" response rather than a "pyrogenic" one. The cause was believed to be the rapid oxidation of the fat, the sudden influx of which temporarily overburdens the ability of the body to store it. On the other hand, Meng⁷³⁹ was able to inject fat in amounts comparable to those of Lambert *et al.*⁷³⁸ without producing a febrile reaction. However, after several months, this emulsion also turned "thermogenic." A number of more recent fat emulsions do not produce the febrile reaction.

(b) *Methods for Stabilizing Emulsions.* Egg lecithin has been widely and successfully employed for producing fat emulsions.^{732,735,740-743} Dunham and Brunschwig⁷³⁵ also used Demal 14, which is a polyglycerol ester. Among the stabilizers tested by McKibbin and associates⁷³⁶ are the following products: Igepon T (oleyl-taurine), cetyl phosphoric acid, edible gelatin, pharmaceutical gelatins, infusion gelatins, commercial soybean phosphatides, and purified soybean phosphatides. Only the emulsion prepared with purified soybean phosphatides proved to be practically non-toxic. In a

⁷³⁶ J. M. McKibbin, A. Pope, S. Thayer, R. M. Ferry, Jr., and F. J. Stare, *J. Lab. Clin. Med.*, **50**, 488-497 (1945).

⁷³⁷ B. G. P. Shafiroff, J. H. Mulholland, and E. Roth, *Proc. Soc. Exptl. Biol. Med.*, **72**, 543-547 (1949).

⁷³⁸ G. F. Lambert, J. P. Miller, and D. D. Frost, *Am. J. Physiol.*, **164**, 490-496 (1951).

⁷³⁹ H. C. Meng, *J. Lab. Clin. Med.*, **37**, 222-228 (1951).

⁷⁴⁰ L. H. Mills and J. R. Murlin, *Proc. Soc. Exptl. Biol. Med.*, **7**, 166-168 (1909-1910).

⁷⁴¹ S. Yamakawa and T. Nomura, *Jikken Ihō*, **523** (1928); cited by T. Nomura, *Tōhoku J. Exptl. Med.*, **12**, 247-253 (1929), p. 248; also by L. E. Holt *et al.*, *J. Pediat.*, **6**, pp. 161, ff.

⁷⁴² T. Nomura, *Tōhoku J. Exptl. Med.*, **12**, 247-253, 388-396, 497-504 (1929); **13**, 51-64 (1929).

⁷⁴³ G. Sato, *Tōhoku J. Exptl. Med.*, **18**, 120-138 (1931).

later study, McKibbin, Ferry, and Stare⁷⁴⁴ modified the stabilizer by adding disodium phosphate and sometimes cholesterol to the soybean phosphatides. Meng and Freeman⁷⁴⁵ tested Span 20 (sorbitan monolaurate (0.5%)), Asolectin (a pure soya phosphatide) (0.4%) and sodium cholate (0.1%), while supersonic radiation has been reported to produce a satisfactory and relatively permanent fat emulsion.⁷⁴⁶ Geyer *et al.*⁷⁴⁷ described a high-pressure method for producing fat emulsions containing droplets with a diameter of less than 2 μ . In the recent assays on toxicity of fat emulsions, Geyer and co-workers⁷⁴⁸ employed rats. A turbidimetric method was used for the determination of fat in the blood. This method gives satisfactory results in testing the blood of the dog, rat, and rabbit.⁷⁴⁹

(c) *Results Obtained with Recent Fat Emulsions.* Collins *et al.*⁷⁵⁰ obtained satisfactory results with a 30% fat emulsion (3 Cal./ml.), which can be given rapidly to adult dogs. However, when the emulsion was stabilized with Asolectin, granulomatous lesions resulted; these were not related to the amount of fat given. Gorens and associates⁷⁵¹ successfully prevented weight loss in patients, and maintained a positive nitrogen and potassium balance over a period as long as twenty-seven days by the daily injection of a 15% fat emulsion (1600 calories per liter) into patients at dosages of 3 g. of fat per kg. body weight in the adults and twice this ratio in the case of an infant. This same emulsion had been widely tested on rats, dogs, and cats prior to its use on human subjects.⁷⁵² More recently, Neptune, Geyer, and others⁷⁵³ administered 64 infusions of a liter or more of 15% fat emulsions to nine patients; the fat was found to be rapidly cleared from the blood stream.

Several recent studies of the utilization of fat by the intravenous route have involved the use of fats labeled with isotopes. Geyer, Chipman, and

⁷⁴⁴ J. M. McKibbin, R. M. Ferry, Jr., and F. J. Stare, *J. Clin. Invest.*, **25**, 679-686 (1946).

⁷⁴⁵ H. C. Meng and S. Freeman, *J. Lab. Clin. Med.*, **33**, 689-707 (1948).

⁷⁴⁶ R. J. Myers and H. Blumberg, *Proc. Soc. Exptl. Biol. Med.*, **35**, 79-84 (1936).

⁷⁴⁷ R. P. Geyer, G. V. Mann, and F. J. Stare, *J. Lab. Clin. Med.*, **33**, 153-162 (1948).

⁷⁴⁸ R. P. Geyer, G. V. Mann, J. Young, T. D. Kinney, and F. J. Stare, *J. Lab. Clin. Med.*, **33**, 163-174 (1948).

⁷⁴⁹ R. P. Geyer, G. V. Mann, and F. J. Stare, *J. Lab. Clin. Med.*, **33**, 175-180 (1948).

⁷⁵⁰ H. S. Collins, L. M. Kraft, T. D. Kinney, C. S. Davidson, J. Young, and F. J. Stare, *J. Lab. Clin. Med.*, **33**, 143-152 (1948).

⁷⁵¹ S. W. Gorens, R. P. Geyer, L. W. Matthews, and F. J. Stare, *J. Lab. Clin. Med.*, **34**, 1627-1633 (1949).

⁷⁵² G. V. Mann, R. P. Geyer, D. M. Watkin, and F. J. Stare, *J. Lab. Clin. Med.*, **34**, 699-712 (1949).

⁷⁵³ E. M. Neptune, R. P. Geyer, I. M. Saslaw, and F. J. Stare, *Surg. Gynecol. Obstet.*, **92**, 365-369 (1951).

Stare⁷⁵⁴ found that trilaurin, in which the lauric acid was labeled on the carboxyl group, was well tolerated when given intravenously to rats. Within two hours, 50% of injected C¹⁴ had been excreted as C¹⁴O₂ in the expired air, while a 70% elimination was observed in the 3.5-hour tests. In the case of tripalmitin labeled on C-6 of the acid, Lerner, Chaikoff *et al.*⁷⁵⁵ likewise reported utilization, but at a slower rate. After two hours, only 7% of the administered C¹⁴ had been excreted in the expired air, while a total of 50% was accounted for by the respiratory route after twenty-four hours. These experiments, carried out with labeled fatty acids, constitute the best proof of the utilization of fat when given by the intravenous route.

Creditor⁷⁵⁶ observed that intravenous fat emulsions produced an increased mechanical fragility of the red blood cells of dog and human blood. Sometimes a spontaneous intravascular hemolysis has been noted in humans who have received these emulsions. It is suggested that the "highly emulsified fat might well distribute itself around the red cell and alter the membrane relationships in such a way that fragility is increased." Waddell and co-workers⁷⁵⁷ reported that the removal of emulsified fat from the blood is a first-order reaction, irrespective of the chemical composition of the oil, except where gelatin is used as an emulsifying agent. Cannon *et al.*⁷⁵⁸ reported that, when rats were injected subcutaneously with protein hydrolysates, intraperitoneal or intravenous fat augmented amino acid utilization.

3. The Oxidation and Metabolism of Glycerol

Just as glucose can be considered to be a mother substance in the synthesis of glycerol, so the oxidation and metabolism of glycerol are bound up with that of glucose. Doerschuk⁷⁵⁹ reported that, when glycerol-1-C¹⁴ was administered to a normal intact rat, the main route of metabolism consisted of phosphorylation and oxidation; over 80% of the total glycerol followed this pathway. The intermediary products, in all probability, involve those suggested in the synthesis of the trihydric alcohol from glucose, and the order of formation is the reverse of the synthetic reaction.

⁷⁵⁴ R. P. Geyer, J. Chipman, and F. J. Stare, *J. Biol. Chem.*, **176**, 1469-1470 (1948).

⁷⁵⁵ S. R. Lerner, I. L. Chaikoff, C. Entenman, and W. G. Dauben, *Science*, **109**, 13 (1949).

⁷⁵⁶ M. C. Creditor, *Proc. Soc. Exptl. Biol. Med.*, **82**, 83-87 (1953).

⁷⁵⁷ W. R. Waddell, R. P. Geyer, I. M. Saslaw, and F. J. Stare, *Am. J. Physiol.* **174**, 39-42 (1953).

⁷⁵⁸ P. R. Cannon, L. E. Frazier, and R. H. Hughes, *Federation Proc.*, **12**, 385 (1953).

⁷⁵⁹ A. P. Doerschuk, *J. Biol. Chem.*, **193**, 39-44 (1951).

Gidez and Karnovsky⁷⁶⁰ reported that, when C¹⁴-tagged glycerol was given to intact rats, only insignificant differences in metabolism were noted, irrespective of whether the alcohol was given intraperitoneally, intragastrically, or intravenously. A constant proportion was oxidized to CO₂ when doses ranging from 30 to 238 mg. were administered. It was calculated that 70 to 100% of the newly-synthesized blood glucose originated from the administered glycerol, while only 15 to 39% of the newly-formed glycogen could be traced to this source.

Moreover, Doerschuk⁷⁵⁹ demonstrated that the organism converts glycerol into the glycerol moiety of the triglycerides as well as into the glycerophosphoric acid portion of the phospholipids, lecithin, and cephalin. However, Gidez and Karnovsky⁷⁶⁰ observed that the liver triglyceride glycerol reached a specific activity about three times that of phosphatide glycerol, although the activity declined more rapidly in the triglyceride than in the phospholipid fraction. It was also noted that tagged glycerol was present in the lipids of most tissues examined, including the brain lipids.

The mechanism of synthesis of glucose from glycerol with isotopically-marked glycerol has been found to be more complex than was originally supposed. Thus, Doerschuk⁷⁶¹ reported that, when glycerol-1,3-C¹⁴ was administered to rats, radioactivity occurred mainly on carbons 1, 3, 4, and 6 of the glucose; it was believed that this indicated a preliminary oxidation and phosphorylation to dihydroxyacetone phosphate or D-3-phosphoglyceraldehyde, which was followed by known reactions of glycolysis.⁷⁶² Schambye and Wood,⁷⁶³ using glycerol-1-C¹⁴, came to similar conclusions. These latter workers reported that the tracer pattern of the carbon chain of glucose was unsymmetrical, since C₃ contained 1.4 to 2.4 times as much C¹⁴ as did C₄. This hypothesis proves that the end-carbons of glycerol are not metabolically identical, and is in harmony with the Ogston hypothesis,⁷⁶⁴ that "the asymmetrical occurrence of isotope in a product cannot be taken as conclusive evidence against its arising from a symmetrical precursor." Swick and Nakao,⁷⁶⁵ on the basis of examination of the glucose formed in the rat after feeding glycerol-1-C¹⁴, demonstrated variations in the radioactivity of the carbons in positions 3 and 4 which contained about 91% of the radioactivity. These workers likewise state that glycerol, like citric acid,

⁷⁶⁰ L. I. Gidez and M. L. Karnovsky, *J. Biol. Chem.*, **206**, 229-242 (1954).

⁷⁶¹ A. P. Doerschuk, *J. Biol. Chem.*, **196**, 423-426 (1952).

⁷⁶² C. F. Cori and W. M. Shine, *Science*, **82**, 134-135 (1935).

⁷⁶³ P. Schambye and H. G. Wood, *J. Biol. Chem.*, **206**, 875-882 (1954).

⁷⁶⁴ A. G. Ogston, *Nature*, **162**, 963 (1948).

⁷⁶⁵ R. W. Swick and A. Nakao, *J. Biol. Chem.*, **206**, 883-886 (1954).

may be regarded as a biologically asymmetrical compound. When glycerol-1-C¹⁴ was metabolized by slices obtained from the livers of fasting normal rats, Teng, Karnovsky *et al.*⁷⁶⁶ were able to account for the fate of 87.5% of the substrate as follows: glucose and glycogen, 71.5%; CO₂, 4.8%; lactate, 3%; barium-precipitable and alcohol-insoluble fractions (phosphorylated intermediates), 4.5%; and lipids, proteins, and ninhydrin-labile CO₂, 3.7%.

When glycerol was oxidized under aerobic conditions by *Streptococcus faecalis* (a species found in the intestines), lactic acid was found to be the terminal product, while glycerophosphate and triosephosphate were believed to be intermediates in the reaction.⁷⁶⁷

⁷⁶⁶ C. T. Teng, M. L. Karnovsky, B. R. Landau, A. B. Hastings, and F. B. Nesbett, *J. Biol. Chem.*, **202**, 705-716 (1953).

⁷⁶⁷ I. C. Gunsalus and W. W. Umbreit, *J. Bacteriol.*, **49**, 347-357 (1945).

CHAPTER IV

THE QUESTION OF THE CONVERSION OF FAT TO CARBOHYDRATE

In contrast to the extensive experimental evidence of the transformation of carbohydrate to fat, the reverse change, namely that of fat $\rightarrow$ carbohydrate, can take place only to an extremely limited extent. In fact, until studies were available, showing the result of the use of isotopes, there was no clear-cut proof of this transformation which could not be matched by equally convincing data which indicated the opposite conclusion. Few fields in science have given rise to such acrimonious debate and have served as the basis for such a large proportion of polemic reports as the question of whether or not fat can be converted to carbohydrate.

The acceptance or rejection of the concept that fat can be converted to carbohydrate has largely been associated with the hypothesis of the cause of diabetes. Investigators who accept the "overproduction" theory argue that hyperglycemia and glycosuria, which occur in diabetes mellitus, result from an increased production of sugar from fat. On the other hand, the advocates of the "non-oxidation" theory of diabetes are of the opinion that glucose accumulates because of an impaired utilization of carbohydrate. Macleod¹ advocated the "overproduction" theory, while Lusk² vigorously denied the possibility that such a change obtains. Although the more recent evidence based upon isotope studies has indicated that some transfer of fatty acid carbon to glucose does occur, equally convincing data indicate that fatty acids do not increase the total carbohydrate³⁻¹⁰ available to the organism. A number of reviews were published before the advent of the

¹ J. J. R. Macleod, *The Fuel of Life*, Princeton Univ. Press, 1928.

² G. Lusk, *Science of Nutrition*, 4th ed., Saunders, Philadelphia, 1928, pp. 642, 644.

³ W. C. Stadie, *Physiol. Revs.*, *25*, 395-441 (1945).

⁴ H. B. Richardson, *Physiol. Revs.*, *9*, 61-125 (1929).

⁵ T. M. Carpenter, *J. Nutrition*, *4*, 281-304 (1931).

⁶ H. C. Geelmuyden, *Ergeb. Physiol.*, *31*, 1-95 (1931).

⁷ M. Dann, *Yale J. Biol. Med.*, *5*, 359-372 (1932-1933).

⁸ H. H. Mitchell, *J. Nutrition*, *6*, 473-491 (1933).

⁹ S. Soskin, *Endocrinology*, *26*, 297-308 (1940).

¹⁰ S. Soskin, *Physiol. Revs.*, *21*, 140-193 (1941).

isotope technics, on the question of the transformation of fat to carbohydrate. Some of these, but not all,^{6,7,10} support the Lusk viewpoint. The more recent summaries on this subject include those of Bloch,¹¹ of Deuel and Morehouse,¹² of Abraham, Chaikoff, and Hassid,¹³ and of Strisower, Chaikoff, and Weinman.¹⁴

1. The Transformation of Glycerol to Glucose

(1) Qualitative Studies

There has been considerable confirmatory evidence, based upon qualitative tests, indicating the transformation of glycerol to glucose. For example, it was early reported that glycerol decreases the ketonuria in diabetic patients;¹⁵⁻¹⁷ Shapiro¹⁸ amply confirmed this finding in the exogenous ketonuria of rats, produced by the administration of sodium acetoacetate to the fasting animals. Moreover, Voegtlin and associates¹⁹ observed that glycerol is an effective agent in relieving the hypoglycemic convulsions produced by the injection of overdoses of insulin.

In the more direct approach to the problem, a number of investigators^{18,20} conclusively demonstrated that glycerol can serve as a glycogenic agent in rats when fed as the free alcohol. However, it has not been possible to demonstrate glycogenesis as conclusively when glycerol is fed as a component of natural fat, to rats.^{21,22} This discrepancy can readily be explained by the fact that only 10% of the triglyceride molecule is glycerol, so that it is difficult to administer comparable doses of the alcohol when it is fed as a component of neutral fat. Moreover, the presence of fat in the gastrointestinal tract delays absorption, as compared with that noted when free glycerol is

¹¹ K. Bloch, *Physiol. Revs.*, **27**, 574-620 (1947).

¹² H. J. Deuel, Jr., and M. G. Morehouse, *Advances in Carbohydrate Chem.*, **2**, 119-160 (1946).

¹³ S. Abraham, I. L. Chaikoff, and W. Z. Hassid, *J. Biol. Chem.*, **195**, 567-581 (1952).

¹⁴ E. H. Strisower, I. L. Chaikoff, and E. O. Weinman, *J. Biol. Chem.*, **192**, 453-463 (1951).

¹⁵ F. Hirschfeld, *Z. klin. Med.*, **28**, 176-209 (1895).

¹⁶ G. Satta, *Beitr. chem. Physiol. Pathol.*, **6**, 376-391 (1905).

¹⁷ R. M. Lang, *Biochem. J.*, **9**, 456-478 (1915).

¹⁸ I. Shapiro, *J. Biol. Chem.*, **108**, 373-387 (1935).

¹⁹ C. Voegtlin, E. R. Dunn, and J. W. Thompson, *Am. J. Physiol.*, **71**, 574-582 (1924-1925).

²⁰ L. F. Catron and H. B. Lewis, *J. Biol. Chem.*, **84**, 553-559 (1929).

²¹ H. J. Deuel, Jr., J. S. Butts, L. F. Hallman, and C. H. Cutler, *J. Biol. Chem.*, **112**, 15-23 (1935-1936).

²² H. J. Deuel, Jr., J. S. Butts, H. Blunden, C. H. Cutler, and L. Knott, *J. Biol. Chem.*, **117**, 119-129 (1937).

fed in aqueous solution, and this retards the appearance of glycogen in the liver. Finally, it is possible that a large part of the glycerol administered in neutral fat is not set free from the fatty acids if the Frazer concept is correct that fat is absorbed largely as triglycerides, together with smaller proportions of diglycerides or monoglycerides. Even if glycerol were completely liberated in the gut, one would expect it, or a comparable amount of newly-synthesized glycerol originating from carbohydrate, to be used in the synthesis of neutral fat from the free fatty acids which would be absorbed. In neither of these situations would glycerol be available to be converted to glucose or, at least, to result in the net synthesis of glucose.

On the other hand, when the triglycerides which are fed cannot be stored in the tissues, the combined glycerol can be utilized as a source of glucose or glycogen. Thus, when neutral fats such as triacetin, tributyrin, tricaproin, or tricaprylin are fed, glycogenesis can be demonstrated.^{21,22} These fats cannot be deposited as such in the fat depots of the rat.^{23,24} However, the feeding of trilaurin results in a deposition of as much as 25% of lauric acid in the depot fat.²⁵ The rapid digestion and absorption of triacetin and tributyrin,²⁶ which exceeds that of all natural fats which have been investigated, would make the glycerol available for glycogenesis within a relatively short period, and so accentuate the extent of glycogen formation. It seems probable that the small amount of liver glycogen which has been reported in the livers of rats previously fasted for twenty-four hours, and then fed butterfat for three to eight days,^{27,28} may be traced to the glycerol available from tributyrin.

Glycogen can also originate from the glycerol moiety of the triglycerides of the short-chain fatty acids having an odd number of carbon atoms; these triglycerides, likewise, cannot be stored in the fat depots. However, in this case, the fatty acids as well as the glycerol serve as the source of carbohydrate. Thus, when tripropionin is fed to rats, the extent of the glycogen deposition considerably exceeds that obtained after triacetin or tributyrin.²²

(2) Quantitative Studies

It has been generally agreed that glycerol is quantitatively converted to glucose in the animal body. Although Cremer,²⁹ using phlorhizinized dogs

²³ H. C. Eckstein, *J. Biol. Chem.*, **81**, 613-628 (1929).

²⁴ H. C. Eckstein, *J. Biol. Chem.*, **84**, 353-357 (1929).

²⁵ M. Powell, *J. Biol. Chem.*, **89**, 547-552 (1930).

²⁶ H. J. Deuel, Jr., and L. Hallman, *J. Nutrition*, **20**, 227-232 (1940).

²⁷ D. E. Gregg, *J. Nutrition*, **4**, 385-398 (1931).

²⁸ C. L. Gemmill and E. G. Holmes, *Biochem. J.*, **29**, 338-349 (1935).

²⁹ M. Cremer, *Münch. med. Wochschr.*, **49**, 944 (1902).

as the test animals, and Lüthje,³⁰ who employed pancreatectomized dogs, were able to account for only approximately 40% of the ingested glycerol as "extra sugar" in the urine, Chambers and Deuel³¹ reported that a complete conversion of glycerol to D-glucose obtains in the phlorhizinized dogs. The incomplete recovery reported earlier may have resulted from an incomplete absorption of the alcohol.

2. The Transformation of Fatty Acids with Odd-Numbered Carbons to Carbohydrate

It has long been recognized that propionic acid is normally converted to carbohydrate. Ringer³² reported that all of the propionic acid molecule (100%) was converted to glucose by the phlorhizinized dog. Further proof of the convertibility of propionic acid to carbohydrate is afforded by the repeated demonstration of glycogenesis after the oral administration of propionate.³³ Moreover, additional confirmation of the glycogenic action of propionate is the demonstration that tripropionin gives rise to more glycogen when fed to rats than obtains after equivalent amounts of triacetin or tributyrin are given.²² As indicated in the last section, this is to be attributed to the fact that both the glycerol and the fatty acid moieties of the triglycerides serve as a source of the glycogen in the case of tripropionin, while only the glycerol behaves in this way in the case of the neutral fats composed of short-chain acids having an even number of carbons.

The longer-chain fatty acids having an uneven number of carbons are likewise convertible to glycogen; it seems most likely that this change occurs to the extent that such odd-chain acids are converted to propionic acid. Ringer³⁴ reported that the five-carbon fatty acid, valeric acid, is converted to glucose to the extent of three carbons by the phlorhizinized dog. The convertibility of valeric (C₅), heptanoic (C₇), and pelargonic (C₉) acids to glycogen was proved when they were fed to rats as their sodium salts²¹ or as their ethyl esters.³⁵ The ethyl ester of undecylic (C₁₁) acid has likewise been shown to yield glycogen in the rat.³⁵ Finally, it has also been reported that the level of liver glycogen deposition is higher for trivalerin or triheptylin than for the triglycerides of the corresponding even-chain fatty acids in which only the glycerol moiety is available for glycogenesis. The

³⁰ H. Lüthje, *Deut. Arch. klin. Med.*, **80**, 98-104 (1904).

³¹ W. H. Chambers and H. J. Deuel, Jr., *J. Biol. Chem.*, **65**, 21-29 (1925).

³² A. I. Ringer, *J. Biol. Chem.*, **12**, 511-515 (1912).

³³ H. C. Eckstein, *J. Biol. Chem.*, **102**, 591-594 (1933).

³⁴ A. I. Ringer, *J. Biol. Chem.*, **14**, 43-52 (1913).

³⁵ J. S. Butts, H. Blunden, W. Goodwin, and H. J. Deuel, Jr., *J. Biol. Chem.*, **117**, 131-133 (1937).

behavior of the simplest representative of the odd-numbered fatty acids, namely formic acid, which is atypical, has only recently been understood. The metabolism of this compound is described in Chapter VI.

Although the evidence is unequivocal that the odd-numbered fatty acids containing from three to eleven carbons are converted to glycogen (probably to the same extent to which they are converted to propionic acid) when they are fed as such or as their triglycerides, one should recognize that these compounds are not physiologic, and that they do not occur in animal or vegetable fats. The conversion of these compounds to glucose cannot be construed as affording evidence of the formation of carbohydrate from natural fats.

3. The Transformation of Fatty Acids with Even-Numbered Carbons to Carbohydrate

Prior to the advent of isotopes, there was no convincing evidence that the even-chain fatty acids could give rise to carbohydrate in the animal body. Thus, it had been demonstrated that, with the exception of acetic acid, the administration of even-numbered fatty acids to fasting rats uniformly resulted in ketonuria,^{36,37} while it has been repeatedly demonstrated that substances which give rise to carbohydrates are not only non-ketogenic but actually counteract an existing ketonuria.

(1) *Experiments Not Involving the Use of Isotopes*

a. Acetic Acid as a Possible Source of Carbohydrate. According to the β -oxidation theory, acetic acid is the two-carbon fragment split off when long-chain fatty acids are metabolized. This supposition has been supported by the recent investigations in which isotopically-labeled fatty acids were employed. Although, as will be reported later, acetic acid can be shown to become incorporated into carbohydrate, there is considerable evidence from a quantitative standpoint that acetate cannot alter the total stores of carbohydrate in the animal body.

In the first place, Stadie and associates³⁸ were unable to obtain any evidence of the presence of acetic acid or of any other steam-distillable fatty acids in the liver slices of depancreatized cats. According to Stadie *et al.*,³⁸ there were no experimental data in the literature previous to 1941 to

³⁶ J. S. Butts, C. H. Cutler, L. Hallman, and H. J. Deuel, Jr., *J. Biol. Chem.*, **109**, 597-613 (1935).

³⁷ H. J. Deuel, Jr., L. F. Hallman, J. S. Butts, and S. Murray, *J. Biol. Chem.*, **116**, 621-639 (1936).

³⁸ W. C. Stadie, J. A. Zapp, Jr., and F. D. W. Lukens, *J. Biol. Chem.*, **137**, 75-87 (1941).

support the hypothesis that acetic acid is formed in the livers of normal or diabetic animals, except those of Cook and Harrison.³⁹ It is suggested by the former workers that, in the diabetic animals, acetic acid can be assumed to originate from the oxidizing agents used in the analyses.

The bulk of the experimental evidence indicates that no increase in carbohydrate reserves results when acetate is given. However, Geelmuyden⁴⁰ reported that acetic acid is converted to glucose in the phlorhizinized dog when fed along with a carbohydrate diet, but the evidence can hardly be considered to be convincing. For example, the experiments are based upon data in which the following D:N ratios² were obtained: fourth day, 7.6; fifth day, 6.8; sixth day (acetate administered), 6.6; and seventh day, 7.0. During these days, the total glucose excretion was 57.0, 55.1, 58.0, and 65.3 g., respectively.^{2,40} In contradistinction to these results, both Ringer and Lusk⁴¹ and Deuel and Milhorat⁴² failed to observe formation of sugar when acetate was given to the phlorhizinized *fasting* dog. Geelmuyden⁴⁰ suggested that acetic acid is not readily utilized, and that its conversion to glucose may be delayed as much as twenty-four hours. It is difficult to accept this hypothesis for a substance as readily metabolized as acetate. Moreover, it is without precedent in a wide range of carbohydrate intermediates and amino acids which have been investigated by the glycogen and phlorhizin technic.

Negative results have been noted by other workers, as regards the ability of acetate to give rise to glycogen. Thus, Ponsford and Smedley-MacLean,⁴³ and also Majer and Reisner,⁴⁴ found no glycogen formation in rats following the administration of fats containing acetic acid. Similar data were recorded by Stöhr⁴⁵ for *fasting* rats, although slightly higher than control values were noted when acetate was fed concomitantly with large doses of glucose. Clutterbuck⁴⁶ also observed that the addition of acetate to minced tissues of rabbits did not result in the formation of succinic acid, which might be an intermediate stage in glucose formation. On the other hand, Thunberg⁴⁷ found that succinic acid may originate from acetic acid in the animal organism. Since the former compound is undoubtedly a

³⁹ R. P. Cook and K. Harrison, *Biochem. J.*, **30**, 1640-1643 (1936).

⁴⁰ H. C. Geelmuyden, *Skand. Arch. Physiol.*, **40**, 211-225 (1920).

⁴¹ A. I. Ringer and G. Lusk, *Z. physiol. Chem.*, **66**, 106-119 (1910).

⁴² H. J. Deuel, Jr., and A. T. Milhorat, *J. Biol. Chem.*, **78**, 299-309 (1928).

⁴³ A. P. Ponsford and I. Smedley-MacLean, *Biochem. J.*, **26**, 1340-1344 (1932).

⁴⁴ E. H. Majer and H. Reisner, *Biochem. Z.*, **263**, 340-352 (1933).

⁴⁵ R. Stöhr, *Z. physiol. Chem.*, **217**, 141-152 (1933); **220**, 27-33 (1933).

⁴⁶ P. W. Clutterbuck, *Biochem. J.*, **21**, 512-521 (1927).

⁴⁷ T. Thunberg, *Skand. Arch. Physiol.*, **25**, 37-84 (1911); **35**, 163-195 (1918); *Physiol. Abst.*, **2**, 665 (1918).

glucose-former,⁴⁸ this would constitute a possible pathway for the biosynthesis of glucose. However, there is a preponderance of evidence that acetic acid does not serve as a source of carbohydrate in the animal body, insofar as can be demonstrated by quantitative experiments.

b. Butyric Acid as a Possible Source of Carbohydrate. Butyric acid is regarded by most workers in the fat field as an intermediate in fat oxidation; therefore, any demonstration of the transformation of this metabolite to sugar should be accepted as direct proof of the fatty acid—carbohydrate transformation. Although there is no convincing proof of the formation of butyric acid in the course of fat catabolism, there is no question that the closely related “ketone bodies” originate directly from the longer-chain fatty acids having an even number of carbon atoms.³⁴ In fact, butyrate has been regularly employed by the Deuel group as the sodium salt to produce exogenous ketonuria in rats. In the case of fasting female rats, the transformation of butyrate to acetone bodies is greater than 50% of that ingested.⁴⁹

The data are quite unanimous in indicating the failure of butyrate to influence the level of tissue carbohydrate quantitatively when given to *fasted* animals. Thus, when butyrate was fed as the sodium salt^{21,33,44} or as its ethyl ester,³⁵ to rats, little or no glycogen formation could be observed in the liver of fasting animals. On the other hand, there are several reports that some additional glycogen formation occurs in *non-fasted* animals when butyrate is fed concomitantly with carbohydrate, as compared with that which was observed when the carbohydrate was given alone.^{45,50,51} In all cases, the increased amounts of glycogen were small; for example, in the experiments of Buchanan *et al.*⁵¹ in which butyric acid-1-C¹⁴ was employed, the butyrate changed to glycogen comprised less than 5% of the butyrate absorbed. Moreover, Dziewiatkowski and Lewis⁵⁰ do not interpret their increased glycogen after butyrate as necessarily an indication of the change of fat to carbohydrate; instead of this, they suggest the possibility that “the butyrate is oxidized readily and thus protects the liver glycogen so that glycogenolysis is diminished” In a further study of the effect of butyrate feeding in the case of fasted rats or of those simultaneously fed glucose, Deuel and co-workers⁵² were unable to demonstrate any augmenta-

⁴⁸ A. I. Ringer, E. M. Frankel, and L. Jonas, *J. Biol. Chem.*, **14**, 539–550 (1913).

⁴⁹ H. J. Deuel, Jr., L. F. Hallman, and S. Murray, *J. Biol. Chem.*, **124**, 385–393 (1938).

⁵⁰ D. D. Dziewiatkowski and H. B. Lewis, *J. Biol. Chem.*, **153**, 49–52 (1944).

⁵¹ J. M. Buchanan, A. B. Hastings, and F. B. Nesbitt, *J. Biol. Chem.*, **150**, 413–425 (1943).

⁵² H. J. Deuel, Jr., C. Johnston, M. G. Morehouse, H. S. Rollman, and R. J. Winzler, *J. Biol. Chem.*, **157**, 135–140 (1945).

tion in the glycogen levels in the livers of rats fed butyrate in addition to glucose over those in the group given glucose alone. Although the possibility of a minimal transformation of butyrate to glucose could not be absolutely ruled out in these latter experiments, it seems most logical to assume that the increased glycogen which has been noted after the administration of butyrate should be ascribed to a sparing action exerted by the fatty acid on glycogenolysis, rather than to a glyconeogenesis from the butyrate molecule. Tidwell and Axelrod⁵³ were unable to correlate hyperglycemia in rats with the injection of acetoacetate.

Another type of investigation which has been employed to follow possible interrelations of butyrate and carbohydrate is the use of isolated tissues. Thus, Blixenkron-Møller⁵⁴ presented evidence that glycogen synthesis occurred when butyrate was artificially perfused through cat liver. It was suggested that only 20% of the butyrate which disappeared was changed to ketone bodies, while the balance was converted to sugar. Haarmann and Schroeder⁵⁵ also reported that several tissues, including the liver, have the ability to transform butyrate to glucose. The following pathway is suggested: butyric acid, crotonic acid, β -hydroxybutyric acid, acetoacetic acid, dihydroxybutyric acid, dihydroxycrotonic acid, diketobutyric acid, methyl glyoxal, pyruvic acid, lactic acid, sugar. The behavior of butyrate in the case of cat liver differs from that observed by Bobbitt and Deuel⁵⁶ with rat liver slices. In the latter case, although ketone bodies were formed when liver slices from fasting rats were immersed in a butyrate medium, the amount of ketones recoverable was less, and the quantity of butyrate disappearing was greater when glycogen was also present in the medium. The implication of this experiment is exactly the opposite of that reported by Weil-Malherbe,⁵⁷ who noted that kidney slices (but not liver slices) were able to form additional reducing substances in a medium containing acetoacetate; it was later shown,⁵⁸ by the osazone test and by the fermentation reaction, that the reducing substance was glucose. It was believed that pyruvic acid acted as an intermediate.

On the other hand, Stadie and associates⁵⁹ failed to confirm the latter investigation. They have likewise pointed out that, in the experiments of Weil-Malherbe, "the excess of newly-formed fermentable substances, pre-

⁵³ H. C. Tidwell and H. E. Axelrod, *J. Biol. Chem.*, **172**, 179-184 (1948).

⁵⁴ N. Blixenkron-Møller, *Z. physiol. Chem.*, **252**, 137-150 (1938).

⁵⁵ W. Haarmann and E. Schroeder, *Biochem. Z.*, **296**, 35-46 (1938).

⁵⁶ B. G. Bobbitt and H. J. Deuel, Jr., *J. Biol. Chem.*, **143**, 1-9 (1942).

⁵⁷ H. Weil-Malherbe, *Biochem. J.*, **32**, 1033-1053 (1938).

⁵⁸ H. Weil-Malherbe, *Biochem. J.*, **32**, 2276-2282 (1938).

⁵⁹ W. C. Stadie, J. A. Zapp, Jr., and F. D. W. Lukens, *J. Biol. Chem.*, **137**, 63-74 (1941).

sumably carbohydrate, . . . formed from acetoacetate over control slices without acetoacetate was (by our calculation) 4.0 ± 2.1 millimoles of wet tissue per g. per hour." This is only 8% of that derived from pyruvate, and scarcely differs from O. Finally, since no lactic acid determinations were made, it is possible that lactate may be the source of the new carbohydrate. Stadie *et al.*⁵⁹ demonstrated that a considerable amount of fermentable carbohydrate can be synthesized by liver slices of diabetic cats from L (*dextro*)-lactic acid.

Another result which contradicts the view that butyric acid is converted to carbohydrate is the antagonism which has repeatedly been demonstrated between the level of urinary ketones and the quantity of carbohydrate ingested. The administration of butyric acid, either in the form of the ethyl ester or as the sodium salt, to fasting rats, always results in the excretion of ketone bodies in the urine.^{37,39,60-67} This ketonuria can be prevented by the administration of glucose in an amount as small as 25 mg. per 100 sq. cm. of surface area, twice daily. Were butyrate normally transformed to glucose in any appreciable amounts under these conditions, not only would it no longer be available for the production of ketonuria, but it would tend to remove any existing ketonuria. This argument is supported by the ketogenic effect of related four-carbon acids. Thus, a ketonuria of similar degree occurs following the administration of acetoacetate^{68,69} to fasting rats, as well as after the feeding of β -hydroxybutyrate⁷⁰ or crotonate^{60,70} to such animals. The ketonuria in these latter cases is likewise prevented by the administration of glucose.^{60,70}

c. Long-Chain Acids and Fats as Possible Sources of Carbohydrate.

The acids longer than butyric having an even number of carbon atoms are also not glycogenic. Thus, completely negative results were noted, insofar as liver glycogen is concerned, when the sodium salt of caprylic acid was fed,^{21,33} or when the ethyl esters of caproic, caprylic, capric, myristic, or

⁶⁰ H. J. Deuel, Jr., C. E. Calbert, L. Anisfeld, H. McKeehan, and H. D. Blunden, *Food Research*, 19, 13-19 (1954).

⁶¹ J. S. Butts and R. O. Sinnhuber, *J. Biol. Chem.*, 139, 963-969 (1941).

⁶² J. S. Butts, M. S. Dunn, and L. F. Hallman, *J. Biol. Chem.*, 123, 711-718 (1938).

⁶³ C. Johnston and H. J. Deuel, Jr., *J. Biol. Chem.*, 149, 117-124 (1943).

⁶⁴ C. E. Vaniman and H. J. Deuel, Jr., *J. Biol. Chem.*, 152, 565-570 (1944).

⁶⁵ J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, 119, 247-255 (1937).

⁶⁶ J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, 124, 709-714 (1938).

⁶⁷ J. S. Butts and R. O. Sinnhuber, *J. Biol. Chem.*, 140, 597-602 (1941).

⁶⁸ J. S. Butts and H. J. Deuel, Jr., *J. Biol. Chem.*, 100, 415-428 (1933).

⁶⁹ J. S. Butts, *J. Biol. Chem.*, 105, 87-96 (1934).

⁷⁰ H. D. Blunden, *The Intermediary Metabolism of Butyric Acid as Determined from the Physiological Behavior of the Various Four-Carbon Acids*. Dissertation, Univ. Southern California, Dept. Biochem. Nutrition, 1938.

oleic acids, or the methyl ester of lauric acid,³⁵ were given. Geelmuyden⁴⁰ likewise found that caproic acid was ineffective as a source of extra glucose in the phlorhizinized dog, but was actually ketogenic. Some investigators reported the formation of glycogen when neutral fats were the principal components of the diet, while the majority failed to find this metabolite. Thus, on the one hand, Takao,⁷¹ Burn and Ling,⁷² and Magnusson⁷³ all reported high values for liver glycogen following fat feeding. However, Takao does not consider that his results furnished any conclusive proof of the conversion of fat to carbohydrate, inasmuch as the glycerol contained in the bacon which was fed may have accounted for the high glycogen level. In the experiments of Burn and Ling,⁷² values as high as 4.6% were recorded for liver glycogen following the feeding of fat to rats over a period of ninety-six hours. On the other hand, negative results were noted by Gregg,²⁷ by Bodey *et al.*,⁷⁴ and by Greisheimer.⁷⁵ Krantz and Carr⁷⁶ found that the glycogen storage in the liver of rats fed dried powdered root of burdock (*Arctium lappa*), in addition to the basal diet of cacao butter, was five or six times that of rats on the basal diet alone (9.79:0.14%). The burdock root contains 50 to 70% carbohydrate.

Although Gregg²⁷ did note some increase in liver glycogen following the administration of butterfat, he ascribes this finding to the glycerol moiety. Gemmill and Holmes²⁸ also found that, when butter was fed to rats on a fast, the liver glycogen was first reduced to zero, but that after the first day it gradually increased until it had reached a level of approximately 1% in the rats sacrificed after four or five days. It is possible to account for such quantities of glycogen after the administration of butterfat without the necessity of a gluconeogenesis. Since tributyrin cannot be stored as such in the animal body, it is probable that the butyrate moiety is oxidized; this leaves the glycerol free for glycogenesis. The occurrence of such a course in the rat is indicated by the demonstration of increased glycogen following the administration of tributyrin to normal fasting rats.²² Deuel *et al.*²² were not able to demonstrate glycogen deposition in rats after trilaurin and natural fats such as Wesson oil, cottonseed oil, peanut oil, or linseed oil had been given, although butterfat and coconut oil were glycogenic. Another possible explanation for the results obtained by Gemmill and Holmes²⁸ is that liver glycogen may have arisen from protein. During fasting, condi-

⁷¹ T. Takao, *Biochem. Z.*, **172**, 272-279 (1926).

⁷² J. H. Burn and H. W. Ling, *Quart. J. Pharm. and Pharmacol.*, **2**, 1-16 (1929).

⁷³ R. Magnusson, *Skand. Arch. Physiol.*, **55**, 278-279 (1929).

⁷⁴ M. G. Bodey, H. B. Lewis, and J. F. Huber, *J. Biol. Chem.*, **75**, 715-723 (1927).

⁷⁵ E. M. Greisheimer, *J. Nutrition*, **4**, 411-418 (1931).

⁷⁶ J. C. Krantz, Jr., and C. J. Carr, *J. Pharmacol.*, **41**, 83-87 (1931).

tions are quite similar to those of fat feeding; there is an initial decrease in nitrogen excretion, but after several days this excretion frequently exceeds the prefasting level, until the more labile deposits have been exhausted.⁷⁷

(2) *Experiments Involving the Use of Isotopes*

From a quantitative standpoint, there is no convincing evidence that acetic acid may be converted to glycogen or to glucose. Thus, acetic acid does not satisfy the classical concept of this transformation, since neither the deposition of glycogen in the liver of a fasting animal nor the glycosuria of the diabetic animal is enhanced. However, Lorber, Lifson, and Wood⁷⁸ demonstrated, by isotope experiments, that acetic acid can furnish the carbon atoms for all positions in the glucose molecule. Lifson *et al.*⁷⁹ likewise proved that the appearance of C¹³-labeled glycogen in the liver of the rat after C¹⁴-labeled butyrate or acetate had been given represented an actual transfer of the fatty acid carbon to glycogen and was not merely incorporated in the carbohydrate molecule by the combination of the carbohydrate precursor with labeled carbon dioxide. In a study in which octanoate labeled with C¹³ and C¹⁴ was fed in conjunction with glucose to fasted rats, the distribution of the isotope was consistent with the transformation of 2-C fragments to glucose.⁸⁰ There was some evidence that the last two carbons of the octanoate molecule are less readily convertible to glycogen than are the first six carbons.

Further evidence that fatty acid carbons may become incorporated into glucose has been reported by the Chaikoff group. Thus, Strisower, Chaikoff, and Weinman¹⁴ reported the presence of C¹⁴-glucose in the urine of alloxan-diabetic rats after feeding labeled palmitic acid; from 3 to 6% of the injected palmitic acid-6-C¹⁴ was recovered in the urine during the first twenty-four hours. On the basis of the known rate of glucose turnover, Strisower *et al.*¹⁴ estimated that 6 to 12% of the injected palmitate was transformed into glucose. It was calculated that from 1.6 to 2.9% of the injected palmitic acid-6-C¹⁴ was present in extracellular glucose in the diabetic rat, and 0.5 to 0.9% in the normal rat, one hour after injection. According to Abraham, Chaikoff, and Hassid,¹³ when palmitic acid-1-C¹⁴ or palmitic acid-6-C¹⁴ was injected into diabetic dogs, approximately 6% of

⁷⁷ H. J. Deuel, Jr., and M. Gulick, *J. Biol. Chem.*, **96**, 25-34 (1932).

⁷⁸ V. Lorber, N. Lifson, and H. G. Wood, *J. Biol. Chem.*, **161**, 411-412 (1945).

⁷⁹ N. Lifson, V. Lorber, W. Sakami, and H. G. Wood, *J. Biol. Chem.*, **176**, 1263-1284 (1948).

⁸⁰ V. Lorber, M. Cook, and J. Meyer, *J. Biol. Chem.*, **181**, 475-479 (1949).

the tagged carbon appeared in the urinary glucose during the first forty-eight hours; about 14% of the isotopic carbon was found in the sugar isolated during the first ninety-six hours. When the 1-C¹⁴-palmitic acid was given, the C¹⁴ was equally divided between the third and fourth carbons. When palmitic acid-6-C¹⁴ was administered, only about 10% of the injected carbons appeared on each of carbons 3 and 4, while the 80% balance was equally distributed between carbons 1, 2, 5, and 6.

According to Buchanan and Hastings,⁸¹ Wood,⁸² and Bloch,¹¹ there is no inconsistency in the fact that a substance like acetic acid can provide carbon atoms for all the positions of the glucose molecule and still be unable to effect an increase in the glycogen level in the animal. Two different processes are involved. In the first case, the precursor affords a net increase in the quantity of the substance formed, since the amount of the precursor present is the limiting factor. This type of reaction can be demonstrated by increased glycogen formation or by the exhibition of a ketolytic effect. This is the classical concept of glycogenesis, and acetic acid obviously does not fit into this category.

In the second type of reaction, the precursor may be partly or wholly incorporated into the reaction product, but its presence is without effect on the rate of formation of the new product. Acetic acid fits into this scheme insofar as glycogen formation is concerned. Acetic acid alone cannot form glucose or pyruvate, but only by becoming incorporated into the tricarboxylic (citric) acid cycle. As a result of this reaction, some of the acetic acid carbons may be transformed into pyruvic acid, which is known to be, without question, an obligatory intermediate in glucose formation. Thus, in conditions of fasting, during which the level of activity of the tricarboxylic acid cycle is at a low ebb, only a small amount of acetate becomes incorporated in citric acid, and it is largely lost as ketone bodies. On the other hand, when ample carbohydrate is available, acetate is readily utilized in citric acid synthesis, ketonuria disappears, and some of the acetate carbon appears in glucose and glycogen. This reaction may occur not only when large amounts of carbohydrate are available, with the result that an increased glycogen deposition obtains, but also when the Krebs cycle is operating at a low level and no change in net glycogen takes place in the tissues. For a further discussion of the relationship of acetate to the Krebs cycle, the reader is referred to the discussion of the relationship of carbohydrate to ketonuria (see page 169).

⁸¹ J. M. Buchanan and A. B. Hastings, *Physiol. Revs.*, **26**, 120-155 (1946).

⁸² H. G. Wood, *Physiol. Revs.*, **26**, 198-246 (1946).

4. General Experimental Evidence on the Question of the Conversion of Fat to Carbohydrate

(1) D:N Ratio

The D:N ratio was one of the earliest constants which was employed to determine whether or not carbohydrate can originate from fat. It is believed that, when a dog is rendered diabetic by phlorhizin and is then subjected to fasting, the major part of the reserve glycogen and glucose is flushed out within the first forty-eight to seventy-two hours. After this is accomplished, any glucose subsequently excreted in the urine could have its origin only in the protein catabolized, or in the fat broken down. According to Stiles and Lusk,⁸³ the D:N ratio under ideal conditions, after the extra glucose from stored carbohydrates has been removed, is 3.65:1; most workers have regarded ratios of 3.9 to 3.3 as acceptable in experiments in which this technic was employed. If one accepts the hypothesis that none of the glucose originated from fat, it can be calculated that approximately 58% of the protein molecule is being converted to glucose. This is a reasonable figure, since there is adequate evidence that some of the amino acids are not glucose formers. However, if one assumes that all the carbon in protein is converted to glucose (other than that excreted in urea), one gram of urinary nitrogen would correspond to 8.2 g. of glucose. However, since the protein nitrogen is largely excreted as urea, one must correct the carbon output from protein available for glucose formation by the quantity lost in the urea molecule. This would bring about a revision of the figure for the total potential glucose per g. nitrogen to 6.5 g. On this basis any D:N ratio during fasting which exceeded 6.5:1 could be interpreted only as indicative of the transformation of fat to carbohydrate.¹²

Although most workers have agreed with Stiles and Lusk⁸³ that the D:N ratio in fasting phlorhizinized dogs approximates 3.6:1, and that it is uninfluenced by the administration of fat, contrary results have been reported. Thus, Hartogh and Schumm⁸⁴ reported D:N ratios as high as 13.0:1 in fasting phlorhizinized dogs between the third and seventh days after the first administration of the glucoside. However, Lusk² strenuously denied the accuracy of these data, and suggested that they might possibly be ascribed to an inaccuracy in the determination of urinary nitrogen. Thus, according to Lusk,² Hartogh and Schumm reported urine nitrogen values of 4.89 and 5.17 g. on the third and seventh days after phlorhizin, respectively,

⁸³ P. G. Stiles and G. Lusk, *Am. J. Physiol.*, 10, 67-79 (1903-1904).

⁸⁴ Hartogh and O. Schumm, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, 45, 11-45 (1901).

in the case of a 55 kg. dog. Such figures compare with values of 29.3 g. for nitrogen, 107.7 g. for sugar and a D:N of 3.61:1 for a dog weighing 40 kg., on the fourth day of fasting, and the second day of phlorhizin, as reported by Mandel and Lusk.⁸⁵ Lusk⁸⁶ denied that the figures of Hartogh and Schumm could be reproduced under proper experimental conditions. Grafe and Wolf⁸⁷ reported similar high D:N ratios which exceeded 5:1 and even reached 11.6 after the ingestion of cereal, in the case of a diabetic patient presumably on an exclusively protein fat diet which was free from carbohydrates. Lusk² suggests that such ratios may be the result of surreptitious ingestion of carbohydrate food. He cites the results of experiments of Greenwald⁸⁸ and of Allen and DuBois⁸⁹ in which similar high values were found in the case of diabetic patients. When the patients were confined to a private room where the food could be checked, ratios approaching 3.65:1 were promptly obtained.^{88,89} The data would seem to indicate unquestionably that, in fasted diabetic animals, there is no evidence, based upon the D:N ratio, that fat serves as a source of carbohydrate.

In the case of fat-fed diabetic animals, also, there is no experimental evidence to confirm the transformation of fat to carbohydrate. Thus, Lusk,² Takao,⁷¹ and Deuel and Milhorat⁴² are unanimous in reporting that fat feeding does not increase the D:N ratio of dogs. On the other hand, Soskin⁹⁰ reported an increased D:N ratio in three depancreatized dogs, two of which had received lecithin and the third olive oil. On this basis, it was suggested that the extra sugar originated from the fatty acids. However, in ten tests no rise in the D:N ratio occurred after 50 g. of fat had been given. In six of the negative tests, the D:N varied between 2.99 and 2.68; these figures are in excellent agreement with the value of 2.8:1 for fasting depancreatized dogs postulated by Minkowski.⁹¹ Moreover, Page and Young⁹² did not find evidence of an increase in the D:N ratio following the injection of phosphatide into phlorhizin-diabetic dogs.

A third criterion in which the D:N ratio has been employed in assembling evidence as to whether or not fat can be changed into carbohydrate is the effect of conditions known to increase fat metabolism on the carbohydrate level. If the metabolism of fat is associated with a marked conversion of

⁸⁵ A. R. Mandel and G. Lusk, *Am. J. Physiol.*, **16**, 129-146 (1926).

⁸⁶ G. Lusk, *Ergeb. Physiol.*, **12**, 315-392 (1912).

⁸⁷ E. Grafe and C. G. L. Wolf, *Deut. Arch. klin. Med.*, **107**, 201-235 (1912).

⁸⁸ I. Greenwald, *J. Biol. Chem.*, **16**, 375-377 (1913).

⁸⁹ F. M. Allen and E. F. DuBois, *Arch. Internal Med.*, **17**, 1010-1059 (1916).

⁹⁰ S. Soskin, *Biochem. J.*, **23**, 1385-1390 (1929).

⁹¹ O. Minkowski, *Naunyn-Schmiedeberg's Arch. expil. Pathol. Pharmacol.*, **31**, 85-189 (1893).

⁹² I. H. Page and F. G. Young, *Biochem. J.*, **26**, 1528-1531 (1932).

this foodstuff to sugar, then any increased consumption of fat should result in an increased production of sugar. In the case of a diabetic animal, this situation would be attended by an increased sugar secretion, with a resultant rise in the D:N ratio. However, Lusk⁹³ reported that, when fat metabolism was considerably increased in the fasting phlorhizinized dog, either by shivering or by working the animals on a treadmill, no increase in glycosuria occurred, provided that all residual glycogen had previously been flushed out of the liver. In addition, it has been demonstrated in an equally convincing manner, by other investigators,^{94,95} that the normal D:N of 2.8:1 observed during fasting, when depancreatized dogs are used, is not changed by subjecting the animals to cold or to work. On the basis of studies of the D:N ratio in fasting diabetic animals, in those which were fed fat, or in those in which fat metabolism was markedly augmented by work or by shivering, the weight of evidence is preponderant against the change of fat to carbohydrate.

(2) Respiratory Quotient

The changes to be expected in the respiratory quotient (R.Q.) if fat were converted to carbohydrate are the opposite of those which have been proved for the carbohydrate $\rightarrow$ fat transformation. In the former case, an oxygen-poor substance (fat) would be converted to an oxygen-rich compound (carbohydrate), with the result that a considerable quantity of oxygen must necessarily be utilized from the respiratory gases. Under these conditions, the observed R.Q. would be considerably below that for fat oxidation, *i.e.*, below 0.707. Pembrey,⁹⁶ in studies of the hibernating marmot, calculated that the fat $\rightarrow$ carbohydrate reaction, if allowed to take place exclusively in the tissues, triolein being converted to glucose, would have a theoretical R.Q. of 0.28, as calculated from the following equation:

Illustrating the Theoretical Conversion of Triolein to Glucose as Postulated by Pembrey¹¹

According to Lusk² the following empirical equation represents the change of palmitic acid to glucose. The theoretical R.Q. as pictured by Lusk would be 0.69–0.70.

⁹³ G. Lusk, *Am. J. Physiol.*, **22**, 163–173 (1908).

⁹⁴ Y. Seo, *Naunyn-Schmiedeberg's Arch. exp'tl. Pathol. Pharmacol.*, **59**, 341–363 (1908).

⁹⁵ W. H. Chambers, H. E. Himwich, and M. A. Kennard, *J. Biol. Chem.*, **108**, 217–225 (1935).

⁹⁶ M. S. Pembrey, *J. Physiol.*, **27**, 66–84 (1901–1902).

Illustrating the Theoretical Conversion of Palmitic Acid to Glucose as Postulated by Lusk²

Thus, the greater the proportion of the total metabolism which is concerned with the fat $\rightarrow$ carbohydrate change, the lower would be the expected non-protein R.Q.²

In a number of reports on the R.Q. of animals, values lower than the theoretical minimum of 0.707 for the non-protein R.Q. of fat have been observed; the latter figure would be obtained when fat is the only non-protein component being metabolized but in which its complete oxidation occurs.

Some of the experimental R.Q. values recorded are exceedingly low. Thus, Pembrey⁹⁶ observed a figure of 0.55 in hibernating marmots, while Voit⁹⁷ reported a minimum value of 0.33 during hibernation in this species. In all of these experiments, the respiratory metabolism was at an extremely low level, so that the actual carbon dioxide and oxygen measured represents a very low quantity of the gases. Under these conditions, inaccuracies might readily be traceable to analytical difficulties. Moreover, the respiratory exchange may not represent the true metabolic picture, inasmuch as many of the hibernating animals exhibit Cheyne-Stokes respiration,⁹⁶ with a consequent derangement of the acid-base balance of the blood. Another suggestion is that such irregularities may be ascribed to differential changes in solubility of carbon dioxide and oxygen which obtain in the colder blood of the hibernating animal.⁹⁸ The solubility of carbon dioxide would increase to a greater extent than that of oxygen in the colder blood; this would cause a greater retention of this gas, with a resultant lower R.Q. In spite of all of these possibilities, Pembrey⁹⁶ reported that the R.Q. of active marmots was 0.70 or higher.

In addition to the analytical difficulty in determining respiratory gases in hibernating animals, because of the small quantities of gases involved, it is also true that most inaccuracies in gas analyses are due to an incorrect determination of oxygen. When a closed circuit apparatus is employed, any leak in the system will result in the calculation of an abnormally high value for oxygen, with a resulting low R.Q. DuBois⁹⁹ listed over 100 low values

⁹⁷ C. Voit, *Z. Biol.*, 14, 57-160 (1878).

⁹⁸ E. Grafe, *Die pathologische Physiologie des Gesamtstoff- und Kraftwechsels bei der Ernährung des Menschen*. J. F. Bergmann, Munich, 1923; reprinted *Ergeb. Physiol.* 21, No. II, 1-499 (1923).

⁹⁹ E. F. DuBois, *Basal Metabolism in Health and Disease*, Lea & Febiger, Philadelphia, third ed., 1936, p. 280.

for R.Q. cited in the literature, in reports from laboratories in which the analytical technic could not be questioned.

A large proportion of the low values were obtained in the case of diabetics; R.Q. values as low as 0.669 for such patients can be justified on theoretical grounds, without the necessity of assuming a transformation of fat to carbohydrate or of an inaccuracy in determination. Thus, if one excludes from the calculation the proportionate amount of carbohydrate formed from protein (since this is not oxidized in the diabetic), the revised protein R.Q.¹⁰⁰ becomes 0.632 instead of 0.802. Magnus-Levy¹⁰¹ estimated the R.Q. of the protein $\rightarrow$ carbohydrate conversion as 0.613, while Geelmuyden¹⁰² proposed a figure of 0.706. The R.Q. of fat is likewise altered in diabetes, since a complete combustion does not obtain due to the attendant ketonuria. According to Allen and DuBois,⁸⁹ and Magnus-Levy,¹⁰¹ the normal R.Q. of fat, 0.707, would be reduced to 0.669 if the β -hydroxybutyrate were not metabolized.² If more than one molecule of ketone bodies were formed from one molecule of long-chain fatty acid,³⁶ the R.Q. would be reduced to a still greater extent, below 0.669. An upset of acid-base balance in the blood and tissues would probably further complicate the calculation of the R.Q. in diabetes.

Hawley, Johnson, and Murlin¹⁰³ have suggested another interesting possibility to explain the low R.Q. during the early period of fat metabolism, which might very well occur normally. Thus, in the oxidation of the fatty acid chains, if desaturation proceeded rapidly and was not accompanied by a rapid completion of the oxidation, absorption of oxygen to oxidize the hydrogens removed might occur without a concomitant production of carbon dioxide. This would result in a reduced R.Q. during the period when this part of the reaction was the predominant one. This as offered is a possible explanation for the low values obtained in human subjects before they have become adjusted to a high-fat diet. Werthessen¹⁰⁴ reported wide variations in the respiratory quotient of rats over a twenty-four-hour period, ranging from 0.27 to 1.70, when the animals were fed their total twenty-four-hour requirement within a period of one to five hours. Similar results were obtained by Markowitz,¹⁰⁵ using himself as subject. Soskin¹⁰ de-

¹⁰⁰ G. Lusk, *Arch. Internal Med.*, 15, 939-944 (1915).

¹⁰¹ A. Magnus-Levy, *Arch. Anat. Physiol.*, 1904, 377-382; *Z. klin. Med.*, 56, 83-99 (1905).

¹⁰² H. C. Geelmuyden, *Ergeb. Physiol.*, 21, No. 1, 274-360 (1923); 22, 51-298 (1923).

¹⁰³ E. E. Hawley, C. W. Johnson, and J. R. Murlin, *J. Nutrition*, 6, 523-557 (1933).

¹⁰⁴ N. Werthessen, *Am. J. Physiol.*, 120, 458-465 (1937).

¹⁰⁵ J. Markowitz, unpublished results cited by S. Soskin, *Physiol. Revs.*, 21, 140-193 (1941), p. 153.

scribed this condition as occurring when the metabolic processes were "caught off balance."

Lusk² reported an R.Q. of 0.687 in a fasting phlorhizinized dog. In Joslin's series of 113 diabetics,¹⁰⁶ only eighteen tests on nine subjects exhibited an R.Q. figure below 0.70. In the evaluation of the low R.Q. figures cited by DuBois,⁹⁹ Richardson⁴ noted that values below 0.65 were in the minority, the lowest figure being 0.64. It is not considered that even the lowest authenticated value represents a situation in which fat is being transformed to carbohydrate. Stewart, Gaddie, and Dunlop,¹⁰⁷ likewise, were unable to obtain any evidence, based upon changes in R.Q., for the conversion of fat to carbohydrate in human subjects undergoing exercise. The results of Hawley¹⁰⁸ on phlorhizin-diabetic dogs, also, offered no evidence of a conversion of fat to carbohydrate under the influence of insulin.

Thus, no convincing data based upon R.Q. are available. The number of authentic values for the non-protein R.Q. which are below the 0.707 are limited, and these are readily explained by the reduction of the protein R.Q., as a result of an incomplete oxidation of the carbohydrate moiety, and of the fat R.Q., which would be attributable to the loss of some of the fat molecule as ketone bodies in the urine. Either changes in acid-base balance or technical difficulties in analyses may also cause the low R.Q. observed in some experiments.

(3) *The Importance of Epinephrine*

One classical viewpoint as to the cause of glycosuria in diabetes was expressed by Eppinger, Falta, and Rudinger.¹⁰⁹ The phenomenon is attributed to an overproduction of carbohydrate from fat, and this was believed to result from an increased stimulation of adrenal secretion. The latter would inhibit insulin production. As evidence for this theory, these investigators¹⁰⁹ cite the largely increased sugar output in depancreatized dogs when epinephrine is administered.

However, a number of workers have been unable to confirm the increased glycosuria which would have followed epinephrine injection if the carbohydrate stores had been removed prior to the administration of the

¹⁰⁶ E. P. Joslin, *Diabetic Metabolism with High and Low Diets*, Carnegie Inst. (Washington), *Publ. No. 323*, 1923.

¹⁰⁷ C. P. Stewart, R. Gaddie, and D. M. Dunlop, *Biochem. J.*, **25**, 733-748 (1931).

¹⁰⁸ E. E. Hawley, *Am. J. Physiol.*, **101**, 185-193 (1932).

¹⁰⁹ H. Eppinger, W. Falta, and C. Rudinger, *Z. klin. Med.*, **66**, 1-52 (1908).

hormone. Ringer¹¹⁰ and others¹¹¹⁻¹¹³ reported that no increased sugar excretion occurs in the urine of phlorrhizinized dogs if the glycogen reserves are first exhausted. Likewise, Kramer and co-workers¹¹⁴ reported similar results on depancreatized dogs. Moreover, no glycosuria occurs in normal dogs following the administration of epinephrine if the glycogen stores have previously been depleted.¹¹⁵

On the other hand, Chaikoff and Weber¹¹⁶ interpreted their data on the effect of repeated injections of epinephrine into fasting depancreatized dogs as indicating a formation of carbohydrate from fatty acids. Macleod,¹ commenting on these experiments, states that they prove "irrefutably the derivation of sugar from fatty acids, at least in diabetic animals." However, a number of investigators have failed to confirm these tests, and one is led to question them. One criticism is that Chaikoff and Weber failed to consider muscle glycogen as a possible source of the sugar excreted. Cori and Cori^{117,118} reported that an increase in blood lactic acid follows the administration of epinephrine to rats, concomitantly with a lowering in the level of muscle glycogen. As a confirmation of this latter viewpoint, one may cite the results of Bollman and collaborators.¹¹⁹ These workers reported that the extra sugar excreted by depancreatized dogs, over and above the 2.8:1 ratio following the administration of epinephrine, was practically identical with that calculated as originating from the muscle glycogen which disappeared. Similar data have been recorded by Chambers and co-workers,⁹⁵ and also by Bachrach, Bradley, and Ivy.¹²⁰ Actually, the latter group calculated the average potential glucose in their depancreatized dogs, after a three-day fast, as 25.8 g., of which 13.9 g. came from muscle glycogen. The actual extra glucose excreted amounted to a mean of only 13.2 g., so that it is entirely unnecessary to postulate that the extra glucose under such conditions arises from fat. Thus, it is apparent that the fat $\rightarrow$ carbohydrate reaction does not gain any substantial support from

¹¹⁰ A. I. Ringer, *J. Exptl. Med.*, **12**, 105-113 (1910).

¹¹¹ R. T. Woodyatt, *J. Biol. Chem.*, **14**, 441-451 (1913).

¹¹² W. D. Sansum and R. T. Woodyatt, *J. Biol. Chem.*, **21**, 1-21 (1915).

¹¹³ R. W. Seuffert and H. Hartmann, *Beitr. Physiol.*, **2**, 199-204 (1924).

¹¹⁴ B. Kramer, J. Marker, and J. R. Murlin, *J. Biol. Chem.*, **27**, 499-515 (1916).

¹¹⁵ F. Hildebrandt, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **88**, 80-112 (1920).

¹¹⁶ I. L. Chaikoff and J. J. Weber, *J. Biol. Chem.*, **76**, 813-832 (1928).

¹¹⁷ C. F. Cori and G. T. Cori, *J. Biol. Chem.*, **79**, 309-319 (1928).

¹¹⁸ C. F. Cori and G. T. Cori, *J. Biol. Chem.*, **84**, 683-698 (1929).

¹¹⁹ J. L. Bollman, F. C. Mann, and C. M. Wilhelmj, *J. Biol. Chem.*, **93**, 83-91 (1931).

¹²⁰ W. H. Bachrach, W. B. Bradley, and A. C. Ivy, *Am. J. Physiol.*, **117**, 203-205 (1936).

the epinephrine experiments, since the negative results explain and far outweigh the positive ones.

(4) *The Importance of Insulin*

Shortly after the discovery of insulin, Wertheimer¹²¹ postulated that this hormone facilitates the conversion of fat to sugar. This hypothesis seemed logical at the time it was proposed, since it had been demonstrated that the deposition of liver glycogen occurred in the diabetic animal following insulin treatment, concomitantly with a decrease in blood sugar, urine sugar, and blood and liver fat.¹²² In Wertheimer's experiments, it was noted that glycogen appeared in the livers of phlorhizinized dogs after the injection of insulin, practically simultaneously with the disappearance of excess fat. Another fact which tended to support this conclusion was the observation that dogs with fatty livers were more resistant to insulin, and that the blood sugar returned to normal more quickly after insulin injection than in control animals with lower fat stores. However, these results are refuted by Hawley,¹⁰⁸ who states that "even when there was a very great need of carbohydrate to save the life of the animal, and when there were ample stores of fat or ample food fat available to meet this need, there was no gluconeogenesis from fat."

(5) *Experimental Data from Isolated Organs*

a. Fat Change and Carbohydrate Content. A number of reports based upon quantitative studies of carbohydrate synthesis by perfused or isolated tissues indicate that fat is converted to carbohydrate. As a result of perfusion studies with isolated livers and with minced livers from normal and diabetic animals, a number of workers¹²³⁻¹²⁶ postulated the origin of carbohydrate from fat in this organ. In these tests, more carbohydrate was formed than could be accounted for by the decrease in known precursors of this foodstuff.¹²⁷ However, Gregg¹²⁸ was unable to confirm these experiments. He concluded that, in order to establish a conversion of fat to carbohydrate in the liver by the use of the balance technic, it would be neces-

¹²¹ E. Wertheimer, *Arch. ges. Physiol. (Pflüger's)*, 213, 298-320 (1926).

¹²² J. J. R. Macleod, *Carbohydrate Metabolism and Insulin*, Longmans Green, London, 1926, pp. 104, 163, ff.

¹²³ H. Heller, *Acta Med. Scand.*, 90, 489-526 (1936).

¹²⁴ J. Seegen, *Arch. ges. Physiol. (Pflüger's)*, 39, 132-142 (1886).

¹²⁵ J. H. Burn and H. P. Marks, *J. Physiol.*, 61, 497-517 (1926).

¹²⁶ H. Jost, *Z. physiol. Chem.*, 197, 90-134 (1931).

¹²⁷ L. Lattes, *Biochem. Z.*, 20, 215-219 (1909).

¹²⁸ D. E. Gregg, *Am. J. Physiol.*, 103, 79-88 (1933).

sary to take into consideration not only glycogen but free sugar, lactic acid, urea, glycerol, and fatty acids, both in the liver and also in the perfusing fluid. Although Stewart and Thompson¹²⁹ noted an increase in carbohydrate in liver slices, along with a decrease in fatty acids, the oxygen consumption was too low to justify the conclusion that a transformation of fatty acid to carbohydrate had occurred.

Although not all these criticisms are valid for all the perfusion experiments which purport to show the transformation of fat to carbohydrate, they have not all been completely refuted. Thus, Richardson⁴ suggests that lactic acid formed in the tissues because of the anaerobic conditions which existed therein prior to the start of the perfusion might well be responsible for a considerable proportion of the new glucose formed when perfusion with oxygenated solution was begun. On the other hand, Page and Young⁹² believed that sufficient glycerol was available to account for the sugar formation in the experiments of Jost.¹²⁶ Lastly, Gregg¹²⁸ is of the opinion that some of the new sugar reported in the case of cats and dogs may have originated from liver glycogen, which had been assumed to be zero but which was probably still present to an appreciable although small extent. Moreover, since the glycogen content of different lobes of the liver varies, one cannot accept the value of any one lobe as representative of the entire liver.

b. Fat Change and Respiratory Quotient. The variations of R.Q. in tissues such as the liver have been interpreted as offering support for the fat $\rightarrow$ carbohydrate transformation. Thus, the R.Q. of liver slices from animals which had been well fed ranged between 0.85 and 0.75. When this organ had been excised from rats whose carbohydrate stores were depleted, either by fasting or by a high-fat diet, values for the R.Q. below 0.70 were recorded by Meyerhof and Lohmann,¹³⁰ and by Soskin and Mirsky.¹³¹ Dickens and Simer¹³² reported R.Q. levels below 0.70 only when the tests were carried out with phosphate- or bicarbonate-buffered Ringer's solution. Gemmill and Holmes²⁸ observed a low R.Q. in liver slices obtained from rats previously on a high-fat regimen. Some increase in fermentable carbohydrate also occurred in the liver during the course of the test. However, Cori and Shine¹³³ also found an increase in fermentable carbohydrate when glycerol or β -glycerophosphate was added. Moreover, Stadie and co-

¹²⁹ C. P. Stewart and J. C. Thompson, *Biochem. J.*, **35**, 245-253 (1941).

¹³⁰ O. Meyerhof and K. Lohmann, *Biochem. Z.*, **171**, 381-402 (1926).

¹³¹ S. Soskin and I. A. Mirsky, *Am. J. Physiol.*, **114**, 106-109 (1935).

¹³² F. Dickens and F. Simer, *Biochem. J.*, **25**, 985-993 (1931).

¹³³ C. F. Cori and W. M. Shine, *Science*, **82**, 134-135 (1935).

workers¹³⁴ accounted for all the oxygen consumed by liver slices in the three processes, deamination, carbon dioxide formation, and ketone body synthesis. On the basis of these results, Stadie¹³⁵ stated that "there was no oxygen whatever available in the metabolism of the diabetic liver slice for this conversion." Similar results were reported later by Stadie and his collaborators^{38,134} in liver slices of fasting, phlorhizinized rats. No gluconeogenesis attributable to fat could be shown by direct analyses in the case of livers of diabetic cats.

5. Résumé of Current Hypotheses Regarding the Fat→Carbohydrate Conversion

One cannot categorically deny the possibility that fatty acid carbons may be transferred to carbohydrate. However, as a result of the studies of a number of investigators who employed isotopes, it is becoming certain that a reaction of this nature is of minor importance. According to Buchanan and co-workers,⁵¹ the proportion of butyrate converted to carbohydrate was less than 5% of the butyrate absorbed. In sharp contrast to this, Wierzuchowski and Ling¹³⁶ calculated that the growing pig may convert over 50% of the ingested carbohydrate (starch) to fat.

The earlier evidence of the non-convertibility of fatty acids to carbohydrate, based upon work carried out before isotope techniques were available, must still be accepted at face value. Experimental evidence which can be adduced from studies on the amount of liver and carcass glycogen following the feeding of even-chain fatty acids or of natural fats can only be interpreted as indicative of the failure of this transformation to occur to any significant degree. Similarly, the data obtained by the employment of the D:N ratio, the R.Q., or the use of epinephrine and insulin can best be explained by the non-transformation of fat to carbohydrate.

On the other hand, no one doubts the ability of the glycerol moiety to contribute to the tissue and liver carbohydrate. This change can readily be demonstrated when glycerol is fed as such or in the form of triglyceride molecules which are incapable of being stored. There is less evidence for the synthesis of glucose from the glycerol moiety of the natural fats, although most workers believe that the reaction does occur when natural fats are oxidized. Fats having an odd number of carbons are likewise sources of glucose to the extent that the fatty acids are convertible to propionic acid;

¹³⁴ W. C. Stadie, J. A. Zapp, Jr., and F. D. W. Lukens, *J. Biol. Chem.*, **132**, 423-443 (1940).

¹³⁵ W. C. Stadie, *J. Clin. Invest.*, **19**, 843-861 (1940).

¹³⁶ M. Wierzuchowski and S. M. Ling, *J. Biol. Chem.*, **64**, 697-707 (1925).

however, one must realize that these fats are artificial, and are not found as natural products.

The most probable explanation for the appearance of the small amounts of fatty acid carbon in the carbohydrate molecule is as follows. Under normal conditions, the acetic acid molecules arising from the oxidation of the fatty acids are combined with oxaloacetate, and they are oxidized *via* the Krebs cycle. When the oxidation is complete, the new oxaloacetate may carry one of the carbons from the original acetic acid. The oxaloacetate is readily converted to pyruvic acid by decarboxylation. The resulting pyruvic acid, still carrying the fatty acid carbon, is in equilibrium with glycogen. However, this reaction is a minor one; it simply means that some of the glycogen carbon is replaced by fatty acid carbon. The total amount of glycogen available remains constant.

CHAPTER V

THE OXIDATION AND METABOLISM OF PHOSPHOLIPIDS, AND THEIR HYDROLYSIS PRODUCTS

The oxidation of the phospholipids is closely associated with that of neutral fat. However, there are certain unique features in the metabolism of these compounds which occur because of the specific nitrogen components in their molecules. The behavior of choline is of particular interest in the latter connection.

1. Hydrolysis of Phospholipids

The first step in the decomposition of all phospholipids would appear to be in the hydrolysis of their molecules. As a result of such changes, fatty acids, glycerol, phosphoric acid, and nitrogenous bases are ultimately set free. Fatty acids and glycerol are believed to follow the same course of metabolism, irrespective of whether they originate from neutral fats or from phospholipids.

The first step in the breakdown of lecithin presumably involves the removal of its two fatty acid residues, resulting in the formation of α -glycerylphosphorylcholine (GPC). Schmidt *et al.*¹ originally isolated this compound from incubated beef pancreas; Schmidt and others² later demonstrated the presence of GPC in aqueous extracts of several tissues, including liver, while a number of investigators³⁻⁵ noted that the corresponding hydrolysis product of phosphatidylethanolamine, namely glycerylphosphorylethanolamine (GPE) likewise occurs in aqueous and ethanol tissue extracts. More recently Dawson⁶ isolated both GPC and GPE chromatographically from rat liver, in rats which had previously been injected with

¹ G. Schmidt, B. Hershman, and S. J. Thannhauser, *J. Biol. Chem.*, **161**, 523-536 (1945).

² G. Schmidt, L. Hecht, P. Fallot, L. Greenbaum, and S. J. Thannhauser, *J. Biol. Chem.*, **197**, 601-609 (1952).

³ P. N. Campbell and T. S. Work, *Biochem. J.*, **50**, 449-454 (1952).

⁴ D. M. Walker, *Biochem. J.*, **52**, 679-683 (1952).

⁵ G. B. Ansell and J. M. Norman, *Biochem. J.*, **55**, 768-774 (1953).

⁶ R. M. C. Dawson, *Biochem. J.*, **57**, xv (1954); **59**, 5-8 (1955).

radioactive phosphate. On the basis of the assumption of Zilversmit and co-workers⁷ that a mathematical relationship exists between the specific radioactivity of the precursor and its immediate metabolic product, after the injection of a single dose of the isotope, Dawson⁶ concluded that GPC and GPE were metabolic products of phosphatidylcholine and phosphatidylethanolamine, respectively; however, it was likewise indicated that they could not serve as major precursors in the synthesis of these phospholipids.

It is believed that GPC and GPE originate from lecithin as the result of the action of specific enzymes, called "lecitholipases"; these are distinct from the usual pancreatic lipases, which act chiefly upon the ester linkages in neutral fat.

The second hydrolysis of the partially decomposed lecithin molecule is believed to involve the release of choline from α -glycerylphosphorylcholine, which would leave α -glycerophosphoric acid. This reasoning is based upon the fact that the ester linkage to choline is readily susceptible to hydrolysis by acid in the α -glycerylphosphorylcholine molecule, just as it is in lecithin; on the other hand, it is very difficult to hydrolyze phosphorylcholine. This suggests that the removal of choline from phosphorylcholine after a prior hydrolysis of the glycerylphosphate linkage would appear to be quite unlikely.

Some light has been thrown on the hydrolysis of lecithin by the investigation of the action of enzyme preparations obtained from a strain of *Serratia plymuthicum* (an aerobic bacterium from water which forms CO_2 and H_2 from glucose, lactose, and sucrose), by Hayaishi and Kornberg.⁸ The following course of metabolism was demonstrated:

The Postulated Course of the Metabolism of Lecithin by Enzyme Preparations of *Serratia plymuthicum*⁸

When cells were cultured on lecithin, the concentration of the lecithin-hydrolyzing enzyme was found to be five times or more greater than that obtained when the cells were grown on ordinary broth.

The pathway for the hydrolysis of the classical cephalin (phosphatidylethanolamine) is considered to be as follows: The hydrolysis of sphingomyelin presents new problems, since this substance does not con-

⁷ D. B. Zilversmit, C. Entenman, and M. C. Fishler, *J. Gen. Physiol.*, **26**, 325-331 (1943).

⁸ O. Hayaishi and A. Kornberg, *J. Biol. Chem.*, **206**, 647-663 (1954).

tain a glycerol component in its molecule. Goebel⁹ reported that

The Postulated Course of the Metabolism of Cephalin by Enzyme Preparations of *Serratia plymuthicum*⁸

sphingomyelin, which had been injected into rabbits and mice, disappeared from the blood, within a relatively short time, which varied with the dose injected. Although a temporary increase in the sphingomyelin content of different organs, and particularly of the liver, resulted, the increase lasted only a short time, and the level of acid-soluble phosphorus in the blood was raised. It was concluded that the destruction of sphingomyelin proceeds first through the cleavage of phosphoric acid. In a later study, Goebel and Seckfort¹⁰ found that human brain and liver were both able to effect the hydrolysis of sphingomyelin. The optimal pH was 7.2 in the liver and 6.2 in the brain. The reaction progressed about three times as rapidly in the liver as in the brain. Here again phosphoric acid was identified as one of the decomposition products, perhaps the only one.

In a more complete study of the hydrolysis of sphingomyelins, Fujino¹¹ suggested the following possibilities for the breakdown of sphingomyelin:

The Hydrolysis of Sphingomyelin as Adapted from Fujino¹¹

⁹ A. Goebel, *Biochem. Z.*, **319**, 196–202 (1948).

¹⁰ A. Goebel and H. Seckfort, *Biochem. Z.*, **319**, 203–208 (1948).

¹¹ Y. Fujino, *J. Biochem. (Japan)*, **39**, 55–62 (1952).

Fujino¹¹ used pig spleen as the source of the enzyme preparation. It was suggested that sphingomyelins and cerebroside are so closely related that they can interchange through the intermediate ceramides. It would appear that phosphorylcholine may be an intermediate in the hydrolysis of sphingomyelin, while it is not believed to function in this capacity in the case of lecithins and cephalins. However, the conditions are different in the former case because glycerol is not present in the sphingomyelin molecule.

2. The Degradation of Phospholipids by Means Other Than by Hydrolysis

O'Connell and Stotz¹² demonstrated that, when hydrolecithin from beef brain or beef lung was oxidized by rat liver homogenates in the presence of ATP, no acetoacetate originated, in spite of the fact that these end-products occur when free fatty acids are the substrates. According to Harper *et al.*,¹³ the rate of utilization of intravenously-injected phospholipid is proportional to the plasma P level in both normal and diabetic dogs. Although the absolute utilization of plasma phospholipid was slightly lower in the diabetic group than in the normal animals, the proportion of plasma phospholipid utilized per hour by the diabetic group was significantly higher. The administration of either insulin or sugar did not increase phospholipid utilization by diabetic dogs; however, it was increased when raw pancreas was given.¹³

The utilization of phospholipid in brain and liver is catalyzed by iron compounds, especially if ascorbic acid is added.¹⁴ In the brain, only the cephalin fraction undergoes oxidation under these conditions. Although several investigators^{15,16} have reported that the turnover of brain lipids (unsaponifiable fraction) is comparatively slow, Sperry¹⁷ found that a decrease in extractable phosphorus occurred during a four-hour incubation period as the result of a cleavage of phospholipids in brain tissue. Stanley¹⁸ reported the formation of acid-soluble phosphate and of inositol when cephalin fractions of brain were treated with homogenates of fresh adult guinea

¹² P. W. O'Connell and E. Stotz, *Proc. Soc. Exptl. Biol. Med.*, **70**, 675-677 (1949).

¹³ P. V. Harper, Jr., W. B. Neal, Jr., and J. R. Rogers, *Proc. Soc. Exptl. Biol. Med.*, **68**, 363-369 (1948).

¹⁴ K. A. Elliott and B. Libet, *J. Biol. Chem.*, **152**, 617-626 (1944).

¹⁵ L. Hahn and G. Hevesy, *Skand. Arch. Physiol.*, **77**, 148-157 (1937).

¹⁶ W. M. Sperry, H. Waelsch, and V. A. Stoyanoff, *J. Biol. Chem.*, **135**, 281-290, 291-296 (1940).

¹⁷ W. M. Sperry, *J. Biol. Chem.*, **170**, 675-686 (1947).

¹⁸ G. H. S. Stanley, *Biochem. J.*, **49**, iv-v (1951).

pig brain. The parent substance was believed to be diphosphoinositide. Although Streicher¹⁹ indicated that the turnover of P^{32} in brain phospholipid is notoriously slow, and is close to zero in the great bulk of such compounds, it is suggested that it may be high in a small fraction or "trace" compound of the phospholipids.

3. The Oxidation and Metabolism of Hydrolysis Products of Phospholipids

(1) Choline

a. Introduction. At least two enzymes are concerned with the oxidation of choline, namely choline oxidase (CO) and choline dehydrogenase (CD). In addition to these, two other enzymes of importance in choline metabolism occur in rat liver, namely, betaine aldehyde dehydrogenase (BAD) and sarcosine oxidase. Christensen and Daniel²⁰ demonstrated that choline dehydrogenase and betaine aldehyde dehydrogenase are different enzymes, inasmuch as, on centrifugation of sucrose homogenates of rat liver, CD was found chiefly in the mitochondrial fraction, while BAD remained in the supernatant fraction.

b. Choline Oxidase. (a) *Distribution of Choline Oxidase.* Bernheim and Bernheim,²¹ in 1933, were the first to recognize that acetylcholine could be oxidized by rat liver, and that one and one-half atoms of oxygen were required for the change. The optimum pH was found to be 7.0, but no enzyme was mentioned. Five years later, these workers²² proved that the destruction of choline was mediated by an enzyme, which they were able to separate in relatively pure form. In the course of the oxidation, it was found that choline is changed to betaine aldehyde, with the uptake of one atom of oxygen, and then to betaine, with the incorporation of two molecules of oxygen in the reaction. The enzyme differed from alcohol oxidase, and was named "choline oxidase." Although Mann and Quastel,²³ in 1937, expressed some doubt whether or not the enzyme responsible for the oxidation of choline was specific, these workers²⁴ came to the conclusion in 1938 that a choline oxidase did exist in rat liver. It is thus evident that

¹⁹ E. Streicher, *Federation Proc.*, **12**, 139-140 (1953).

²⁰ J. R. Christensen and L. J. Daniel, *Federation Proc.*, **12**, 189-190 (1953).

²¹ F. Bernheim and M. L. C. Bernheim, *Am. J. Physiol.*, **104**, 438-439 (1933).

²² F. Bernheim and M. L. C. Bernheim, *Am. J. Physiol.*, **121**, 55-60 (1938).

²³ P. J. G. Mann and J. H. Quastel, *Biochem. J.*, **31**, 869-878 (1937).

²⁴ P. J. G. Mann, H. E. Woodward, and J. H. Quastel, *Biochem. J.*, **32**, 1024-1032 (1938).

both the British and the American groups of workers are to be credited with the discovery of the existence of choline oxidase.

The enzyme is present in high concentration in the liver. Kensler and Langemann,²⁵ using the centrifugation technic, found that the choline oxidase activity was concentrated in the mitochondrial fraction (78%), while only insignificant amounts were present in the nuclear (9%) and supernatant (13%) fractions. The distribution was found to be similar to that of succinoxidase, although probably not identical with it. The absence of CO in the isolated nuclei of liver cells confirms an earlier report of Lan.²⁶

(b) *Chemical Nature of Choline Oxidase.* Apparently choline oxidase is a protein, since it has been shown to be inactivated by trypsin and papain.²⁷ Williams²⁸ presented evidence to indicate that mitochondrial CO requires a heat-stable co-factor, since aging has been shown to decrease the CO activity of mitochondrial suspensions.

The inhibitory effect of folic acid upon the activity of CO appears to be indirect;²⁹ evidently its action consists in regulating the synthesis of the enzyme protein, rather than in exerting a direct inhibitory effect. Williams³⁰ found that folic acid and aminopterin had no effect upon the CO activity of rat liver when added *in vitro*, although when they were fed they inhibited the action of this enzyme. On the other hand, Dinning and collaborators³¹ reported that the bone-marrow of chickens, which normally exhibits CO activity, did not have this activity if the birds had previously been treated with aminopterin, which is generally accepted as a folic acid inhibitor. This is interpreted as evidence of the displacement of folic acid (pteroylglutamic acid) from its position in the enzyme system by aminopterin. Dinning, Keith, and Day³² likewise recorded that the CO content of the livers and kidneys of folic acid-deficient monkeys is reduced; presumably this results because folic acid is the prosthetic group of CO. Williams³³ states that only folic acid and ascorbic acid stimulate choline oxidation in normal rat livers. The results indicate that folic acid (PGA), *Leuconostoc citrovorum* factor (LCF), vitamin B₁₂, and ascorbic acid are all important in maintaining choline oxidase.

²⁵ C. J. Kensler and H. Langemann, *J. Biol. Chem.*, **192**, 551-554 (1951).

²⁶ T. H. Lan, *J. Biol. Chem.*, **151**, 171-175 (1943).

²⁷ F. Bernheim, *J. Biol. Chem.*, **133**, 141-144 (1940).

²⁸ J. N. Williams, Jr., *J. Biol. Chem.*, **197**, 709-715 (1952).

²⁹ J. N. Williams, Jr., M. L. Sunde, W. W. Cravens, and C. A. Elvehjem, *J. Biol. Chem.*, **185**, 895-901 (1950).

³⁰ J. N. Williams, Jr., *J. Biol. Chem.*, **187**, 47-54 (1950).

³¹ J. S. Dinning, C. K. Keith, P. L. Davis, and P. L. Day, *Arch. Biochem.*, **27**, 89-93 (1950).

³² J. S. Dinning, C. K. Keith, and P. L. Day, *Arch. Biochem.*, **24**, 463-464 (1949).

³³ J. N. Williams, Jr., *J. Biol. Chem.*, **192**, 81-85 (1951).

Although there is considerable support for the hypothesis that folic acid is the coenzyme of CO, Williams³⁴ stated that the closely related folinic acid (the so-called *Leuconostoc citrovorum* factor, LCF) is the actual coenzyme. However, synthetic LCF was shown to have less effect upon the CO system *in vitro* than did folic acid plus ascorbic acid. Williams³⁵ observed that the activity of CO *in vitro* was reduced with a low vitamin C concentration and increased with a high vitamin C content. According to Williams,³⁵ when aminopterin was fed to rats, liver ascorbic acid was reduced to only 50% of normal. Although Mann *et al.*²⁴ postulated that hydrogen is transferred from CO to the cytochrome oxidase system, Williams and co-workers³⁶ did not find that added cytochrome exerted any effect on CO activity. Williams *et al.*³⁶ demonstrated that a whole liver homogenate furnished an adequate CO system for assay without additional co-factors. Normal rat liver has a choline oxidase activity equivalent to 76.5 μ l of O₂ per ten min. per 167 mg. liver.³⁶

(c) *Mechanism of Action of Choline Oxidase.* Choline oxidase functions by catalyzing the oxidation of choline to betaine according to the following scheme:

The Oxidation of Choline to Betaine by Choline Oxidase

Barrenscheen and Pantlitschko³⁷ reported that, in order to function as a transmethyating agent, choline must be oxidized to trimethylamine oxide ((CH₃)₃N:O). Dubnoff³⁸ confirmed the fact that the oxidation of choline must occur before it can function in transmethylation; the latter worker suggested that it is converted to betaine rather than to trimethylamine oxide. Thus, it would appear that the transfer of methyl groups from choline is indirectly controlled by CO. Barrenscheen and v. Vályi-Nagy³⁹ had demonstrated earlier in *in vitro* tests with muscle, liver, brain, kidney, and adrenal gland that a similar oxidation of methionine to a sul-

³⁴ J. N. Williams, Jr., *J. Biol. Chem.*, **191**, 123-127 (1951).

³⁵ J. N. Williams, Jr., *Proc. Soc. Exptl. Biol. Med.*, **77**, 315-317 (1951).

³⁶ J. N. Williams, Jr., G. Litwack, and C. A. Elvehjem, *J. Biol. Chem.*, **192**, 73-80 (1951).

³⁷ H. K. Barrenscheen and M. Pantlitschko, *Z. physiol. Chem.*, **284**, 250-256 (1949).

³⁸ J. W. Dubnoff, *Arch. Biochem.*, **24**, 251-262 (1949).

³⁹ H. K. Barrenscheen and T. v. Vályi-Nagy, *Z. physiol. Chem.*, **283**, 91-99 (1948).

foxide must precede transmethylation. Only liver and adrenal are able to synthesize choline from methionine and ethanolamine or serine. It has also been reported that, before choline can be formed from methionine and ethanolamine (or serine) in the liver, the preparation must be activated by ascorbic acid-cobalt in the presence of CO; however, ascorbic acid alone does not bring about this synthesis,⁴⁰ and in fact has an inhibitory effect.

(d) *Factors Altering the Activity or Concentration of Choline Oxidase.* According to Kensler and Langemann,⁴¹ a marked species variation in CO activity obtains in the tissues. Thus the CO activity and the capacity to utilize choline for the methylation of homocysteine are low or absent in fresh human liver, just as in the liver tissue of guinea pig or rabbit. Handler⁴² likewise observed that no CO is present in guinea pig liver; it is possible that this may be one of the reasons why fatty livers cannot be produced in this species. However, the livers of the species examined (rat, mouse, guinea pig, rabbit, chicken, dog, and man) were all found to be capable of utilizing betaine and its sulfur analogue, dimethylthetin, to methylate homocysteine to form methionine.⁴¹

Bernheim⁴³ was the first to demonstrate that the activity of CO is inhibited by the presence of fatty acids. This inhibitory effect of fats on CO was found to be quite specific, inasmuch as CO is by far the most sensitive (61%); the inhibition of other oxidases by stearic acids was considerably less: succinoxidase (19%), cytochrome (13%), *d*-amino acid oxidase (8%), amine oxidase (0%). It is suggested that, since CO is especially active in effecting the oxidation of choline in normal phospholipid catabolism, sufficient choline might not be available for phospholipid synthesis unless there were some inhibitory action on CO. The results of Humoller and Zimmerman⁴⁴ likewise support the finding that a high concentration of fatty acids greatly inhibits the action of CO. Thus, in rats fed a diet high in fats and low in choline, the CO activity decreased precipitously during the first week of the diet, concomitantly with the increase in liver fat. Moreover, the CO activity could not be augmented in the fatty livers when choline or betaine was injected two hours before the animals were sacrificed.

The activity of CO has been shown to be inhibited by oxidized fatty acids. Thus, Bernheim and co-workers⁴⁵ reported that the extent of inac-

⁴⁰ H. K. Barrrenscheen and I. Skudrzyk, *Z. physiol. Chem.*, **284**, 228-236 (1949).

⁴¹ C. J. Kensler and H. Langemann, *Proc. Soc. Exptl. Biol. Med.*, **85**, 364-367 (1954).

⁴² P. Handler, *Proc. Soc. Exptl. Biol. Med.*, **70**, 70-73 (1949).

⁴³ F. Bernheim, *J. Biol. Chem.*, **133**, 291-292 (1940).

⁴⁴ F. L. Humoller and H. J. Zimmerman, *Am. J. Physiol.*, **174**, 199-202 (1953).

⁴⁵ F. Bernheim, K. M. Wilbur, and C. B. Kenaston, *Arch. Biochem.*, **38**, 177-184 (1952).

tivation parallels the amount of oxidized fatty acids formed, as measured by the thiobarbituric acid reagent. Quercetin, which protects the fat from oxidation, prevents this inactivation.

It has been reported that ethionine, $\text{C}_2\text{H}_5\text{S}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, causes a decrease in activity of two systems concerned with choline oxidation, namely CO and sarcosine oxidase.^{46,47} On the other hand, succinoxidase is not affected by this analogue of methionine. Other methionine analogues, methoxinine, and methionine sulfoximine, do not influence CO activity when added *in vitro* to preparations of liver.⁴⁷ Swendseid *et al.*⁴⁷ suggest that the actual inhibitor is not ethionine but an enzymatically produced derivative. Ethionine likewise exerts an inhibitory effect upon CO *in vivo*.⁴⁶

Barron and associates⁴⁸ noted that the nitrogen mustards inhibit all enzymes concerned with choline metabolism, including CO. The action of the latter enzyme system was found to be inhibited by as small an amount as $1 \times 10^{-6} M$ solutions of HN_2 , methyl-bis (β -chloroethyl) amine HCl, or HN_3 , methyl-tris (β -chloroethyl) amine HCl. Boyland⁴⁹ suggests that the inhibition is probably due to the action of the ethylene imonium form of the bases, $\text{R}-\overset{+}{\text{N}}=(\text{CH}_2\cdot\text{CH}_2)$, in which form the nitrogen mustards have a convulsant and rapidly lethal action.⁵⁰ This, in turn, might be ascribed to the inhibition of the enzymes concerned with choline metabolism.⁴⁸ According to Howe and associates,⁵¹ choline affords no protection against nitrogen mustard intoxication. The aliphatic chloroethyl amines react with most cell constituents,⁵² including enzymes. CO is inhibited by these substances.⁵³ This effect may be counteracted by the addition of choline.⁵³

According to Williams,²⁸ CO is partially inactivated by freezing and thawing; a potent, heat-labile inhibitor of CO is present in the supernatant fraction.⁵⁴ Moreover, boiled extracts of CO were found to increase the ac-

⁴⁶ M. E. Swendseid, A. L. Swanson, and F. H. Bethell, *Federation Proc.*, **10**, 257 (1951).

⁴⁷ M. E. Swendseid, A. L. Swanson, and F. H. Bethell, *J. Biol. Chem.*, **201**, 803-809 (1953).

⁴⁸ E. S. G. Barron, G. R. Bartlett, and Z. B. Miller, *J. Exptl. Med.*, **87**, 489-501 (1948).

⁴⁹ E. Boyland, *Ann. Rev. Biochem.*, **18**, 217-242 (1949).

⁵⁰ E. Boyland, *Brit. J. Pharmacol. Chemotherap.*, **1**, 247-254 (1946).

⁵¹ G. W. Howe, H. N. Marvin, and C. L. Spurr, *Texas Repts. Biol. and Med.*, **10**, 39-44 (1952).

⁵² J. S. Fruton, W. H. Stein, M. A. Stahmann, and C. Golumbic, *J. Org. Chem.*, **11**, 471-580 (1946).

⁵³ E. S. G. Barron, G. R. Bartlett, Z. B. Miller, J. Meyer, and J. E. Seegmiller, *J. Exptl. Med.*, **87**, 503-519 (1948).

⁵⁴ J. N. Williams, Jr., *J. Biol. Chem.*, **194**, 139-142 (1952); **198**, 579-585 (1952).

tivity of partially-inactivated extracts.²⁸ Riboflavin deficiency was shown to decrease the activity of CO in weanling rat liver, while vitamin B₆ and thiamine deficiencies were without effect.⁵⁵ The rate of inactivation of CO in washed liver homogenates was increased by cystine and by semicarbazide.⁵⁶

Several investigators⁵⁷⁻⁵⁹ reported that the incorporation of carcinogenic azo dyes such as dimethylaminoazobenzene (butter yellow, DMAB) into the diet of animals produces a pronounced drop in CO activity. This may result in a block in the transmethylation cycle, since oxidation of choline is a prerequisite for methyl transfer. Kensler *et al.*⁶⁰ observed that the CO activities of transplantable mouse liver tumors examined were only 15, 33, and 50% of that present in normal liver. It is also of interest that, according to Kensler and associates,⁶¹ CO is low in the livers of newborn rats, and increases to only one-third of the adult level by the time of weaning. Williams and co-workers²⁹ reported the presence of CO in the livers of the chick embryo.

c. The Course of Choline Metabolism. Müller⁶² was of the opinion that choline chloride is broken down to trimethylamine, since increased amounts of this compound were found in the urine after the feeding of choline chloride or lecithin. The methyl groups did not originate from creatine. On the basis of a study of liver slices, Artom and Crowder⁶³ reported that the major pathway of choline metabolism was involved in the formation of water-soluble combinations which were resistant to mild hydrolysis. These resemble phosphorylcholine rather than lecithin or glycerylphosphorylcholine, which are easily hydrolyzable. Boyd⁶⁴ noted that, during the development of the chick embryo, the labile methyl groups in the liver decreased about 25%. Since it is known that a CO system exists,²⁹ dimethylaminoacetic acid is probably formed. However, it does not accumulate, and therefore it is believed that the demethylation product must be rapidly metabolized.⁶⁵ An alternative pathway for the metabolism of cho-

⁵⁵ D. A. Richert and W. W. Westerfeld, *J. Biol. Chem.*, **199**, 829-833 (1952).

⁵⁶ G. S. Eadie and F. Bernheim, *J. Biol. Chem.*, **185**, 731-739 (1950).

⁵⁷ G. Viollier, *Helv. Physiol. Pharmacol. Acta*, **8**, C34-C36, C37-C39 (1950).

⁵⁸ C. J. Kensler and H. Langemann, *Cancer Research*, **11**, 264 (1951).

⁵⁹ G. E. Woodward, *Cancer Research*, **11**, 918-921 (1951).

⁶⁰ C. J. Kensler, M. Rudden, and H. Langemann, *Cancer Research*, **12**, 274 (1952).

⁶¹ C. J. Kensler, M. Rudden, E. Shapiro, and H. Langemann, *Proc. Soc. Exptl. Biol. Med.*, **79**, 39-42 (1952).

⁶² H. Müller, *Z. physiol. Chem.*, **263**, 243-258 (1940).

⁶³ C. Artom and M. Crowder, *Arch. Biochem.*, **29**, 227-228 (1950).

⁶⁴ G. S. Boyd, *Biochem. J.*, **47**, xlvii-xlviii (1950).

⁶⁵ G. S. Boyd, *Biochem. J.*, **49**, vii (1951).

line suggested by Siekevitz and Greenberg⁶⁶ involves the transfer of methyl C to the β -carbon of serine.

Mills⁶⁷ reported an increased choline requirement in the case of rats kept in a high environmental temperature. Johnson *et al.*⁶⁸ reported that only approximately 1% of the choline intake was excreted as choline by man. It was found to be present in undiluted sweat to the extent of 2.7 to 15.3 $\mu\text{g. \%}$. However, the latter workers found no increase in choline excretion by four adult male subjects kept under "hot moist" conditions. It is therefore uncertain whether or not the choline requirement in the human subject is augmented at high environmental temperatures.

Busset⁶⁹ reported that, four hours after the administration of labeled choline to a dog, it had passed through the wall of the small intestine, the blood and the bile, where it was present in the amount of 7.2%. However, there was no evidence of oxidation of this substance. Byerrum and Wing⁷⁰ reported that the methyl carbons of choline can be transferred to yield the methyl groups in nicotine in the North American wild tobacco plant (*Nicotiana rustica*). The methyl carbons of choline and of methionine appeared to be donated to this plant at about the same rate. Since there were no detectable amounts of phospholipids, it was not believed that choline is normally involved in the synthesis of these substances.

d. Choline as a Source of Glycine. Choline is considered to be a precursor of glycine in the intact rat. Soloway and Stetten⁷¹ noted that, when N^{15} -labeled choline was injected into rats together with sodium benzoate, a considerable amount of the excreted hippuric acid contained the glycine fragment labeled with N^{15} . Betaine was equally active as a precursor, but dimethylethanolamine was found to be far less efficient in this respect. It was demonstrated by the use of doubly-labeled choline that the ethanolic residue of choline, together with the attached nitrogen, is transformed intact into glycine. It was postulated that an obligatory oxidation of choline occurs, to yield betaine, prior to its demethylation to glycine.⁷¹

(2) Ethanolamine

a. Introduction. Ethanolamine, $\text{HOCH}_2\cdot\text{CH}_2\cdot\text{NH}_2$, is of considerable importance, inasmuch as it constitutes the nitrogenous constituent of the

⁶⁶ P. Siekevitz and D. M. Greenberg, *J. Biol. Chem.*, **186**, 275-286 (1950).

⁶⁷ C. A. Mills, *Arch. Biochem.*, **1**, 73-81 (1942).

⁶⁸ B. C. Johnson, T. S. Hamilton, and H. H. Mitchell, *J. Biol. Chem.*, **159**, 5-8 (1945).

⁶⁹ R. Busset, *Compt. rend. soc. biol.*, **147**, 711-713 (1953).

⁷⁰ R. U. Byerrum and R. E. Wing, *J. Biol. Chem.*, **205**, 637-642 (1953).

⁷¹ S. Soloway and De W. Stetten, Jr., *J. Biol. Chem.*, **204**, 207-214 (1953)

group of phospholipids originally referred to as "cephalins," and now identified more specifically as "phosphatidylethanolamine."

b. Sources of Ethanolamine in the Animal Body. Several compounds which can be readily synthesized physiologically in the animal body may serve as mother substances for ethanolamine. These include glycine,⁷² and possibly serine.⁷² Presumably glycine is first converted to serine, and this compound becomes ethanolamine when decarboxylation has been effected. There is ample proof that glycine and serine are interconvertible. Shemin⁷³ was the first to prove that glycine can originate from serine, while a number of workers⁷⁴⁻⁷⁷ proved the reverse reaction, namely the glycine $\rightarrow$ serine reaction. According to Sakami,⁷⁸ formate condenses with glycine to form serine; the formate carbon becomes the β carbon of the newly-synthesized amino acid. This was demonstrated experimentally by using C¹⁴-formate and C¹³-1-glycine as the reactants, as follows:

The Combination of Glycine and Formate to Form Serine⁷⁸

Siekevitz and Greenberg⁷⁷ reported that one molecule of glycine may contribute the α -carbon to form formate, which then condenses with a second molecule of glycine to yield serine.

The conversion of serine to ethanolamine was first suggested by Folch and Schneider,⁷⁹ who based their opinion upon the probable presence of serine in the cephalin fraction. This relationship was further supported by the observation of Nord⁸⁰ that anaerobic bacteria decarboxylate serine to yield ethanolamine. Levine and Tarver,⁸¹ and Arnstein,⁸² further confirmed this reaction by proving that serine labeled in the β position with C¹⁴ gives rise to β -labeled ethanolamine.

c. Degradation of Ethanolaminé in the Animal Body. Undoubtedly, the major metabolic route for ethanolamine is its conversion to choline.

⁷² De W. Stetten, Jr., *J. Biol. Chem.*, **138**, 437-438 (1941); **140**, 143-152, cxxvii (1941).

⁷³ D. Shemin, *J. Biol. Chem.*, **162**, 297-307 (1946).

⁷⁴ P. D. Goldsworthy, T. Winnick, and D. M. Greenberg, *J. Biol. Chem.*, **180**, 341-343 (1949).

⁷⁵ T. Winnick, I. Moring-Claesson, and D. M. Greenberg, *J. Biol. Chem.*, **175**, 127-132 (1948).

⁷⁶ P. Siekevitz, T. Winnick, and D. M. Greenberg, *Federation Proc.*, **8**, 250 (1949).

⁷⁷ P. Siekevitz and D. M. Greenberg, *J. Biol. Chem.*, **180**, 845-856 (1949).

⁷⁸ W. Sakami, *J. Biol. Chem.*, **176**, 995-996 (1948).

⁷⁹ J. Folch and H. A. Schneider, *J. Biol. Chem.*, **137**, 51-62 (1941).

⁸⁰ F. F. Nord, *Biochem. Z.*, **95**, 281-285 (1919).

⁸¹ M. Levine and H. Tarver, *J. Biol. Chem.*, **184**, 427-436 (1950).

⁸² H. R. V. Arnstein, *Biochem. J.*, **48**, 27-32 (1951)

This reaction can be brought about by transmethylation with methionine. On the other hand, Stetten⁷² was of the opinion that no appreciable amount of ethanolamine is converted to glycine, although the reverse reaction readily occurs. However, White *et al.*,⁸³ in their recent textbook, have pictured the conversion of ethanolamine to glycine by the pathway illustrated below.

The Conversion of Ethanolamine to Glycine⁸³

The relationship of these several nitrogenous bases to each other, as suggested by Stetten, is illustrated in the accompanying schematic diagram.

Schematic Diagram to Illustrate the Synthesis of Choline from Betaine, Glycine, and Ethanolamine⁷²

When ethanolamine or choline is included in the diet, it is rapidly incorporated into the phospholipids;⁷² it was found that these compounds replaced an equal amount in the tissue phospholipids. Insofar as choline is concerned, the reaction proceeded most rapidly in the liver¹ and was slowest in the brain.

(3) Serine

Serine, $\text{HOCH}_2\text{·CH(NH}_2\text{)·COOH}$, has only recently been recognized as a normal component of one type of phospholipids; thus, Folch and Schneider⁷⁹ proved the presence of this amino acid combined in the phosphatide molecule. This variety of compound is now referred to as "phosphatidylserine."

As indicated in the preceding section, serine is readily converted to ethanolamine, as well as to glycine.⁷³ It has also long been recognized that the

⁸³ A. White, P. Handler, E. L. Smith, and De W. Stetten, Jr., *Principles of Biochemistry*, McGraw-Hill, New York, 1954, pp. 491, 554.

residue of serine, remaining after deamination, is largely converted to glucose and to glycogen. On the basis of the phlorhizin technic, Dakin⁸⁴ showed that serine, cysteine, proline, ornithine, and arginine yield large amounts of glucose. These results are supported by the qualitative experiments of Butts *et al.*⁸⁵ and of Schofield and Lewis,⁸⁶ who demonstrated that glycogen stores increased after the feeding of serine to fasted rats. After conversion to glucose, the serine carbons follow the usual fate of the carbohydrate molecule in the animal body. Barnet and Wick⁸⁷ fed C¹⁴-labeled glycine to rats, and found that the carboxyl carbon supplied approximately one carbon to each twenty-eight carbon atoms incorporated into glycogen. One carbon atom in every 8.5 carbon atoms in the glycogen molecule was derived from the α -carbon of the fed glycine. This is compatible with the pathway of conversion of glycine to glycogen suggested by Sakami.⁷⁸

(4) Sphingosine

Sphingosine, $\text{CH}_3 \cdot (\text{CH}_2)_{12} \cdot \text{CH} : \text{CH} \cdot \text{CHOH} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2\text{OH}$, is the nitrogenous base present in the sphingomyelin molecule. Although Zabin and Mead⁸⁸ reported that sphingosine is synthesized from acetate, little is known concerning the degradation products. It is established that sphingosine is readily reduced to dihydrosphingosine, $\text{CH}_3 \cdot (\text{CH}_2)_{14} \cdot \text{CHOH} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2\text{OH}$, but it is not known whether this is an intermediate in the decomposition of sphingosine or whether it is merely a side-product. Apparently, dihydrosphingosine is a normal product, since it is found in nerve tissue and brain;⁸⁹ its concentration was shown to be higher in spinal cord than in brain.⁹⁰ Lesuk and Anderson⁹¹ likewise recorded its presence in the larval form (*Cysticercus fasciolaris*) of the cat tapeworm (*Taenia crassicolis*). However, White *et al.*⁸³ stated in 1954 that "Neither the origin nor the fate of . . . sphingosine and dihydrosphingosine is known. Since they are not essential in the diet, these nitrogenous bases presumably can be synthesized in the mammalian organism."

⁸⁴ H. D. Dakin, *J. Biol. Chem.*, **14**, 321-333 (1913).

⁸⁵ J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, **124**, 709-714 (1938).

⁸⁶ F. A. Schofield and H. B. Lewis, *J. Biol. Chem.*, **169**, 373-378 (1947).

⁸⁷ H. N. Barnet and A. N. Wick, *J. Biol. Chem.*, **185**, 657-661 (1950).

⁸⁸ I. Zabin and J. F. Mead, *J. Biol. Chem.*, **205**, 271-277 (1953).

⁸⁹ H. E. Carter and W. P. Norris, *J. Biol. Chem.*, **145**, 709 (1942).

⁹⁰ H. E. Carter, W. P. Norris, F. J. Glick, G. E. Phillips, and R. Harris, *J. Biol. Chem.*, **170**, 269-283 (1947).

⁹¹ A. Lesuk and R. J. Anderson, *J. Biol. Chem.*, **189**, 457-469 (1941).

4. The Effect of Deficiency of the Phospholipids

(1) *The Effect of Phospholipid Deficiency in General*

Phospholipids play so many varied roles in metabolism that their deficiency in the diet may cause many abnormalities, and may actually result in death. However, the body is able to synthesize phospholipids from the various building stones to such a great extent that it is only when there is a prolonged deficiency of some of the compounds incapable of synthesis in the animal body that a deficiency in phospholipid itself occurs. Glycerol and the saturated and monoethenoid acids are always readily available for phospholipid synthesis, as are also phosphate and ethanolamine. The only substances, shortages of which may become critical, since they cannot be synthesized in the body and must be furnished in the diet, are the essential fatty acids, choline (under certain circumstances), and sphingomyelin.

When facilities for the synthesis of a sufficient amount of phospholipids are lacking, the most obvious abnormalities which occur involve the transportation of the fats. For example, fatty livers develop. In extreme cases of phospholipid deficiency, insufficient amounts of these compounds are present to serve as cell membranes, with the result that serious difficulties arise, which may result in hematuria and even in death. In addition to the role of phospholipids in the transport and storage of fats, which was described in *The Lipids*, Volume II, in the section on fatty livers, pages 633-697, one of the phospholipid components of thromboplastin has an added function, in that it activates the conversion of prothrombin to thrombin in the process of blood coagulation. A deficiency of this component would interfere with the conversion. The phospholipids assure permeability of the cellular membranes as far as ions are concerned, and also appear to play a role in the transport of metals, according to Leulier *et al.*⁹² These workers found that brain lipids contain about 0.5% of sodium and potassium. In the absence of phospholipids, obviously this function is disturbed. Furthermore, the phospholipids appear to be the factor in some animal sera which is capable of inactivating certain types of virus. This was found to be true of the lecithin-like and cephalin-like fractions of serum lipids in psittacosis (*Bacterium psittacosis*).⁹³ Elson⁹⁴ showed that certain phospholipids have both an inhibitory and a "killing" action on the effect of propamidine (4:4'-diamidino-1:3-diphenoxypropane) on disease organisms; the sub-

⁹² A. Leulier, B. Drevon, and A. Bernard, *Compt. rend. soc. biol.*, 142, 1131-1134 (1948).

⁹³ J. P. Utz, *Proc. Soc. Exptl. Biol. Med.*, 69, 186-189 (1948).

⁹⁴ W. O. Elson, *J. Biol. Chem.*, 154, 717-718 (1944).

stance is effective in the chemotherapy of wounds. This action can be reversed by certain phospholipids. For example, soya lecithin protected *Staphylococcus aureus* against the inhibitory effect of propamidine.

(2) *The Effect of Choline Deficiency*

In the absence of sufficient choline in the diet, the oxidative metabolism is impaired. According to Abdon and Borglin,⁹⁵ the oxygen consumption of the muscle tissue of choline-deficient animals is decreased to two-thirds of its normal value. When choline is added *in vitro* no improvement is noted, but when it is injected *in vivo* it is able to correct the impaired function. It is believed that choline or its methyl groups are used in the formation of an unknown coenzyme. According to Artom,⁹⁶ choline also plays a role in the oxidation of the long-chain fatty acids. When palmitate-1-C¹⁴ was fed to choline-deficient rats, the rate of oxidation was lower than was the case when choline was injected before the animals were sacrificed. This suggests that the choline accelerates the oxidation of long-chain fatty acids as well as that of octanoic acid. It is also possible that the lipotropic action of choline may be due at least in part to the increased catabolism of fatty acids in the liver. Handler and Bernheim⁹⁷ demonstrated that, when choline was absent from the diet of young rats for only one week, a systematic hypertension developed.⁹⁷ On the other hand, the hypertension of partially nephrectomized rats disappeared when they were fed a choline-deficient diet, while it was promptly restored by the administration of ACTH.⁹⁸ Baxter and Campbell⁹⁹ demonstrated that the renal lesions and the mortality, in the case of animals given a purified diet deficient in choline, could be largely prevented by supplementing the diet with rather high levels of crystalline aureomycin, this also appeared to reduce the extent of the fatty infiltration of the liver. These effects produced by aureomycin were apparently not due to changes in food consumption, and did not appear to involve the endocrine systems. Hartcroft *et al.*¹⁰⁰ reported that lesions occurred in the aortas, the carotid and the coronary arteries of young rats which had been maintained on low-choline diets for periods up to 216 days. These initial lesions, as identified microscopically, involved the endothelial cells of the intima. In later stages a

⁹⁵ N. O. Abdon and N. E. Borglin, *Nature*, 158, 793-794 (1946).

⁹⁶ C. Artom, *XIX Intern. Physiol. Congr., Montreal*, Aug.-Sept., 1953, *Abst.*, 176-177.

⁹⁷ P. Handler and F. Bernheim, *Am. J. Physiol.*, 162, 189-192 (1950).

⁹⁸ P. Handler and F. Bernheim, *Am. J. Physiol.*, 162, 375-378 (1950).

⁹⁹ J. H. Baxter and H. Campbell, *Proc. Soc. Exptl. Biol. Med.*, 80, 415-419 (1952).

¹⁰⁰ W. S. Hartcroft, J. H. Ridout, E. A. Sellers, and C. H. Best, *Proc. Soc. Exptl. Biol. Med.*, 81, 384-393 (1952).

proliferation of intimal cells took place, so that small plaques resulted. It is not known whether or not this is applicable to atheroma in man.

Guggenheim and Diamant¹⁰¹ found that the livers of choline-deficient rats maintained on either a low-fat (5%) or a high-fat (20%) diet showed a reduction of the pitressin-inactivating capacity. Diuresis was also retarded, regardless of the amount of protein in the diet. Supplementation of the choline-deficient diet with either vitamin B₁₂ (60 and 1000 μ g./kg. of ration) or aureomycin (50 mg./kg. of ration) did not reduce the fat content of the liver. However, the pitressin-inactivating capacity of the liver was significantly increased by the addition of vitamin B₁₂ to the low-fat diet, and also to the high-fat ration when the amount of 1000 μ g./kg. of ration was given. Aureomycin added to the high-fat, choline-deficient diet was equally effective. It appears, therefore, that the antilipotropic effect of choline deficiency does not necessarily accompany impairment of the pitressin-inactivating capacity of the liver.

¹⁰¹ K. Guggenheim and E. J. Diamant, *J. Nutrition*, 57, 249-260 (1955).

CHAPTER VI

THE ROLE OF ACETIC ACID, FORMIC ACID, AND PROPIONIC ACID IN THE INTERMEDIARY METABOLISM OF FATS

The metabolism of acetic and formic acids plays a commanding role in the intermediary metabolism of fats and of other foodstuffs. It would appear to be more appropriate to consider the metabolism of these fatty acids apart from the general discussion of fatty acid metabolism rather than to attempt to correlate this information with the general pattern of synthesis and oxidation of fat.

1. Acetic Acid and Acetate

Acetic acid plays a key role in the metabolic processes of the animal body. On the one hand, it serves as the main building stone in the synthesis of long-chain fatty acids. On the other hand, it is the fragment which is broken off from the fatty acid chain by β -oxidation. In the presence of carbohydrate, the acetate, so formed, combines with oxaloacetate to become incorporated into the tricarboxylic acid cycle, to complete the oxidation of the fatty acid to CO_2 and H_2O . In the absence of carbohydrate, two acetate molecules combine to form the ketone bodies.

In addition to these all-important functions in the synthesis and oxidation of fatty acids, acetic acid is known to be built up into the steroid nucleus (see Chapter VIII). Another important function, which it serves, is that of acetylation, whereby it reduces the toxicity and physiologic activity of the amines. It may also function in the synthesis of porphyrin and uric acid, while it can form glucose to a limited extent. There is considerable evidence to suggest that, in all these reactions, acetic acid is present in a biologically active form, which has been identified as acetyl CoA (see Chapter II). Bloch¹ has written a comprehensive review of all phases of the metabolism of acetic acid.

¹ K. Bloch, *Physiol. Revs.*, 27, 574-620 (1947).

(1) The Occurrence of Acetic Acid in the Animal Body

The presence of the acetate molecule in the tissues has been generally accepted since the development of the theory of β -oxidation. As a result of the demonstration by Knoop² that phenyl-substituted fatty acids are oxidized to benzoic or to phenylacetic acids, one must conclude that the acetic acid would be a normal by-product of this reaction. The supposition is that two-carbon compounds, presumably molecules of acetic acid, are successively split off as oxidation proceeds. The results of Dakin³ likewise supported this hypothesis.

In spite of the fact that acetic acid is believed to be continuously formed as a result of metabolic reactions, the quantities in the blood are barely detectable.⁴ This acid has been recognized as a normal constituent of horse urine⁵ and of human urine.^{6,7} In fact, Proust⁷ recognized its presence as early as 1800. The failure to demonstrate the presence of acetic acid in tissues may be due to the fact that it is so rapidly metabolized. Smyth⁸ reported that only minimal amounts of acetic acid are excreted in the urine after the intravenous injection of acetate, and that this excretion accounts for only a small proportion of the acetate disappearing from the blood stream. The liver was shown to be the tissue most active in destroying the acetate, while the kidney is no more important than other extrahepatic organs in acetate metabolism. Acetate is used to some extent by muscle,⁸ as well as by the beating heart.⁹ Another suggestion to explain the failure to demonstrate acetic acid in tissues is that it may be bound with protein.^{10,11}

The volatile acids, which include propionic and butyric acid, as well as acetic, are present in the highest amount in the cecum and colon of the horse, pig, and rabbit; in the sheep, which is a ruminant, an even greater amount is present in the reticulum and rumen. Thus, Barcroft and associates¹² found the following average weights for volatile acids in the

² F. Knoop, *Beitr. chem. Physiol., Pathol.*, **6**, 150-162 (1905).

³ H. D. Dakin, *J. Biol. Chem.*, **6**, 203-219, 221-233 (1909).

⁴ J. F. McClendon, *J. Biol. Chem.*, **154**, 357-360 (1944).

⁵ C. Schotten, *Z. physiol. Chem.*, **7**, 375-383 (1882-1883).

⁶ J. L. W. Thudichum, *J. Chem. Soc.*, **23**, 400-409 (1870); *Arch. ges. Physiol.* (Pflüger's), **15**, 12-26 (1877).

⁷ M. Proust, *Ann. chim.* (ser. 1), **36**, 258-277 (1800); D. L. Proust, *Ibid.* (ser. 2), **14**, 257-288 (1820).

⁸ D. H. Smyth, *J. Physiol.*, **105**, 299-315 (1947).

⁹ J. Barcroft, R. A. McAnally, and A. T. Phillipson, *Biochem. J.*, **38**, iv (1944).

¹⁰ A. L. Lehninger, *J. Biol. Chem.*, **143**, 147-157 (1942).

¹¹ E. M. MacKay, personal communication to A. L. Lehninger, *J. Biol. Chem.*, **143**, 147-157 (1942), p. 150.

¹² J. Barcroft, R. A. McAnally, and A. T. Phillipson, *Biochem. J.*, **38**, ii (1944).

cecum and colon: sheep (56 to 86 kg.), 7.9 to 9.1 g.; horse (458 to 662 kg.), 249 to 355 g.; pig (19 to 21 kg.), 2.3 to 8.7 g.; rabbit (approximately 1 kg.), 0.21 to 0.67 g. In the case of the sheep, the diverticulum of the stomach, the rumen, and the reticulum contained from 26 to 91 g. of volatile acids. The concentration of these acids was negligible in the stomach of the horse and rabbit, but small amounts were found in the stomach of the pig, and greater concentrations in that of the sheep. In the latter animals, the various parts of the gastrointestinal tract were found to have the following volatile fatty acid content: rumen and reticulum, 6.0 to 9.7 g. %; omasum, 1.1 to 2.1 g. %; abomasum, 0.1 to 0.2 g. %. Thus, the volatile acids disappear from the ingesta before they pass into the glandular portion of the stomach, namely, the abomasum. The concentration of volatile acids has usually been found to be negligible in the small intestine. It is believed that the volatile acids originate from the fermentation of cellulose or of hemicellulose.¹³ According to Gray and Pilgrim,¹³ only a small portion of the butyric acid arises directly from this fermentation. Another fraction originates by synthesis of the acetate also produced, and some may be derived from the protein fraction of the fodder. Moyle and Baldwin¹⁴ demonstrated the presence of acetic, propionic, and butyric acids, as well as of traces of formic acid, in the perienteric fluid of the round-worm (*Ascaris lumbricoides*). An optically active 2-methylbutyric acid was also found to be present.

However, the low concentration of acetic acid in tissues other than those of the gastrointestinal tract has been cited as evidence against the β -oxidation theory.¹⁵ Bloch¹ is of the opinion that the failure to detect acetic acid in the tissues is not an argument against the theory of its action as an intermediate. The condition which must rather be satisfied, to establish it as a metabolite lying on the main metabolic path, is the capacity of the tissues to metabolize it as rapidly as it is formed. Under such circumstances, its concentration at any one time will not be relevant. There are many commonly accepted intermediates in the catabolism of carbohydrate or protein which are present in concentrations as low as that of acetic acid; in spite of the fact that the demonstration of the former compounds in normal tissues is not experimentally feasible, they have been generally regarded as normal intermediates.

One method for establishing the occurrence of a metabolite, which ordinarily does not accumulate in sufficient amounts to permit analytical

¹³ F. V. Gray and A. F. Pilgrim, *Nature*, 170, 375-376 (1952).

¹⁴ V. Moyle and E. Baldwin, *Biochem. J.*, 51, 504-510 (1952).

¹⁵ W. C. Stadie, *Physiol. Revs.*, 25, 395-441 (1945).

detection, is by the use of the isotope dilution technic. When an isotopically marked analogue of the metabolite under consideration is administered to an animal, or is added to an isolated system, and the isotopically marked atoms are subsequently recovered in a free or combined state, the test substance will be diluted with unlabeled compound in proportion to that which is available in the tissues. Thus, Bernhard¹⁶ reported that, when foreign amines such as sulfanilamide and deuterioacetic acid were given to rabbits, the acetylated sulfanilamide excreted contained only 12% of the labeled acetate. In tests on human subjects, it was found that 9% of the acetyl excreted was derived from the deuterioacetic acid fed. The conclusion was that the fraction of acetyl groups contributed by exogenous acetate was in proportion to its contribution to the total acetate available. The acetyl precursor, therefore, was a mixture of endogenous acetate and the labeled acetate which had been fed. A similar approach was made by Bloch and Rittenberg¹⁷ in determining the proportion of isotope present in acetylamine ($\text{CH}_3\cdot\text{CONH}_2$) in the intact animal after the injection of deuterioacetic acid. The results obtained justified the conclusion that the acetylation process can be attributed to a merging of labeled dietary acetate and that normally arising during metabolism. On the basis of the application of the equation for isotope dilution,¹⁸ the quantity of acetate formed in the rat over a twenty-four-hour period was calculated to be 15 to 20 mM (0.9 to 1.2 g.) per 100 g., or approximately 1% of the body weight.¹⁷ The accuracy of this figure is based upon the assumption that ingested acetic acid mixes adequately with the acetate in the metabolic pool. Moreover, the correctness of the figure is supported by the fact that the isotope concentration of excreted acetyl was found to be directly proportional to the dosage of isotopic acetate; this would indicate that the concentration of acetate calculated as available *in vivo* was independent of the dosage variation over a wide range. Thus, there was a rapid merging of the ingested (exogenous) acetate with the acetic acid formed in the tissues (endogenous).

Lorber *et al.*¹⁹ tested the acetate concentration in the intact rat, by perfusion of the isolated heart with labeled acetic acid, and obtained figures which correspond closely with those obtained by the isotope dilution method. By means of calculations of the acetate required for fatty acid regeneration over given periods, as determined by the method of Bernhard

¹⁶ K. Bernhard, *Z. physiol. Chem.*, **267**, 91-98 (1940).

¹⁷ K. Bloch and D. Rittenberg, *J. Biol. Chem.*, **159**, 45-58 (1945).

¹⁸ D. Rittenberg and G. L. Foster, *J. Biol. Chem.*, **133**, 737-744 (1940).

¹⁹ V. Lorber, N. Lifson, H. G. Wood, and J. Barcroft, *Am. J. Physiol.*, **145**, 557-560 (1946).

and Bullet,²⁰ it is possible to estimate how much acetate should arise if it is assumed that the fatty acids are the sole cause of acetic acid. The results agree closely with those obtained by the isotope dilution technic.¹

One criticism of the use of the isotope dilution method, based upon the concentration of isotopically-labeled acetylamine, for determining the endogenous acetic acid production, is that this may account for only the hepatic acetate. According to Klein and Harris,²¹ the acetylation reaction is confined to the liver, and only the acetate formed outside the liver which escapes oxidation at the site of synthesis may be expected to be included in the hepatic acetate pool. That extrahepatic oxidation of fat takes place is indicated by many experimental facts, such as R.Q. studies of hepatectomized animals and of heart-lung preparations. For a review of this subject, see page 194.

(2) Sources of Acetic Acid in the Animal Body

a. Formation from Even-Chain Fatty Acids. There is very little direct evidence in the literature to substantiate the fact that acetic acid arises in the oxidation of fats. Leloir and Muñoz²² did detect acetic acid in the course of oxidation of octanoate by the liver, but the amounts were insignificant in comparison with the quantity of acetoacetate. Acetoacetate has been demonstrated on feeding the even-chain fatty acids to fasted rats,^{23,24} and the quantities available per molecule of fatty acids are progressively increased with a lengthening of the carbon chain. Similar results have been reported when fatty acids are metabolized by liver slices.²⁵⁻²⁸ These results might indicate that acetoacetate rather than acetate is the final product which originates from fatty acids.

However, a number of experimental facts prove that a two-carbon fragment, presumably acetate, is the primary degradation product of fatty acids. For example the change of stearic acid $\rightarrow$ palmitic acid has been demonstrated *in vivo* unequivocally by Schoenheimer and Rittenberg.²⁹

²⁰ K. Bernhard and F. Bullet, *Helv. Chim. Acta*, **26**, 1185-1189 (1943).

²¹ J. R. Klein and J. S. Harris, *J. Biol. Chem.*, **124**, 613-626 (1938).

²² L. F. Leloir and J. M. Muñoz, *Biochem. J.*, **33**, 734-736 (1939).

²³ H. J. Deuel, Jr., L. F. Hallman, J. S. Butts, and S. Murray, *J. Biol. Chem.*, **116**, 621-639 (1939).

²⁴ J. S. Butts, C. H. Cutler, L. Hallman, and H. J. Deuel, Jr., *J. Biol. Chem.*, **109**, 597-613 (1935).

²⁵ M. Jowett and J. H. Quastel, *Biochem. J.*, **29**, 2159-2180 (1935).

²⁶ P. P. Cohen, *J. Biol. Chem.*, **119**, 333-346 (1937).

²⁷ J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **27**, 1753-1762 (1933).

²⁸ A. L. Lehninger, *J. Biol. Chem.*, **157**, 363-381 (1945).

²⁹ R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **120**, 155-165 (1937).

Their data, likewise, indicated that the change, palmitic acid $\rightarrow$ myristic acid, occurs in tissues.¹ In both of these reactions, a two-carbon fragment is broken off. Likewise, Weinhouse and collaborators,³⁰ who investigated the oxidation of carboxyl-labeled octanoic acid, demonstrated that the acetoacetate formed contained C¹³ in both the carboxyl and the carbonyl group; these data could only be interpreted as indicating that the acetoacetate had originated by random conjugation of two C₂ fragments which had been the primary degradation products.

A more direct proof of the origin of acetic acid in fatty acid oxidation has been obtained by the use of the acetylation reaction. Since the acetyl group, CH₃·CO, is readily derived from acetic acid, its presence affords an excellent criterion as to the availability of the acetate molecule. Bloch and Rittenberg³¹ demonstrated that, when deuteriobutyric acid, deuterio-*n*-valeric acid, alanine, and deuteriomyristic acid were fed, deuterioacetyl groups were present in the acetylated amines. On the other hand, tagged propionic acid and undecylic acids failed to yield acetyl groups. The results were in harmony with the assumption that β -oxidation occurs, and that the acetyl groups originate on removal from the β -keto acids.

b. Formation from Odd-Chain Fatty Acids. The odd straight-chain fatty acids are ordinarily not found in animal tissues, but they can be oxidized as readily as the even-chain acids. In contradistinction to other odd-chain acids with more than three carbons, propionic acid cannot form ketone bodies; it is also a source of glycogen, and it exhibits other properties in harmony with this behavior. Bloch and Rittenberg³¹ likewise support this viewpoint, since propionic acid was shown to be incapable of yielding acetyl groups for the acetylation of phenylaminobutyric acid. This latter fact also indicates that propionic acid is not metabolized *via* pyruvic acid, inasmuch as the keto acid was found to be a potent source of the acetyl radicle.

The experiments with *n*-valeric acid (CH₃·CH₂·CH₂·CH₂·COOH), in which the acid was tagged with deuterium, indicate that the acid gives rise to acetic acid to the extent of one molecule.³¹ The remaining three-carbon fragment is the source of glucose.^{32,33} On the other hand, isovaleric acid ((CH₃)₂:CH·CH₂·COOH) was shown by Cohen,²⁶ by the use of liver slices, to be a much more potent source of acetate than was its normal

³⁰ S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **155**, 143-151 (1944).

³¹ K. Bloch and D. Rittenberg, *J. Biol. Chem.*, **155**, 243-254 (1944).

³² J. S. Butts, H. Blunden, W. Goodwin, and H. J. Deuel, Jr., *J. Biol. Chem.*, **117**, 131-133 (1937).

³³ H. J. Deuel, Jr., J. S. Butts, L. F. Hallman, and C. H. Cutler, *J. Biol. Chem.*, **112**, 15-23 (1935-1936).

isomer. In harmony with this observation, isovalerate was found to give rise to more acetyl groups than was the case with *n*-valerate.³⁴ Undecylic acid, labeled with deuterium on carbons 10 and 11, did not yield acetyl.³¹

In general, the odd-carbon acids are believed to be degraded by β -oxidation to a C₃ (propionic acid) fragment, which can be converted to carbohydrate,³⁵ and to a two-carbon unit which can combine to form acetoacetate.³⁶ However, the resulting ketonuria will be determined by the relative proportion of ketogenic and ketolytic molecules. The longer the odd-chain acid, the greater will be the proportion of acetoacetate formed in comparison with the carbohydrate originating from propionate. This is in harmony with the observation that practically no ketonuria results in fasting rats fed valeric acid, and only small amounts obtain after sodium heptanoate is fed.^{23,24}

c. Formation from Pyruvate. Although a number of investigators have demonstrated the conversion of pyruvic acid to acetic acid both aerobically^{37,38} and anaerobically by dismutation,^{39,40} this does not appear to be the main pathway of breakdown, as the acetic acid is oxidized very slowly. The oxidation product of pyruvate is in all probability a two-carbon compound more active than acetate. A phosphoroclastic splitting of pyruvate into acetylphosphate and formate, or CO₂ + H₂O, which Lipmann⁴¹ observed in *Bacillus acidificans longissimus* (*Lactobacillus delbrueckii*) from fermenting mash, would meet the qualifications. This type of splitting has not been demonstrated in animal tissue.

There are several examples of the accumulation of acetate following the metabolism of pyruvate. Thus, Barron *et al.*⁴² reported the formation of acetate when kidney or liver slices oxidized pyruvate in the presence of fluoroacetate. It was suggested that acetate fails to be oxidized because of competition of fluoroacetate with the active enzyme.⁴³ Bloch¹ suggests that fluoroacetate may exert its inhibiting effect by blocking condensations involving the reactive acetyl compound, which then becomes stabilized as

³⁴ K. Bloch, *J. Biol. Chem.*, **155**, 255-263 (1944).

³⁵ G. Lusk, *The Elements of the Science of Nutrition*, 4th ed., Saunders, Philadelphia and London, 1928, pp. 219, 660.

³⁶ E. M. MacKay, A. N. Wick, and C. P. Barnum, *J. Biol. Chem.*, **136**, 503-507 (1940).

³⁷ H. A. Krebs and W. A. Johnson, *Biochem. J.*, **31**, 645-660 (1937).

³⁸ C. Long, *Biochem. J.*, **32**, 1711-1717 (1938).

³⁹ H. Weil-Malherbe, *Biochem. J.*, **31**, 2202-2216 (1937).

⁴⁰ F. Lipmann, *Skand. Arch. Physiol.*, **76**, 255-272 (1937).

⁴¹ F. Lipmann, *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 248-259 (1939).

⁴² E. S. G. Barron, G. R. Bartlett, and G. Kalnitsky, *Federation Proc.*, **5**, 120-121 (1946).

⁴³ G. R. Bartlett and E. S. G. Barron, *J. Biol. Chem.*, **170**, 67-82 (1947).

acetate. However, Kalnitsky⁴⁴ reported that, in the presence of oxaloacetate, a marked accumulation of citrate in the fluoroacetate-poisoned kidney took place, without the accumulation of acetate. Liébecq and Peters⁴⁵ likewise found increased citrate without an accumulation of acetate when fumarate was present. Elliott and Kalnitsky⁴⁶ reported that the inhibition of acetate synthesis was reversed by oxaloacetate. It was suggested that the inhibition of acetate is the result of the formation of fluorocitrate, which inhibits citrate oxidation.

As a parallel to the possible transformation of pyruvate to acetic acid, one may cite the evidence of the formation of a closely related compound, namely acetoacetate, from pyruvate⁴⁷ and from fatty acids as well, under certain conditions. Embden and collaborators⁴⁸⁻⁵⁰ found that acetone bodies were formed when the livers of healthy dogs were perfused with butyric, caproic, and isovaleric acids, and that the level of acetone production was raised in the livers of phlorhizin-treated or depancreatized dogs⁵¹ when they were perfused with beef blood; acetone bodies were increased in the livers of normal dogs following perfusion with beef and dog blood.⁵² Gorr and Wagner⁵³ noted the presence of acetone bodies in liver brei when pyruvate was added to the digest. According to Annau,⁵⁴ the formation of acetoacetate from pyruvate is to be ascribed to the condensation of two pyruvate molecules, with subsequent decarboxylation and oxidation. It has been shown that acetoacetate accumulates in amounts up to 25-28% of the pyruvate oxidized,⁵⁵ when liver slices break down pyruvate in the presence of malonate. Ammonium chloride also increases the production of acetoacetic acid from pyruvate.^{54,56}

Moreover, pyruvate is quantitatively converted to acetoacetate by the washed-cell liver preparation of Lehninger.^{28,57} When fumarate was added

⁴⁴ G. Kalnitsky, *J. Biol. Chem.*, **179**, 1015-1025 (1949).

⁴⁵ C. Liébecq and R. A. Peters, *J. Physiol.*, **108** (*Proc. Physiol. Soc.*) 11P, June 1948; *Biochim. et Biophys. Acta*, **3**, 215-230 (1949).

⁴⁶ W. B. Elliott and G. Kalnitsky, *J. Biol. Chem.*, **186**, 487-493 (1950).

⁴⁷ G. Embden and M. Oppenheim, *Biochem. Z.*, **45**, 186-206 (1912); **55**, 335-340 (1913).

⁴⁸ G. Embden and A. Marx, *Beitr. chem. Physiol. Pathol.*, **11**, 318-322 (1908).

⁴⁹ G. Embden and H. Engel, *Beitr. chem. Physiol. Pathol.*, **11**, 323-326 (1908).

⁵⁰ G. Embden, H. Salomon, and F. Schmidt, *Beitr. chem. Physiol. Pathol.*, **8**, 129-155 (1906).

⁵¹ G. Embden and L. Lattes, *Beitr. chem. Physiol. Pathol.*, **11**, 327-331 (1908).

⁵² G. Embden and F. Kalberlah, *Beitr. chem. Physiol. Pathol.*, **8**, 121-128 (1906).

⁵³ G. Gorr and J. Wagner, *Biochem. Z.*, **254**, 5-7 (1932).

⁵⁴ E. Annau, *Z. physiol. Chem.*, **224**, 141-149 (1934).

⁵⁵ E. A. Evans, Jr., *Biochem. J.*, **34**, 829-837 (1940).

⁵⁶ N. L. Edson, *Biochem. J.*, **29**, 2082-2094 (1935).

⁵⁷ A. Lehninger, *J. Biol. Chem.*, **165**, 131-145 (1946).

to the washed-cell preparations, acetoacetate formation was suppressed in favor of a greater production of di- and tricarboxylic acids. Since the *in vitro* conditions which affected the relative yields of acetoacetate and polycarboxylic acids were the same for octanoic acid and for pyruvic acid, it was believed that an acetyl-like C_2 compound was common to both fats and carbohydrates.

Bloch¹ points out that, in spite of the evidence from *in vitro* tests that pyruvic acid under certain conditions is a source of ketone bodies, this reaction is contrary to known data obtained with intact animals. Pyruvate has the ability to yield glycogen and to suppress ketonuria in the fasting rat,⁵⁸ whereas even-chain fatty acids provoke a ketonuria. Under such conditions the catabolites of fat and pyruvate do not follow a single pathway. The derivation of acetoacetate from pyruvate may be a favored reaction under special conditions *in vitro*; however, if it occurs at all, it is quantitatively insignificant in the pyruvate metabolism of the intact animal. Bloch¹ concludes that "The hypothesis that a C_2 fragment arises in the oxidation of both carbohydrate and fat would seem to be untenable unless it is assumed that the C_2 fragments are not identical."

There are a number of reactions in which the behavior of pyruvate differs from that of fatty acids. Thus, whereas acetate serves as an excellent source of the acetyl group both for aromatic amines (such as sulfanilamide) and for α -amino acids (such as phenylaminobutyric acid), pyruvate, when administered as tagged alanine, can acetylate α -amino acids only.¹⁷ Another difference is that pyruvate (alanine) does not serve as a building stone for cholesterol either *in vivo*¹⁷ or *in vitro*.⁵⁹ An additional variation between the physiological behavior of acetate and that of lactate (which is in equilibrium with pyruvate) is that acetate may serve as the source of carbons 2 and 8 in the purine nucleus, while lactate cannot be incorporated into the uric acid molecule. Thus, if pyruvic acid or lactic acid were converted to the same C_2 fragment as originates from acetate, then should show identical reactions in the acetylation of aromatic amines and in the synthesis of cholesterol and uric acid. Since this does not occur, one must conclude that no important part of pyruvate metabolism proceeds by way of the acetyl group.

d. Formation from Amino Acids. It is generally recognized that α -amino acids are usually deaminated with the formation of an α -keto acid; this is followed by decarboxylation, with the ultimate formation of a fatty acid having one less carbon than the original amino acid. This non-nitrogenous moiety is subsequently oxidized in a manner similar to that of

⁵⁸ I. Shapiro, *J. Biol. Chem.*, 108, 373-387 (1935).

⁵⁹ D. Rittenberg, E. Borek, and K. Bloch, *Federation Proc.*, 5, 151 (1946).

ordinary fatty acids. Bloch⁵⁴ reported that leucine and isovaline both yielded acetyl groups to the same degree, thereby indicating that isovaline is an intermediate in leucine catabolism. On the other hand, valine and isobutyric acids, which are non-ketogenic, were likewise found to be ineffective as sources of the acetyl group.

(3) *Acetylation Reactions in the Animal Body*

Acetylation is a process by which the acetyl group is introduced into a molecule. It occurs in the case of many amines before their elimination from the body. It is generally considered to be a method of detoxication, whereby the body tends to modify foreign substances in such a way as to tender them less harmful to the cell. This may be accomplished, either by increasing their solubility in biologic fluids, thus preventing deposition in the tissues, or by their conversion to substances which have less affinity for enzyme systems.

However, in some cases, as that of acetylsulfanilamide, the acetylated compound has a greater toxicity, and is more insoluble than is the original product.⁶⁰ Acetylation, therefore, cannot be considered as a detoxication process,^{61,62} but must rather be classed as one of the intermediary reactions in the metabolism of the product under question. Bloch¹ is of the opinion that "detoxication" reactions are mediated by enzymes, normally present in the tissues, which catalyze reactions with foreign compounds similar to those observed in normally-occurring metabolites. Thus, the acetylation of a foreign amines is brought about by the enzyme which has as its normal function the acetylation of normally-occurring compounds.

In addition to the dependence of the degree of acetylation upon dosage,⁶³ one of the most interesting features of the acetylation reaction is the species difference. For example, rats excrete sulfanilamide partly unchanged, partly as *N*-acetylsulfanilamide, and partly in combination with glucuronic acid after the formation of an hydroxyl compound.¹ No acetylation of aromatic amines occurs in the case of dogs or rabbits.^{1,64} Litchfield⁶⁵ studied the excretion of sulfanilamide in several lower vertebrates, and found that it appeared in the urine in a conjugated form in the fish and chicken, but that no conjugated derivative capable of acid hydrolysis was

⁶⁰ E. K. Marshall, Jr., W. C. Cutting, and K. Emerson, Jr., *J. Am. Med. Assoc.*, **110**, 252-257 (1938).

⁶¹ J. A. Stekol, *Ann. Rev. Biochem.*, **10**, 265-284 (1941).

⁶² P. Handler and W. A. Perlzweig, *Ann. Rev. Biochem.*, **14**, 617-642 (1945).

⁶³ H. G. Bray, F. C. Neale, and W. V. Thorpe, *Biochem. J.*, **40**, 406-413 (1946).

⁶⁴ R. Cohn, *Z. physiol. Chem.*, **17**, 274-310 (1893).

⁶⁵ J. T. Litchfield, *J. Pharmacol.*, **67**, 212-223 (1939).

excreted by the frog. According to Marshall alone⁶⁶ and with others,⁶⁷ the dog excretes *p*-aminobenzenesulfonamide (sulfanilamide) in an unchanged form, whereas in rabbits it is excreted mainly as acetylamino-benzenesulfonamide. Mice excrete the unchanged form,⁶⁸ although Marshall⁶⁶ found that a conjugated form was also present in the urine. This conjugated form, probably acetylsulfanilamide, was excreted by the fish, chicken, rat, guinea pig, cat, pig, cow, horse, and monkey, as well as by the human subject and rabbit.⁶⁶ As much as one-half of the sulfanilamide excreted by man may be in the form of the acetylated compound.⁶⁹ The acetylation reaction of sulfanilamide is given below:

Acetylation of Sulfanilamide⁶⁹

In the case of sulfapyridine, a species variation in metabolism has not been noted. The drug may be excreted in the free form, as its acetyl derivative, or it may be oxidized to a hydroxy derivative which is excreted either free, acetylated, or conjugated with sulfuric or glucuronic acids. The occurrence of oxidation was first demonstrated by Seudi⁷⁰ in the dog, with the isolation of hydroxysulfapyridine; apparently a similar reaction obtains in the rat, since an increased glucuronic acid excretion was noted in this species after the administration of sulfapyridine.⁷¹⁻⁷³ Finally, Bray *et al.*⁶³ isolated an hydroxy derivative from the urine of rabbits fed sulfapyridine which is believed to be similar to the product isolated from the urine of the dog after the aforementioned drug was administered.

⁶⁶ E. K. Marshall, Jr., *Physiol. Revs.*, **19**, 240-269 (1939).

⁶⁷ E. K. Marshall, Jr., K. Emerson, Jr., and W. C. Cutting, *J. Am. Med. Assoc.*, **108**, 953-957 (1937).

⁶⁸ A. T. Fuller, *Lancet*, **1937**, *I*, 194-198.

⁶⁹ E. S. West and W. R. Todd, *Textbook of Biochemistry*, Macmillan, New York, 1955, p. 512.

⁷⁰ J. V. Seudi, *Science*, **91**, 486 (1940).

⁷¹ J. V. Seudi and H. J. Robinson, *Am. J. Med. Sci.*, **201**, 711-717 (1941).

⁷² C. J. Weber, J. J. Lulich, and R. H. Major, *Proc. Soc. Exptl. Biol. Med.*, **53**, 190-192 (1943).

⁷³ J. V. Seudi, *Proc. Soc. Exptl. Biol. Med.*, **55**, 197-199 (1944).

The ability of the animal body to bring about an acetylation is dependent upon the presence of an adequate supply of CoA. Thus, Shils *et al.*⁷⁴ reported that, in rats whose tissues had a subnormal CoA content as a result of a diet deficient in pantothenic acid, the acetylation of sulfanilamide was markedly depressed, although this function remained normal in thiamine deficiency. Riggs and Hegsted⁷⁵ noted that the acetylation of *p*-aminobenzoic acid was dramatically reduced in rats suffering from pantothenic acid or riboflavin deficiency; some reduction was also observed when the amount of thiamine was deficient.

a. Acetylation of Amino Acids and Amides. Knoop⁷⁶ originally believed that acetylation was a normal process in the metabolism of amino acids. He based this conclusion upon the following series of experiments: When DL-phenylaminobutyric acid ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$) was fed to dogs, the optically active acetyl compound, acetyl-D-phenylaminobutyric acid ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHNH}(\text{COCH}_3) \cdot \text{COOH}$), appeared in the urine.⁷⁷ After the corresponding keto compound ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{COOH}$) was fed, the same acetyl compound was recovered from the urine as had been excreted after the ingestion of the racemic amino acid. On the other hand, when acetyl-DL-phenylaminobutyric acid was administered to dogs, an excess of acetyl-L-phenylaminobutyric acid appeared in the urine.⁷⁸ This showed that the body was able to oxidize the acetyl-D compound better than the acetyl-L-acid, and the conclusion was drawn that the dextrorotatory amino acid was probably the one corresponding in spatial configuration to the naturally occurring amino acid series.

In a further consideration of these results, Knoop and Blanco⁷⁸ reasoned that the DL-amino acid could not have been directly acetylated, or the acetyl-L acid would have appeared in the urine, rather than the acetyl-D compound which was actually found. Knoop was of the opinion that the isolation of acetyl-D-phenylaminobutyric acid affords excellent support for the hypothesis that the amino acid is first deaminated to the keto acid; the acetyl-D-phenylaminobutyric acid then arises by asymmetric synthesis. The results obtained with phenylaminoacetic acid ($\text{C}_6\text{H}_5 \cdot \text{CHNH}_2 \cdot \text{COOH}$) were similar to those in the case of the higher homolog.

However, in a continuation of their work, Knoop and Blanco⁷⁸ found that the compound excreted in the urine after the feeding of acetyl-DL-phenylalanine was the levorotatory acetylphenylalanine. These workers inter-

⁷⁴ M. E. Shils, H. M. Seligman, and L. J. Goldwater, *J. Nutrition*, **37**, 227-235 (1949).

⁷⁵ T. R. Riggs and D. M. Hegsted, *J. Biol. Chem.*, **178**, 669-675 (1949).

⁷⁶ F. Knoop, *Z. physiol. Chem.*, **67**, 489-502 (1910).

⁷⁷ F. Knoop and E. Kertess, *Z. physiol. Chem.*, **71**, 252-265 (1911).

⁷⁸ F. Knoop and J. G. Blanco, *Z. physiol. Chem.*, **146**, 267-275 (1925).

preted the data as indicating that, in this instance, the animal could utilize the acetylated derivative of the unnatural isomer more readily than that of the natural isomer. On the basis of these results, Knoop and Blanco were led to abandon the theory that acetylation is a normal intermediary process in the metabolism of amino acids.

du Vigneaud and Meyer⁷⁹ were able to establish the fact that the acetyl-D-phenylalanine has a specific rotation, $[\alpha]_D^{26} = -51^\circ$, which is identical with that of the compound isolated by Knoop and Blanco. It thus appeared that the acetyl derivative was that of the unnatural form of phenylalanine. Subsequently, du Vigneaud and Irish⁸⁰ proved that the dog can oxidize the natural acetyl-L-phenylalanine more readily than acetyl-D-phenylalanine. Since this obstacle to the acceptance of the original theory of Knoop was removed, du Vigneaud and Irish⁸⁰ were in favor of reestablishing the acetyl theory for the *in vivo* synthesis of an amino acid from the corresponding keto acid. The *in vivo* conversion of an unnatural to a natural amino acid is believed to occur by formation of the keto acid, followed by formation of the natural acetylated amino acid. The intermediary formation of the keto acid in the *in vivo* conversion of D-phenylaminobutyric acid to acetyl-L-phenylaminobutyric acid has been proved by the demonstration that practically all of the nitrogen of the original D-acid is replaced by new nitrogen in the L-acetyl compound.⁸¹ Acetic acid, therefore, becomes a most important intermediate in amino acid metabolism.

Lipmann⁸² reported that acetate is about twice as effective as acetoacetate or pyruvate in effecting acetylation of sulfanilamide, when liver homogenates or extracts are used as the source of the enzyme. The greater effectiveness of acetate over that of the other acetyl precursors is to be expected, since they must necessarily be converted to acetate before they can be active. In addition to the enzyme, adenylyl pyrophosphate is required.

Acetylation proceeds most effectively under aerobic conditions. Thus, Lipmann⁸² found that five times as much acetylsulfanilamide was formed when air was present, as under anaerobic conditions. Inhibitors of oxidation, such as arsenious oxide or iodoacetate, likewise abolish acetylation.¹⁹ Although oxygen is not required in the direct acetylation reaction, it is apparently essential to supply the energy needed for condensation. How-

⁷⁹ V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **98**, 295-308 (1932).

⁸⁰ V. du Vigneaud and O. J. Irish, *J. Biol. Chem.*, **122**, 349-370 (1937).

⁸¹ V. du Vigneaud, M. Cohn, G. B. Brown, O. J. Irish, R. Schoenheimer, and D. Rittenberg, *J. Biol. Chem.*, **131**, 273-296 (1939).

⁸² F. Lipmann, *J. Biol. Chem.*, **160**, 173-190 (1945).

ever, acetylation will proceed under anaerobic conditions if an "energy-rich" phosphate source such as adenylyl pyrophosphate is present.⁸² This may function by bringing about the formation of acetylphosphate.

A different enzyme is required for acetylation of α -amino acids than for sulfanilamide. This enzyme was found to be present in much higher concentration in slices of rat liver than in those of guinea pig or pigeon liver.¹ This enzyme brought about acetylation, not only of L-phenylaminobutyric acid, but also of the natural amino acids, leucine and phenylalanine.⁸³

b. Formation of Mercapturic Acid. When cysteine is employed for the detoxication of bromobenzene, acetylation apparently precedes the formation of *p*-bromophenylmercapturic acid,⁶⁹ which is the end-product excreted in the urine.

Synthesis of *p*-Bromophenylmercapturic Acid

According to Stekol, mercapturic acids are formed after the administration of *p*-bromobenzyl bromide,⁸⁴ monobromobenzene,⁸⁵⁻⁸⁸ naphthalene,⁸⁷⁻⁹¹ anthracene,⁹¹⁻⁹² and benzyl chloride.⁹¹ The synthesis of mercapturic acids has been reported in many species of animals, including mice,⁸⁵ rats,^{87,88,90-94} rabbits,^{87-89,92,93,95-97} cats,⁹⁸ dogs,^{85-89,91,99,100} and swine.^{87,88}

⁸³ K. Bloch and E. Borek, *J. Biol. Chem.*, **164**, 483-484 (1946).

⁸⁴ J. A. Stekol, *J. Biol. Chem.*, **138**, 225-229 (1941).

⁸⁵ J. A. Stekol, *Proc. Soc. Exptl. Biol. Med.*, **33**, 115-119; 119-121 (1935).

⁸⁶ J. A. Stekol, *J. Biol. Chem.*, **117**, 147-159 (1937); **118**, 155-160 (1937).

⁸⁷ J. A. Stekol, *J. Biol. Chem.*, **113**, 675-682 (1936).

⁸⁸ J. A. Stekol, *J. Biol. Chem.*, **121**, 87-91 (1937).

⁸⁹ J. A. Stekol, *J. Biol. Chem.*, **110**, 463-469 (1935).

⁹⁰ J. A. Stekol, *J. Biol. Chem.*, **122**, 333-342 (1938).

⁹¹ J. A. Stekol, *J. Biol. Chem.*, **124**, 129-133 (1938); **128**, 199-205 (1939).

⁹² E. Boyland and A. A. Levi, *Biochem. J.*, **30**, 1225-1227 (1936).

⁹³ N. R. Lawrie, *Biochem. J.*, **25**, 1037-1044 (1931).

⁹⁴ A. White and R. W. Jackson, *J. Biol. Chem.*, **111**, 507-513 (1935).

⁹⁵ E. Abderhalden and E. Wertheimer, *Arch. ges. Physiol. (Pflüger's)*, **207**, 215-221 (1925).

⁹⁶ M. C. Bourne and L. Young, *Biochem. J.*, **28**, 803-808 (1934).

⁹⁷ K. Nishimura, *Acta Schol. Med. Univ. Kioto*, **12**, 73-78 (1929); *Chem. Abst.*, **24**, 410 (1930).

⁹⁸ R. W. Virtue, *Proc. Soc. Exptl. Biol. Med.*, **34**, 425-427 (1936).

⁹⁹ E. Baumann and C. Preusse, *Ber.*, **12**, 806-810 (1879).

¹⁰⁰ M. Jaffe, *Ber.*, **12**, 1092-1098 (1879).

Lawrie⁹³ found that mercapturic acids resulted following the feeding of iodobenzene to rats and rabbits, and Jaffe¹⁰⁰ observed the presence of these acids in the urine of dogs following the administration of chlorobenzene. An apparently analogous selenium mercapturic acid, in which selenium replaced the sulfur of cysteine, was reported in selenium-treated dogs following the administration of bromobenzene.¹⁰¹ A similar phenomenon apparently obtains in selenium-poisoned steers; it was possible to remove selenium practically completely from the blood by feeding bromobenzene. Lemley¹⁰² used bromobenzene feeding in the treatment of a case of selenium poisoning in man, with excellent results.

c. Acetylation of Choline. Acetylcholine, $\text{CH}_3 \cdot \text{CO} \cdot \text{OCH}_2 \cdot \text{CH}_2 \cdot \text{N}^+ : (\text{CH}_3)_3$, is a potent compound, produced at the nerve endings when the nerves are stimulated. As discussed in *The Lipids*, Vol. II, Chap. II, there are several types of cholinesterases which can act on acetylcholine to hydrolyze it and so to dissipate its main physiologic activity. However, to effect the opposite reaction, which will result in the synthesis of acetylcholine, another enzyme system is required. Nachmansohn and Machado¹⁰³ studied the mechanism of the acetylation of choline. The presence of an enzyme in cell-free brain extract, which was called *choline acetylase*, was described by Nachmansohn and collaborators.^{103,104} In the presence of ATP, choline acetylase can convert choline into acetylcholine. Lipmann *et al.*^{105,106} showed that, as in other acetylations, pantothenic acid serves as a coenzyme, presumably by virtue of its presence in CoA (see page 15).

(4) Metabolic Reactions Involving Acetate

Acetic acid is widely used in the synthesis of a variety of essential components in the tissues. Although certain compounds required for the functioning of the tissues, such as essential fatty acids, vitamins, and the so-called essential amino acids, must be furnished as such from exogenous sources, many of the remaining compounds may be synthesized from the condensation of numerous small-sized units such as acetate, rather than by the utilization and rearrangement of preformed large molecules. The more important compounds which are known to originate more or less from

¹⁰¹ A. L. Moxon, A. E. Schaefer, H. A. Lardy, K. P. Du Bois, and O. E. Olson, *J. Biol. Chem.*, **132**, 785-786 (1940).

¹⁰² R. E. Lemley, *J. Lancet*, **60**, 528-531 (1940).

¹⁰³ D. Nachmansohn and A. L. Machado, *J. Neurophysiol.*, **6**, 397-403 (1943).

¹⁰⁴ D. Nachmansohn and H. M. John, *J. Biol. Chem.*, **158**, 157-171 (1945).

¹⁰⁵ F. Lipmann, *Harvey Lectures*, **44**, 99-123 (1948).

¹⁰⁶ F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle, and B. M. Guirard, *J. Biol. Chem.*, **167**, 869-870 (1947).

acetate include glycogen, fatty acids, dicarboxylic acids, cholesterol, protoporphyrin, and uric acid. The demonstration of the synthesis of these compounds from acetate has been made possible by the use of labeled acetic acid. Further information as to the mechanism of synthesis can be gleaned by determining the particular carbon atoms involved, by degradation of the newly synthesized compounds by certain prescribed methods. Thus, by various chemical and microbiological techniques (*Lactobacillus casei*, lactic acid bacillus from milk and cheese), the isotope concentrations of the several carbons in the glucose molecule have been determined.^{107, 108} The portion of the cholesterol molecule affected in the synthetic reaction has been ascertained by degradation of the sterols into the nucleus and side chain.¹⁰⁹

a. Glycogen Synthesis. In discussing the precursors of any substance, according to Bloch,¹ one must differentiate between (1) those which afford a net increase in the quantity of the product, *viz.*, reactions in which the quantity of the precursor is the limiting factor, and (2) reactions in which substances become totally or partly incorporated into a product without affecting the rate of formation.

(a) *The Effect on Total Glycogen.* According to the classical concept, a glycogenic substance is one which increases glycogen deposition in fasted animals, which causes the excretion of "extra sugar" in diabetic animals, or which reduces ketonuria. When measured by any of these yardsticks, acetic acid cannot be classed as a glycogenic compound. Thus, neither Ringer and Lusk¹¹⁰ nor Deuel and Milhorat¹¹¹ could demonstrate any increased D:N ratio in phlorhizinized dogs after the administration of fairly large doses of sodium acetate. Although Geelmuyden¹¹² did report an increased D:N ratio which he interpreted as indicative of glucose formation, the D:N ratio of the dogs in the control period was high, and it is impossible to determine from his data whether or not glucose was being formed.

Although sodium acetoacetate, as well as the saturated even-carbon acids from C₄ to C₁₄, and also oleic acid, are not glycogen formers,³² triacetin was found to serve as a source of liver glycogen.¹¹³ However, all

¹⁰⁷ H. G. Wood, N. Lifson, and V. Lorber, *J. Biol. Chem.*, **159**, 475-489 (1945).

¹⁰⁸ De W. Stetten, Jr., and M. R. Stetten, *J. Biol. Chem.*, **165**, 147-155 (1946).

¹⁰⁹ K. Bloch and D. Rittenberg, *J. Biol. Chem.*, **145**, 625-636 (1942).

¹¹⁰ A. J. Ringer and G. Lusk, *Z. physiol. Chem.*, **66**, 106-119 (1910).

¹¹¹ H. J. Deuel, Jr., and A. T. Milhorat, *J. Biol. Chem.*, **78**, 299-309 (1928).

¹¹² H. C. Geelmuyden, *Skand. Arch. Physiol.*, **40**, 211-225 (1920).

¹¹³ H. J. Deuel, Jr., J. S. Butts, H. Blunden, C. H. Cutler, and L. Knott, *J. Biol. Chem.*, **117**, 119-129 (1937).

evidence points to the fact that the source of the carbohydrate, in this case, is the glycerol of the triacetin molecule and not the acetic acid moiety. Moreover, when acetic acid is fed to fasting rats, it provokes ketonuria, and does not act as a source of ketolytic material.^{114,115} Further evidence that the course of metabolism of glucose is quite independent of that of acetate is obtained from the experiments of Drury and Wick.¹¹⁶ Although the presence of acetate was found to reduce the rate of oxidation (but not of disappearance) of glucose in the insulin-treated, eviscerated rabbit, these factors did not influence the rate of oxidation of the acetate. This is interpreted to mean that neither acetate nor any of its derivatives forms a pool with any derivative of glucose.

(b) *The Effect on the Composition of Glycogen.* It has been demonstrated by Buchanan and Hastings¹¹⁷ and by Wood¹¹⁸ that radioactive carbon from labeled acetate becomes incorporated into glycogen after the test material has been fed. In fact, Lorber, Lifson, and Wood¹¹⁹ demonstrated that radioactive carbon was present in all six carbons of glucose. However, it was found that acetic acid cannot form glycogen spontaneously,¹²⁰ but only as a result of becoming incorporated in the tricarboxylic acid cycle. Under such conditions, it yields pyruvate, which is an obligatory intermediate in glucose synthesis.^{1,118} Strisower *et al.*¹²¹ reported that liver slices from normal rats were able to change acetate-1-C¹⁴ to glucose to the extent of 3 to 6%, while acetate-2-C¹⁴ was transformed to carbohydrate to the extent of 10 to 19%. The extent of incorporation of C¹⁴ into glucose was somewhat augmented in liver slices from diabetic animals; on the other hand, the incorporation of acetate into glucose was greatly reduced by insulin. The C¹⁴O₂ production was two to three times as great with carboxyl-labeled acetate as it was in the case of the methyl-tagged acid. The variations in CO₂ production were not great between normal and diabetic liver slices, but the C¹⁴O₂ recovery from both substrates was reduced by 50% in the livers from the insulin-treated diabetic rats.

b. Fatty Acid Synthesis. Acetic acid is the building stone for both fatty

¹¹⁴ E. M. MacKay, R. H. Barnes, H. O. Carne, and A. N. Wick, *J. Biol. Chem.*, **135**, 157-163 (1940).

¹¹⁵ M. E. Swendseid, R. H. Barnes, A. Hemingway, and A. O. Nier, *J. Biol. Chem.*, **142**, 47-52 (1942).

¹¹⁶ D. R. Drury and A. N. Wick, *J. Biol. Chem.*, **203**, 411-417 (1953).

¹¹⁷ J. M. Buchanan and A. B. Hastings, *Physiol. Revs.*, **26**, 120-155 (1946).

¹¹⁸ H. G. Wood, *Physiol. Revs.*, **26**, 198-246 (1946).

¹¹⁹ V. Lorber, N. Lifson, and H. G. Wood, *J. Biol. Chem.*, **161**, 411-412 (1945).

¹²⁰ N. Lifson, V. Lorber, and H. G. Wood, *Federation Proc.*, **4**, 47 (1945).

¹²¹ E. H. Strisower, G. D. Kohler, and I. L. Chaikoff, *J. Biol. Chem.*, **198**, 115-126 (1952).

acids and cholesterol.¹⁷ This synthesis occurs without the intermediary formation of carbohydrate. The variation in the enzyme systems required to effect these syntheses is indicated by the fact that, in the presence of sulfite, steroid formation is inhibited,¹²² while that of fatty acids is unaffected. It is suggested that acetaldehyde might be the intermediate in cholesterol synthesis, while acetate might condense directly on the fatty acid chain. In the case of yeast, Sonderhoff and Thomas¹²³ noted that deuterioacetate was metabolized in such a way as to indicate that carbohydrate is not an intermediate in the lipid synthesis from acetate. The mechanism of synthesis of fatty acids from acetate and its relation to CoA are discussed at length on pages 10 to 26.

c. Cholesterol Synthesis. In the synthesis of the unsaponifiable fraction in yeast from deuterioacetate, Sonderhoff and Thomas¹²³ noted that the deuterium concentration was twice as high as in the fatty acid fraction, and twenty times that in the yeast carbohydrate. These results are also interpreted to indicate that the synthesis of the yeast steroids proceeds directly. The detailed discussion of the mechanism of cholesterol synthesis is given in Chapter VIII.

d. Protoporphyrin Synthesis. Bloch and Rittenberg¹⁷ reported that hemin isolated from the red blood cells of animals which received deuterioacetate contained appreciable amounts of deuterium. Since the protoporphyrin moiety of hemoglobin is synthesized at a relatively slow rate, the isotope concentration in hemin is of a low order in short-term feeding tests.¹²⁴ However, when the feeding is continued over several weeks, the concentration of deuterium in the hemin reaches a level which is 50% of that in the acetic acid available in the metabolic pool. According to unpublished work of Bloch and Rittenberg cited by Bloch,¹ at least one-half of the hydrogen atoms in the porphyrin molecule originate from acetic acid.

Although the above experiments prove that acetate is a source of carbons in the protoporphyrin molecule, they do not indicate which parts of the molecule are involved. Since no hydrogens are attached to the carbons of the pyrrole rings, it is not possible to determine whether or not acetate enters the pyrrole ring structure. However, carbon-bound hydrogen occurs in the methine bridges which link the pyrrole rings, as well as in the pyrrole side chains. Bloch¹ is of the opinion that the acetate molecule enters into

¹²² I. Smedley-MacLean and D. Hoffert, *Biochem. J.*, **20**, 343-357 (1926).

¹²³ R. Sonderhoff and H. Thomas, *Ann.*, **530**, 195-213 (1937).

¹²⁴ D. Shemin and D. Rittenberg, *J. Biol. Chem.*, **159**, 567-568 (1945); **166**, 621-625 (1945).

the synthesis of the pyrrole rings. Bloch and Rittenberg, in unpublished work cited by Bloch,¹ noted that deuteriopropionate was not incorporated into hemin; on the other hand, butyrate was about one-half as effective as was acetate. The effect of this acid is presumably the result of the formation of acetyl groups.

e. Uric Acid Synthesis. Sonne *et al.*¹²⁵ reported that the carboxyl carbon of acetate was incorporated into the two ureido carbons (2 and 8) of uric acid excreted by the pigeon. On the other hand, the carboxyl of glycine was shown to give rise to carbon 4, while CO₂ was found to be the source of carbon 6 of the purine nucleus. The difference in origin of carbons 2 and 8 (which are derived from acetate) and that of 4 (which originates from glycine) indicates that acetate and glycine are not interconvertible in the pigeon.¹

f. Amino Acid Synthesis. Gilvorg and Bloch¹²⁶ reported that brewery yeast (*Saccharomyces cerevisiae*) is able to utilize acetate as a source of carbon for the amino acids. Thus, when the yeast was grown on a medium containing glucose and labeled acetate, it was found that the isotopic C was present in most of the amino acids with the exception of phenylalanine, tyrosine, and glycine, which were believed to be derived rather directly from glucose. Acetic acid was found to be the major source of the carbon skeleton of lysine. Arnstein and Neuberger¹²⁷ reported that both carbons of acetate appear in the glycine molecule synthesized to detoxicate administered benzoic acid. It has likewise been proved that the carboxyl C of acetate is converted into the dicarboxylic amino acids without first being transformed to CO₂.¹²⁸

g. Miscellaneous Syntheses Other Than *via* the Tricarboxylic Acid Cycle. In addition to the aforementioned specific compounds which originate from acetate, the synthesis of other compounds from acetate was discussed earlier. This includes the ketone bodies (especially acetoacetic acid, but also β -hydroxybutyric acid), and the several polycarboxylic acids of the tricarboxylic acid cycle, such as citric, isocitric, α -ketoglutaric, malic, succinic, fumaric, and oxaloacetic acids. Although the partial synthesis of these several acids by the incorporation of acetic acid into the tricarboxylic acid cycle is generally accepted, there is some evidence of syntheses of these compounds from acetate by other pathways.

¹²⁵ J. C. Sonne, J. M. Buchanan, and A. M. Delluva, *J. Biol. Chem.*, **166**, 395-396 (1946).

¹²⁶ C. Gilvorg and K. Bloch, *J. Biol. Chem.*, **193**, 339-346 (1951).

¹²⁷ H. R. V. Arnstein and A. Neuberger, *Biochem. J.*, **45**, iii-iv (1949).

¹²⁸ C. B. Anfinsen, A. Beloff, and A. K. Solomon, *J. Biol. Chem.*, **179**, 1001-1013 (1949).

(a) *Succinic Acid*. Thunberg¹²⁹ was the first to suggest that succinic acid might arise by the linking of the methyl groups of two acetate molecules as given below:

Wieland and Sonderhoff¹³⁰ likewise suggested that the conversion of acetate to succinate by yeast in the absence of oxaloacetate proceeded by a dehydrogenative coupling. Moreover, Sonderhoff and Thomas,¹²³ employing deuterioacetate, interpreted their results as evidence of the direct synthesis of succinic acid; citric acid was also found to contain a large proportion of deuterium.

According to Bloch,¹ proof of the synthesis of succinate by dehydrogenative coupling would be conclusive only if the concentration of deuterium in succinate approached four deuterium atoms instead of the two atoms actually found. Although it is possible that several pathways for the synthesis of succinate may be available, there is no proof that the reaction does not involve exclusively synthesis *via* the tricarboxylic acid cycle. This suggestion is strengthened by the fact that, in no case in which succinate is synthesized is it the sole product; α -ketoglutarate and tricarboxylic acids also accumulate. Guzman-Barron and Ghiretti¹³¹ suggested that the formation of succinate from acetate in *Corynebacterium creatinovorans* (an aerobic, non-motile bacterium of the *Mycobacteriaceae*), *Aerobacter aerogenes* (from grains, plants, and the intestinal tract of man and animals, which uses citric acid as the sole source of carbon), *Pseudomonas fluorescens* (baker's yeast), and other molds may occur as the result of the oxidative condensation of acetyl-CoA.

(b) *Acetopyruvic Acid*. The condensation of acetic acid and of pyruvic acid to yield five-carbon dicarboxylic acids can theoretically take place by two pathways, give rise to acetopyruvic acid or to α -ketoglutaric acid. When the carboxyl group of acetic acid condenses with the methylene group of pyruvic acid, acetopyruvic acid is formed. Krebs and Johnson¹³² suggested the following equation for this synthetic reaction:

¹²⁹ T. Thunberg, *Skand. Arch. Physiol.*, **40**, 1-91 (1920).

¹³⁰ H. Wieland and R. Sonderhoff, *Ann.*, **499**, 213-228 (1932).

¹³¹ E. S. Guzman-Barron and F. Ghiretti, *Biochim. Biophys. Acta*, **12**, 239-249 (1953).

¹³² H. A. Krebs and W. A. Johnson, *Biochem. J.*, **31**, 772-779 (1937).

Acetopyruvic acid is believed to be immediately converted to acetoacetic acid in the liver, as follows:

Lehninger¹³³ demonstrated the conversion of acetopyruvic acid to acetone bodies in the intact rat, as well as in isolated tissues. Moreover, although no liver glycogen was deposited in rats after the administration of this di-keto acid, acetopyruvic acid was able to protect rats from toxic doses of insulin. Lehninger suggests that this conversion to glucose may occur during a critical demand on blood sugar. It is similar to the metabolic fate of isoleucine, which Butts *et al.*¹³⁴ reported as being both ketogenic and glucose-forming. When acetopyruvic acid acts in its glucose-forming capacity, the following reaction may be postulated:

However, several facts cause one to question whether or not acetopyruvic acid can normally arise from the condensation of acetic and pyruvic acids. In the first place, pyruvic acid has repeatedly been found to be a powerful glycogenic agent, as well as one which is ketolytic in the presence of acetone bodies.⁵⁸ Moreover, Weinhouse and collaborators³⁰ reported that labeled octanoic acid is oxidized in such a way as to preclude the participation of pyruvate in the synthesis of acetoacetate. It is therefore certain that acetopyruvate is not an obligatory intermediate in ketone body formation; in fact, it is highly improbable that the direct synthesis of this compound from acetate and pyruvate actually occurs in the tissues.

(c) *α-Ketoglutaric Acid*. According to Krebs,¹³⁵ when an "end-to-end" condensation of acetic and pyruvic acids occurs, *α*-ketoglutarate is the compound which results. On the basis of his findings, the following equation to illustrate this change is suggested:

¹³³ A. L. Lehninger, *J. Biol. Chem.*, **148**, 393-404 (1943).

¹³⁴ J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, **120**, 289-295 (1937).

¹³⁵ H. A. Krebs, *Nature*, **138**, 288-289 (1936).

¹³⁶ S. Weinhouse, G. Medes, N. F. Floyd, and L. Noda, *J. Biol. Chem.*, **161**, 745-746 (1945).

The experiments of Weinhouse *et al.*¹³⁶ have been interpreted as indicative of the dehydrogenative coupling of acetic acid and of pyruvic acid to form α -ketoglutaric acid.¹¹⁸ When isotopic acetate was incubated with kidney slices or kidney mince, it was found, at the end of the experiment, that no isotope was incorporated in the citric acid and *cis*-aconitate which were added as carriers, in spite of the presence of aconitase.¹³⁶ While this might be considered as evidence for the direct synthesis of α -ketoglutarate, Bloch¹ suggests that the data exclude only citric acid itself and not necessarily the tricarboxylic acid cycle. The possibility exists that, in systems containing tricarboxylic acids and aconitase, the conversion of *cis*-aconitate proceeds more rapidly to isocitrate than to citrate; the conversion of isocitrate to α -ketoglutarate is likewise a rapid reaction.

In a later report of Weinhouse and co-workers,¹³⁷ it was shown that, when labeled acetoacetate and ordinary oxaloacetate were incubated with kidney, the citric acid contained enough isotopic carbon to indicate that two-thirds of the citrate arose from acetoacetate. Since the latter compound is directly converted to acetate, it is difficult to explain the diametrically opposite results in the two sets of experiments. Wood¹¹⁸ is of the opinion that there are insufficient data to rule out the direct dehydrogenative synthesis of α -ketoglutarate. However, although there are reactions in which carbon-to-carbon bonds are formed, such as the formation of hexose from triose, or of acetoacetate from acetate, or of the carboxylation of pyruvate to oxaloacetate, all these reactions involve at least one carbonyl group in the reacting molecules. On the other hand, Bloch¹ was unable to cite a single instance in which the formation of a carbon-to-carbon group was established by the elimination of hydrogen between methyl or methylene groups. This would seem to render it highly improbable that a direct dehydrogenative synthesis of α -ketoglutarate results from acetate and pyruvate.

h. The Formation of Citric Acid. As indicated in the equations on page 171, citric acid is formed by the condensation of acetate with oxaloacetic acid; the six-carbon tricarboxylic acid is then successively degraded to α -ketoglutaric acid and to each of the four-carbon dicarboxylic acids. This series of reactions is now recognized as a cycle whereby the acetate molecule can be oxidized in the animal body. Virtanen and Pulkki¹³⁸ were the first to suggest this method of synthesis of citric acid from acetate by way of sugar in the fungus (*Aspergillus niger*). A similar pathway was

¹³⁷ S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **166**, 691-703 (1946).

¹³⁸ A. I. Virtanen and L. Pulkki, *Ann. Acad. Sci. Fennicae (Suomalainen Tiedeakatemia, Helsingfors, Toimikasia)*, Ser. A. **33**, No. 3, 1-19 (1930).

shown to exist in yeast, since both citrate and succinate accumulated when acetate was the sole nutrient.¹³⁰ Moreover, when deuterioacetate was employed, the citrate and succinate were found to contain approximately 50% of the original deuterium concentration in bound form.¹²³ Lynen¹³⁹ also demonstrated that the citric acid cycle was involved in the formation of succinate from acetate. Inasmuch as the inhibition of malonate synthesis is overcome by additional oxaloacetate or fumarate, the participation of C₄ acids is required in acetate synthesis (with the formation of a C₆ acid). Wieland and Rosenthal¹⁴⁰ were able to obtain maximum yields of citric acid when acetoacetate and oxaloacetate were incubated in the presence of kidney brei; these authors actually established the identity of the newly-formed citric acid by direct isolation. Two molecules of extra citric acid were formed for each mole of acetoacetate which disappeared.¹⁴¹ Lehninger⁵⁷ was able to account for all carbon as acetoacetate when octanoic acid was oxidized with washed rat liver cells; however, when fumarate was present, the citrate and α -ketoglutarate were shown to accumulate at the expense of the acetoacetate. The formation of labeled citrate from acetoacetate has also been demonstrated by Weinhouse *et al.*¹³⁷

One remarkable feature of the disappearance of acetate from the medium is the importance of other metabolites present in the incubation medium. The presence of added oxaloacetate markedly increases the removal of the acetate and the rate of synthesis of citric acid by yeast.¹⁴² Buchanan and co-workers¹⁴³ likewise reported the stimulatory effect of citrate, α -ketoglutarate, and of the several C₄-dicarboxylic acids on the aerobic disappearance of acetoacetate in contact with kidney homogenates. Although the stimulatory effect on the formation of citrate is exhibited not only by oxaloacetate but also by succinate, malate, and fumarate, Bloch¹ opines that the effect is attributable in all cases to oxaloacetate.

Although acetate and acetoacetate are generally considered to function in an identical manner in the Krebs cycle, this condition does not invariably obtain. When variations do occur, acetoacetate is usually the more reactive molecule. Thus, in the balance studies with kidney preparations,^{140,141} as well as in a heart muscle preparation,⁵⁷ only acetoacetate was found to be a precursor of citrate. Under such conditions acetylphosphate

¹³⁹ F. Lynen, *Ann.*, 554, 40-68 (1943).

¹⁴⁰ H. Wieland and C. Rosenthal, *Ann.*, 554, 241-260 (1943).

¹⁴¹ F. E. Hunter and L. F. Leloir, *J. Biol. Chem.*, 159, 295-310 (1945).

¹⁴² R. Sonderhoff and M. Deffner, *Ann.*, 536, 36-43, 44-50 (1938).

¹⁴³ J. M. Buchanan, W. Sakami, S. Gurin, and D. W. Wilson, *J. Biol. Chem.*, 159, 695-709 (1945).

was likewise found to be ineffective in bringing about citrate synthesis.^{57,141,144} Buchanan *et al.*¹⁴³ also observed that acetate and acetoacetate were equally effective as precursors of α -ketoglutarate when kidney homogenates were used.

Although it has generally been considered that acetate condenses with oxaloacetate to form isocitric acid, it has been suggested that acetoacetate may be the compound which condenses with oxaloacetate.¹⁴⁰ The greater reactivity of acetoacetate as compared with acetate could then be explained by the fact that the rate of the reaction, acetate $\rightarrow$ acetoacetate, might to some extent be a limiting factor. Wieland and Rosenthal¹⁴⁰ postulated that acetoacetate combines with oxaloacetate to yield pro-citric acids such as citroylacetic or acetylcitric acid. This might likewise explain the fact that Weinhouse *et al.*, using kidney homogenates, were able to demonstrate citric acid formation when acetoacetate was the substrate,¹³⁷ but only α -ketoglutarate when acetate was present in the incubation medium.¹³⁶

There are several arguments against the hypothesis that acetoacetate is required for condensation with oxaloacetate. Thus, citrate is formed in yeast by the tricarboxylic synthesis from acetate, but this fungus cannot change acetate to acetoacetate. According to Medes, Floyd, and Weinhouse,¹⁴⁵ acetate, which is completely oxidized by heart muscle, does not pass through acetoacetate as an intermediate; the same is at least partly true of the acetate utilized by kidney. Moreover, Lehninger⁵⁷ reported that, in washed liver cells, acetoacetate, in contradistinction to octanoic acid, was incapable of yielding citrate. This may be due to the inability of this tissue to convert acetoacetate to the active two-carbon fragment. On the opposite side of the picture, Buchanan *et al.*¹⁴⁴ showed that, when labeled acetoacetate and unlabeled acetate were incubated with rabbit kidney, the acetate remaining had a lower isotope content than did the succinate resulting from the oxidation of α -ketoglutarate. Bloch¹ suggests that, from thermodynamic considerations, an energy-rich acetyl would be expected to arise more readily from acetoacetate than from acetate. Lehninger,⁵⁷ in testing for the identity of the compound which combines with oxaloacetic acid, showed that the following compounds are inactive: glycolate ($\text{HOCH}_2\cdot\text{COOH}$), glyoxalate ($\text{OCH}\cdot\text{COOH}$), oxalate, acetaldehyde, ethanol, acetamide ($\text{CH}_3\cdot\text{CONH}_2$), glycine ($\text{NH}_2\text{CH}_2\cdot\text{COOH}$), acetyl phosphate ($\text{CH}_3\text{COPO}_3\text{H}_2$), and acetyl pyrophosphate ($\text{CH}_3\text{CO}(\text{PO}_3)_2\text{H}$).

¹⁴⁴ J. M. Buchanan, W. Sakami, S. Gurin, and D. W. Wilson, *Federation Proc.*, **5**, 126 (1946).

¹⁴⁵ G. Medes, N. F. Floyd, and S. Weinhouse, *J. Biol. Chem.*, **112**, 1-9 (1946).

i. **The Formation of Acids Related to Citric Acid.** The identity of the six-carbon acid formed in the citric acid cycle has been a matter of dispute. The tricarboxylic acids, which are known to be formed, namely, citric, isocitric, and *cis*-aconitic, are interconvertible as shown below:

Interconversions of Tricarboxylic Acids

When the enzyme, aconitase, is present, all three acids will appear in the system.^{146,147} Although Krebs and Johnson¹⁴⁷ originally proposed that citric acid was the initial condensation product, this hypothesis had to be abandoned when Evans and Slotin,¹⁴⁸ as well as Wood *et al.*,¹⁴⁹ demonstrated that the labeled carbon in newly-synthesized α -ketoglutarate formed from labeled CO_2 and pyruvate in pigeon liver had the isotopic carbon only in the carboxyl group adjacent to the carbonyl group. The tricarboxylic acids could not have originated from a symmetrical citric acid molecule, which would have been formed according to the original hypothesis. This must also be the case with the precursors of the dicarboxylic acids obtained after the reaction of acetate or acetoacetate and oxaloacetate.^{137,143}

Although it is certain that a symmetrical tricarboxylic acid is not the initial condensation product, it is not definite which unsymmetrical acid is the primary product. Lynen¹⁵⁰ points out that the formation of labeled citrate and succinate in yeast¹²² also rules out symmetrical citric acid. However, the presence of citric acid in yeast, which does not contain the enzyme, aconitase, and which is necessary for transforming isocitrate or *cis*-aconitate to citrate, would seem to indicate that citric acid is the primary condensation product in this case. According to Ochoa,¹⁵¹ oxalosuccinic

¹⁴⁶ C. Martius, *Z. physiol. Chem.*, **257**, 29-42 (1938).

¹⁴⁷ H. A. Krebs and W. A. Johnson, *Enzymologia*, **4**, 148-156 (1937).

¹⁴⁸ E. A. Evans, Jr., and L. Slotin, *J. Biol. Chem.*, **141**, 439-450 (1941).

¹⁴⁹ H. G. Wood, C. H. Werkman, A. Hemingway, and A. O. Nier, *J. Biol. Chem.*, **142**, 31-45 (1942).

¹⁵⁰ F. Lynen, *Ann.*, **552**, 270-306 (1942).

¹⁵¹ S. Ochoa, *J. Biol. Chem.*, **159**, 243-244 (1945).

acid is another possible tricarboxylic acid which may be an intermediate, since the oxalosuccinic acid $\rightarrow$ isocitric acid change is a reversible one.

(5) *Résumé of the Metabolism of Acetic Acid*

It has long been recognized that acetic acid is readily metabolized by the animal, but the pathway of this degradation remained an enigma until the advent of the radioisotope technic. Lusk¹⁵² reported that the dog apparently could oxidize large amounts of acetic acid, since the acid which was administered disappeared; it was without effect in changing the CO₂-combining power of the blood. It is not converted to "extra sugar" in the phlorhizinized dog,^{110,111} and the amount of glycogen is not increased by its administration. However, there is some evidence, based upon tracer studies, that acetate can be incorporated into the glycogen stores of an animal without increasing the total amount of this polysaccharide.^{117,118}

It was formerly believed that acetate was oxidized directly to carbon dioxide and water. However, no one has been able to prove any intermediate stages in this oxidation. Thus, Kleinzeller¹⁵³ reported that neither glycolic acid, glyoxylic acid, nor glycine can be considered as an intermediate. Guzman-Barron and Ghiretti¹³¹ demonstrated that enzymes are present in microorganisms which are active in the synthesis of citric acid and in the oxidation of isocitric acid. They found the enzymes in the bacillus from plants and animals, *Aerobacter aerogenes*, in the animal and plant pathogen, *Corynebacterium creatinovorus*, in black bread mold, *Rhizopus nigricans*, and in reactions in which the oxidation of acetate had been presumed to occur *via* glycolic acid, as is the case with the dry wood-rot molds, *Merulius tremellosus* and *M. niveus*. The present concept is that acetic acid is oxidized by being incorporated into the tricarboxylic acid cycle. There is some disagreement as to whether it combines with oxaloacetate as the acetate or as the acetoacetate group. However, it is known that acetate must be in an active form to effect the condensation; this requires the intervention of an enzyme, as well as of a coenzyme, which is known as Coenzyme A. Elliott and Kalnitsky¹⁵⁴ demonstrated that, when acetate is oxidized by homogenates of rabbit kidney cortex, Mg⁺⁺ or Mn⁺⁺, inorganic phosphate, and ATP, as well as oxaloacetate and pantothenate, are also required for the oxidation. The oxidation of acetate was found to be inhibited by malonate, arsenite, iodoacetate, and fluoroacetate.

¹⁵² G. Lusk, *J. Biol. Chem.*, **49**, 453-478 (1921).

¹⁵³ A. Kleinzeller, *Biochem. J.*, **37**, 674-677 (1943).

¹⁵⁴ W. B. Elliott and G. Kalnitsky, *J. Biol. Chem.*, **186**, 477-486 (1950).

Although citric acid and related compounds have been shown to be formed from acetate in yeast,¹²³ as well as in such isolated tissues as kidney,¹⁴⁰ the intermediates have not been proved in the intact animal. However, Rittenberg and Bloch¹⁵⁵ demonstrated that labeled glutamic acid ($\text{HOOC} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$), and aspartic acid ($\text{HOOC} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{COOH}$), can be isolated from the liver proteins of rats and mice after the feeding of C^{13} -labeled acetate. It is suggested that these compounds originate from α -ketoglutarate and from a four-carbon dicarboxylic acid with which the amino acids are in equilibrium. This should constitute adequate proof of the operation of the tricarboxylic cycle in the intact animal.

In addition to the incorporation of acetic acid into the components of the tricarboxylic acid cycle, as occurs in oxidation, acetate readily disappears from the tissues by becoming incorporated into many other new compounds. Thus, it serves in the synthesis of fatty acids, cholesterol, protoporphyrin, uric acid, acetoacetate, and, to a limited extent, of glycogen. Moreover, acetate may be used to combine with amino groups to form the acetylated compounds.

Acetate is being continually formed by oxidation of fatty acids, amino acids, and pyruvic acid (under certain specific conditions). Despite its continued production, it can be detected in the tissues only in traces. Presumably, the failure to detect acetic acid is related to its rapid utilization in one of the various metabolic pathways available for it.

2. Formic Acid and Formate Metabolism

(1) Introduction

Little was known of the participation of formic acid in metabolic processes prior to the advent of the isotope technic. In 1930, and again later, Edlbacher and co-workers^{156,157} demonstrated that a molecule of formic acid, together with one of glutamic acid and two of ammonia, were formed when histidine was broken down by the enzyme, histidase. This reaction was the only one in which formate was known to occur until the findings of Sonne *et al.*¹⁵⁸ in 1948. These workers studied the utilization of acetate in ureide formation, and suggested that acetate is the biologic source of the ureide carbon, and that formate is either an intermediate or may readily be converted into an intermediate of this reaction.

¹⁵⁵ D. Rittenberg and K. Bloch, *J. Biol. Chem.*, **157**, 749-750 (1945).

¹⁵⁶ S. Edlbacher and J. Kraus, *Z. physiol. Chem.*, **191**, 225-242 (1930); **195**, 267-272 (1931).

¹⁵⁷ S. Edlbacher and S. Neber, *Z. physiol. Chem.*, **224**, 261-272 (1934).

¹⁵⁸ J. C. Sonne, J. M. Buchanan, and A. M. Delluva, *J. Biol. Chem.*, **173**, 69-79 (1948).

(2) *The Nature of the Active Formyl Group*

It now appears that the "formyl" group behaves in a manner similar to that of the "acetyl" group. Thus, for the participation of formate in various syntheses, it is necessary that it occur as "active formate." It is believed that this "active" compound is the citrovorum factor (CF), or *Leuconostoc citrovorum* factor (LCF). This is also referred to as folinic acid-SF, and as leucovorin. Although there is no absolute proof that CF actually transports the formate radicle, there is considerable circumstantial evidence to support this hypothesis. Thus, a *N*-formyl group actually occurs in the CF molecule; the chemical designation for this factor is 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid, or 5-formyl-5,6,7,8-tetrahydrofolic acid. Moreover, it is known that this formyl group may be lost without markedly altering the CF activity.¹⁵⁹ If the CF molecule functions in reactions through which the formyl radicle is alternately gained and lost while it still maintains its other functions, the possibility that it acts as a transformylating agent is greatly increased. An equation illustrating how CF may participate in this reaction is given below:

The Transformation of CF to 5,6,7,8-Tetrahydropteroylglutamic Acid with the Formation of Formate. The reverse reaction also occurs. The figures in the circles represent the position numbers adopted for this compound.

¹⁵⁹ H. P. Broquist, M. J. Fahrenbach, J. A. Brockman, Jr., E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem. Soc.*, **73**, 3535-3536 (1951).

In more recent studies on the enzyme system involved in formylations, Rauen and Jaenicke¹⁶⁰ demonstrated that folic acid can also serve as the transformylating agent. It is now recognized that folic acid (also called "pteroylglutamic acid") is changed in the animal to a compound which is called folinic acid. The latter compound appears in the urine after the administration of folic acid. It can also be synthesized by the liver *in vitro*. Folinic acid appears to be a reduced form of folic acid, and it is identical with LCF (or CF).

The enzyme system which mediates the reaction was prepared from liver homogenates. Mutton liver was found to be the most active source, followed in order by that of the pig, ox, and calf; dog liver was inactive. Enzymatic formylation of folic acid depends upon an intact respiration, and hence it is inhibited by cyanide, azide, and iodoacetate. ATP increases the rate of reaction, while DPN is without effect. Serine and histidine were found to be the most active formyl donors; uric acid, acetate, formate, thymine, uracil, and hypoxanthine were less active in this respect. Glycine had only a slight activity, and methionine, choline, and betaine were completely inactive.

The (N) (12) formyl-folic acid was shown by Rauen and Jaenicke¹⁶⁰ to be relatively stable under aerobic conditions, even in the presence of formyl acceptors. On the other hand, the transfer of the formyl group occurred slowly under anaerobic conditions, in the absence of acceptors, while the reaction proceeded rapidly when acceptors were present. Glycine, pyruvic acid, and uracil were the best acceptors. ATP and DPN were likewise required for the completion of the transformylation. Folic acid antagonists inhibited the synthesizing but not the decomposition reactions. Since both the energy of respiration and ATP are required for the continuance of the reaction, it is suggested that this formyl group should be referred to as "activated formate."

Formate, presumably in the active form, is now known to be involved in many synthetic reactions. Presumably, CF may function as a donor of the formyl group, while one may also postulate that 5,6,7,8-tetrahydropteroylglutamic acid might act as an acceptor of formyl groups which have been set free. The reactions may be somewhat complex, and they may require the assistance of cofactors other than those obtained from folic acid. Welch and Nichol¹⁶¹ illustrated the several reactions involving the formation or utilization of formic acid, in the diagram presented below:

¹⁶⁰ H. M. Rauen and L. Jaenicke, *Z. physiol. Chem.*, 293, 46-47 (1953).

¹⁶¹ A. D. Welch and C. A. Nichol, *Ann. Rev. Biochem.*, 21, 633-686 (1952).

The Various Reactions in Which the Formyl Radicle Has Been Shown to Participate¹⁶¹

(3) Reactions in Which Formate Plays a Role

a. Purine Synthesis (Reactions 1 and 2). Sonne and co-workers¹⁵⁸ and Karlsson and Barker¹⁶² reported that formic acid contributes to the ureide carbons 2 and 8 of the uric acid synthesized and excreted by pigeons. This same reaction also occurs in the rat,¹⁶³ an animal which synthesizes much less purine than does the bird. In the case of the fowl, it has been recognized that practically all excreted nitrogen is converted into uric acid before elimination. Skipper *et al.*¹⁶⁴ reported that an antagonist-induced folic acid deficiency in mice diminishes the incorporation of C¹⁴-formate into the nucleic acid purines. This inhibition appears to be specific for formic acid, as the incorporation of C¹⁴O₂ into purines is only partially blocked, and tissue CO₂ fixation is not affected.

Greenberg^{165,166} likewise demonstrated that, in the synthesis of hypoxanthine, inosine-5-phosphate is one of the first purine derivatives to be detected. Thus, it was found that, in the presence of a dialyzed extract of pigeon liver, glycine, CO₂, glutamine, CF, biotin, and ribose-1-phosphate

¹⁶² J. L. Karlsson and H. A. Barker, *J. Biol. Chem.*, **177**, 597-599 (1949).

¹⁶³ M. R. Heinrich and D. W. Wilson, *J. Biol. Chem.*, **186**, 447-460 (1950).

¹⁶⁴ H. E. Skipper, J. H. Mitchell, Jr., and L. L. Bennett, Jr., *Cancer Research*, **10**, 510-512 (1950).

¹⁶⁵ G. R. Greenberg, *J. Biol. Chem.*, **190**, 611-631 (1951).

¹⁶⁶ G. R. Greenberg, *Federation Proc.*, **10**, 192 (1951).

brought about the incorporation of C^{14} -formate into the inosine-5-phosphate provided ATP was present as a source of energy. It was shown that, in this reaction, the ureide 2 carbon of inosine-5-phosphate behaved differently from that of the ureide 8 carbon; thus, when a dialyzed liver preparation was incubated with inosine-5-phosphate and C^{14} -formate, a rapid interchange in carbons took place in position 2, but no transfer occurred in position 8.^{167,168} Moreover, the interchange was augmented by CF, which is another indication of its participation in the transfer of formate-carbon.¹⁶¹ Although this discrepancy between the incorporation of formate carbon in position 2, as distinguished from position 8, is very definite in *in vitro* tests, it does not occur in the intact animal, in which both carbons 2 and 8 appear to be derived from formate. It may take place in the former case (*in vitro*) because of some special condition such as a high concentration of inosinic acid.

However, in spite of the relatively clear-cut evidence that the formyl radicle participates in purine synthesis, the results of Totter and co-workers¹⁶⁹ on chicks render the mechanism obscure. Thus, in folic acid-deficient chicks, the incorporation of C^{14} -formate into the purines of the viscera did not differ from that noted in control birds.¹⁶⁹ On the other hand, Drysdale *et al.*¹⁷⁰ reported that, in the case of rats made folic acid-deficient by the administration of a purified diet containing 2% of succinylsulfathiazole, much less labeled formate was present in the purines of the nucleic acid in the liver than was found in control rats which had previously received PGA (pteroylglutamic or folic acid). However, the level of the purines of the extrahepatic tissues (kidneys, spleen, pancreas, heart, testes, and intestines) of the folic acid-deficient rats was not depressed below those of the PGA-supplemented rats; in fact, the specific activity of these purines was about three times that of the liver purines in the PGA-treated animals. The results of Skipper *et al.*,¹⁶⁴ which demonstrated that the incorporation of formate into the purines of the nucleic acid of mice was depressed by the folic acid antagonist, aminopterin, have been confirmed and extended in rats by Goldthwait and Bendich.¹⁷¹ Although these divergent results are somewhat confusing, the discrepancy may have been related to variations in the extent of deficiency of folic

¹⁶⁷ J. M. Buchanan, *Abstracts Am. Chem. Soc., 119th Meeting*, (Boston, Mass., April, 1951), p. 136-146.

¹⁶⁸ M. P. Schulman and J. M. Buchanan, *Federation Proc.*, **10**, 244-245 (1951).

¹⁶⁹ J. R. Totter, E. Volkin, and C. E. Carter, *J. Am. Chem. Soc.*, **73**, 1521-1522 (1951).

¹⁷⁰ G. R. Drysdale, G. W. E. Plaut, and H. A. Lardy, *J. Biol. Chem.*, **193**, 533-538 (1951).

¹⁷¹ D. A. Goldthwait and A. Bendich, *Federation Proc.*, **10**, 190 (1951).

acid rather than to fundamental differences in the mechanism of synthesis of purines. One is forced to conclude that formate furnishes two carbons for this molecule and that its incorporation in the molecule is related to the function of CF.

b. Thymine Synthesis (Reaction 3). Formate has been shown to be the precursor of the methyl group in the pyrimidine, 5-methyluracil, or thymine, in chicks,¹⁶⁹ as well as in rats.¹⁷² Thus, Elwyn and Sprinson¹⁷² found that the C¹⁴ of L-serine-3-C¹⁴ and of glycine-2-C¹⁴, both of which are known formate precursors, became incorporated in the CH₃ group of thymine.

c. Serine Synthesis (Reaction 5). Plaut and associates¹⁷³ were the first to demonstrate the fact that formate participates in the synthesis of serine; it is incorporated in the β-position (C-3). Thus, rats with a folic acid deficiency utilized less formate for serine synthesis, and produced less C¹⁴O₂, than did pair-fed control rats which received folic acid. Moreover, it was noted that biotin deficiency does not influence the synthesis; therefore, folic acid deficiency specifically interferes with formate utilization.

Sakami¹⁷⁴ reported that when C¹³-glycine and formate-C¹⁴ were fed to rats, serine as well as liver glycogen containing both C¹³ and C¹⁴ were isolated from the liver hydrolysates. This reaction is pictured as follows:

The Transformation of Glycine and of Formate to Serine and to Glycogen¹⁷⁴

It has also been demonstrated that the formate used in the synthesis of serine originates from glycine.^{175,176} It is postulated that the following changes occur in the transformation of glycine to formate:

The Transformation of Glycine to Formate¹⁷⁵

As early as 1946, Shemin¹⁷⁷ demonstrated by *in vivo* tests that the β-carbon of serine breaks down to yield glycine and formic acid.

¹⁷² D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.*, **72**, 3317-3318 (1950).

¹⁷³ G. W. E. Plaut, J. J. Bethel, and H. A. Lardy, *J. Biol. Chem.*, **184**, 795-805 (1950).

¹⁷⁴ W. Sakami, *J. Biol. Chem.*, **176**, 995-996 (1948).

¹⁷⁵ W. Sakami, *Federation Proc.*, **8**, 246 (1949).

¹⁷⁶ P. Siekevitz, T. Winnick, and D. M. Greenberg, *Federation Proc.*, **8**, 250 (1949).

¹⁷⁷ D. Shemin, *J. Biol. Chem.*, **162**, 297-307 (1946).

d. Formate as a Source of the Labile Methyl Group (Reactions 6 and 8). Sakami and Welch¹⁷⁸ reported, on the basis of *in vitro* tests with liver slices, that the rat is able to synthesize the labile methyl group of methionine or of choline from C¹⁴-formate. This was the first demonstrated that this important radicle can be synthesized in the liver; it was previously believed to be available only from dietary sources, and from intestinal synthesis. Folic acid apparently plays as important a role in the synthesis of these labile methyl groups as it does in other syntheses. For example, liver slices from rats deficient in folic acid converted less C¹⁴-formate to methionine-methyl than did slices from the livers of rats which had received PGA shortly before the rat was sacrificed. Moreover, an appreciably lower amount of C¹⁴-formate was incorporated into the choline-methyl when it was fed to folic acid-deficient rats than when it was given to animals having an adequate amount of this vitamin.¹⁷⁹ The conversion of formate to labile methyl groups has been repeatedly confirmed both *in vitro* and *in vivo*.¹⁸⁰⁻¹⁹⁰ It was shown to occur in germ-free rats.^{186,191} Both formaldehyde¹⁸⁹ and methanol^{187,192,193} serve as precursors, but bicarbonate does not.^{184,186,189} Du Vigneaud *et al.*¹⁸⁷ noted that 69 to 75% of the deuterium was lost in the synthesis of choline methyl from doubly labeled methanol, C¹⁴D₃OH. This is consistent with the hypothesis that formic acid is an intermediate.

Stekol *et al.*¹⁹⁴ reported that vitamin B₁₂ increased the choline synthesis from formate, while Verly and associates,¹⁹³ as well as Arnstein and Neu-

¹⁷⁸ W. Sakami and A. D. Welch, *J. Biol. Chem.*, **187**, 379-384 (1950).

¹⁷⁹ J. A. Stekol, S. Weiss, and K. Weiss, *Abst. Am. Chem. Soc., 120th Meeting* (New York, N. Y., Sept. 1951), 21 C.

¹⁸⁰ P. Siekevitz and D. M. Greenberg, *J. Biol. Chem.*, **186**, 275-286 (1950).

¹⁸¹ S. Jonsson and W. A. Mosher, *J. Am. Chem. Soc.*, **72**, 3316 (1950).

¹⁸² P. Berg, *J. Biol. Chem.*, **190**, 31-38 (1951).

¹⁸³ S. Kirkwood and L. Marion, *Can. J. Chem.*, **29**, 30-36 (1951).

¹⁸⁴ V. du Vigneaud, W. G. Verly, and J. E. Wilson, *J. Am. Chem. Soc.*, **72**, 2819-2820 (1950).

¹⁸⁵ H. G. Wood, *Harvey Lectures*, **45**, 127-148 (1949-1950).

¹⁸⁶ V. du Vigneaud, C. Ressler, and J. R. Rachele, *Science*, **112**, 267-271 (1950).

¹⁸⁷ V. du Vigneaud, W. G. L. Verly, J. E. Wilson, J. R. Rachele, C. Ressler, and J. M. Kinney, *J. Am. Chem. Soc.*, **73**, 2782-2785 (1951).

¹⁸⁸ C. Mitoma and D. M. Greenberg, *Federation Proc.*, **10**, 225 (1951).

¹⁸⁹ W. G. L. Verly and V. du Vigneaud, *J. Am. Chem. Soc.*, **72**, 2819-2820 (1950).

¹⁹⁰ C. Ressler, J. R. Rachele, and V. du Vigneaud, *J. Biol. Chem.*, **197**, 1-5 (1952).

¹⁹¹ V. du Vigneaud, C. Ressler, J. R. Rachele, J. A. Reyniers, and T. D. Luckey, *J. Nutrition*, **45**, 361-376 (1951).

¹⁹² V. du Vigneaud and W. G. Verly, *J. Am. Chem. Soc.*, **72**, 1049 (1950).

¹⁹³ W. G. Verly, J. E. Wilson, J. M. Kinney, and J. R. Rachele, *Federation Proc.*, **10**, 264 (1951).

¹⁹⁴ J. A. Stekol, K. W. Weiss, and S. Weiss, *Federation Proc.*, **10**, 252 (1951).

berger,^{195,196} found that a deficiency in vitamin B₁₂ had little or no effect upon the above synthesis. Vitamin B₁₂ is apparently required for the conversion of glycine to serine.^{179,194,196} Serine forms the aminoethanol moiety of choline.^{194,197} Since it is well known that formate participates in the synthesis of serine from glycine, it is evident that more formate carbon would appear in the choline molecule than can be accounted for in the methyl groups.

Although the weight of evidence would seem to indicate that vitamin B₁₂ does not appreciably influence the conversion of the β -carbon of serine to choline-methyl groups, it is apparently concerned with the conversion of glycine to a form suitable for the acceptance of "formate" to yield serine. This vitamin may be needed for the activation of another compound which reacts with glycine to form serine. It is possible that sulfhydryl compounds such as homocysteine or glutathione are required; according to Dubnoff,^{198,199} vitamin B₁₂ is necessary to maintain these reducing compounds in their reduced state.

Moreover, folic acid does not take part in the formation of cystathionine from homocysteine and serine.¹⁹⁴ There is considerable question, therefore, as to whether or not folic acid or CF plays a definite role in serine metabolism.²⁰⁰ One may conclude that folic acid functions in the biosynthesis of choline in the reactions leading to the synthesis of methionine,¹⁷⁸ and in reactions involved in the synthesis of L-serine, which is the precursor of the aminoethanol moiety.¹⁷⁸

e. Formate in the Catabolism of Histidine (Reaction 12). The reaction discovered by Edlbacher and Kraus¹⁵⁶ in 1930 without the use of isotopes, namely the formation of formic acid when histidine is decomposed by histidase, has been studied more completely by the use of isotopes. It was shown by Soucy and Bouthillier²⁰¹ that heavily-labeled serine resulted when histidine-2-C¹⁴-imidazole was fed to rats. Moreover, radioactive allantoin, uric acid, and choline were found to be synthesized in rats after histidine-2-C¹⁴ was metabolized.²⁰² Carbon-2 of histidine has been found

¹⁹⁵ H. R. V. Arnstein and A. Neuberger, *Biochem. J.*, **48**, ii-iii (1951).

¹⁹⁶ H. R. V. Arnstein and A. Neuberger, *Biochem. J.*, **50**, xxxviii (1952).

¹⁹⁷ H. R. V. Arnstein, *Biochem. J.*, **48**, 27-32 (1951).

¹⁹⁸ J. W. Dubnoff, *Federation Proc.*, **9**, 166 (1950); **10**, 178 (1951).

¹⁹⁹ J. W. Dubnoff, *Arch. Biochem.*, **27**, 466-467 (1950).

²⁰⁰ D. Elwyn, A. Weissbach, and D. B. Sprinson, *J. Am. Chem. Soc.*, **73**, 5509-5510 (1951).

²⁰¹ R. Soucy and L. P. Bouthillier, *Rev. can. biol.*, **10**, 290-291 (1951).

²⁰² J. C. Reid and M. O. Landefeld, *Arch. Biochem. Biophys.*, **34**, 219-221 (1951).

to be an especially effective source of carbons 2 and 8 of uric acid in pigeons.
202,203

f. Miscellaneous Compounds Related to Formate. Acetone (Reaction 11) is able to contribute the carbon of its methyl groups to the methyl groups of choline. Sakami²⁰⁴ is of the opinion that this transfer involves the formation of a formate-like intermediate.

Stekol *et al.*¹⁹⁴ reported that formate or glycine is involved in the synthesis of cysteine. In deficiency of folic acid or vitamin B₁₂, this synthesis was found to be depressed.

Serine can originate from carbon-2 of threonine by way of glycine,²⁰⁵ and formate from tryptophane *via* formylkynurenine.^{206,207} It is thus evident that four of the so-called essential amino acids, namely, histidine, methionine, threonine, and tryptophane, are sources of the one-carbon intermediate, formate, which appears to be necessary in the synthesis of many essential biologic products.

(4) *Factors Involved in Formate Utilization*

Berg²⁰⁸ prepared a cell-free extract from pigeon liver which was able to catalyze the anaerobic incorporation of formate-C¹⁴ into cystathionine, methionine, the β -carbon of serine, and the ureide carbon of purines. It was shown that all reactions were catalyzed individually; serine and cystathionine were not believed to be intermediates in the conversion of formate to the methyl group of methionine. Homocysteine markedly increased the incorporation of formate not only into serine and purines, but also into the methionine methyl groups. When compounds are added which promote the formation of different formate acceptors, the relative distribution of formate between the several compounds listed above may be altered. It is suggested that an intermediate formed from homocysteine and formate is a common precursor in the serine, purine, and methionine synthesis. Berg²⁰⁸ also reported that one or more heat-labile factors are required for the conversion of formate to methionine methyl groups.

Arnstein and Neuberger²⁰⁹ found that, when young rats were fed C¹⁴-containing precursors, the radioactivity of the methionine methyl groups

²⁰³ D. B. Sprinson and D. Rittenberg; unpublished results cited by D. B. Sprinson, *Abst. Am. Chem. Soc., 119th Meeting*, (Boston, Mass., April, 1951), 11C-12C.

²⁰⁴ W. Sakami, *J. Biol. Chem.*, **187**, 369-378 (1950).

²⁰⁵ H. L. Meltzer and D. B. Sprinson, *Federation Proc.*, **9**, 204 (1950).

²⁰⁶ W. E. Knox and A. H. Mehler, *J. Biol. Chem.*, **187**, 419-430 (1950).

²⁰⁷ A. H. Mehler and W. E. Knox, *J. Biol. Chem.*, **187**, 431-438 (1950).

²⁰⁸ P. Berg, *J. Biol. Chem.*, **205**, 145-162 (1953).

²⁰⁹ H. R. V. Arnstein and A. Neuberger, *Biochem. J.*, **55**, 259-271 (1953).

greatly exceeded that of the choline methyl groups; this suggests that most of the newly-synthesized methyl groups are first incorporated into methionine and are then transferred to choline by transmethylation. Cobalamin (B_{12} , or "animal protein factor") was isolated in 1948 and was found to be a cobalt coördination compound, or "chelate." A second homologous form was isolated in 1949. These forms were identified as cyano (B_{12}) and hydroxo ($B_{12}^b = B_{12}^b$) forms of the organometallic molecule containing cobalt.²¹⁰ Cobalamin was found to increase the conversion of β - C^{14} -serine, α - C^{14} -glycine, and formate into the methyl group of methionine and into both the methyl groups and the ethanolamine moiety of choline.

Little is known about the appearance of formate in animals. Kendal and Ramanathan²¹¹ reported the appearance of formate in the urine of man after the administration of methanol. When ethanol was ingested, the elimination of formate was completely inhibited in repeated experiments. Formate appeared in the urine within one or two hours after the cessation of ethanol ingestion; its appearance was coincident with the rapid decline in the body methanol concentration which ensues.

According to Sperling *et al.*,²¹² carbonate and formate have the same general pattern of excretion and distribution after their intraperitoneal injection into rats. More than 90% of the injected dose of C^{14} -carbonate was excreted in twenty-four hours, while an excess of 80% of C^{14} -formate was excreted during the same period. The retained C^{14} was distributed throughout all the tissues in both groups of animals.

3. The Metabolism of Propionic Acid

In contradistinction to the formate and acetate residues, which are transferred and metabolized as units, and which serve as intermediates in the synthesis of many biologically-important compounds, propionic acid does not have a similar metabolic function; the metabolism of propionic acid is closely related to that of the various three-carbon intermediates.

Propionic acid does not give rise to ketone bodies^{23,24,36} in the fasting rat; on the other hand, propionic acid is converted to glycogen both when fed as the sodium salt of the acid³³ and when administered as the ethyl ester.³² Moreover, other odd-chain acids are believed to be oxidized to

²¹⁰ P. B. Hawk, B. L. Oser, and W. M. Summerson, *Practical Physiological Chemistry*, 13th ed., Blakiston, New York, Toronto, 1954, pp. 1207 ff.

²¹¹ L. P. Kendal and A. N. Ramanathan, *Biochem. J.*, 54, 424-426 (1953).

²¹² F. Sperling, E. S. Maxwell, and W. F. Van Oettingen, *Am. J. Physiol.*, 174, 33-38 (1953).

propionate under normal conditions, after which the three-carbon residues follow the same metabolic pathway as does preformed propionate. Further evidence that propionate is a source of carbohydrate is to be inferred from the fact that the administration of tripropionin to rats is followed by the deposition of a much greater amount of liver glycogen than occurs when a similar amount of triacetin or of tributyrin is fed.¹¹³ This is because glycerol alone serves as a source of carbohydrate in the latter cases, while both glycerol and propionic acid are potential sources of glycogen in the case of tripropionin.

The metabolism and oxidation of propionic acid would thus appear to be similar to that of glucose and of glucose intermediates. Thus, Mahler and Huennekens²¹³ reported that, when labeled propionate was oxidized in the presence of large pools of unlabeled L-lactate, D-lactate, and pyruvate, the label appeared in these intermediates. This is interpreted to mean that they are in equilibrium with the actual intermediates. In a study of the role of propionate as a precursor of milk constituents of the intact dairy cow, Kleiber *et al.*²¹⁴ noted that the decarboxylation rate of propionate appeared to be higher than that of acetate, whereas the relative oxidation rates of the resulting two-carbon residues were similar for acetate and propionate. The formation of lactose from propionate is, for the most part, direct, rather than *via* CO₂; this is suggested by the fact that only 30% of the C¹⁴ transferred from C¹⁴-1-propionic acid passes through the carbonate pool in the case of the cow. Less than 10% of the carbon transfer from C¹⁴-2-propionic acid, which is converted to lactose, is involved in the carbonate pool. A similar pattern was noted for the C¹⁴ transferred to casein.

²¹³ H. R. Mahler and F. M. Huennekens, *Biochim. Biophys. Acta*, **11**, 575-583 (1953).

²¹⁴ M. Kleiber, A. L. Black, M. A. Brown, and B. M. Tolbert, *J. Biol. Chem.*, **203**, 339-346 (1953).

CHAPTER VII

THE METABOLISM OF BRANCHED-CHAIN ACIDS, HYDROXY AND KETO ACIDS, DICARBOXYLIC ACIDS, TRICARBOXYLIC ACIDS, FATTY ACID AMIDES, FATTY ACID ALDEHYDES, AND HYDROCARBONS

When the nature of a fatty acid is altered by the presence of a side chain, of an hydroxyl or of a ketone group, its metabolism may be markedly altered from that of the normal straight-chain acid. The presence of more than one carboxyl group poses a new problem in oxidation, and unique properties and a separate metabolism may be expected of the polycarboxylic acids. Likewise, the behavior of the fatty acid residue is altered when the carboxyl group is replaced by the amide group, as in the fatty acid amides, or by the aldehyde group (fatty aldehydes). Finally, a still greater alteration in metabolism is to be expected in the hydrocarbons than in any of the fatty acid derivatives listed above.

1. Branched-Chain Acids

The metabolism of branched-chain acids has been extensively investigated in animals and in man. The most important factor which influences the course of the metabolism is the position of the side chain in relation to the carboxyl group. The nature of the substituent group likewise provides a situation which results in a variation in metabolism. The alteration in the metabolism of the branched-chain acids from that of the straight-chain compounds will likewise be related to the extent to which the usual enzyme systems may be displaced and prevented from combining with the substrate by the altered configuration of the molecule.

In contradistinction to the disturbing influence of the side chain on the metabolism of the fatty acid in the animal, such variations in structure have little influence on the utilization of the fatty acids by bacteria. Ac-

according to Weitzel and Schraufstätter,¹ the utilization of the fatty acids by bacteria is inhibited to a progressively greater degree as the chain length increases, but this is not influenced by methyl-substitution in the side chain. In the case of the molds, such as *Penicillium glaucum* (blue-green cheese mold), Thaler *et al.*² reported that the α -methyl group caused no poisoning action on growth. Thus, the mold grew well in the presence of α -methylbutyric, α -methylcaproic, or α -methyllauric acids, with the formation of ketones. It was suggested that the course of metabolism involves the following changes: saturated fatty acid, α,β -unsaturated fatty acid, β -hydroxy-fatty acid, β -keto-fatty acid, and methylketone. On the other hand, β -methyl- β -hydroxypelargonic acid and β -methyl- β -hydroxylauric acid could not serve as sources of carbon in the culture medium for *P. glaucum*; moreover, no methylketones resulted from mold action on these derivatives.

(1) α -Substituted Acids

Kay and Raper³ early called attention to the aberrant behavior of several α -phenyl-substituted acids. Thus, α -phenylpropionic acid (hydratropic acid), $\text{CH}_3\cdot\text{CH}(\text{C}_6\text{H}_5)\cdot\text{COOH}$, was oxidized to the extent of 70%, while the unoxidized fraction was excreted as the glucuronate. α -Phenyl- β -hydroxypropionic acid (inactive tropic acid), $\text{HOCH}_2\cdot\text{CH}(\text{C}_6\text{H}_5)\cdot\text{COOH}$, was excreted unchanged to the extent of 90%. When the hydroxyl group was also on the α -carbon, as in inactive atrolactic acid ($\text{CH}_3\cdot\text{C}(\text{OH})(\text{C}_6\text{H}_5)\cdot\text{COOH}$), 80% of the resulting compound was excreted. Finally, atropic acid, $\text{C}_6\text{H}_5\cdot\text{C}(\text{:CH})\cdot\text{COOH}$, was completely oxidized when administered in small doses.

When a methyl group is substituted in the α -position, the acids are readily oxidized in the body to yield the same end-products as the corresponding unsubstituted acids.^{4,5} Since the α -methyl-substituted acids have two β -carbon atoms, oxidation may involve either one. If it attacks the methyl group, it will be removed, and oxidation of the resulting straight-chain acid will be normal. If oxidation results on the second β -group, the cleavage of a three-carbon fragment will occur.

Kay and Raper³ were of the opinion that the first pathway best explained

¹ G. Weitzel and E. Schraufstätter, *Z. physiol. Chem.*, **285**, 172–182 (1950).

² H. Thaler, A. Schottmayer, I. Stählin, and H. Beck, *Biochem. Z.*, **320**, 87–98 (1949).

³ H. D. Kay and H. S. Raper, *Biochem. J.*, **16**, 465–474 (1922).

⁴ H. D. Kay and H. S. Raper, *Biochem. J.*, **18**, 153–160 (1924).

⁵ M. J. Coon and N. S. B. Abrahamsen, *J. Biol. Chem.*, **195**, 805–812 (1952).

their results. Thus, the oxidation of α -phenylpropionic acid to yield phenylacetic acid was believed to occur as follows:

Suggested Pathway of Metabolism of α -Phenylpropionic (Hydratropic) Acid⁶

Although the results on α -phenylpropionic acid indicated a demethylation and the formation of the corresponding straight-chain acid, this compound has only one aliphatic β -carbon. This would automatically preclude the second pathway postulated above, namely, oxidation on the second β -carbon with the cleavage of a three-carbon fragment which includes the α -methyl group. Kay and Raper⁴ answered this criticism by experiments with α -methyl- β -phenylpropionic acid, $\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \underset{\text{CH}_3}{\underset{|}{\text{CH}}} \cdot \text{COOH}$, and α -methyl- γ -phenylbutyric acid, $\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \underset{\text{CH}_3}{\underset{|}{\text{CH}}} \cdot \text{COOH}$. Both of these acids have two β -aliphatic carbons. When these acids were given in moderate doses to dogs, the end-products were benzoic and phenylacetic acid, which are likewise the intermediates after phenylpropionic or phenylbutyric acids are fed. In all cases, these acids were found to be conjugated with glycine or glucuronic acid before being excreted in the urine. When α -methylphenylpropionic acid was fed to cats, α -methylcinnamic acid and benzoic acid were present in the urine.

The Pathway of Oxidation of α -Methyl- β -Phenylpropionic Acid in Cats⁴

These results strongly support the hypothesis that oxidation of α -methyl acids occurs on the β (non-methyl)-carbon, provided it is an aliphatic carbon. Carter⁶ obtained substantially identical results, and postulated the following general scheme of oxidation of α -methyl-substituted acids:

General Scheme for Oxidation of an α -Methyl-Substituted Acid⁶

⁶ H. E. Carter, *Biol. Symposia*, 5, 47-63 (1941).

However, ω -phenyl-substituted acids with groups larger than methyl in the α -position are resistant to oxidation in the animal body. Carter⁶ reported that both α -ethyl and α -propyl- β -phenylpropionic acids ($\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}(\text{C}_2\text{H}_5)\cdot\text{COOH}$ and $\text{C}_6\text{H}_5\cdot\text{CH}_2\text{CH}(\text{C}_3\text{H}_7)\cdot\text{COOH}$) were excreted unchanged. Likewise, Kuhn and Livada⁷ noted that α -ethylcinnamic acid ($\text{C}_6\text{H}_5\text{CH}:\text{C}(\text{C}_2\text{H}_5)\cdot\text{COOH}$ and α -benzylcrotonic acid ($\text{CH}_3\cdot\text{CH}:\text{C}(\text{CH}_2\cdot\text{C}_6\text{H}_5)\cdot\text{COOH}$) were excreted unchanged by dogs and rabbits. Carter⁶ suggests that, when the α -substituted group is larger than a methyl, steric hindrance prevents enzymatic attack on the carbon chain.

When a simple α -methyl-substituted acid such as α -methylbutyrate ($\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}(\text{CH}_3)\cdot\text{COOH}$) is metabolized, the mechanism of β -oxidation is presumably still operative. Thus, Coon and Abrahamsen⁵ are of the opinion that β -oxidation occurs on the longer chain to produce "acetate" and a three-carbon acid fragment. These results are in accord with the belief that methylbutyrate is an intermediate in isoleucine metabolism, and they account for the weakly ketogenic action of this acid as established by Butts *et al.*⁸ If the three-carbon acid is propionic, it would also furnish evidence of a source of the glycogen noted after the feeding of isoleucine.⁸

(2) β -Methyl-Substituted Acids

β -Methyl-substituted acids are ordinarily resistant to demethylation. Thus, Kuhn and Livada⁷ reported that β -phenylbutyric acid ($\text{C}_6\text{H}_5\cdot\text{CH}(\text{CH}_3)\cdot\text{CH}_2\cdot\text{COOH}$) is conjugated with glycine or glucuronic acid by rabbits, and is excreted.⁶ The fact that some β -phenylcrotonic acid ($\text{C}_6\text{H}_5\cdot\text{C}(\text{CH}_3):\text{CH}\cdot\text{COOH}$) is present as an excretion product is taken to mean that desaturation, rather than formation of a β -keto acid, is the first step in β -oxidation. Carter⁶ confirmed the results of Kuhn and Livada.⁷ Carter recognized that the substituted cinnamic acid can originate by the introduction of an hydroxyl radicle on the β -carbon, followed by removal of a molecule of water, as well as by primary desaturation in the α,β -positions; however, he favors the second possibility.

ω -Oxidation is sometimes called into play to effect the oxidation of β -methyl-substituted acids. Thus, Hirohata and collaborators⁹ reported that, when L-rhodinic acid was administered to dogs, cats, rabbits, or human subjects, it underwent ω -oxidation to a large extent, and was excreted as dihydro-Hildebrandt acid. Moreover, after the administration of geranic acid to man, as well as after that of tetrahydrogeranic acid, both

⁷ R. Kuhn and K. Livada, *Z. physiol. Chem.*, **220**, 235-246 (1933).

⁸ J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, **120**, 289-295 (1937).

Hildebrandt and dihydro-Hildebrandt acids were eliminated in the urine. The occurrence of ω -oxidation is believed to result from the hindrance to β -oxidation by the branched-chain on the α - or β -carbon, or from its presence in both positions. These reactions are illustrated below:

The Formation of Dicarboxylic Acids by ω -Oxidation after the Administration of L-Rhodinic Acid, Geranic Acid, or Tetrahydrogeranic Acid, Based upon the Findings of Hirohata *et al.*⁹

(3) β -Phenyl-Substituted Acids

When the phenyl group occurs on the β -position, β -oxidation of the fatty acid is likewise hindered or entirely blocked; under such conditions, ω -oxidation may also occur as an emergency method of disposing of these fatty acids.^{10,11} Stevens¹² also showed that, when a phenyl group is intro-

⁹ R. Hirohata, S. Yamasaki, T. Iseki, T. To, S. Iseda, and M. Shojima, *J. Biochem. (Japan)*, **38**, 135-146 (1951).

¹⁰ B. Flaschenträger, K. Bernhard, C. Löwenberg, and M. Schläpfer, *Z. physiol. Chem.*, **225**, 157-167 (1934).

¹¹ R. Kuhn, F. Köhler, and L. Köhler, *Z. physiol. Chem.*, **242**, 171-197 (1936); **247**, 197-219 (1937).

¹² C. M. Stevens, *I. Azlactones. II. Metabolism of Fatty Acids*. Thesis, Univ. Illinois, 1941; also cited by H. E. Carter, *Biol. Symposia*, **5**, 47-63 (1941), p. 60.

duced in the β -position of lauric acid, a much larger proportion undergoes ω -oxidation than is normally the case. Presumably, ω -oxidation is called into play because the partial storage in the fat depots available to lauric acid is not open to the phenyl-substituted lauric acid, and it must be removed from the body by any method of oxidation which can be made available.

(4) *Miscellaneous Substituted Acids*

Aliphatic acids which contain a tertiary carbon atom are oxidized with greater difficulty in the animal body. Moreover, they may be detoxicated by conjugation with glucuronic acid, and are the only instances which have been reported of the conjugation of glucuronic acid with an *aliphatic* fatty acid. Thus, Dziewiatkowski and Lewis¹³ reported that pivalic or trimethylacetic acid,

and tertiary butylacetic acid,

were practically completely excreted in unoxidized form by both rats and rabbits. Moreover, it was shown that they were excreted largely in the form of labile conjugated glucuronides. In a later study, Dziewiatkowski *et al.*¹⁴ reported that methylneopentylacetic acid,

was oxidized with difficulty in the organism of the rat and rabbit; its administration likewise led to the excretion of large amounts of extra glucuronic acid, presumably in combination with the unchanged acid. However, the excretion of aliphatic fatty acids as glucuronides was apparently not limited to those acids having a tertiary carbon, since it was likewise

¹³ D. D. Dziewiatkowski and H. B. Lewis, *J. Biol. Chem.*, 158, 77-87 (1945).

¹⁴ D. D. Dziewiatkowski, A. Venkataraman, and H. B. Lewis, *J. Biol. Chem.*, 178, 169-177 (1949).

observed that, following the administration of α -ethyl-substituted acids such as α -ethylbutyric acid, $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{COOH}$, and α -ethyl-*n*-caproic acid, $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{COOH}$ considerable amounts of glucuronides were also excreted. These results are in harmony with those of Carter,⁶ in which the failure of α -ethyl and α -propyl acids to be metabolized was noted. Akiya and Yamakawa¹⁵ reported that a number of acids, including:

2-methylcaprylic acid $(\text{CH}_3 \cdot (\text{CH}_2)_5 \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH})$

3-methylnonanoic acid $(\text{CH}_3 \cdot (\text{CH}_2)_5 \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{COOH})$

3-ethylcaproic acid $(\text{CH}_3 \cdot (\text{CH}_2)_2 \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{CH}_2 \cdot \text{COOH})$

dibutylacetic acid $((\text{CH}_3(\text{CH}_2)_3)_2 \cdot \text{CH} \cdot \text{COOH})$

dipropylpropionic acid $((\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2)_2 \cdot \text{CH} \cdot \text{CH}_2 \cdot \text{COOH})$

were excreted unchanged by rabbits, and also as the dicarboxylic acids after ω -oxidation.

In the comprehensive studies of Keil and co-workers,¹⁶⁻¹⁸ it was found that methyl-substituted fatty acids were largely metabolized. Thus, after the feeding of

α -methyl-dodecanoic acid $(\text{CH}_3 \cdot (\text{CH}_2)_9 \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH})$

β -methyl-dodecanoic acid $(\text{CH}_3 \cdot (\text{CH}_2)_8 \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{COOH})$

δ -methyl-dodecanoic acid $(\text{CH}_3 \cdot (\text{CH}_2)_6 \cdot \text{CH}(\text{CH}_3) \cdot (\text{CH}_2)_3 \cdot \text{COOH})$

to dogs in the form of triglycerides, without added fat, there was only a slight increase in the urinary acids excreted, as contrasted with the results of the administration of unbranched dodecanoic acid or tridecanoic acid. Weitzel and co-workers¹⁹ demonstrated that one methyl group attached to carbon-atom 2 (α) hindered oxidation more than did three methyl groups on a long-chain fatty acid at positions 3, 7, and 11 to the carboxyl. However, when ethyl-substituted acids were fed to dogs in a 1:3 mixture in cocoa fat, as triglycerides, amounts of ether-soluble metabolic end-products up to 50% of the weight of the ingested acids were found to be excreted in the urine. The following end-products were obtained from the several acids administered:¹⁶

¹⁵ S. Akiya and T. Yamakawa, *J. Pharm. Soc. Japan*, 70, 477-80 (1950); *Chem. Abst.*, 45, 3479 (1951).

¹⁶ W. Keil, *Z. physiol. Chem.*, 274, 175-185 (1942).

¹⁷ H. Appel, H. Böhm, W. Keil, and G. Schiller, *Z. physiol. Chem.*, 274, 186-205 (1942).

¹⁸ W. Keil, *Z. physiol. Chem.*, 276, 26-32 (1942).

¹⁹ G. Weitzel, A. Fretzdorff, J. Wojahn, W. Savelsberg, and K. Thomas, *Naturz*, 163, 406-407 (1949).

When the methyl-dodecanoic acids were fed in cocoa fat, the dogs receiving δ -methyl-dodecanoic acid excreted 35% of the weight of ingested triglyceride as fatty material in the urine,¹⁶⁻¹⁸ which was almost equal to the amount of ethyl-substituted acids excreted. Methyl-sebacic acid was also found in the urine. Although Dziewiatkowski *et al.*¹⁴ reported that α -ethylbutyric and α -ethylcaproic acids were excreted largely unchanged as the glucuronide by the dog, somewhat different evidence was presented earlier as to the fate of such compounds. Thus, Blum and Koppel,²⁰ on the basis of experiments on dogs, reported the excretion of methylpropyl ketone ($\text{CH}_3\text{CH}_2 \cdot \text{CO} \cdot \text{CH}_3$) when β -ethylbutyric acid was injected subcutaneously. It was also reported by Baer and Blum²¹ that this acid gives rise to acetoacetic acid when perfused through the liver. It has likewise been reported that α -ethylbutyric acid results in the formation of urinary acetone bodies in human subjects. However, Dziewiatkowski and co-workers¹⁴ were unable to find any evidence of the formation of ketone bodies after α -ethylbutyric or α -ethylcaproic acids had been fed to rabbits or rats.

In later work by Keil and Schiller,²² it was found that the propyl-branched fatty acids, when fed in a 1:3 mixture with cocoa fat, were excreted as partially metabolized end-products to the extent of 10 to 25% of the material fed. However, the propyl-substituted compounds were better utilized than were the ethyl-substituted ones. The following reactions were shown to occur with some of the substituted acids:

²⁰ L. Blum and M. Koppel, *Ber.*, 44, 3576-3578 (1911).

²¹ J. Baer and L. Blum, *Naunyn-Schmiedeberg's Arch. exp. Pathol. Pharmacol.*, 55, 89-115 (1906); 56, 92-100 (1907).

²² W. Keil and G. Schiller, *Z. physiol. Chem.*, 282, 135-136, 137-142 (1947).

and isobutyrate in rumen contents; it is believed that these probably arise from the bacterial degradation of isoleucine, leucine, and valine.

Grafflin and Green²⁷ reported that isocaproic acid $((\text{CH}_3)_2\text{CH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH})$ can be oxidized by a kidney enzyme system which contains, in addition, ATP (or adenosine monophosphate), Mg^{++} , and inorganic phosphate. Atchley,²⁸ likewise making use of the kidney enzyme system, proved that the intermediate in the oxidation of isocaproic acid was isobutyric acid, as would be expected from the β -oxidation theory. Isobutyric acid was isolated from the reaction mixture by the use of the counter-current distribution, and was identified. The course of this reaction is indicated below:

The Oxidation of Isocaproic Acid by a Kidney Fatty Acid Oxidizing System²⁸

When isobutyric acid was oxidized by the kidney fatty acid oxidizing system, propionic acid was identified as one of the end-products, by isolation with counter-current distribution. Atchley²⁸ pictured the possible pathway of breakdown of isobutyric acid as follows:

The Postulated Pathway of Oxidation of Isobutyric Acid (I) through β -Hydroxyisobutyric Acid (II), Methylmalonic Semialdehyde (III), Propionaldehyde (IV) to Propionic Acid (V)²⁸

Since propionic acid is quantitatively transformed to glucose, this pathway is in agreement with the finding of Ringer, Frankel, and Jonas²⁹ that isobutyric acid gives rise to glucose and that isobutyl alcohol yields an amount of glucose corresponding to the conversion of three of its carbons when it is given to a phlorhizinized dog. The ketogenic activity of branched-chain fatty acids has been discussed earlier (see page 130).

²⁷ A. L. Grafflin and D. E. Green, *J. Biol. Chem.*, **176**, 95-115 (1948).

²⁸ W. A. Atchley, *J. Biol. Chem.*, **176**, 123-131 (1948).

²⁹ A. I. Ringer, E. M. Frankel, and L. Jonas, *J. Biol. Chem.*, **14**, 525-538 (1913).

2. Hydroxy and Keto Acids

Important intermediates in the catabolism of the fatty acids, as well as in the degradation of glucose and amino acids, include hydroxy and keto acids.

(1) α -Hydroxy and α -Keto Acids

There are several common α -hydroxy acids in the animal body. One of these is lactic acid ($\text{CH}_3\cdot\text{CHOH}\cdot\text{COOH}$), which originates from carbohydrate, as well as from the hydrolytic deamination of alanine. More complex α -hydroxy fatty acids include cerebronic acid ($\text{CH}_3\cdot(\text{CH}_2)_{21}\cdot\text{CHOH}\cdot\text{COOH}$) and hydroxynervonic acid ($\text{CH}_3\cdot(\text{CH}_2)_7\cdot\text{CH}:\text{CH}\cdot(\text{CH}_2)_{12}\cdot\text{CHOH}\cdot\text{COOH}$), both of which are important components of cerebrosides.

Keto acids may be formed by controlled oxidation of the corresponding hydroxy acids. Lactic acid is readily converted to pyruvic acid, while the reverse change is also known to occur. The prime importance of α -keto acids in the metabolic picture is realized when one recalls that pyruvic acid, and several members of the tricarboxylic acid cycle, such as α -ketoglutaric and oxaloacetic, are α -keto acids.

Most of the naturally occurring amino acids are α -amino acids. Since it is known that the first step in their catabolism is deamination, it is obvious that hydroxy and keto acids can be formed from them. Thus, when hydrolytic deamination occurs, α -hydroxy acids are formed and, when oxidative deamination obtains, the α -keto acids result.

The commonest method for the oxidation of α -hydroxy acids involves (1) conversion to an α -keto acid, (2) decarboxylation with the formation of an aldehyde having one less carbon than the original acid, (3) oxidation of aldehyde to acid, and (4) oxidation of the resulting acid by β -oxidation.

Both hydroxy and keto acids may likewise be converted to the corresponding amino acids by amination. As proof of these syntheses, one may cite the fact that the essential acids, tryptophane, valine, and histidine, may be replaced in the diet by corresponding α -hydroxy acids. Moreover, the α -keto acids, as well as the α -hydroxy acids, have been shown to replace phenylalanine, leucine, and isoleucine.

Since the hydroxy acids contain an asymmetric carbon, they are optically active. Although it is recognized that only one of the optical isomers occurs naturally, the appropriate form can usually be synthesized by the body. The ability of α -keto acids to serve as a source of the amino acids which likewise contain an asymmetric α -carbon is evidence for this *in vivo* capacity.

Baker³⁰ demonstrated that both optical isomers of straight-chain α -hydroxy acids from C_3 to C_8 , and also α -hydroxyisovaleric acid, α -hydroxyisocaproic acid, and L- β -imidazole lactic acid can be oxidized by enzyme preparations from rat liver and rat kidney, and from hog kidney. At least two enzyme systems, distinct from the lactic dehydrogenase system and the cyclophorase-racemase system, were separated from washed particle and supernatant preparations of liver and kidney. One of these is specific for oxidation of the L-isomer, and the other is specific for that of the D-isomer of the α -hydroxy acids.

a. α -Hydroxyisobutyric Acid. When a hydroxyl group is introduced into the α -carbon of isobutyric acid, a marked change in properties obtains. Thus, it was shown by Dziewiatkowski and co-workers¹⁴ that hydroxyisobutyric or acetonic acid, $(CH_3)_2 \cdot C(OH) \cdot COOH$, was largely excreted as such without conjugation with glucuronic acid. Moreover, no ketone formation occurred. This is in striking contrast to the behavior of isobutyric acid, $(CH_3)_2 \cdot CH \cdot COOH$, and isobutyl alcohol, which Ringer and associates²⁹ found was converted to glucose by the phlorhizinized dog to the extent of three carbon atoms. Acetoacetic acid was also shown to be formed when β -hydroxyisovaleric acid, $(CH_2)_3 : C(OH) \cdot CH_2COOH$, was perfused through the surviving liver;³¹ this latter compound has the same grouping $((CH_3)_2 \cdot C(OH) \cdot)$ as is present in hydroxyisobutyric acid.

(2) β -Hydroxy and β -Keto Acids

The most common acids in this category are β -hydroxybutyric acid and acetoacetic (diacetic) acid. The hydroxy and keto acids in this category are interconvertible in the animal body. Thus, Breusch and Tulus³² found that not only is the α -keto acid, pyruvic acid, reduced by minced tissue, but the same is true of the β -keto acids, β -ketobutyric acid and β -ketocaprylic acid $(CH_3 \cdot (CH_2)_4 \cdot CO \cdot CH_2 \cdot COOH)$. It will be recalled that the oxidation of fatty acids regularly proceeds by oxidation of the β -carbon atom to a keto group. In the case of long-chain acids, a rupture of the acid occurs at the β -carbon, yielding an acetic acid molecule and an aldehyde having two less carbons than the original acid. The aldehyde is oxidized to an acid, which again is subject to β -oxidation, with the formation of a new β -keto acid which is metabolized similarly. When the fatty acid chain is reduced to four carbons, the sequence of events is interrupted

³⁰ C. G. Baker, *Arch. Biochem. Biophys.*, **41**, 325-332 (1952).

³¹ E. Friedmann, *Beitr. chem. Physiol. Pathol.*, **11**, 365 (1908).

³² F. L. Breusch and T. Tulus, *Arch. Biochem.*, **11**, 499-506 (1946).

in the absence of carbohydrate. Under such circumstances, a considerable portion of the keto acid is reduced to the hydroxy acid, and the mixture of these two acids is excreted in the urine.

Further confirmation of the ease of oxidation of the β -hydroxy and β -keto acids can be obtained from the classical studies of Grafflin and Green.²⁷ These investigators reported that a large number of β -hydroxy and β -keto acids, as well as α,β -unsaturated fatty acids, which have been considered as intermediates in fatty acid catabolism according to the β -oxidation theory, were as readily oxidized by the kidney cyclophorase system as were the corresponding fatty acids, and that, furthermore, their end-products were also identical with those of the parent acids. When the oxidation of the fatty acid involved the cleavage of a single C_2 group, and particularly when one of the products of oxidation was inhibitory, as, for example, propionic acid from valeric acid, oxidation occurred satisfactorily when increased amounts of the "sparker" were present. The hydroxy-acids tested included the following DL-acids: β -hydroxybutyric; β -hydroxyvaleric, $CH_3 \cdot CH_2 \cdot CHOH \cdot CH_2 \cdot COOH$; β -hydroxyisocaproic, $(CH_3)_2 \cdot CH \cdot CHOH \cdot CH_2 \cdot COOH$; β -hydroxyeaproic, $CH_3 \cdot CH_2 \cdot CH_2 \cdot CHOH \cdot CH_2 \cdot COOH$; and β -hydroxycaprylic; $CH_3 \cdot (CH_2)_4 \cdot CHOH \cdot CH_2 \cdot COOH$. All these acids were readily oxidized. On the other hand, β -hydroxylauric acid ($CH_3 \cdot (CH_2)_8 \cdot CHOH \cdot CH_2 \cdot COOH$) was found to be a powerful inhibitor of fatty acid oxidase, even at concentrations of less than 0.1 micromole per ml. Here again, 75% of this acid was oxidized when highly potent enzyme preparations were employed.

The β -keto acids tested by Grafflin and Green²⁷ were likewise readily oxidized. The samples tested were the following: β -ketovaleric acid, $CH_3 \cdot CH_2 \cdot CO \cdot CH_2 \cdot COOH$; β -ketoisocaproic acid, $(CH_3)_2 \cdot CH \cdot CO \cdot CH_2 \cdot COOH$; and β -ketooctanoic acid, $CH_3 \cdot (CH_2)_4 \cdot CO \cdot CH_2 \cdot COOH$. The several intermediates of fatty acid oxidation were similarly broken down by a liver cyclophorase. Similar results were obtained by Witter *et al.*³³ with their washed liver homogenates which were able to metabolize β -hydroxyhexanoate, β -ketoheptanoate, and, to some extent, β -hydroxy- γ -hexenoate ($CH_3 \cdot CH : CH \cdot CHOH \cdot CH_2 \cdot COOH$), but not α,β -dihydroxyhexanoate ($CH_3 \cdot CH_2 \cdot CH_2 \cdot CHOH \cdot CHOH \cdot COOH$) or triacetic acid ($CH_3 \cdot CO \cdot CH_2 \cdot CO \cdot CH_2 \cdot COOH$).

These experiments are all consistent in demonstrating that the β -hydroxy and β -keto acids are readily metabolized products which follow the same course of metabolism as do their parent acids.

³³ R. F. Witter, E. H. Newcomb, and E. Stotz, *J. Biol. Chem.*, 185, 537-548 (1950).

(3) *Miscellaneous Hydroxy and Keto Acids*

Breusch and Tulus³² reported that only α - and β -keto acids can be attacked by tissue enzymes. In compounds such as γ -ketovaleric acid ($\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$) and δ -ketocaproic acid ($\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$), in which the ketones were further removed from the carboxyl group, the tissue enzymes were unable to metabolize them.

Polyketonic acids are readily metabolized in some cases. In fact, according to the multiple alternate oxidation theory as proposed by Hurlley³⁴ and later by Jowett and Quastel,³⁵ and supported by Stadie,³⁶ polyketonic acids are believed to be formed in the catabolism of fatty acids; such compounds can then break down simultaneously at several points to smaller fragments.

The α, γ -diketo acids are somewhat difficult to break down. However, the simplest member of this group, acetylpyruvic acid ($\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{COOH}$), is apparently rapidly metabolized by animal tissues,³⁷ and a specific enzyme which produces pyruvic acid from this compound has been partially purified by Meister and Greenstein.³⁸ On the other hand, α, γ -diketooctanoic acid ($\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{COOH}$), is only slowly metabolized by broken cell preparations of liver, in contradistinction to the rapid catabolism of octanoic acid.³⁹ There is no evidence from the tests of Weinhouse and co-workers³⁹ with carboxyl-labeled octanoic acid that an α, γ -diketo acid is formed.

The β, δ -diketo acid has been shown to be readily metabolized. Thus, Witter and Stotz⁴⁰ found that rat liver homogenate and an enzyme prepared from liver were able to break down triacetic acid into a molecule of acetate and one of acetoacetate, as indicated below:

The corresponding ethyl ester of triacetic acid was metabolized in a similar manner to the free acid, after the preliminary action of an esterase. Breusch and Ulusoy⁴¹ reported that a δ -lactone of triacetic acid was also metab-

³⁴ W. H. Hurlley, *Quart. J. Med.*, **9**, 301-408 (1915-1916).

³⁵ M. Jowett and J. H. Quastel, *Biochem. J.*, **29**, 2159-2180 (1935).

³⁶ W. C. Stadie, *Physiol. Revs.*, **25**, 395-441 (1945).

³⁷ A. Lehninger, *J. Biol. Chem.*, **148**, 393-404 (1943).

³⁸ A. Meister and J. P. Greenstein, *Federation Proc.*, **7**, 173 (1948).

³⁹ S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, **155**, 143-151 (1949).

⁴⁰ R. F. Witter and E. Stotz, *J. Biol. Chem.*, **176**, 501-510 (1948).

⁴¹ F. L. Breusch and E. Ulusoy, *Arch. Biochem.*, **14**, 183-191 (1947).

olized by the liver to acetoacetic acid; it was found that 1.3 moles of acetoacetate were formed per mole of triacetic acid, (β,δ -diketohexanoic acid). On the other hand, kidney, muscle, and lung were unable to attack the diketonic acid. The results are interpreted as offering support for the multiple alternate oxidation theory, as far as liver is concerned, while they exclude the validity of this concept in the peripheral organs.

3. Polycarboxylic Acids

(1) Dicarboxylic Acids

a. Functions of the Dicarboxylic Acids. Although the dicarboxylic acids are not important components of the food, and are not generally considered as direct intermediates in fat utilization, two important functions are now recognized. Thus, dicarboxylic acids form some of the essential components of the tricarboxylic acid (or Krebs) cycle, which assists in the oxidation of acetic acid and of carbohydrate intermediates; dicarboxylic acids may normally also be formed, in some cases, as intermediates in the breakdown of the fatty acids of moderate chain-length by the so-called process of ω -oxidation.

(a) *The Role of Dicarboxylic Acids in the Tricarboxylic Acid Cycle.* The successful functioning of the tricarboxylic acid cycle in activating the production of energy is undoubtedly related to the ability of the dicarboxylic acids to interchange, and to build up to the tricarboxylic acids, isocitric, citric, and aconitic. Oxaloacetic acid, $\text{HOOC}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH}$, appears to be the key acid in the functioning of the cycle. This acid is conjugated with a two-carbon compound to yield, in turn, the six carbon acids, citric, *cis*-aconitic, isocitric, and oxalosuccinic. α -Ketoglutaric acid, $\text{HOOC}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$, is formed next; the following members of this cycle are, in succession, the four-carbon dicarboxylic acids, succinic ($\text{HOOC}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$), fumaric ($\text{HOOC}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$), and malic ($\text{HOOC}\cdot\text{CH}_2\cdot\text{CHOH}\cdot\text{COOH}$); malic acid is readily converted into oxaloacetic acid, and the cycle can be restarted.

The discovery and recognition of this cycle as a method for promoting the oxidation of carbohydrate and fat intermediates is largely due to the work of Krebs⁴² alone and with Johnson.⁴³ However, somewhat earlier, Annau *et al.*⁴⁴ and Stare and Baumann⁴⁵ had reported that the four-carbon

⁴² H. A. Krebs, *Advances in Enzymol.*, **3**, 191-252 (1943).

⁴³ H. A. Krebs and W. A. Johnson, *Enzymologia*, **4**, 148-156 (1937).

⁴⁴ E. Annau, I. Banga, B. Gözsy, St. Huszák, K. Laki, B. Straub, and A. Szent-Györgyi, *Z. physiol. Chem.*, **236**, 1-68 (1935); E. Annau, I. Banga, A. Blazsó, V. Bruckner, K. Laki, F. B. Straub, and A. Szent-Györgyi, *Ibid.*, **244**, 105-152 (1936).

⁴⁵ F. J. Stare and C. A. Baumann, *Proc. Roy. Soc. London, B* **121**, 338-357 (1936).

dicarboxylic acids, succinic, fumaric, malic, and oxaloacetic, and pyruvic acid, had a powerful catalytic influence in increasing oxygen uptake and carbon dioxide production by muscles and by other tissues. It was recognized that the succinate $\rightarrow$ fumarate reaction is inhibited by malonate,⁴⁴ while the fumarate $\rightarrow$ malate conversion is blocked by arsenite.

Martius⁴⁶ was the first to demonstrate that citric, isocitric, and *cis*-aconitic acids are readily interconvertible in the tissues, while Martius and Knoop⁴⁷ and Martius alone⁴⁸ observed that citric acid can be transformed into α -ketoglutaric acid. Ochoa and Weisz-Tabori⁴⁹ discovered the enzyme, oxalosuccinic carboxylase, which is capable of causing the decarboxylation of oxalosuccinic acid, with the resultant formation of α -ketoglutaric acid. This enzyme also catalyzes the reverse reaction by causing the incorporation of CO₂ into α -ketoglutaric acid to form oxalosuccinic acid. Apparently the enzyme is widely distributed in tissues.

Another key reaction is the conversion of α -ketoglutaric acid to succinic acid.⁵⁰ This is accomplished by the intermediation of the α -ketoglutaric acid dehydrogenase system. According to Ochoa,⁵¹ both ATP and the cytochrome system are required for the completion of this reaction. The details of the mechanism of condensation of oxaloacetic acid and of acetic acid to yield citric acid have been recorded by Stern and Ochoa.^{52,53}

(b) *The Role of Dicarboxylic Acids as Intermediary Products in Fatty Acid Oxidation.* Omega-oxidation, with the resultant formation of a dicarboxylic acid, is believed to afford an alternate pathway for the oxidation of fatty acids. Verkade and co-workers⁵⁴ reported that the fatty acids of medium length, when fed to man as their triglycerides, were in part excreted as dicarboxylic acids of the same chain length, or as dicarboxylic acids with two or four fewer carbons than the original acids. This reaction occurs principally with C₉, C₁₀, and C₁₁ acids, but C₈ and C₁₂ also exhibit a limited amount of this type of oxidation. This course of oxidation for capric acid is pictured on the following page:

⁴⁶ C. Martius, *Z. physiol. Chem.*, **257**, 29-42 (1938).

⁴⁷ C. Martius and F. Knoop, *Z. physiol. Chem.*, **246**, I-II (1937).

⁴⁸ C. Martius, *Z. physiol. Chem.*, **247**, 104-110 (1937).

⁴⁹ S. Ochoa and E. Weisz-Tabori, *J. Biol. Chem.*, **159**, 245-246 (1945); **174**, 123-132 (1948).

⁵⁰ H. A. Krebs and W. A. Johnson, *Biochem. J.*, **31**, 645-660 (1937).

⁵¹ S. Ochoa, *J. Biol. Chem.*, **155**, 87-100 (1944).

⁵² J. R. Stern and S. Ochoa, *J. Biol. Chem.*, **179**, 491-492 (1949).

⁵³ J. R. Stern and S. Ochoa, *Federation Proc.*, **9**, 234-235 (1950).

⁵⁴ P. E. Verkade, M. Elzas, J. van der Lee, H. H. de Wolff, A. Verkade-Sandbergen, and D. Van der Sande, *Proc. koninkl. Akad. Wetenschap.*, Amsterdam, **35**, Afd. Nat.-urk., 251-266 (1932).

Successive Stages in the Oxidation of Capric Acid by ω -Oxidation, Followed by Two Successive β -Oxidations²³

It is believed that ω -oxidation proceeds under opposite conditions from the usual β -oxidation. Whereas fatty acids are believed to proceed by β -oxidation to the ketone bodies in the case of conditions which engender ketonuria, diaciduria is fostered on high carbohydrate diets in the absence of ketonuria.

Although ω -oxidation of fatty acids higher than C_{12} cannot ordinarily be demonstrated, the oxidation of myristic (C_{14}) and stearic (C_{18}) acids by this pathway can be shown when the usual β -oxidation is blocked by the presence in the α -position of a side-chain more than one carbon in length.²³ Under these conditions, long-chain dicarboxylic acids have been demonstrated in the urine. For a further discussion of ω -oxidation, the reader is referred to the preceding chapter.

b. The Occurrence and Metabolism of Dicarboxylic Acids. (a) *Introduction.* The most complete information in this category is available concerning the metabolism of the four-carbon dicarboxylic acids; of these acids, the evidence at hand mainly concerns succinic acid. Oxalic acid, $\text{HOOC} \cdot \text{COOH}$, the simplest dicarboxylic acid, is oxidized to only a limited extent. When administered in large amounts to the animal, it is toxic. It appears in the urine of man to the extent of 20 to 40 mg. daily.⁵⁵ Malonic acid, $\text{HOOC} \cdot \text{CH}_2 \cdot \text{COOH}$, is much more completely utilized, although it is not generally regarded as a normally occurring metabolite. This acid may act as an antagonist to ordinary tissue oxidation. The subject of utilization of these and of other dicarboxylic acids has been reviewed by Dakin⁵⁶ and by Flaschenträger.⁵⁷

⁵⁵ P. Müller, *Verhandl. Ver. schweiz. Physiol.*, 11, 8-9 (1937); *Chem. Abst.*, 32, 6308 (1938); *Physiol. Abst.*, 22, 349 (1937).

⁵⁶ H. D. Dakin, *Oxidations and Reductions in the Animal Body*, 2nd ed., Longmans, Green, London & New York, 1922.

⁵⁷ B. Flaschenträger, *Z. physiol. Chem.*, 159, 297-308 (1926).

(b) *Succinic Acid*. Employing a method for the isolation of succinic acid worked out by Thomas and Weitzel,⁵⁸ Weitzel⁵⁹ reported that man excretes, by way of the kidney, 2 to 12 mg. of succinic acid daily.

A number of factors result in an increase in the excretion of succinic acid. For example, the elimination of this dicarboxylic acid in man was augmented by the ingestion of 250 mg. of glucose.⁵⁹ The higher dicarboxylic acids may likewise serve as sources of urinary succinate. Thus, Flaschen-träger and Bernhard⁶⁰ demonstrated the presence of small amounts of succinic acid anilide and succinic aniline after the feeding of adipic acid or the half anilide of sebacic acid to the dog. Weitzel⁶¹ also reported the greatest increase in succinate after adipic acid (C₆) and pimelic acid (C₇) had been given; the author is of the opinion that suberic (C₈) and azelaic (C₉) acids also exhibit some activity. Glutaric (C₅) acid was found to be entirely inactive insofar as succinate formation is concerned.

The fatty acids having an odd number of carbon atoms or having branched chains are very important sources of succinate. Thomas and Weitzel⁶² observed that the ingestion of synthetic fats, produced from petroleum in Germany during the war, increased succinic acid excretion; these fats were characterized by a high proportion (50%) of fatty acids having an odd number of carbon atoms. After the administration of one such fat to nine subjects, the succinic acid output was markedly increased; in a second series following the administration of another synthetic fat, no alteration in the normal excretion of succinic acid could be noted. These authors suggest that the increased succinic acid output results from a disturbance in enzymic decomposition brought about by branched monocarboxylic acids, or by normal and abnormal branched dicarboxylic acids. Similar results have been reported by Hanson.^{63,64}

The effect of alkylated succinic acid derivatives on the urine succinic acid varies with the chain length of the substituent. Thus, Emmrich, Neumann, and Emmrich-Glaser⁶⁵ found that considerable amounts of succinic acid were present in the urine of the dog after methyl-, ethyl-, *n*-propyl-, or *n*-butyl-succinic acid had been fed; the utilization of *n*-hexyl-succinic acid was somewhat greater than that of its homologues. In a later

⁵⁸ K. Thomas and G. Weitzel, *Z. physiol. Chem.*, **282**, 170-174 (1947).

⁵⁹ G. Weitzel, *Z. physiol. Chem.*, **282**, 174-180 (1947).

⁶⁰ B. Flaschenträger and K. Bernhard, *Z. physiol. Chem.*, **256**, 71-74 (1938).

⁶¹ G. Weitzel, *Z. physiol. Chem.*, **282**, 185-191 (1947).

⁶² K. Thomas and G. Weitzel, *Z. physiol. Chem.*, **282**, 180-185 (1947).

⁶³ H. Hanson, *Ernährung*, **6**, 273-277 (1941); *Chem. Zentr.*, **113**, II, 2153 (1942).

⁶⁴ H. Hanson, *Z. ges. expit. Med.*, **113**, 226-244 (1943).

⁶⁵ R. Emmrich, P. Neumann, and I. Emmrich-Glaser, *Z. physiol. Chem.*, **267**, 228-241 (1941).

study of higher homologues of succinic acid, Thomas, Weitzel, and Neumann⁶⁶ reported that these compounds are readily utilizable by the dog when given in dosages of 0.1 to 0.2 g. per kg. body weight per day. The following amounts of succinic acid were isolated from the urine after dosages of 12 or 18 g. of the alkyl succinates: 12 g. heptylsuccinate, 9.7 mg. succinate; 18 g. octylsuccinate, 63.6 mg.; 12 g. nonylsuccinate, 4.3 mg.; 18 g. decylsuccinate, 133.8 mg.; 18 g. undecylsuccinate, 158.0 mg.; 18 g. dodecylsuccinate, 112.4 mg.; and 12 g. cetylsuccinate, 45.4 mg. Lincke⁶⁷ also reported increased succinate after similar alkylated succinates had been given. Moreover, these synthetic compounds were found to be largely utilized, and no traces of them were excreted in the urine. The latter investigation indicated that some increase in oxalic acid elimination occurred after the alkylated succinates had been fed.

Emmrich⁶⁸ found that methyl succinic acid ($\text{HOOC}\cdot\text{CH}(\text{CH}_3)\cdot\text{CH}_2\cdot\text{COOH}$) is difficultly utilized by the dog; after the administration of 20 g. of this compound, as much as 43.8% was excreted in the urine. It was also noted that a small amount (2.2%) was excreted as methyl fumarate ($\text{HOOC}\cdot\text{C}(\text{CH}_3):\text{CH}\cdot\text{COOH}$). In an extension of these studies, Weitzel⁶⁹ reported that symmetric dimethylsuccinate ($\text{HOOC}\cdot\text{CHCH}_3\cdot\text{CHCH}_3\cdot\text{COOH}$) is excreted to the extent of about 60% by the dog; it is thus utilized with more difficulty than is the monomethyl derivative of succinic acid. Moreover, no difference in tolerance could be demonstrated between the meso- and the racemic forms of the symmetrical compound. Since an increased excretion of normal succinate followed the administration of dimethylsuccinic acid, Weitzel⁶⁹ postulates that the latter compound disturbs the normal succinate metabolism in the tissues. Bodur⁷⁰ observed that the unsymmetrical dimethylsuccinate is even less efficiently utilized by the dog than is the symmetrical isomer, since as much as 85% of the ingested unsymmetrical product was isolated in the crude form. However, there was no evidence that the unsymmetrical dimethylsuccinate was converted to trimethylacetic acid ($(\text{CH}_3)_3\text{C}\cdot\text{COOH}$), as had been assumed.

Succinate itself is rapidly oxidized. Flaschenträger and Hosoda⁷¹

⁶⁶ K. Thomas, G. Weitzel, and P. Neumann, *Z. physiol. Chem.*, **282**, 192-200 (1947).

⁶⁷ H. Lincke, *Das Stoffwechselverhalten alkylierter Bernsteinsäuren beim Hunde*, Dissertation, Zurich, 1945; cited by K. Thomas, G. Weitzel, and P. Neumann, *Z. physiol. Chem.*, **282**, 192-200 (1947) on basis of personal communication from K. Bernhard.

⁶⁸ R. Emmrich, *Z. physiol. Chem.*, **261**, 61-70 (1939).

⁶⁹ G. Weitzel, *Z. physiol. Chem.*, **282**, 200-205 (1947).

⁷⁰ H. Bodur, *Z. physiol. Chem.*, **282**, 206-207 (1947).

⁷¹ B. Flaschenträger and T. Hosoda, *Z. physiol. Chem.*, **282**, 215-220 (1947).

reported that, when a large dose (17.5 g.) was given to a dog weighing 24.8 kg., and the animal was sacrificed 90 minutes thereafter, the dicarboxylic acid was largely utilized. For example, only 4.43 g. of the 17.5 g. injected could be accounted for. This was isolated as follows: urine, 1.66 g.; blood, 1.38 g.; total muscle, 1.05 g.; liver, 0.17 g., and the balance of the succinate widely distributed in a number of tissues.

There is also considerable evidence that succinic acid is convertible to glucose. Thus, Ringer *et al.*,⁷² and MacKay and Barnes⁷³ reported that succinic acid yields glucose in the phlorhizinized dog. Moreover, this dicarboxylic acid forms liver glycogen,⁷⁴⁻⁷⁷ although Kutscher and Krabbenhöft⁷⁷ concluded that C₄ dicarboxylic acids in general do not increase the glycogen formation. Succinic acid is also antiketogenic.⁷⁵⁻⁷⁸ The only conflicting evidence is that of Deuel, Murray, and Hallman,⁷⁹ who were unable to demonstrate any ketolytic effect comparable to that of glucose. It is possible that the negative results obtained with succinate in the latter tests may be attributed to the fact that the dicarboxylic acid was fed as its sodium salt. If succinate were oxidized, sodium bicarbonate would remain, producing an appreciable ketonuria.⁸⁰

(c) *Dicarboxylic Acids Having More Than Four Carbons.* Although α -ketoglutaric acid is a most important component of the tricarboxylic acid cycle, glutaric acid has an independent fate. In the case of the dog, it appears to be completely oxidized⁵⁶ (apparently by the tricarboxylic acid cycle); on the other hand, Rose⁸¹ reported that it is nephrotoxic for the rabbit.^{81,82}

Adipic acid can be recovered in the urine of rabbits,⁸³ dogs,⁵⁷ and man,⁵⁷ to the extent of 50 to 60% of that administered, when it is given orally or parenterally. It also appears in small amounts in the urine after the

⁷² A. I. Ringer, E. M. Frankel, and L. Jonas, *J. Biol. Chem.*, **14**, 539-546 (1913).

⁷³ E. M. MacKay and R. H. Barnes, *Proc. Soc. Exptl. Biol. Med.*, **38**, 417-419 (1938).

⁷⁴ A. P. Ponsford and I. Smedley-MacLean, *Biochem. J.*, **26**, 1340-1344 (1932).

⁷⁵ E. M. MacKay, J. W. Sherrill, and R. H. Barnes, *J. Clin. Invest.*, **18**, 301-305 (1939).

⁷⁶ R. Stöhr, *Klin. Wochschr.*, **17**, 1663-1664 (1938).

⁷⁷ W. Kutscher and F. Krabbenhöft, *Z. physiol. Chem.*, **282**, 54-60 (1947).

⁷⁸ A. Korányi and A. Szent-Györgyi, *Orvosi Hetilap*, **81**, 615-618 (1937); *Chem. Abst.*, **31**, 6335 (1937).

⁷⁹ H. J. Deuel, Jr., S. Murray, and L. Hallman, *Proc. Soc. Exptl. Biol. Med.*, **37**, 413-414 (1937).

⁸⁰ J. S. Butts, H. J. Deuel, Jr., and L. Hallman, *Proc. Soc. Exptl. Biol. Med.*, **32**, 898-899 (1935).

⁸¹ W. C. Rose, *J. Pharmacol. Exptl. Therap.*, **24**, 123-146, 147-158 (1924).

⁸² W. C. Rose, C. J. Weber, R. C. Corley, and R. W. Jackson, *J. Pharmacol. Exptl. Therap.*, **25**, 59-64 (1925); W. C. Rose, and P. S. Dimmitt, *Ibid.*, **25**, 65-73 (1925).

⁸³ Y. Mori, *J. Biol. Chem.*, **35**, 341-351 (1918).

feeding of suberic acid,⁸⁴ sebacic acid,⁸⁵ or the C_{16} or C_{18} dicarboxylic acids.⁸⁶ However, the corresponding cyclic compound, cyclohexanol, probably cannot be converted to adipic acid. Although Weitzel⁸⁷ showed that cyclohexanol or 1,3-dimethyl-5-cyclohexene was easily oxidized *in vitro* by appropriate means to adipic acid, or to α,β -dimethyladipic acid, respectively, he was unable to demonstrate any evidence of this transformation in the dog when either of these compounds was fed in doses of one g./kg. daily. On the other hand, Bernhard⁸⁸ was unable to detect either phenol, which would result from dehydrogenation, or adipic acid, which would result from an oxidative splitting, after cyclohexanol had been injected into dogs in doses of 0.238 g./kg./day. However, he was of the opinion that adipic acid would arise following increased doses of cyclohexanol. Weitzel⁸⁷ also obtained negative results, insofar as adipic acid formation is concerned, with another cyclic compound, camphoric acid. After feeding 76 g. of the pure compound to a dog over a six-day period, this investigator was able to recover 68.4 g. of pure acid from the urine. The comparative structure of these compounds is shown below:

When alkyl side chains are introduced on the adipic acid molecule, considerable changes in the metabolism of the resulting compound occur. α,β' -Dimethyladipic acid ($\text{HOOC} \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{COOH}$), was less well utilized by dogs than was α -methyladipic acid ($\text{HOOC} \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$).⁸⁷ When the substituent alkyl group was introduced into the β -position, Weitzel⁸⁷ observed the following "tolerances" (g./kg./day) for the several adipic acid derivatives: n -adipic acid, 6.27; β -methyladipic acid, 0.16; β -ethyladipic acid, 0.13; β - n -propyladipic acid, 0.11; β - n -butyladipic acid, 0.15; β - n -hexyladipic acid, 0.40;

⁸⁴ K. Bernhard and M. Andreae, *Z. physiol. Chem.*, **245**, 103-106 (1937).

⁸⁵ B. Flaschenträger and K. Bernhard, *Z. physiol. Chem.*, **238**, 221-232 (1936).

⁸⁶ R. Emmrich and I. Emmrich-Glaser, *Z. physiol. Chem.*, **266**, 183-192 (1940).

⁸⁷ G. Weitzel, *Z. physiol. Chem.*, **285**, 58-77 (1950).

⁸⁸ K. Bernhard, *Z. physiol. Chem.*, **248**, 256-276 (1937).

and β -*n*-decyladipic acid, practically completely utilized. Emmrich⁶³ obtained almost identical results on feeding β -methyladipic acid to dogs. The increased tolerance obtained on lengthening the alkyl residue was likewise reported by Bernhard for alkylmalonates⁸⁹ and by Emmrich *et al.*⁶⁵ and Bernhard and Lincke⁹⁰ for alkylsuccinates. The tolerance values obtained by the former investigators⁶⁵ for alkylsuccinates were as follows: methylsuccinic acid, 0.35 g.; ethylsuccinic acid, 0.23 g.; *n*-propylsuccinic acid, 0.09 g.; *n*-butylsuccinic acid, 0.09 g.; and *n*-hexylsuccinic acid, 0.09 g. When the dicarboxylic acids having long-chain alkyl residues are fed, tricarboxylic acids can be detected, presumably as a result of ω -oxidation of the alkyl substituent. Such phenomena have been observed after octyl-, nonyl-, or decylmalonate⁸⁹ or nonyl- or decylsuccinate⁹⁰ has been fed. After the feeding of nonylsuccinate, $\text{HOOC}\cdot\text{CH}((\text{CH}_2)_8\cdot\text{CH}_3)\cdot\text{CH}_2\cdot\text{COOH}$, the side chain was shortened three times by β -oxidation of the side chain after ω -oxidation;

β -carboxy- γ -oxyadipic acid,

was isolated from the urine.

According to Flaschenträger,⁵⁷ the several dicarboxylic acids were excreted in the urine unchanged by dogs in the following proportions after they had been injected: adipic, 40%; suberic, 60%; and sebacic, 61%. After the injection of each of the several C_6 to C_{10} dicarboxylic acids into phlorhizinized dogs, Baer and Blum⁹¹ reported the following recoveries: adipic, 12%; pimelic, 47%; suberic, 62%; azelaic, 50%; and sebacic, 46 and 13.7%. Although the figures for the extent of diaciduria are probably of little importance, since they would be expected to vary with the size of the dose administered, they do indicate that the tolerance for all these acids is exceedingly low. Rose^{81,82} noted that adipic, pimelic, suberic, and azelaic acids produce a nephritis in rabbits qualitatively similar to that caused by glutaric and by tartaric acids; however, quantitatively, the extent of the nephritis was less severe when the higher dicarboxylic acids were given than following the shorter-chain dicarboxylic acids. Mucic acid ($\text{HOOC}\cdot(\text{CHOH})_4\cdot\text{COOH}$) was likewise found to have a

⁸⁹ K. Bernhard, *Z. physiol. Chem.*, **269**, 135–145 (1941).

⁹⁰ K. Bernhard and H. Lincke, *Helv. Chim. Acta*, **29**, 1457–1466 (1946).

⁹¹ J. Baer and L. Blum, *Beitr. chem. Physiol. Pathol.*, **11**, 101–108 (1908).

nephrotoxic action.^{81,82,92} Flaschenträger and Bernhard⁹³ reported that sebacic acid (C₁₀) was utilized only to the extent of 40%, and that the rest of the injected acid appeared in the urine; however, when one acid group was blocked, the acid was largely utilized, and only 10% was excreted in the urine.

Azelaic acid (C₉) was investigated by Smith.⁹⁴ This acid is of considerable importance, since it is formed when the unsaturated acids having a double bond in the C₉-C₁₀ position (which includes most physiologic unsaturated fatty acids) are oxidized by potassium permanganate. If a rupture at the unsaturated linkage occurs in the case of oleic acid, one would expect azelaic acid to be one of the intermediates in its biologic oxidation. Smith⁹⁴ reported that azelaic acid was oxidized by dogs in amounts smaller than would be produced if oxidation took place at the double bond. It was utilized at an average rate of only 58% (with a range of 29 to 92%) when the doses varied from 10.5 to 44.9 g. It was therefore indicated that oxidation of the 9,10-unsaturated acids does not normally involve a rupture at the double bond. The fact that no glycogen is formed from the nonanoic (pelargonic) acid, which would be produced simultaneously with azelaic acid, has likewise been interpreted as precluding the 9,10-rupture of oleic acid as a normal *in vivo* pathway of oxidation.⁹⁵

The mechanism of oxidation of the dicarboxylic acids which do disappear is not clear. Most of the evidence for ω -oxidation has indicated that β -oxidation proceeds from the carboxyl group, with the formation of dicarboxylic acids having two less carbon atoms than the parent acid. This was proved by Bernhard,⁹⁶ who demonstrated a dilution of the deuteriosuberic acid eliminated in the urine after the feeding of deuteriosuberic acid, when C₁₂ or C₁₆ dicarboxylic acids were also fed. However, since the odd-numbered diacids, pimelic (C₇) and azelaic (C₉), are apparently no more toxic than are the even-chain acids, adipic (C₆) and suberic (C₈), Bloor⁹⁷ suggests that it is improbable that a direct β -oxidation of these acids occurs. Baer and Blum⁹² reported that adipic (C₆), pimelic (C₇), and suberic (C₈) acids behaved like glutaric (C₅) acid in inhibiting

⁹² J. Baer and L. Blum, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **65**, 1-33 (1911).

⁹³ B. Flaschenträger and K. Bernhard, *Z. physiol. Chem.*, **240**, 19-22 (1936).

⁹⁴ H. G. Smith, *J. Biol. Chem.*, **103**, 531-535 (1933).

⁹⁵ J. S. Butts, H. Blunden, W. Goodwin, and H. J. Deuel, Jr., *J. Biol. Chem.*, **117**, 131-133 (1937).

⁹⁶ K. Bernhard, *Helv. Chim. Acta*, **24**, 1412-1425 (1941).

⁹⁷ W. R. Bloor, *Biochemistry of the Fatty Acids*, Reinhold, 1943, p. 321.

sugar excretion by phlorhizinized animals; this, of course, might possibly be attributed to their nephropathic action. Mazza⁹⁸ brought forward the direct proof that tissues can oxidize the dicarboxylic acids by demonstrating this phenomenon in liver slices with the following acids: succinic (C₄); adipic (C₆); suberic (C₈); azelaic (C₉); sebacic (C₁₀); and hexane dicarboxylic (C₁₆). Only glutaric acid (C₅) was not attacked by liver slices, while kidney slices did not break the latter acid down to an appreciable extent.

(2) Tricarboxylic Acids

The metabolism of tricarboxylic acids is intimately bound up with that of the shorter-chain dicarboxylic acids through the Krebs cycle. Thus, citric acid has been repeatedly proved to be a glucose former. Baer and Blum⁹² demonstrated that an increase in glucose obtained when citric acid was fed to a phlorhizinized dog. Greenwald⁹⁹ also reported that sodium citrate augmented the glucose excretion, both in the case of phlorhizinized dogs and in the diabetic human patient. MacKay, Carne, and Wick¹⁰⁰ have demonstrated in an unequivocal manner that citric acid is as effective as glucose in relieving the symptoms of hypoglycemia in albino rats; this acid was also shown to function as a glycogenic agent, and to exert an antiketogenic action. On the other hand, Fürth and co-workers¹⁰¹ were unable to demonstrate either a glycosuric or an antiketogenic effect on the part of citric acid in the phlorhizinized dog; moreover, no increase in liver glycogen could be demonstrated in rats after feeding them citric acid. Although the majority of experimental data point to the conversion of citric acid to glucose, the single negative report makes one cautious about interpreting such data. Because of the interrelations of the several four-carbon dicarboxylic acids, α -ketoglutaric acid and the several six-carbon dicarboxylic acids other than citrate in the tricarboxylic acid cycle, it would seem quite probable that they must all be considered as glucose formers. The finding by Orten and Smith¹⁰² that the injection of all the compounds in the citric acid cycle increased the citrate excretion would seem to support this thesis. Krebs *et al.*¹⁰³ obtained a similar result, and demonstrated that the excretion of α -ketoglutarate was also augmented when any components of the citric acid cycle were administered.

⁹⁸ F. P. Mazza, *Arch. sci. biol. (Italy)*, **22**, 307-326 (1936).

⁹⁹ I. Greenwald, *J. Biol. Chem.*, **18**, 115-121 (1914).

¹⁰⁰ E. M. MacKay, H. O. Carne, and A. N. Wick, *J. Biol. Chem.*, **133**, 59-65 (1940).

¹⁰¹ O. Fürth, H. Minnibeck, and E. Edel, *Biochem. Z.*, **269**, 379-396 (1934).

¹⁰² J. M. Orten and A. H. Smith, *J. Biol. Chem.*, **117**, 555-567 (1937).

¹⁰³ H. A. Krebs, E. Salvin, and W. A. Johnson, *Biochem. J.*, **32**, 113-117 (1938).

4. Fatty Acid Amides

The replacement of the carboxyl group by an acid amide group has been found to influence, to an important extent, the rate of disappearance of the corresponding fatty acids. Bernhard^{88,104} reported that the substitution of methyl-, ethyl-, or propylamide of benzoic acid, or of hexahydrobenzoic acid, did not influence the course of intermediary metabolism. In the case of dicarboxyl acids which are metabolized with difficulty, it was found that the transformation to the corresponding amide resulted in a more readily utilized compound. Thus, Flaschenträger⁵⁷ found that sebacic acid amide ($\text{HOOC} \cdot (\text{CH}_2)_8 \cdot \text{CONH}_2$) was better tolerated in the dog than was sebacic acid, while Bernhard¹⁰⁵ observed that sebacic acid-mono-*N*-methanamide ($\text{COOH} \cdot (\text{CH}_2)_8 \cdot \text{CONH} \cdot \text{CH}_3$), sebacic acid-bis-*N*-methanamide ($\text{CH}_3 \cdot \text{NH} \cdot \text{OC} \cdot (\text{CH}_2)_8 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_3$), and sebacic acid-mono-*N*-propylamide ($\text{HOOC} \cdot (\text{CH}_2)_8 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_3$), as well as the mono-*N*-propylamide of suberic acid ($\text{HOOC} \cdot (\text{CH}_2)_6 \cdot \text{CONH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_3$), were more slowly utilized than were the corresponding free acids.

The amides of the normal aliphatic saturated and also straight-chain unsaturated monocarboxylic acids were found, by Kuhn and Löw,¹⁰⁶ to be readily metabolized. After the feeding of caproylamide ($\text{CH}_3 \cdot (\text{CH}_2)_6 \cdot \text{CONH}_2$), lauramide ($\text{CH}_3 \cdot (\text{CH}_2)_{10} \cdot \text{CONH}_2$) or palmitamide ($\text{CH}_3 \cdot (\text{CH}_2)_{14} \cdot \text{CONH}_2$) to rabbits, Kuhn and Löw¹⁰⁶ reported that no trace of amide or any decomposition product could be found in the urine. The same results were noted with several unsaturated acid amides, including α, β -hexenoamide ($\text{CH}_3 \cdot (\text{CH}_2)_2 \cdot \text{CH} : \text{CH} \cdot \text{CONH}_2$), β, γ -hexenoamide ($\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH} : \text{CH} \cdot \text{CH}_2 \cdot \text{CONH}_2$) and 3,5-octadienoic acid amide ($\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH} : \text{CH} \cdot \text{CH} : \text{CH} \cdot \text{CH}_2 \cdot \text{CONH}_2$).¹⁰⁶ However, small amounts of the ingested substance were excreted in the case of lauric acid anilide ($\text{CH}_3 \cdot (\text{CH}_2)_{10} \cdot \text{CONHC}_6\text{H}_5$) and lauric acid methyl anilide ($\text{CH}_3 \cdot (\text{CH}_2)_{10} \cdot \text{CON}(\text{CH}_3)\text{C}_6\text{H}_5$) and lauric acid methyl anilide ($\text{CH}_3 \cdot (\text{CH}_2)_{10} \cdot \text{CON}(\text{CH}_3)\text{C}_6\text{H}_5$), as well as after caprinyln-D,L-alanine ($\text{CH}_3 \cdot (\text{CH}_2)_8 \cdot \text{CONH} \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH}$).

In some instances, the presence of the amide group may limit the oxidation of the compound. Thus, β -methyl- β -propylacrylic acid amide (I) is not completely oxidized; as much as 13% of the dose administered was recovered by Kuhn and Löw¹⁰⁶ as the monamide of 2-methyl-butene (1,2)-1,4-dicarboxylic acid (II) as indicated below:

The Metabolism of β -Methyl- β -Propylacrylic Acid Amide (I) by ω -Oxidation¹⁰⁶

¹⁰⁴ K. Bernhard, *Z. physiol. Chem.*, **251**, 47-51 (1938).

¹⁰⁵ K. Bernhard, *Z. physiol. Chem.*, **256**, 65-70 (1938).

¹⁰⁶ R. Kuhn and I. Löw, *Z. physiol. Chem.*, **259**, 182-193 (1939).

The oxidation of the amide is interfered with by the methyl group on the β -carbon atom; the body in this emergency reverts to ω -oxidation.

Another instance in which an inhibition of oxidation has been noted through amide formation is in the case of the polyene acid amide. Kuhn, Köhler, and Köhler¹¹ observed that β -oxidation was inhibited, and ω -oxidation was strongly augmented in rabbits, under such conditions, with the resultant formation of polyene- ω,ω' -dicarboxylic acid amides. Thus, the amide of octatrienoic acid gave rise to a dicarboxylic acid to the extent of 42%, as follows:

The Inhibition of Complete Oxidation of Fatty Acid Amides in Polyene Acids

TABLE 1
THE EXTENT OF ABSORPTION OF FATTY ACID AMIDES
AND THE QUANTITY EXCRETED AS DICARBOXYLIC ACIDS BY DOGS^a

Substance fed	Melting point, °C.	Amount fed, g.	Recovered		
			Unchanged in feces		As dicarboxylic acid in urine, mg.
			Crude, g.	Pure, g.	
<i>n</i> -Decanoic acid amide	99-99.5	45.0	39.4	13.0	300
		67.5	35.6	4.5	901
<i>n</i> -Dodecanoic acid amide	101-101.5	117.0	67.0	21.5	780
<i>n</i> -Decanoic acid- <i>N</i> -methyl-amide	67-68	35.0	9.5	—	140
<i>n</i> -Dodecanoic acid- <i>N</i> -methyl-amide	57-58	24.0	7.0	0.05	560
2-Methylnonanoic acid amide	75.5-76	109.0	16.7	2.95	3014
4-Methyldodecanoic acid amide	75.0-76	25.0	17.8	0.3	2560
2-Methyltridecanoic acid amide	94.0-95	25.0	19.7	3.72	580
5-Methyltetradecanoic acid amide	61.5-62	22.5	—	—	1540

^a Adapted from G. Weitzel, H. Schön, and H. Kalbe, *Z. physiol. Chem.*, 295, 173-187 (1953), pp. 176, 178.

In an extended study of the metabolism of various straight-chain and methyl-substituted acid amides, Weitzel, Schön, and Kalbe¹⁰⁷ reported

¹⁰⁷ G. Weitzel, H. Schön, and H. Kalbe, *Z. physiol. Chem.*, 295, 173-187 (1953).

that a considerable diaciduria developed, even in cases in which the fatty acids themselves were not diacidogenic. Table 1 records the quantitative results.

The following excretion compounds were found to result after the administration to dogs of comparatively large amounts of lauric acid monoamide:

The several products were identified by paper chromatography.¹⁰⁷ Similarly, after 2-methylnonanoic acid amide ($\text{CH}_3 \cdot (\text{CH}_2)_6 \cdot \text{CH}(\text{CH}_3) \cdot \text{CONH}_2$) was fed, 2-methylazelaic acid amide, 2-methylpimelic acid amide, and 2-methylglutaric acid amide were demonstrated. In fact, the corresponding malonic acid derivative, $\text{HOOC} \cdot \text{CH}(\text{CH}_3) \cdot \text{CONH}_2$, probably accounted for the C_4 fraction recovered, which was absent from normal urine.¹⁰⁷

It would thus appear that the presence of the amide group renders fatty acids readily susceptible to diacidogenesis. Even those acids which do not normally undergo this change may be induced to follow this method of oxidation if the acid is bound up with an amide. The introduction of a methyl side chain markedly increases the susceptibility of these acid amides to an oxidation pattern of this nature. There is considerable evidence that, after the formation of the diacid, the resulting compound can undergo sufficient β -oxidations so that short-chain dicarboxylic acids are the final product of oxidation.

5. Fatty Aldehydes

The importance of the aldehydes of fatty acids in the tissues was first emphasized by Feulgen and his collaborators,¹⁰⁸⁻¹¹¹ and by Behrens alone.¹¹² It was shown that these aldehydes have a wide-spread distribu-

¹⁰⁸ R. Feulgen and K. Voit, *Arch. ges. Physiol. (Pflüger's)*, **206**, 389-410 (1924).

¹⁰⁹ R. Feulgen, K. Imhäuser, and M. Behrens, *Z. physiol. Chem.*, **180**, 161-179 (1929).

¹¹⁰ R. Feulgen and M. Behrens, *Z. physiol. Chem.*, **256**, 15-20 (1938).

¹¹¹ R. Feulgen and T. Bersin, *Z. physiol. Chem.*, **260**, 217-245 (1939).

¹¹² M. Behrens, *Z. physiol. Chem.*, **191**, 183-186 (1930).

tion in the tissues, and it is believed that they are normal metabolic products of fats. Anchel and Waelsch¹¹³ reported that the aldehydes isolated from beef muscle, beef brain, rat muscle, and rat brain (which were chiefly C₁₆ and C₁₈ compounds) were similar, as judged from the analytical results of their derivatives. They were separated in amounts varying between 0.05 and 0.2% of the tissue used.

Ehrlich and Waelsch¹¹⁴ attempted to ascertain whether or not the fatty aldehydes are intermediates in the oxidation of fatty acids. On the basis of tests in which deuterium was fed to rats on a low-fat diet, or to animals receiving deuterio-fats, it was found that the deuterium content of the aldehydes was invariably less than that of the corresponding fatty acids. This was taken to indicate that the fatty aldehydes are not involved in the over-all synthesis or transport of fatty acids, but rather are associated with some special aspects of fatty acid metabolism. The fatty aldehydes appear to be in highest concentration in the skin, as indicated by the high deuterium content of the unsaponifiable fraction of these lipids; the proportion of the unsaponifiable fraction was much lower in the muscle lipids.

The fatty aldehydes have also been found in the plant kingdom. Thus, the C₈ and C₉ aldehydes, octanal (CH₃·(CH₂)₆·CHO) and nonanal (CH₃·(CH₂)₇·CHO), are well-known components of essential oils. Decanal (CH₃·(CH₂)₈·CHO) is present in definite quantities in coriander oil,^{115,116} and dodecanal, or lauric aldehyde (CH₃·(CH₂)₁₀·CHO), has been isolated from the oil obtained from the lily of the valley.¹¹⁷ An aldehyde containing eighteen carbons was prepared from the pulp of olives by Marcelet.¹¹⁸ Some of the floral odors can be traced to the shorter-chain aldehydes, each of which has its own characteristic aroma. In some cases these are unsaturated, as, for example, 2,6-nonadienal, CH₃·CH₂·CH:CH·CH₂·CH₂·CH:CH·CHO, which occurs in violet leaf oil.¹¹⁹ Information on the chemistry of the fatty aldehydes is to be found in *The Lipids*, Vol. I, pages 124 to 132.

¹¹³ M. Anchel and H. Waelsch, *J. Biol. Chem.*, 145, 605-613 (1943).

¹¹⁴ G. Ehrlich and H. Waelsch, *J. Biol. Chem.*, 163, 195-202 (1946).

¹¹⁵ P. P. Shorygin and V. P. Osipova, *Sintezy Dushistykh Veshchestv., Sbornik Stat'ei*, 1939, 246-247; *Khim. Referat. Zhur.*, 1940, No. 4, 117; *Chem. Abst.*, 36, 3781 (1942).

¹¹⁶ L. Y. Bryusova, R. Y. Shagalova, and N. Novikova, *Sintezy Dushistykh Veshchestv., Sbornik Stat'ei*, 1939, 247-252; *Khim. Referat. Zhur.*, 1940, No. 117-118; *Chem. Abst.*, 36, 3781 (1942).

¹¹⁷ W. Hannemann, *Deut. Parfüm.-Ztg.*, 9, No. 8/9, 7 (Apr. 15, 1923); *Chimie & Industrie*, 11, 941 (1924).

¹¹⁸ H. Marcelet, *Compt. rend.*, 206, 529-530 (1938).

¹¹⁹ E. Späth and F. Keszler, *Ber.*, 67B, 1496-1500 (1934).

6. Hydrocarbons

The hydrocarbons, which reputedly are transformed with difficulty by mild chemical treatment in the laboratory, are equally resistant to biologic transformations. In spite of this, some hydrocarbons do represent compounds of physiologic importance. In most cases the parent substances are insoluble in water, blood, or tissue fluids. However, they may be oxidized to alcohols or to acids, whereby they may become partially soluble in aqueous solutions. The chief hydrocarbons which possess biologic importance include both aliphatic and aromatic compounds. The chemical nature and distribution of these compounds are summarized in *The Lipids*, Vol. I, pages 400 to 404.

(1) Aliphatic Hydrocarbons

a. Paraffin Hydrocarbons and Related Compounds. A chance observation of Chibnall and Channon,^{120,121} that the phosphatide fraction of cabbage leaves also contained a large amount of a white crystalline material, which was shown to be a mixture of *n*-nonacosane and *n*-nonacosan-15-one (di-*n*-tetradecylketone), led to the concept that these compounds may be normal metabolic products which arise from fatty acids in the plant. Thus, it was believed that two molecules of fatty acids condense to yield a ketone which, on reduction, would be converted into paraffins as shown below:

The Possible Mechanism for the Origin of Hydrocarbons and Ketones in Plants^{121,122}

Clenshaw and Smedley-MacLean¹²³ made a similar suggestion as regards the origin of the hydrocarbon, *n*-hentriacontane, from spinach leaves.

(a) *The Presence of Paraffin Hydrocarbons in Plant and Insect Waxes.* In recent years, hydrocarbons have been found to have an especially wide

¹²⁰ A. C. Chibnall and H. J. Channon, *Biochem. J.*, **23**, 176-184 (1929).

¹²¹ H. J. Channon and A. C. Chibnall, *Biochem. J.*, **23**, 168-175 (1929).

¹²² A. C. Chibnall and S. H. Piper, *Biochem. J.*, **28**, 2209-2219 (1934).

¹²³ E. Clenshaw and I. Smedley-MacLean, *Biochem. J.*, **23**, 107-109 (1929).

distribution in fruit and vegetable waxes and in insect waxes. *n*-Eicosane ($C_{22}H_{42}$) was first reported in red-berry bryony oil (*Bryonia dioica*), in 1892, by Étard.¹²⁴ It has been more recently reported in laurel berry fat (*Laurus* spp.)¹²⁵ and in parsley seed oil (*Petroselinum sativum*).¹²⁶

n-Pentacosane ($C_{25}H_{52}$), and the C_{27} , C_{29} , and C_{31} normal hydrocarbons, were recorded in beeswax (*Apis mellifica*), by Chibnall and Piper,¹²² although the C_{25} -hydrocarbon has not been identified in plant waxes. *n*-Heptacosane ($C_{27}H_{56}$) was found not only in apple cuticle wax,^{122, 127, 128} but also in carnauba wax (*Copernicia cerifera*).¹²² *n*-Nonacosane ($C_{29}H_{60}$) has been identified in cabbage lipids,^{121, 129} in apple peel wax,^{130, 131} in pear wax,¹³¹ in grapefruit peel wax,¹³² in Bing cherry skin wax (*Prunus avium* L.),¹³³ and in brussels sprouts (*Brassica oleracea gemmifera*).¹³⁴ Chibnall and Piper¹²² also noted the presence of nonacosane in the non-cuticular leaf waxes of cabbage (*Brassica oleracea capitata*), Swede turnip or rutabaga (*Brassica napobrassica*), white mustard (*Brassica hirta*, *Sinapis alba*), and tobacco (*Nicotiana tabacum*).

n-Hentriacontane ($C_{31}H_{64}$) has a similarly wide distribution in plants, which include brussels sprouts,¹³⁴ cabbage lipids,^{122, 135} grapefruit peel wax,¹³² and spinach lipids (*Spinacia oleracea*).^{122, 129} Chibnall and Piper¹²² likewise listed *n*-hentriacontane as a component of the leaf cuticle wax from candelilla or wax slipperflower (*Pedilanthus pavonis*), and in such non-cuticular leaf waxes as Swede turnip, white mustard, and tobacco. The presence of this C_{31} -hydrocarbon in Chinese insect wax (*Coccus ceriferus*) was observed by Collins.¹³⁶

n-Tritriacontane ($C_{33}H_{68}$) has been found in candelilla wax,¹²² as well as in cactus and tobacco leaf waxes.¹²² The C_{35} -hydrocarbon, pentatriacon-

¹²⁴ A. Étard, *Compt. rend.*, **114**, 364-366 (1892).

¹²⁵ H. Matthes and H. Sander, *Arch. Pharm.*, **246**, 165-177 (1908).

¹²⁶ H. Matthes and W. Heintz, *Ber. deut. pharm. Ges.*, **19**, No. 9, 325-328 (1909).

¹²⁷ A. C. Chibnall, S. H. Piper, A. Pollard, J. A. B. Smith, and E. F. Williams, *Biochem. J.*, **25**, 2095-2110 (1931).

¹²⁸ A. C. Chibnall, S. H. Piper, A. Pollard, J. A. B. Smith, E. F. Williams, and P. N. Sahai, *Biochem. J.*, **28**, 2189-2208 (1934).

¹²⁹ D. L. Collison and I. Smedley-MacLean, *Biochem. J.*, **25**, 606-613 (1931).

¹³⁰ K. S. Markley and C. E. Sando, *J. Biol. Chem.*, **101**, 431 (1933).

¹³¹ K. S. Markley, S. B. Hendricks, and C. E. Sando, *J. Biol. Chem.*, **98**, 103-107 (1932); **111**, 135-146 (1935).

¹³² K. S. Markley, E. K. Nelson, and M. S. Sherman, *J. Biol. Chem.*, **118**, 433-441 (1937).

¹³³ K. S. Markley and C. E. Sando, *J. Biol. Chem.*, **119**, 641-645 (1937).

¹³⁴ P. N. Sahai and A. C. Chibnall, *Biochem. J.*, **26**, 403-412 (1932).

¹³⁵ J. Ozaki, *J. Agr. Chem. Soc. Japan*, **6**, 773-782 (1930); *Chem. Abst.*, **25**, 2754 (1931).

¹³⁶ F. J. E. Collins, *J. Soc. Chem. Ind.*, **54**, 33-35 T (1935).

tane ($C_{35}H_{72}$), is also a component of cactus, as well as of sugar cane wax (*Saccharum officinarum*).¹³⁷ *n*-Heptatriacontane ($C_{37}H_{76}$) has been shown to occur in cactus.

Pristane, $C_{15}H_{32}$, is an example of a saturated hydrocarbon with a branched chain, which has a wide distribution in liver oils of fishes. This hydrocarbon was first separated from the liver lipid of the basking shark by Tsujimoto^{138,139} in the amount of 6 to 10%. Toyama¹⁴⁰ suggested that pristane is present to a small extent in most liver oils which contain squalene; among the fish-liver oils which have been shown to contain pristane are those from the liver of sardine, herring, and the sperm whale (*Physeter macrocephalus*).¹⁴¹

(b) *The Presence of Compounds Related to Hydrocarbons in Plant and Insect Waxes.* In addition to hydrocarbons, primary alcohols, ketones, and fatty acids have been demonstrated in plant and insect waxes, where they are believed to be concerned with the intermediary metabolism. Some of the products which have been observed are listed in the following sections.

a'. Primary Alcohols: In contradistinction to the naturally-occurring hydrocarbons, which usually are composed of odd-carbon chains, the primary alcohols found in various plant and insect waxes consist of those having an even number of carbon atoms. Thus, of the insect waxes, honey-beeswax (*Apis mellifica*) is known to contain the C_{24} -, C_{26} -, C_{28} -, C_{30} -, C_{32} -, and C_{34} -alcohols, while in lac wax from the shield-louse (*Coccus lacca*)¹²² alcohols having C_{26} to C_{36} have been observed. In Chinese insect wax (*Coccus ceriferus*), the C_{26} -, C_{28} -, and C_{30} -alcohols are present. There is also a wide distribution of the even-chain primary alcohols in leaf cuticle waxes as shown below: carnauba wax, C_{26} - C_{34} ; candelilla wax, C_{28} - C_{34} ; raphia wax and pisang wax (from the plantain, *Musa paradisiaca*), C_{28} - C_{32} .¹²² Fruit and seed coat cuticle waxes consist of a large proportion of these even-chain primary alcohols, as indicated by the composition of apple cuticle wax (C_{26} - C_{30}) and cotton wax (C_{24} - C_{34}).¹²²

Chibnall and Piper¹²² likewise recorded a high primary alcohol content in non-cuticular leaf waxes, as follows: brussels sprouts, C_{26} - C_{32} ; white mustard, C_{28} - C_{32} ; cactus, C_{26} - C_{32} ; and wild white clover (*Trifolium repens*), C_{28} , C_{30} .

¹³⁷ N. L. Vidyarthi and M. Narasingarao, *J. Indian Chem. Soc.*, **16**, 135-143 (1939).

¹³⁸ M. Tsujimoto, *Bull. Chem. Soc. Japan*, **10**, 144-148, 149-153 (1935); *J. Chem. Soc. Japan*, **55**, 702-741 (1934); *Chem. Abst.*, **28**, 6484 (1934).

¹³⁹ M. Tsujimoto, *Ind. Eng. Chem.*, **9**, 1098-1099 (1917).

¹⁴⁰ Y. Toyama, *Chem. Umschau Gebiete Fette, Öle, Wachse u. Harze*, **30**, 181-187 (1923); *Chem. Abst.*, **17**, 3616 (1923).

¹⁴¹ Y. Toyama and T. Tsuchiya, *J. Soc. Chem. Ind. Japan*, **38**, suppl., 254-258 (1935).

b'. Secondary Alcohols: Several secondary alcohols have likewise been demonstrated in plant products. In contradistinction to the primary alcohols, these have an odd number of carbon atoms. One secondary alcohol, *d-n-nonacosan-10-ol*, was originally isolated by Kawamura¹⁴² from the fruit of the maidenhair tree (*Ginkgo biloba*), and was called ginnol. Chibnall and Piper¹²² proved that the secondary alcohol is *d-n-nonacosan-10-ol*. This alcohol was likewise isolated from apple cuticle wax,¹²⁷ as well as from the leaves of the *Ginkgo biloba*.¹⁴³

Sahai and Chibnall¹³⁴ discovered another secondary alcohol, namely *n-nonacosan-15-ol*, in the leaf wax of brussels sprouts; the presence of the same alcohol was later recorded in the Swede turnip.¹²²

c'. Ketones: The possibility that ketones may be intermediates in the oxidation of fatty acids, primary alcohols, and hydrocarbons, as suggested by Chibnall and Piper,¹²² is supported by the discovery that *n-nonacosan-15-one* occurs in brussels sprouts leaf wax,¹³⁴ as well as in cabbage.¹²²

b. Unsaturated Hydrocarbons in Plants and Animals. (a) *Squalene*. Squalene is a highly unsaturated hydrocarbon, with an empirical formula of $C_{30}H_{50}$. It is widely distributed in the animal and plant kingdoms. Squalene was first discovered as an important component of the liver oil of sharks of the *Squalidae* family, but it has since been found to be as widely distributed in the animal kingdom. Moreover, it is now recognized as a normal component in higher animals.

Tsujimoto¹⁴⁴ first reported the presence of an unsaturated hydrocarbon in the liver oil of a black shark of the genus *Zameus*; this was later shown to be squalene.¹⁴⁵ Squalene was found to be a component of the liver oils of sixteen of thirty-six species of elasmobranch fish examined, all from Japanese waters,¹⁴⁶ including the basking shark (*Cetorhinus maximus*).^{138,139} Hilditch¹⁴⁷ also reported the presence of squalene in the liver oils of some of the family *Squalidae* (sharks) and also of other shark families. Heilbron and co-workers¹⁴⁸ observed the presence of this hydrocarbon in the undeveloped eggs (but not in the mature eggs) of the dogfish, *Etmopterus spinax*, which is a member of the *Squalidae*. This would suggest that

¹⁴² J. Kawamura, *Japan. J. Chemistry*, 3, 89-92 (1928).

¹⁴³ S. Furukawa, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, 19, 27-38, 39-42 (1932).

¹⁴⁴ M. Tsujimoto, *J. Soc. Chem. Ind. Japan*, 9, 953 (1906); cited by M. Tsujimoto, *Ind. Eng. Chem.*, 8, 889-896 (1916).

¹⁴⁵ M. Tsujimoto, *Ind. Eng. Chem.*, 8, 889-896 (1916).

¹⁴⁶ M. Tsujimoto, *Ind. Eng. Chem.*, 12, 63-73 (1920).

¹⁴⁷ T. P. Hilditch, *The Chemical Constitution of the Natural Fats*, Wiley, New York, 1947.

¹⁴⁸ I. M. Heilbron, E. D. Kamm, and W. M. Owens, *J. Chem. Soc.*, 1926, 1630-1644.

squalene plays a role in embryologic development. According to Channon,¹⁴⁹ the presence of squalene is largely limited to *Squalidae*; this worker recorded its occurrence in three of five species examined, but noted that it was not present in eleven different elasmobranch families, in fourteen species of teleostid fishes, or in any of a number of phyto- or zoöplanktons examined. André and Bloch¹⁵⁰ observed that the liver oil of the shark, "Darkie Alf" (*Dalatias licha*) contained as much as 57% of squalene, as compared with a total glyceride content of only 21.7% and a glyceryl ether percentage of 21.3%. Bloor⁹⁷ suggested that, in the case of the shark liver oils, squalene replaces the unsaturated fatty acids. Squalene has likewise been reported to be a component of the sebum of man,¹⁵¹ where it is being constantly excreted, together with cholesterol and traces of fatty acids. According to Wheatley,¹⁵² squalene may be present to the extent of 5% in human forearm sebum.

In addition to animal fats, at least two plant fats have been reported to contain squalene. Thus, Thorbjarnarson and Drummond¹⁵³ found it in various olive oils; the non-saponifiable extract of Palestinian, Tunisian, Spanish, and Turkish olive oils contained 64, 38, 31, and 58% of this hydrocarbon, respectively. Although no significant amount of squalene could be demonstrated in teaseed oil,¹⁵³ Täufel and co-workers¹⁵⁴ recorded that squalene comprises 16% of yeast fat.

The importance of squalene in intermediary metabolism has recently been emphasized by the suggestion that it participates in cholesterol synthesis. Considerable difficulty exists in establishing the intermediate position, inasmuch as the metabolic turnover is very rapid. The maximum acetate carbon is incorporated into squalene in thirty minutes.¹⁵⁵ Langdon and Bloch¹⁵⁵ found that, when labeled acetate was administered together with natural squalene, the recovered squalene was partially radioactive. When labeled C¹⁴-squalene prepared biosynthetically from acetate was administered to mice, it was found to be efficiently converted to choles-

¹⁴⁹ H. J. Channon, *Biochem. J.*, **22**, 51-59 (1928).

¹⁵⁰ E. André and A. Bloch, *Bull. soc. chim.* [5], **2**, 789-802 (1935); *Compt. rend.*, **196**, 618-620 (1933).

¹⁵¹ R. M. B. MacKenna, V. R. Wheatley, and A. Wormall, *J. Investigative Dermatol.*, **15**, 33-47 (1950).

¹⁵² V. R. Wheatley, *Livre jubilaire, 1901-1951, de la Société belge de Dermatologie et de Syphilographie*, Brussels, 1952, pp. 91-103; cited by R. P. Cook, *Comparative Aspects of Lipid Absorption and Excretion*, in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia, No. 9, 14-29 (1952).

¹⁵³ T. Thorbjarnarson and J. C. Drummond, *Analyst*, **60**, 23-29 (1935).

¹⁵⁴ K. Täufel, H. Thaler, and H. S. Shreyegg, *Z. Untersuch. Lebensm.*, **72**, 394-404 (1936).

¹⁵⁵ R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 129-134 (1953).

terol.¹⁵⁶ Another indication that squalene is an intermediate in the synthesis of cholesterol from acetate is the finding that feeding squalene to rats for two days leads to a nearly complete suppression of the acetate $\rightarrow$ cholesterol transformation, concomitantly with an increase in liver sterols.¹⁵⁷

On the other hand, some negative evidence in regard to the hypothesis that squalene is an intermediate in cholesterol synthesis has been adduced. Thus, Tomkins *et al.*¹⁵⁸ were unable to demonstrate the *in vitro* or *in vivo* conversion of synthetically-prepared squalene into cholesterol by the liver. There was some evidence of its oxidation to CO₂. However, in a later study by Tomkins and co-workers,¹⁵⁹ in which natural squalene was fed for nine days, in place of regenerated squalene, a marked reduction in the conversion of the added C¹⁴ to cholesterol resulted. This leads these authors to conclude that natural squalene is a cholesterol precursor. Although Popják¹⁶⁰ demonstrated the biosynthesis of both squalene and cholesterol from acetate-1-C¹⁴ by the liver slices of the rat and by the ovarian tissues of the laying hen, the relationship of the specific activity was believed to be such as to exclude squalene *per se* as the immediate precursor of cholesterol. Kritchevsky *et al.*¹⁶¹ likewise reported that squalene does not induce a significant increase in atheroma, as does cholesterol, even though it is believed to be an immediate precursor in the biosynthesis of cholesterol. Schwenk, Todd, and Fish¹⁶² isolated squalene-C¹⁴ from the extracts obtained from the livers of pigs previously perfused with C¹⁴-labeled acetate, which was converted by rats into cholesterol-C¹⁴ after intraperitoneal injection or after feeding. This was the first instance in which squalene was isolated from a mammalian liver. The presence of a number of intermediates of the acetate $\rightarrow$ cholesterol reaction in the non-saponifiable fraction of the perfused livers was also observed. This evidence is so overwhelming for the participation of squalene in the acetate $\rightarrow$ cholesterol reaction that one can accept it as a proven fact. For a further discussion, see page 384.

On the other hand, little is known concerning the metabolism of squalene, other than its conversion to cholesterol and its probable oxidation to carbon

¹⁵⁶ R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 135-144 (1953).

¹⁵⁷ R. G. Langdon and K. Bloch, *Federation Proc.*, **12**, 235-236 (1953).

¹⁵⁸ G. M. Tomkins, I. L. Chaikoff, W. G. Dauben, H. L. Bradlow, and P. A. Srere, *J. Am. Chem. Soc.*, **74**, 6145-6146 (1952).

¹⁵⁹ G. M. Tomkins, W. G. Dauben, H. Sheppard, and I. L. Chaikoff, *J. Biol. Chem.*, **202**, 487-489 (1953).

¹⁶⁰ G. Popják, *Arch. Biochem. Biophys.*, **48**, 102-106 (1954).

¹⁶¹ D. Kritchevsky, A. W. Moyer, and W. C. Tesar, *Arch. Biochem. Biophys.*, **44**, 241 (1953).

¹⁶² E. Schwenk, D. Todd, and C. A. Fish, *Arch. Biochem. Biophys.*, **49**, 187-206 (1954).

dioxide under certain conditions. Langdon and Bloch¹⁵⁵ reported that this highly unsaturated hydrocarbon is readily absorbed from the intestine of the rat, and that 5 to 10% can be recovered unchanged from the tissues. Yamasaki¹⁶³ observed that, in rabbits, squalene is oxidized to a small extent to succinic and levulinic acids. Some is eliminated by way of the kidney and gastrointestinal tract, and another small part may be stored for over three months, principally in the liver. Yamasaki postulates that the oxidation of squalene proceeds with difficulty. This may also be true of other aliphatic hydrocarbons. The diffusion of this compound through animal cells would appear to be very slow.

(b) *Unsaturated Hydrocarbons Other Than Squalene.* Several unsaturated aliphatic hydrocarbons, which have been isolated from natural sources, have been reported, both in liver oils and in plant lipids. Thus, gadusene, $C_{18}H_{32}$, which was separated from wheat-germ oil by Drummond *et al.*,¹⁶⁴ is presumably the same compound isolated earlier by Tsujimoto¹⁶⁵ from the liver oil of the Japanese ishinagi (*Stereolepis ishinagi*). Gadusene has likewise been shown to comprise an important proportion of the unsaponifiable fraction of rice-germ oil, soybean oil, and of some fish liver oils.¹⁶⁶ Oleastane, $C_{21}H_{36}$, was reported by Sani¹⁶⁷ to occur in the olive, while zamene, $C_{18}H_{36}$, has been prepared from the liver oil of the basking shark (*Cetorhinus maximus*).¹³⁸ According to Marcelet,¹⁶⁸ hypogene, $C_{15}H_{30}$, and arachidene, $C_{19}H_{38}$, occur in the non-saponifiable fraction of peanut oil. The following unsaturated hydrocarbons have likewise been identified in olive oils: oleatridecadiene ($C_{13}H_{24}$); oleahexadecadiene ($C_{16}H_{30}$); oleanonadecadiene ($C_{36}H_{16}$); oleatricosatriene ($C_{23}H_{42}$); and oleaoctacosatetraene ($C_{28}H_{50}$).¹⁶⁹ Two saturated hydrocarbons were likewise reported from the same source, namely oleatetracosane ($C_{24}H_{50}$) and oleahexacosane ($C_{26}H_{54}$).

c. *The Metabolism of Aliphatic Hydrocarbons and of Related Compounds.* The most extensive study of the origin of the hydrocarbons and of the several related compounds in the plant waxes was carried out by Chibnall, and is summarized in the paper of Chibnall and Piper.¹²² The salient suggestions for the origin of the several components of the waxes in their scheme are as follows:

¹⁶³ S. Yamasaki, *J. Biochem. (Japan)*, **37**, 99-104 (1950).

¹⁶⁴ J. C. Drummond, E. Singer, and R. J. MacWalter, *Biochem. J.*, **29**, 456-471 (1935).

¹⁶⁵ M. Tsujimoto, *Bull. Chem. Soc. Japan*, **6**, 237-239 (1931); *Chem. Abst.*, **26**, 612-613 (1932).

¹⁶⁶ Z. Nakamiya, *Sci. Papers Inst. Phys. Chem. Research, Tokyo*, **28**, 16-26 (1935).

¹⁶⁷ G. Sani, *Atti accad. naz. Lincei*, **12**, 238-242 (1950).

¹⁶⁸ H. Marcelet, *Compt. rend.*, **202**, 1809-1811 (1936).

¹⁶⁹ H. Marcelet, *Bull. soc. chim.* [5], **3**, 1156-1160; 2055-2057 (1936); *Compt. rend.*, **202**, 867-869 (1936).

(1) Primary alcohols are formed by the reduction of the corresponding fatty acids.

(2) Paraffins, secondary alcohols, and ketones originate by indirect decarboxylation of the corresponding $(n + 1)$ acids.

(3) Saturated acids, ketonic acids, and hydroxy-acid sorigate by synthesis from shorter intermediates.

Fig. 1. The origin of the primary alcohols and hydrocarbons by reduction of fatty acids or by their decarboxylation according to Chibnall and Piper.¹²²

Fig. 2. Suggested origin of cocceryl alcohol in waxes.¹²²

The accompanying chart indicates how the large number of C_{33} - to C_{24} -intermediates presumably originate by β -oxidation, reduction, and/or decarboxylation from octatriacontanoic acid ($CH_3 \cdot (CH_2)_{33} \cdot COOH$), as shown in Figure 1.

Fig. 3. Suggested origin of *n*-nonacosan-10-ol in waxes.¹²²

Fig. 4. Suggested origin of *n*-nonacosan-15-ol in waxes.¹²²

The suggested pathway for the origin of cocceryl alcohol is indicated in Figure 2, that of *n*-nonacosan-10-ol is given in Figure 3, and that of *n*-nonacosan-15-ol is shown in Fig. 4.

(2) *Aromatic Hydrocarbons*

With the exception of the cyclopentenophenanthrene nucleus and the triterpenes, the distribution of the aromatic hydrocarbons in biologic materials is limited. Such aromatic hydrocarbons as do exist are quite resistant to chemical changes in the animal body. The reactions which occur usually involve oxidations to form hydroxyl or carboxyl groups; these new compounds are water-soluble, and may become conjugated with detoxicating products. Williams,¹⁷⁰ in his excellent monograph entitled *Detoxication Mechanisms*, described the metabolic fate of a large group of organic compounds, including the hydrocarbons. A second excellent collection of articles, also edited by Williams,¹⁷¹ entitled *Biological Oxidation of Aromatic Rings*, has been published in Biochem. Soc. Symposia No. 5.

a. Benzene and Related Compounds. Although benzene as such can scarcely be considered to be a physiologic substance, the body possesses certain mechanisms for its oxidation and metabolism when it does gain access to the body. The general pattern of these reactions varies not only with the cyclic compound employed but even, in the case of benzene, with the site of substitution.

(a) *Benzene.* a'. The Elimination of Unchanged Benzene: A considerable proportion of the administered benzene can be eliminated from the body through the lungs and by way of the kidney, especially after large doses of the hydrocarbon have been fed. As early as 1883, Nencki and Sieber¹⁷² stated that about one-third of the quantity of benzene given was excreted by the respiratory route. Lehmann and co-workers¹⁷³ later placed the figure at 40 to 45% of the quantity absorbed. In the latest experiments of Parke and Williams,¹⁷⁴ in which C¹⁴-labeled benzene was employed, 43% of the total benzene was excreted unchanged in the expired air by the rabbit within two days after a single oral dose. Earlier, these investigators¹⁷⁵ had reported an excretion of ordinary benzene *via* the lungs amounting to 40% when the dose employed was 0.25 and 0.50 g. per kg., and to 64% when it was given at a level of 1.0 g. per kg.

The kidney is a much less important route of elimination of unchanged

¹⁷⁰ R. T. Williams, *Detoxication Mechanisms, The Metabolism of Drugs and Allied Organic Compounds*, 2nd ed., Wiley, New York, 1949.

¹⁷¹ R. T. Williams, *Biological Oxidation of Aromatic Rings*, Biochem. Soc. Symposia, No. 5, Cambridge Univ. Press, 1950, pp. 1-96.

¹⁷² M. Nencki and N. Sieber, *Arch. ges. Physiol. (Pflüger's)*, 31, 319-349 (1883).

¹⁷³ K. B. Lehmann, S. O. Gundermann, O. Stöhr, and R. Kleiner, *Arch. Hyg.*, 72, 307-326 (1910).

¹⁷⁴ D. V. Parke and R. T. Williams, *Biochem. J.*, 54, 231-238 (1953).

¹⁷⁵ D. V. Parke and R. T. Williams, *Biochem. J.*, 46, 236-243 (1950).

benzene than is the lung. When less than 3 ml. (about 2.6 g.) of benzene was given to rabbits, no benzene was found in the urine, although some was detected when larger doses of this hydrocarbon were given.¹⁷⁶ However, Parke and Williams¹⁷⁴ were unable to detect any radioactive unchanged benzene in the urine of rabbits after feeding C¹⁴-labeled benzene, although an average of 32.6% of the ingested material was accounted for in the form of oxidized products (phenol, catechol, quinol, hydroxyquinol, *trans-trans*-muconic acid and L-phenylmercapturic acid). The failure to detect unchanged benzene may be related to the size of the dose employed in the latter tests. Almost half of the radioactive benzene fed by Parke and Williams¹⁷⁴ could be accounted for as oxidation products. Only 1.5% was excreted as C¹⁴O₂ over a period of two days following the feeding of this tagged compound.

b'. Oxidation of Benzene to Phenol: The presence of phenols in normal urine has long been recognized. The most important member of the group is phenol itself (C₆H₅OH), while the dihydroxybenzenes, catechol (*o*-dihydroxybenzene) and quinol (*p*-dihydroxybenzene), and 1,2,4-trihydroxybenzene (hydroxyquinol), which are grouped under phenols, account for an appreciable proportion of the ingested benzene. In the tests of Parke and Williams¹⁷⁴ with C¹⁴-labeled benzene, the following proportion of the administered benzene was isolated as the several phenols: phenol, 23.5%; catechol, 2.2%; quinol, 4.8%; and hydroxyquinol, 0.3%.

A portion of the phenols is eliminated in conjugation with sulfuric acid, while another part may be present in combination with glucuronic acid. A small amount may also appear as the free phenol. The total phenol excretion by normal men on a standard mixed diet is given by Hawk, Oser, and Summerson¹⁷⁷ as 0.2 g. per day.

Schultzen and Naunyn¹⁷⁸ were the first to report the fact that benzene is converted to phenol in the animal body. Munk¹⁷⁹ confirmed this finding and, in addition, reported that the level of urinary phenols is increased in proportion to the amount of benzene administered. Since some phenol ordinarily occurs in urine in the absence of preformed benzene in the diet, the latter data were necessary to establish the fact that the source of some of the urinary phenol was benzene itself. The fact that an appreciable proportion of benzene is oxidized to phenol has been amply confirmed by a number of workers. Thus, one-third of the benzene absorbed was con-

¹⁷⁶ J. C. Drummond and I. L. Finar, *Biochem. J.*, **32**, 79-84 (1938).

¹⁷⁷ P. B. Hawk, B. L. Oser, and W. H. Summerson, *Practical Physiological Chemistry*, 13th ed., Blakiston, New York & Toronto, 1954, p. 437.

¹⁷⁸ O. Schultzen and B. Naunyn, *Arch. Anat. Physiol.*, **1867**, 349-357.

¹⁷⁹ O. Munk, *Arch. ges. Physiol. (Pflüger's)*, **12**, 142-151 (1876).

verted to phenols in man,¹⁸⁰ and in rabbits.¹⁸¹ In the case of the dog, Schmiedeberg¹⁸² noted that an excretion of 1.884 g. as phenol and 0.445 g. as dihydroxyphenols occurred after 21 g. of benzene had been administered. This amounts to a total excretion of only 8.5% of the administered benzene in the form of phenols.

c'. Oxidation of Benzene to Di- and Trihydroxyphenols: In addition to the formation of the monohydroxy derivative, at least two dihydroxyphenols and one trihydroxyphenol are now recognized as oxidation products of benzene. Catechol, *o*-dihydroxybenzene,

and hydroquinone, *p*-dihydroxybenzene,

were isolated by Brieger¹⁸³ from 40 liters of urine obtained from hospital patients treated with phenol, but no reference is made to the quantities isolated, or to the amount of phenol administered. Nencki and Giacosa¹⁸⁴ isolated catechol in the crystalline state from the urine of both dogs and men after relatively large doses of benzene (5 to 6 g. per day) had been given.

Hydroquinone was isolated, many years ago, from dog urine by Baumann and Preusse,^{185,186} after phenol had been given. Nencki and Giacosa¹⁸⁴ did not detect quinol in dog urine, but they did demonstrate its presence qualitatively in human urine. However, Baernstein,¹⁸⁷ using newer methods for the determination of the several separate phenols, was able to prove that catechol, phenol, and hydroquinone occur in the urine of

¹⁸⁰ H. Boruttau and E. Stadelmann, *Biochem. Z.*, **61**, 372-386 (1914).

¹⁸¹ A. E. Braunstein, A. N. Parschin, and O. D. Chalisowa, *Biochem. Z.*, **235**, 311-327 (1931).

¹⁸² O. Schmiedeberg, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **14**, 288-312 (1881).

¹⁸³ L. Brieger, *Arch. Anat. Physiol. Suppl.*, 1879, 61-68.

¹⁸⁴ M. Nencki and P. Giacosa, *Z. physiol. Chem.*, **4**, 325-338, 339-344 (1880).

¹⁸⁵ E. Baumann and C. Preusse, *Arch. Anat. Physiol., Physiol. Abt.*, 1879, 245-249.

¹⁸⁶ E. Baumann and C. Preusse, *Z. physiol. Chem.*, **3**, 156-160 (1879).

¹⁸⁷ H. D. Baernstein, *J. Biol. Chem.*, **161**, 685-692 (1945).

rabbits after the injection of benzene. The presence of an additional polyhydroxyquinol has been indicated in more recent work.^{175,188} Since it is a quinol derivative, the only possible formula is

Garton and Williams,¹⁸⁸ on the basis of earlier work of Porteous and Williams,^{189,190} and of their own,^{191,192} postulated the following scheme to illustrate the sequence of the formation of the several phenols in the oxidation of benzene:

According to the scheme of Garton and Williams,¹⁸⁸ it is the free phenol rather than conjugated phenol which gives rise to the polyphenols. These workers offered extensive evidence of the validity of these reactions.

Porteous and Williams¹⁹⁰ observed that, when benzene is oxidized, quinol and catechol are produced in equal amounts, while ten times as much quinol is generated as catechol when phenol itself is oxidized. This led Garton and Williams¹⁸⁸ to adopt the theory that a part of the benzene is first oxidized to an unknown intermediate, which is converted to catechol without the intermediation of phenol. It is suggested that the unknown intermediate may possibly be 1,2-dihydro-1,2-dihydroxybenzene or dihydroxycyclohexadiene. The suggestion that this compound may serve in the above capacity is based upon the fact that compounds with similar structures have been isolated as metabolites of naphthalene,^{193,194} anthracene,^{195,196} and phenanthrene.¹⁹⁷ When benzene was administered orally

¹⁸⁸ G. A. Garton and R. T. Williams, *Biochem. J.*, **45**, 158-163 (1949).

¹⁸⁹ J. W. Porteous and R. T. Williams, *Biochem. J.*, **44**, 46-55 (1949).

¹⁹⁰ J. W. Porteous and R. T. Williams, *Biochem. J.*, **44**, 56-61 (1949).

¹⁹¹ G. A. Garton and R. T. Williams, *Biochem. J.*, **43**, 206-211 (1948).

¹⁹² G. A. Garton and R. T. Williams, *Biochem. J.*, **44**, 234-238 (1949).

¹⁹³ L. Young, *Biochem. J.*, **41**, 417-422 (1947).

¹⁹⁴ J. Booth and E. Boyland, *Biochem. J.*, **41**, xxix (1947).

¹⁹⁵ E. Boyland and A. A. Levi, *Biochem. J.*, **29**, 2679-2683 (1935).

¹⁹⁶ E. Boyland and A. A. Levi, *Biochem. J.*, **30**, 728-731, 1225-1227 (1936).

¹⁹⁷ E. Boyland and G. Wolf, *Biochem. J.*, **42**, xxxii (1948).

to rabbits, Porteous and Williams¹⁸⁹ observed that the ratio of phenol:total polyphenols (catechol, quinol, and hydroxyquinol) was approximately one;¹⁸⁸ on the other hand, when phenol was administered, the above ratio changed to 5 or 6. The explanation offered by Garton and Williams¹³³ for this phenomenon is that, when phenol is administered, it is rapidly conjugated with sulfate or glucuronic acid, and such conjugated compounds are excreted unchanged. This would leave a relatively small proportion of phenol in the free state to undergo further oxidation to the polyphenols.¹⁹²

The oxidation of benzene to phenol has been shown to take place largely in the liver,¹⁹⁸ both in the case of warm-blooded animals such as the cat and the rabbit, and in the classical example of cold-blooded animals, namely the frog. It is believed¹⁹⁸ that both classes of animals likewise possess extrahepatic sites where this oxidation may occur. Both free and conjugated phenols have been isolated from the livers of benzene-poisoned warm-blooded animals.¹⁹⁸

d'. Conjugation of Phenols: Phenol and the dihydroxy- and trihydroxyphenols are toxic substances. If more than small amounts of the free phenols accumulate, the animals will exhibit severe symptoms. It has long been known that the animal possesses the capacity to conjugate the phenols with products which render them non-toxic, and which may readily be excreted *via* the kidney without causing any deleterious symptoms. It has repeatedly been demonstrated that almost all of the phenols originating after the administration of benzene or after the feeding of free phenol itself are excreted in conjugated form, and that practically no free phenol is present. Although normal human urine contains practically no free phenols (5 to 10 mg. conjugated phenols per liter), normal rabbit urine is made up of 2 to 6 mg. of free phenols and 10 to 13 mg. of total phenols per liter.¹⁸⁹ After feeding benzene, Porteous and Williams¹⁸⁹ recovered 9.2% as phenol and 11.5% as other phenols from the urine of rabbits (total 20.7%); 9.5% of the products of benzene was excreted in the form of ethereal sulfates, and 11.2% as glucuronides (total 20.7%). It is evident that phenol itself and the other phenols are conjugated both with sulfuric acid and with glucuronic acid.

The proportion of the total phenol combined depends upon the nature of the diet; it is highest on acid diets and lowest on basic regimens.^{199,200} The amount excreted is decreased following repeated injections.^{170,200} Diuresis influences the ratio of the conjugated phenols.^{170,200}

¹⁹⁸ A. M. Tschernikow, I. D. Gadaskin, and I. I. Gurewitsch, *Arch. exptl. Pathol. Pharmacol.*, **154**, 222-227 (1930).

¹⁹⁹ A. E. Braunstein and A. N. Parschin, *Biochem. Z.*, **235**, 334-343 (1931).

²⁰⁰ A. E. Braunstein and A. N. Parschin, *Biochem. Z.*, **235**, 344-352 (1931).

a". Conjugation with Sulfuric Acid. As early as 1876, Baumann²⁰¹ proved that administered phenol is conjugated with sulfuric acid to form phenyl sulfate.

The Conjugation of Phenol with Potassium Acid Sulfate to Form Phenyl Sulfate

Because of the relative difficulty in hydrolysis of the phenyl sulfate, it is evident that the bond is firmer than that possessed by the inorganic salts. Such compounds constitute the so-called "ethereal sulfate" fraction of the urine. In addition to phenol itself, quinol, catechol, and hydroxyquinol have been shown to be present in the ethereal sulfate fraction of the urine.¹⁸⁸

A number of workers, including Christiani,²⁰² Rhode,²⁰³ Sperber,²⁰⁴ and Garton and Williams,¹⁸⁸ showed that, when the potassium or sodium salts of phenylsulfuric acid were fed or injected, they were excreted unchanged in a practically quantitative amount. Garton and Williams¹⁸⁸ confirmed the presence of small amounts of quinol (estimated as 0.3% of the dose) in the urine of rabbits after the feeding of phenyl sulfate. Moreover, extremely small quantities of catechol and hydroxyquinol were also detected.¹⁸⁶ The presence of the quinol and of other polyphenols could have resulted from a slight hydrolysis of the phenyl sulfate through the action of the phenyl sulfatase in the gut. Although the direct oxidation of the phenyl sulfate to the quinol derivative cannot be completely excluded, it is believed that the polyphenols result from the oxidation of free phenol rather than from that of conjugated phenol. The unusually rapid disposal of the injected sodium or potassium salts of phenyl sulfuric acid is indicated by the fact that Sperber²⁰⁴ accounted for 75% of the amount injected in the urine of hens within eighty minutes after the injection was made.¹⁸⁸

b". Conjugation with Glucuronic Acid. Glucuronic acid affords an important detoxicating mechanism, not only for phenol itself and for the

²⁰¹ E. Baumann, *Arch. ges. Physiol. (Pfluger's)*, 13, 285–308 (1876).

²⁰² A. Christiani, *Z. physiol. Chem.*, **2**, 273-287 (1878).

²⁰³ H. Rhode, *Z. physiol. Chem.*, **124**, 15-36 (1923).

²⁰⁴ I. Sperber, *Kgl. Lantbruks.-Högskol. Ann. (Ann. Roy. Agr. Coll., Sweden)*, **15**, 317-349 (1948); *Chem. Abst.*, **42**, 6440 (1948); cited by G. A. Garton and R. T. Williams, *Biochem. J.*, **45**, 158-163 (1949); pp. 158, 159.

other higher phenols, but also for benzoic acid, sex hormones, and many other biologic products. The fact that phenol conjugates with glucuronic acid was reported in 1883 by Külz,²⁰⁵ and was confirmed later.²⁰⁶ Neuberg and Neimann²⁰⁷ and Salkowski and Neuberg²⁰⁸ were among the first to describe this reaction.

The Conjugation of Glucuronic Acid with Phenol, and the Resulting Formation of Phenylglucuronide

When phenylglucuronide is injected, it is quantitatively excreted in the urine by rabbits¹⁸⁸ and by hens.²⁰³ On the other hand, when this compound is given orally, it undergoes considerable hydrolysis, and some of it is destroyed. Only approximately 50% of the dose can be accounted for in the urine under these conditions.¹⁸⁸ A sharp rise in the ethereal sulfate fraction of the urine occurs on the first day, and 15 to 20% of the dose of glucuronide appears as the ethereal sulfate.¹⁸⁸ Moreover, Garton and Williams¹⁸⁸ proved that the ethereal sulfate fraction under these conditions contained quinol, catechol, and hydroxyquinol, in addition to phenol. The several changes which take place are graphically represented in Figure 5.

The fact that no phenylsulfate appears in the urine after phenylglucuronide is injected, and that the phenylglucuronide is quantitatively recovered in the urine under these conditions, indicates that it does not undergo hydrolysis when it is administered parenterally. Glucuronidase apparently does not exert its hydrolytic function in the intact animal under these conditions.¹⁸⁸

e'. Oxidation of Benzene to Muconic Acid: In 1909, Jaffé²⁰⁹ first isolated muconic acid, $\text{HOOC}\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$, from the urine of

²⁰⁵ E. Külz, *Arch. ges. Physiol. (Pflüger's)*, 30, 484-485 (1883).

²⁰⁶ E. Külz, *Z. Biol.*, 27 [n.s. 9], 247-258 (1890).

²⁰⁷ C. Neuberg and W. Neimann, *Z. physiol. Chem.*, 44, 114-126 (1905).

²⁰⁸ E. Salkowski and C. Neuberg, *Biochem. Z.*, 2, 307-311 (1907).

Fig. 5. The metabolic changes occurring when phenylglucuronide is given orally.¹⁸⁸

rabbits and dogs which had received benzene. According to chemical nomenclature, it is described as hexa-2:4-diene-1:6-dioic acid. Muconic acid can occur in three different isomeric forms, which are identified as *cis-cis*, *cis-trans*, and *trans-trans*. Various findings have been reported.²¹⁰⁻²¹⁷ All the samples of muconic acid isolated from the urine by Thierfelder and Klenk,²¹⁰ by Drummond and Fonar,¹⁷⁶ and by Parke and Williams,^{174,214} were *trans-trans*. Mori⁸³ and Neumaerker²¹² failed to find the acid in the urine of rabbits after the administration of high doses of benzene. Bernhard and Gressly²¹¹ found traces, following its injection into animals. Fuchs and Soós²¹³ found muconic acid, but gave no definite evidence as to its isomeric form. Böeseken *et al.*²¹⁵⁻²¹⁷ considered it to be *cis-cis*. Kleinzeller and Fencel²¹⁸ reported that an unnamed microorganism isolated from waste waters, which was related to *Micrococcus sphaeroides* (from manure and soil) oxidized benzene solely to *trans-trans*-muconic acid.

It would be expected that *cis-cis* muconic acid would be the isomer which should be present in the urine if the muconic acid originated from

²⁰⁹ M. Jaffé, *Z. physiol. Chem.*, **62**, 58-67 (1909).

²¹⁰ H. Thierfelder and E. Klenk, *Z. physiol. Chem.*, **141**, 29-32 (1924).

²¹¹ K. Bernhard and E. Gressly, *Helv. Chim. Acta*, **24**, 83-87 (1941).

²¹² H. Neumaerker, *Z. physiol. Chem.*, **126**, 203-209 (1923).

²¹³ D. Fuchs and A. v. Soós, *Z. physiol. Chem.*, **98**, 11-13 (1916).

²¹⁴ D. V. Parke and R. T. Williams, *Biochem. J.*, **51**, 339-348 (1952).

²¹⁵ J. Böeseken and R. Engelberts, *Proc. Acad. Sci., Amsterdam*, **34**, 1292 (1931); **35**, 750-755 (1932).

²¹⁶ J. Böeseken and C. L. M. Kerkhoven, *Rac. trav. chim.*, **51**, 964-970 (1932).

²¹⁷ J. Böeseken and G. Sloof, *Proc. Acad. Sci. Amsterdam*, **32**, 1043-1045 (1929).

²¹⁸ A. Kleinzeller and Z. Fencel, *Chem. Listy*, **46**, 300-302 (1952); *Chem. Abst.*, **47**, 4290 (1953).

$ \begin{array}{c} \text{HCOH} \\ \\ \text{HCOH} \\ \\ \text{HOCH} \\ \\ \text{HOCH} \\ \\ \text{HC} \\ \\ \text{CH}_2\text{OH} \end{array} $	$ \begin{array}{c} \text{COOH} \\ \\ \text{HC} \\ \\ \text{CH} \\ \\ \text{CH} \\ \\ \text{HC} \\ \\ \text{COOH} \end{array} $	$ \begin{array}{c} \text{COOH} \\ \\ \text{HC} \\ \\ \text{HC} \\ \\ \text{HC} \\ \\ \text{HC} \\ \\ \text{COOH} \end{array} $	$ \begin{array}{c} \text{COOH} \\ \\ \text{HC} \\ \\ \text{CH} \\ \\ \text{HC} \\ \\ \text{HC} \\ \\ \text{COOH} \end{array} $
Galactose	<i>trans-trans-</i> Muconic acid (m.p., 301°C. decomp.)	<i>cis-cis-</i> Muconic acid (m.p., 195°C.)	<i>cis-trans-</i> Muconic acid (m.p. unknown)

²²⁰ O. Hayaishi and K. Hashimoto, *J. Biochem. (Japan)*, **37**, 371-374 (1950).

isomers were recovered from the urine. These investigators therefore postulate that *trans-trans*-muconic acid is formed directly in the course of oxidation of benzene, and does not result from the isomerization of the *cis-cis* form *in vivo*.

The quantities of muconic acid recovered have shown marked variations. Jaffé²⁰⁹ was able to isolate only 0.3% of the benzene administered as muconic acid, compared with figures of 3.7% cited by Thierfelder and Klenk,²¹⁰ 0.4% by Drummond and Finar,¹⁷⁶ 0.5% by Bernhard and Gressly,²¹¹ and practically zero by Neumaerker²¹² and by Mori.⁸³ Fuchs and Soós²¹³ reported the presence of 0.11% of this unsaturated hexadienoic acid in the urine of patients treated with benzene for leukemia.

Although Jaffé²⁰⁹ was able to recover only about 0.3% of the benzene administered as benzoic acid, he postulated that 25 to 30% of the benzene is metabolized by this route. This hypothesis was based upon the fact that, when muconic acid itself was injected, only approximately 1% could be isolated from the urine; presumably the remaining muconic acid, which is unaccounted for, is oxidized, demonstrating that it is a readily oxidizable metabolite. However, there is some controversy as to how readily muconic acid is metabolized when it is given to rabbits. In contrast to the positive results of Jaffé,²⁰⁹ Bernhard and Gressly²¹¹ were unable to detect any of the administered muconic acid in the urine. However, Neumaerker,²¹² Mori,⁸³ and Drummond and Finar¹⁷⁶ obtained recoveries of 53, 73, and 56%, respectively, of administered muconic acid in the urine. These varying results led Williams¹⁷⁰ to question whether or not muconic acid may arise normally as a direct metabolite of benzene; he suggested that it may originate because of an abnormal metabolism brought about by benzene.

However, the fact that *trans-trans*-muconic acid is a normal product seems to have been confirmed by the use of labeled benzene. Thus, Bernhard and Gressly²¹¹ showed that, when deuteriobenzene was injected into rabbits, deuteriomuconic acid was excreted in the urine. It was later proved by Parke and Williams¹⁷⁴ that C¹⁴-labeled *trans-trans*-muconic acid could be recovered from the urine of rabbits fed C¹⁴-containing benzene to the extent of 1.3% of the benzene fed.

Although a considerable proportion of benzene is eliminated as the mono- or diphenolic compounds, definite amounts of the benzene disappear, presumably by the rupture of the phenyl residue, with the formation of muconic acid and its ultimate oxidation to carbon dioxide and water. According to Williams,¹⁷⁰ on the basis of the findings of Drummond and Finar,¹⁷⁶ the course of the oxidation of benzene may proceed as follows:

The Postulated Course of the Oxidation of Benzene in the Animal Body^{170, 176}

The course of the oxidation of muconic acid to CO_2 and H_2O is not entirely clear. It is believed that muconic acid is oxidized by β -oxidation, inasmuch as Hensel and Riesser²²¹ observed that this acid (presumably the *trans-trans* isomer, since this was the only one known in 1913) was converted to acetone when perfused through the surviving liver. According to Raizon and Yamamoto,²²² *trans-trans*-muconic acid gives rise to acetone bodies when perfused through dog liver, while the *cis-cis* form does not. No other products resulting from the oxidation of muconic acid have been demonstrated. Mori³³ observed that the administration of *trans-trans*-muconic acid did not increase the output of oxalic acid in rabbits. Moreover, Parke and Williams¹⁷⁴ were unable to demonstrate C^{14} -labeling in acetic or fumaric acids after C^{14} -labeled benzene had been fed; no radioactivity was observed in succinic, oxalic, adipic, or *mesotartaric* acids isolated from the urine under these conditions.

According to Kuhn *et al.*,²²³ small amounts (0.1–0.2%) of *trans-trans*-muconic acid also originate after the feeding of sorbic acid, $\text{CH}_3\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$, to rabbits. Muconic acid likewise originates following the administration of ethyl sorbate (0.5%), but not after that of the methyl ester; sorbamide, $\text{CH}_3\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{CONH}_2$, gives a 32% yield of the *trans-trans* acid, while the methyl amide, $\text{CH}_3\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{CONH}(\text{CH}_3)$, and the anilide of sorbic acid, $\text{CH}_3\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{CONH}\cdot\text{C}_2\text{H}_5$, give rise to 44% and 36%, respectively, of muconic acid. The presence of the amide group decreases the loss of sorbic acid by β -oxidation. A similar decrease in the oxidation of sorbic acid obtains in the β -methyl derivative, which can be converted to the corresponding muconic acid compound to the extent of 62%.

²²¹ M. Hensel and O. Riesser, *Z. physiol. Chem.*, **88**, 38–43 (1913).

²²² T. Raizon and I. Yamamoto, *Osaka Igakkai, Zasshi (J. Osaka Med. Assoc.)*, **39**, 525–526 (1940); *Chem. Abst.*, **36**, 5559 (1942); *Japan. J. Med. Sci.*, **II**, *Biochem.*, **4**, No. 4, 105–106 (1941).

²²³ R. Kuhn, F. Köhler, and L. Köhler, *Z. physiol. Chem.*, **247**, 197–219 (1937).

The Conversion of β -Methylsorbamide to the Corresponding Muconic Acid²²³

f'. Transformation of Benzene to Phenylmercapturic Acid: The formation of phenylmercapturic acid by the rabbit indicates another possible route for the detoxication of benzene. Thiophenol, which is probably a derivative of phenylmercapturic acid, was formed when the urine of rabbits which had received benzene was treated with hydrochloric acid. On the basis of this finding, Drummond and Finar¹⁷⁶ postulated the possible formation of phenylmercapturic acid, although they failed to isolate this compound by the methods used. This would involve the following reaction:

Witter²²⁴ reached the erroneous conclusion that the rabbit does not excrete phenylmercapturic acid in the urine. This compound is also formed from benzene, and is excreted by the rat. In spite of its ready decomposition, Zbarsky and Young²²⁵ succeeded in isolating it from the urine after the administration of phenyl-L-cysteine; in fact, as much as 38% of the theoretical yield of the L-phenylmercapturic acid was isolated²²⁵ after the feeding of phenyl-L-cysteine.²²⁶ Moreover, Zbarsky and Young²²⁵ recorded experiments on rats in which 0.37% of the theoretical yield was isolated from the urine of rats when benzene was fed in corn oil (to delay absorption), while 0.11% of that expected was excreted when benzene was given without a diluent. Although Williams¹⁷⁰ originally considered that phenylmercapturic acid is only a minor metabolite of benzene, Parke and Williams²²⁷ were later able to prove that 1% of the benzene fed was excreted as phenylmercapturic acid when the dose level was 0.5 g./kg., and 0.8% when the dose level was 1 g./kg. This figure corresponds to 2.2% and 1.7%, respectively, of the benzene catabolized and excreted as mercapturic acid when this hydrocarbon was fed at the two levels. According

²²⁴ R. F. Witter, *Univ. Microfilms Ann Arbor*, 6, No. 1, p. 21 (1945); cited by R. T. Williams, *Detoxication Mechanisms*, Wiley, New York, 1949, p. 38.

²²⁵ S. H. Zbarsky and L. Young, *J. Biol. Chem.*, 151, 487-492 (1943).

²²⁶ S. H. Zbarsky and L. Young, *J. Biol. Chem.*, 151, 211-215, 217-219 (1943).

²²⁷ D. V. Parke and R. T. Williams, *Biochem. J.*, 48, 624-629 (1951).

to Parke and Williams,²²⁷ the following balance sheet can be drawn up when benzene is fed at 0.5 g./kg.

Benzene eliminated unchanged.....	39%
Benzene oxidized (excreted as glucuronides or as ethereal sulfates).....	21%
Benzene excreted as phenylmercapturic acid.....	1%

After C¹⁴-labeled benzene was fed to rabbits, Parke and Williams¹⁷⁴ were able to account for 0.5% of the dose administered as radioactive L-phenylmercapturic acid. Although phenylmercapturic acid would appear to be a minor detoxication product of benzene, there can be no doubt that it plays a definite role in this reaction.

TABLE 2

THE METABOLIC FATE OF A SINGLE ORAL DOSE OF C¹⁴-LABELED BENZENE IN A RABBIT
TWO TO THREE DAYS AFTER DOSING^a

Nature of metabolite	Individual determination (average), % of dose	Total in each category, % of dose
In expired air		
Unchanged benzene	43.0	
Respiratory CO ₂	1.5	
Total	—	44.5
In urine		
Phenol ^b	23.5	
Catechol ^b	2.2	
Quinol ^b	4.8	
Hydroxyquinol ^b	0.3	
Muconic acid ^c	1.3	
L-Phenylmercapturic acid (urinary radioactivity)	0.5 (34.5)	
Total	—	32.6
In tissues		
Metabolized benzene	4.5-9.5	4.5-9.5
In feces	0.5	0.5
Total accounted for		
In expired air and urine	77	79
In all sources	82-87	84-89

^a Adapted from D. V. Parke and R. T. Williams, *Biochem. J.*, 54, 231-238 (1954), p. 236.

^b Excreted in conjugated form.

^c *Trans-trans* acid.

g'. Résumé of the Metabolism of Benzene: There is ample evidence that benzene can be metabolized in the body by oxidation to phenol, catechol, quinol, and hydroxyquinol. These intermediates can be con-

jugated with sulfuric acid or glucuronic acid, followed by their excretion in the urine. Phenol or catechol can be oxidized before conjugation to *trans-trans*-muconic acid. Presumably, the latter compound is mainly oxidized to carbon dioxide and water; acetone bodies are possible intermediates. Some benzene is combined directly with mercapturic acid to form the detoxication product, phenylmercapturic acid. The metabolic fate of a single oral dose of C¹⁴-labeled benzene was reported by Parke and Williams¹⁷⁴ as indicated in Table 2.

(b) *Toluene and Other Methyl-Substituted Benzenes*. When one introduces a methyl group on the benzene nucleus, the compound is readily oxidized on the aliphatic side-chain. As early as 1867, Schultzen and Naumyn¹⁷⁸ reported that the methyl substituent was oxidized to a carboxyl group in the animal body. The benzoic acid so formed is then excreted as hippuric acid. Toluene, C₆H₅·CH₃, behaves in the following way:

The Detoxication of Toluene in the Animal Body

According to Epstein and Braunstein,²²⁸ up to 70% of the administered toluene was found to be converted to benzoic acid. Knoop and Gehrke²²⁹ were able to isolate 12 g. of hippuric acid from the urine after 7 g. of toluene had been administered to a dog; this figure constitutes an 80 to 90% conversion of toluene. The fate of the balance of the toluene is not certain. Presumably it is not converted to phenols, as no increased excretion of ethereal sulfate occurs. Possibly it is changed to benzoic acid and is excreted in conjugation with glucuronic acid.

In the case of benzene derivatives having more than one methyl group attached to the ring, only one of the methyl groups is oxidized; in some cases, a hydroxyl group is also introduced into the ring. Some of the reactions proved experimentally are the following:

The Metabolism of *m*-Xylene in the Dog and Rabbit^{178,230}

²²⁸ I. S. Epstein and A. E. Braunstein, *Biochem. Z.*, 235, 328-333 (1931).

²²⁹ F. Knoop and M. Gehrke, *Z. physiol. Chem.*, 146, 63-71 (1925).

²³⁰ E. Filippi, *Arch. farmacol. sper.*, 18, 178-192, 193-211 (1914).

(c) *Higher Homologues of Benzene.* When the aliphatic side-chain is lengthened, the metabolic changes become largely dependent upon the nature of the substituent. Williams¹⁷⁰ outlined the several types of reaction which may be expected, as related to structure, as follows:

(1) When the side-chain is a normal alkyl group such as methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl, the oxidation proceeds on the side-chain which is oxidized to the carboxyl ($-\text{COOH}$) or to the acetate ($-\text{CH}_2\cdot\text{COOH}$) group. No change takes place in the benzene ring. The aromatic acids are excreted as conjugates with glycine (hippuric or phenaceturic acid), or possibly with glucuronic acid as benzoyl glucuronide, or as the phenylaceturic glucuronide.

(2) When there is no aliphatic side-chain (*i.e.*, in benzene) or if the side-chain is branched, as in isopropyl, no oxidation occurs in the side-chain, and the benzene nucleus is oxidized to phenol; the latter product is excreted in combination with sulfuric acid, as evidenced by the increased ethereal sulfate excretion.

(3) When both a methyl group and a branched side-chain are combined with the benzene nucleus, the methyl group is oxidized to carboxyl, while no reaction takes place in the ring or in the branched side-chain.

a'. Ethylbenzene: It was first demonstrated by Nencki and Giacosa,¹⁸⁴ in 1880, that, in the case of the dog, ethylbenzene is oxidized to benzoic acid, which is excreted as hippuric acid. Methylphenylcarbinol was found by Neubauer²³⁴ to be an intermediate in the rabbit, and mandelic acid was later identified as another metabolite²³⁵ in this animal. Presumably no oxidation occurs in the benzene nucleus, as the urine was found to contain the normal amount of ethereal sulfate after the administration of ethylbenzene.¹⁸⁴ Methylphenylcarbinol is excreted in combination with glucuronic acid,¹³⁵ and the conjugate corresponds to that found when the closely-related acetophenone, $\text{C}_6\text{H}_5\cdot\text{CO}\cdot\text{CH}_3$, is metabolized.¹⁷⁰

The Oxidation of Ethylbenzene in the Case of the Dog and the Rabbit

²³⁴ O. Neubauer, *Naunyn-Schmiedeberg's Arch. expil. Pathol. Pharmacol.*, 46, 133-154 (1901).

²³⁵ H. Thierfelder and K. Daiber, *Z. physiol. Chem.*, 130, 380-396 (1923).

b'. *n*-Alkylbenzenes: The metabolism²³⁷ of the compounds having *n*-alkyl side-chains longer than ethyl is identical with that of the corresponding aliphatic acids having a phenyl group in the ω -carbon. The compounds with an odd-numbered aliphatic side-chain, *n*-propylbenzene ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_3$),^{184, 210} *n*-amylbenzene ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_3$),²¹⁰ are oxidized to benzoic acid and excreted as hippuric acid. On the other hand, the aromatic compounds with an even-numbered aliphatic side-chain (*n*-butylbenzene ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_3$)) are oxidized to phenylacetic acid, to be excreted as phenaceturic acid. In the case of *n*-alkyl derivatives having three or more carbons in the side-chain, the oxidation of the side-chain proceeds by β -oxidation, exactly as occurs when the aliphatic substituent is an acid. (See pages 292-296.)

c'. Iso-Alkylbenzenes: In contradistinction to the *n*-alkylbenzenes, the iso-compounds are not oxidized to the aromatic acids, but to phenols. Nencki and Giacosa¹⁸⁴ observed an increased amount of ethereal sulfate in the urine following the administration of these iso-compounds, while no other aromatic acids were present. The products studied included isopropylbenzene (cumene), $\text{C}_6\text{H}_5 \cdot \text{CH}(\text{CH}_3)_2$, the isobutylbenzenes, dimethylbenzylmethane ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}(\text{CH}_3)_2$), and methylethylphenylmethane ($\text{C}_6\text{H}_5 \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CH}_3$).

In the case of *p*-cymene (*p*-isopropyltoluene), which has both a methyl and an isopropyl group attached to the benzene ring, the methyl group is oxidized to a carboxyl while the isopropyl residue is unchanged; this results in the formation of cumic acid, which is excreted after the feeding of *p*-cymene to sheep, man, or dogs.^{184, 232, 236, 237}

The Metabolism of *p*-Cymene (*p*-Isopropyltoluene) in Sheep and Dogs

(d) *Halogenated Aromatic Hydrocarbons*. Although the halogenated aromatic compounds are not to be considered as physiologic, their metabolism is of importance because of the reactions they evoked in the animal organism. Two pathways of metabolism are possible for the halogenated hydrocarbons, either oxidation to a phenol, which is excreted in conjuga-

²³⁶ J. M. Harvey, M. White, and T. G. H. Jones, *Univ. Queensland, Dept. Chem., Coll. Papers*, 1, No. 23, 1-10 (1942).

²³⁷ E. Ziegler, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, 1, 65-72 (1873).

tion with sulfuric acid or glucuronic acid, or synthesis of a mercapturic acid.

The Metabolism of Bromobenzene by Oxidation to a Phenol (1) or by Synthesis of Bromomercapturic Acid (2)¹⁷⁰

The formation of the mercapturic acids was first described in 1879 by Baumann and Preusse¹⁸⁵ and by Jaffé.²³⁸ Similar reactions have been noted in the case of all four monohalogen benzenes (fluorobenzene, chlorobenzene, bromobenzene, iodobenzene), in that each yields a specific mercapturic acid.^{239,240} Monohalogen naphthalenes and anthracenes also form mercapturic acids.

o- and *m*-Dichlorobenzenes (and presumably the other corresponding halogen derivatives) likewise give rise to mercapturic acid²⁴¹ but *p*-dichlorobenzene does not.²³⁹ Likewise, mono- and dichlorotoluenes do not form mercapturic acids.²³⁹ Callow and Hele²⁴¹ confirmed the fact that *o*-chlorotoluene has no influence upon the sulfur metabolism of the dog.

²³⁸ M. Jaffé, *Ber.*, 12, 1092-1098 (1879).

²³⁹ E. Baumann, *Z. physiol. Chem.*, 8, 190-197 (1883).

²⁴⁰ L. Young and S. H. Zbarsky, *J. Biol. Chem.*, 154, 389-395 (1944).

²⁴¹ E. H. Callow and T. S. Hele, *Biochem. J.*, 20, 598-605 (1926).

Coombs and Hele²⁴² reported that the following compounds do not give rise to mercapturic acids in the dog: *o*-, *m*-, and *p*-chlorophenol, *o*- and *p*-chloroanisole, *m*-chloroanisole, and *p*-chloroacetanilide. When the halogen is in the side-chain, as for example in benzylchloride ($\text{C}_6\text{H}_5 \cdot \text{CH}_2\text{Cl}$), mercapturic acid formation occurs,²⁴³ with the elimination of the halogen and the attachment of the S directly to the side-chain, as follows:

The ability of animals to form mercapturic acid is a general one, but is not universal. Thus dogs, rabbits, and rats (Stekol,²⁴³⁻²⁵⁰ du Vigneaud *et al.*,²⁵¹ Stekol and Foy,²⁵² Conway²⁵³), cats (Virtue²⁵⁴), and mice (Stekol²⁵⁵) are able to effect this synthesis, but pigs are unable to do so (Coombs and Hele²⁵⁶).

b. Naphthalene and Related Products. (a) *Naphthalene*. The metabolism of naphthalene presents many similarities to that of benzene. Thus, it may be oxidized to monophenolic compounds, although in some cases diphenols or quinones arise. Under such conditions, the positions attacked usually differ from those which, according to Berenblum and Schoental,²⁵⁷ are the most active in chemical substitutions *in vitro*. Secondly, naphthalene may be reduced, resulting in a partly reduced aromatic ring.¹⁷⁰ Although naphthalene is less toxic than phenol, it has been shown to produce opacity of the crystalline lens in the case of the rabbit; this is a condition resembling human senile cataract.

²⁴² H. I. Coombs and T. S. Hele, *Biochem. J.*, **20**, 606-612 (1926).

²⁴³ J. A. Stekol, *J. Biol. Chem.*, **128**, 199-205 (1939).

²⁴⁴ J. A. Stekol, *J. Biol. Chem.*, **110**, 463-469 (1935).

²⁴⁵ J. A. Stekol, *J. Biol. Chem.*, **117**, 147-158 (1937).

²⁴⁶ J. A. Stekol, *J. Biol. Chem.*, **121**, 87-91 (1937).

²⁴⁷ J. A. Stekol, *J. Biol. Chem.*, **122**, 333-342 (1938).

²⁴⁸ J. A. Stekol, *J. Biol. Chem.*, **124**, 129-133 (1938).

²⁴⁹ J. A. Stekol, *J. Biol. Chem.*, **138**, 225-229 (1941).

²⁵⁰ J. A. Stekol, *Ann. Rev. Biochem.*, **2**, 150-157 (1943); **10**, 265-284 (1941).

²⁵¹ V. du Vigneaud, J. L. Wood, and O. J. Irish, *J. Biol. Chem.*, **129**, 171-177 (1939).

²⁵² J. A. Stekol and J. R. Foy, *J. Biol. Chem.*, **118**, 155-160 (1937).

²⁵³ W. J. Conway, *J. Biol. Chem.*, **121**, 27-29 (1937).

²⁵⁴ R. W. Virtue, *Proc. Soc. Exptl. Biol. Med.*, **34**, 425-427 (1936).

²⁵⁵ J. H. Stekol, *Proc. Soc. Exptl. Biol. Med.*, **33**, 115-119 (1935).

²⁵⁶ H. I. Coombs and T. S. Hele, *Biochem. J.*, **21**, 611-622 (1927).

²⁵⁷ I. Berenblum and R. Schoental, *Biochem. J.*, **39**, lxiv-lxv (1945).

a'. Oxidation of Naphthalene to Naphthol: Bourne and Young²⁵⁸ demonstrated that naphthalene is partly oxidized in the α -position by dogs and by human subjects, to yield α -naphthol; this phenol is conjugated with glucuronic acid or sulfuric acid, and is then excreted in the urine as the glucuronide or as ethereal sulfate. The fact that naphthalene, when administered to human subjects and to dogs, gives rise to a compound with glucuronic acid was reported by Lesnik and Nencki²⁵⁹ in 1886, and later by Lesnik.²⁶⁰ Bourne and Young²⁶¹ found this compound in the case of rabbits; however, only minimal amounts were isolated. Lesnik²⁶⁰ was able to isolate small amounts of the α -glucuronide from the urine of a man after 12 g. of naphthalene had been administered. However, Umezawa and Masamune²⁶² confirmed this finding in the case of the rabbit. The reactions involved in the formation of the α -naphthol and its detoxication are pictured below:

The Detoxication of Naphthalene by Oxidation to α -Naphthol, and Its Conjugation with Glucuronic Acid or with Potassium Hydrogen Sulfate²⁵⁸

b'. Formation of L- α -Naphthylmercapturic Acid: When naphthalene is given to rabbits, one product formed in a considerable amount is L- α -naphthylmercapturic acid. This compound is analogous to the L-phenylmercapturic acid formed in the metabolism of benzene. Bourne and

²⁵⁸ M. C. Bourne and L. Young, *Chemistry & Industry*, 52 (11), 271 (1933).

²⁵⁹ M. Lesnik and M. Nencki, *Ber.*, 19, 1534-1538 (1886).

²⁶⁰ M. Lesnik, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, 24, 167-179 (1888).

²⁶¹ M. C. Bourne and L. Young, *Biochem. J.*, 28, 803-808 (1934).

²⁶² S. Umezawa and H. Masamune, *J. Biochem. (Japan)*, 28, 487-493 (1938).

Young²⁶¹ first isolated the mercapturic acid derivative of naphthalene from rabbit urine in 1934, and their work has been confirmed by others for dogs²⁴⁴ and for rabbits.²⁶³

The Formation of L- α -Naphthylmercaptopuric Acid in the Metabolism of Naphthalene in Rabbits

c'. 1,2-Dihydro-2-Naphthol as an Oxidation Product: Bourne and Young,²⁶¹ have suggested that the parent substance of naphthalene, first obtained by Baumann and Herter²⁶⁴ when acidified "naphthalene urine" was distilled, is 1,2-dihydro-2 (or β)-naphthol. Bourne and Young²⁶¹ and Stekol²⁴⁶ confirmed the fact that acidified urine yields naphthalene under these conditions. Baumann and Herter²⁶⁴ were of the opinion that this proved the fact that free naphthalene was excreted in the urine. However, Bourne and Young²⁵⁸ demonstrated that naphthalene was not formed when "naphthalene urine" was made alkaline before being distilled. Pure 1,2-dihydro-2-naphthol, which was first synthesized by Bamberger and Lodter,²⁶⁵ has been shown to be converted to naphthalene by mineral acids, and presumably it is the substance giving rise to naphthalene when the acidified solution is distilled. According to Bourne and Young,²⁵⁸ a considerable portion of this compound may be excreted. A similar type of product has been demonstrated in the urine following the administration of anthracene.¹⁷⁰

The Conversion of 1,2-Dihydro-2-Naphthol to Naphthalene by Heating in the Presence of Mineral Acids^{258, 261}

d'. 1,2-Dihydroxy-1,2-Dihydronaphthalene as a Product of Naphthalene Metabolism: Lesnik and Nencki²⁵⁹ were the first to call attention to the presence of dihydroxynaphthalenes as oxidation products of this

²⁶³ H. R. Ing, M. C. Bourne, and L. Young, *Biochem. J.*, **28**, 809-810 (1934).

²⁶⁴ E. Baumann and E. Herter, *Z. physiol. Chem.*, **1**, 244-269 (1877).

²⁶⁵ E. Bamberger and W. Lodter, *Ann.*, **288**, 74-133 (1895).

hydrocarbon. More recently, Young^{193,266} reported that a levorotatory diol was excreted by rats which had been given naphthalene orally or subcutaneously. This diol was identified as 1,2-dihydronaphthalene-1,2-diol. When decomposed with acid, this diol gives rise to α -naphthol.²⁶⁷

The Conversion of 1,2-Dihydronaphthalene-1,2-diol to α -Naphthol by Heating in the Presence of Mineral Acid²⁶⁷

This diol has been shown to have the *trans* configuration.^{268,269} It has likewise been demonstrated by Boyland and Wiltshire²⁷⁰ that naphthalene is slowly converted to 1,2-dihydroxy-1,2-dihydronaphthalene by liver slices; however, there was no evidence for the formation of α -naphthol. The rate of the *in vitro* reaction was of sufficient magnitude to account for the metabolic production of the diol in rats.

e'. *Résumé of the Metabolism of Naphthalene*: The metabolism of naphthalene resembles that of benzene in the formation of α -naphthol, which is detoxicated and excreted both as the glucuronide and as the ethereal sulfate. A further resemblance in the metabolic pathway of benzene and of naphthalene is in their formation of mercapturic acids. On the other hand, no unchanged naphthalene is excreted, as occurs with benzene. Moreover, there is no evidence of rupture of the aromatic ring in the case of naphthalene, such as occurs when benzene is broken down to muconic acid. A further dissimilarity is in the partial saturation of the naphthalene nucleus concomitantly with the formation of the monohydroxy or dihydroxy compounds. These metabolic products are 1,2-dihydro-2-naphthol and 1,2-dihydroxy-1,2-dihydronaphthalene. α -Naphthaquinone has also been cited as a metabolite of naphthalene. Although

²⁶⁶ L. Young, *Can. Chem. Process Inds.*, **30**, 124 (1946).

²⁶⁷ L. Young, *The Oxidation of Polycyclic Hydrocarbons in the Animal Body*, in R. T. Williams, *Biological Oxidation of Aromatic Rings*, Biochem. Soc. Symposia, No. 5, Cambridge Univ. Press, 27-39 (1950).

²⁶⁸ J. W. Cook, J. D. Loudon, and W. F. Williamson, *26th Rept. Brit. Empire Cancer Campaign*, p. 249 (1949); cited by L. Young, *The Oxidation of Polycyclic Hydrocarbons in the Animal Body*, in R. T. Williams, *Biological Oxidation of Aromatic Rings*, Biochem. Soc. Symposia, No. 5, Cambridge Univ. Press, 27-39 (1950), p. 35.

²⁶⁹ J. Booth and E. Boyland, *Biochem. J.*, **44**, 361-365 (1949).

²⁷⁰ E. Boyland and G. H. Wiltshire, *Biochem. J.*, **53**, 424-426 (1953).

Penzoldt²⁷¹ reported the presence of this product in "naphthalene urine" as early as 1886, no investigators have since confirmed or denied this statement.

(b) *Hydrogenated Naphthalenes*. Considerable information is available concerning the two hydrogenated naphthalenes, namely tetrahydronaphthalene or tetralin,

and decahydronaphthalene or decalin,

In the case of tetralin, Pohl and Rawicz²⁷² observed that tetralylglucuronic acid, a dihydronaphthalene of unknown composition, and naphthalene, were present in the urine. Röckemann²⁷³ reported that different glucuronides are formed by dogs and by rabbits, respectively, when tetralin is fed; in the first case, *ac*- α -tetralylglucuronide (tetralol) is formed, while in the second instance *ac*- β -tetralylglucuronide is the product excreted.

Bernhard²⁷⁴ was unable to isolate the glucuronide after feeding 30 g. of tetralin, although he did prove the presence of glucuronic acid in the urine concentrate.

²⁷¹ F. Penzoldt, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **21**, 34-40 (1886).

²⁷² J. Pohl and M. Rawicz, *Z. physiol. Chem.*, **104**, 95-104 (1919).

²⁷³ W. Röckemann, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **92**, 52-67 (1922).

²⁷⁴ K. Bernhard, *Z. physiol. Chem.*, **257**, 54-60 (1938).

In the case of decalin, Bernhard²⁷⁴ was able to account for 33% of the *cis*- and *trans*-hydrocarbons administered, in the form of decahydronaphthol. However, little glucuronide formation took place, and Bernhard²⁷⁴ believed that most of the decalin was completely oxidized in the animal.

c. Three-Ringed Hydrocarbons. (a) *Anthracene*. Although anthracene is a compound having a coefficient of digestibility of only 31 according to Chang,²⁷⁵ the proportion which is absorbed follows metabolic pathways not too remote from those of benzene and naphthalene.²⁷⁶ The products excreted in the urine include 1,2-dihydroxy-1,2-dihydroanthracene and its glucuronide, a hydroxy-dihydroanthracene, 1-anthrylmercapturic acid, and possibly 9,10-anthraquinone.^{195,196} Elson *et al.*²⁷⁷ reported that no ethereal sulfate was formed as a result of the metabolism of anthracene in the rat. Boyland and Levi^{195,196} have been extremely active in investigating the metabolism of anthracene.

a'. Dihydroxydihydroanthracene: Boyland and Levi¹⁹⁵ isolated and identified 1,2-dihydroxy-1,2-dihydroanthracene (also called 1,2-dihydroanthracene-1,2-diol) in the urine of rats and rabbits which had received anthracene. Booth and Boyland²⁶⁹ are of the opinion that this compound has a *trans* configuration.

The Conversion of Anthracene to 1,2-Dihydroanthracene-1,2-Diol in the Animal Body, and Its Further Conversion to 1-Hydroxyanthracene by Heating with Dilute HCl

1,2-Dihydroanthracene-1,2-diol differs from anthracene by having an additional H₂O₂. The formation of such a compound is referred to as "perhydroxylation."

Boyland and Levi¹⁹⁶ also isolated a monoglucuronide of 1,2-dihydroxyanthracene-1,2-diol from the urine of rats and rabbits which had been treated with anthracene. The compound was shown to have the glucuronic acid combined with the 1-hydroxyl, since on treatment with acid it yielded 1-anthrylglucuronide, which in turn could be changed to 1-hydroxyanthracene by hydrolysis.

²⁷⁵ L. H. Chang, *J. Biol. Chem.*, **151**, 93-99 (1943).

²⁷⁶ A. A. Levi and E. Boyland, *Chemistry & Industry*, **56** (15), 446 (1937).

²⁷⁷ L. A. Elson, F. Goulden, and F. L. Warren, *Biochem. J.*, **39**, 301-308 (1945).

The Conversion of 1,2-Dihydroanthracene-1,2-diol Monoglucuronide to 1-Anthrylglucuronide by Treatment with Acid, Followed by Hydrolysis to 1-Hydroxyanthracene¹⁹⁶

The samples of 1,2-dihydroanthracene-1,2-diol obtained from the urine of the rat and the rabbit appear to differ in physical properties.¹⁹⁵ However, both yield the same naphthalene-2,3-dicarboxylic acid on oxidation with potassium permanganate. The yield of the diol is about 0.5% of the anthracene fed. As might be expected, the properties of the glucuronides of the diol also differ in rabbit and rat urine, respectively.

b'. Anthrylmercapturic Acid: In common with benzene and naphthalene, as well as with many other aromatic compounds, anthracene also gives rise to a mercapturic acid derivative when metabolized in the animal body. Boyland and Levi¹⁹⁶ proved that the increase in the quantity of organic sulfur excreted in the urine of rats and rabbits receiving anthracene was due in part to the presence of anthrylmercapturic acid, which they likewise isolated. In contradistinction to the glucuronides, the mercapturic acid derivatives in the urine of rats and of rabbits were identical. The sulfur-containing excretion product was shown to be α (or 1)-anthrylmercapturic acid; the structure was proved by its conversion to α, α' -dianthryldisulfide by alkaline hydrolysis, followed by oxidation with hydrogen peroxide. This compound was changed, by means of nitric acid, to anthraquinone-sulfonic acid, which was transformed, by reaction with HCl and KClO₃, to α -chloroanthraquinone. The structure of the latter is known.

c'. Anthraquinone: Boyland and Levi¹⁹⁶ reported the isolation of small amounts of 9,10-anthraquinone,

from the urine of rats and rabbits which had received anthracene. It is not known as yet whether or not the anthraquinone is a true metabolic product of the anthracene, or whether it was a contaminant present in the anthracene administered.

(b) *Phenanthrene*. Phenanthrene is an isomer of anthracene in which no carbons separate the attachment of the two benzene groups to the central ring, in contrast to the separation of these two rings by one carbon in the case of anthracene.

Phenanthrene

However, little is known about the metabolism of phenanthrene, other than that it yields a phenanthrol which is excreted as a strongly levorotatory glucuronide ($[\alpha]_D = -35^\circ$).²⁷⁸ Elson *et al.*²⁷⁷ observed that, in rats, a metabolic product (presumably a phenanthrol) results, which increases the ethereal sulfate fraction of the urine.

(c) *Acenaphthene*. Acenaphthene differs from anthracene and phenanthrene in that the third ring is a partially saturated five-carbon ring which is similar to that present in such powerful carcinogenic agents as cholanthrene and 20-methylcholanthrene.

Chang and Young²⁷⁹ observed that, when acenaphthene was given orally to rats, its five-carbon ring underwent fission, with the formation of naphthalic anhydride, which was eliminated in the urine. A similar metabolic fate resulted when acenaphthene was given by subcutaneous injection.²⁸⁰ Since naphthalic acid (naphthalene-1,8-dicarboxylic acid) is readily convertible to its anhydride,²⁸⁰ it is only reasonable to suppose that the acid is an intermediate in the oxidation of acenaphthene. Young²⁶⁷ suggests the possibility that the dicarboxylic acid is the true metabolic product formed in these cases, and that the anhydride originates during its separation from the urine.

²⁷⁸ P. Bergell and R. Pschorr, *Z. physiol. Chem.*, **38**, 16-38 (1903).

²⁷⁹ L. H. Chang and L. Young, *J. Biol. Chem.*, **151**, 87-91 (1943).

²⁸⁰ L. Young and A. R. Morrison, unpublished observations, 1949; cited by L. Young, *The Oxidation of Polycyclic Hydrocarbons in the Animal Body*, in R. T. Williams, *Biological Oxidation of Aromatic Rings*, Biochem. Soc. Symposia No. 5. Cambridge Univ. Press, 27-39 (1950), p. 29.

The Conversion of Acenaphthene to Naphthalic Acid (Naphthalene-1,8-dicarboxylic Acid) in Rats, and Then to Naphthalic Anhydride (the Latter Compound May Be Formed during the Isolation of the Naphthalic Acid from the Urine)

d. Higher Aromatic Hydrocarbons. Because of the carcinogenic action of a number of the higher aromatic hydrocarbons, considerable information has been amassed in recent years concerning the intermediary metabolism of these compounds. According to Boyland and Levi,^{195,196} Dobriner *et al.*,²⁸¹ Berenblum and Schoental,^{282,283} and Berenblum *et al.*,²⁸⁴ the polycyclic hydrocarbons usually undergo oxidation to yield phenolic derivatives. The positions of oxidation *in vivo* differ from those known to be the most active *in vitro*, which, in the case of anthracenoid substances, are the *meso*-positions.

(a) *Four-Ringed Hydrocarbons.* a'. Chrysene: This is a non-carcinogenic hydrocarbon, which is most reactive in mono-substitutions in position 2, while other substitutions lead mainly to 6-position derivatives.²⁸⁵ Cook and Schoental²⁸⁶ were able to identify all six possible monochrysols. According to Berenblum and Schoental,^{287,287} 3-methoxychrysenol is formed in the rat from chrysene, and the substitution does not proceed beyond the mono-stage. The 3-position is α to the naphthalene nucleus. Chrysene is not metabolized to products which increase the ethereal sulfate output of rats.²⁷⁷

b'. 1,2-Benzanthracene: Berenblum and Schoental²⁸³ demonstrated that intraperitoneally injected 1,2-benzanthracene is eliminated in the feces of mice and rats as 4'-hydroxy-1,2-benzanthracene. Dickens²⁸⁸

²⁸¹ K. Dobriner, C. P. Rhoads, and G. I. Lavin, *Cancer Research*, **2**, 95-107 (1942).

²⁸² I. Berenblum and R. Schoental, *Cancer Research*, **3**, 145-150 (1943).

²⁸³ I. Berenblum and R. Schoental, *Cancer Research*, **3**, 686-688 (1943).

²⁸⁴ I. Berenblum, D. Crowfoot, E. R. Holiday, and R. Schoental, *Cancer Research*, **3**, 151-158 (1943).

²⁸⁵ M. S. Newman and J. A. Cathcart, *J. Org. Chem.*, **5**, 618-622 (1940).

²⁸⁶ J. W. Cook and R. Schoental, *J. Chem. Soc.*, 1945, 288-293.

²⁸⁷ I. Berenblum and R. Schoental, *Biochem. J.*, **44**, 604-606 (1949).

²⁸⁸ F. Dickens, *22nd Rept. Brit. Empire Cancer Campaign*, p. 55; cited by L. Young, *Oxidation of Polycyclic Hydrocarbons in the Animal Body*, in R. T. Williams, *Biological Oxidation of Aromatic Rings*, Biochem. Soc. Symposia, No. 5, Cambridge Univ. Press, 27-39 (1950), p. 33.

The Oxidation of Chrysene in the Rat^{257,267} to 3-Hydroxychrysene (The numbers on the chrysene molecule indicate the designation for the several positions)

found that 9,10-dimethyl-1,2-benzanthracene is converted to a similar 4'-hydroxy derivative in rats.

The Metabolism of 1,2-Benzanthracene in Mice and Rats to the Corresponding 4 Hydroxyl Derivative^{170,283}

(b) *Five-Ringed Hydrocarbons.* a'. 3,4-Benzpyrene: 3,4-Benzpyrene is an extremely potent carcinogenic agent; it has been identified as one of the substances in coal tar which is capable of producing cancer, although Cook and co-workers²⁸⁹ found that 1,2 benzpyrene was the most active carcinogenic agent in tar, especially in the case of mice. Rabbits are less susceptible.^{170,290}

According to Chang,²⁷⁵ 3,4-benzpyrene is digested by rats to the extent of approximately 56%. When benzpyrene is administered to rats and mice, the feces exhibit a blue-green fluorescence,²⁹¹ while the bile and fatty tissues show a blue-violet fluorescence.²⁹²⁻²⁹⁴ The substances responsible for the fluorescence were designated "BPF" (feces) and "BPX" (bile),

²⁸⁹ J. W. Cook, C. L. Hewett, and I. Hieger, *J. Chem. Soc.*, 1933, 395-405.

²⁹⁰ C. Sannié, C. Oberling, M. Guérin, and P. Guérin, *Compt. rend. soc. biol.*, 120, 1196-1198 (1935).

²⁹¹ J. G. Chalmers, *Biochem. J.*, 32, 271-278 (1938).

²⁹² P. R. Peacock, *Brit. J. Exptl. Pathol.*, 17, 164-172 (1936).

²⁹³ P. R. Peacock and S. Beck, *Brit. J. Exptl. Pathol.*, 19, 315-319 (1938).

²⁹⁴ P. R. Peacock, *Am. J. Cancer*, 40, 251-254 (1940).

respectively. Chalmers and Crowfoot²⁹⁵ isolated a small amount of "BPX" as a crystalline compound, and proved it to be identical with "BPF." It was shown to be a phenolic compound, but was neither 4-hydroxy-3,4-benzpyrene nor 6-hydroxy-3,4-benzpyrene. However, it was later identified as 8-hydroxy-3,4-benzpyrene, by Berenblum and co-workers.^{283,284} A quinone, identified as 3,4-benzpyrene-5,8-quinone,²⁸⁴ has likewise been isolated from the feces of mice and rats which had received 3,4-benzpyrene intraperitoneally.²⁸²

The Metabolism of 3,4-Benzpyrene in Rats to 8-Hydroxy-3,4-Benzpyrene and to 3,4-Benzpyrene-5,8-Quinone

Weigert and Mottram²⁹⁶ believe that there are several intermediates in the conversion of 3,4-benzpyrene to 8-hydroxy-3,4-benzpyrene. These workers considered "BPX" and "BPF" to be different, and they have suggested two new intermediates (BPX) and (BPF). The relationship is given in Figure 6.

(1) *In vivo*, local production in skin, kidney, lung, and liver (hence in bile).

(2) *In vivo*, in the large intestine and occasionally in the lung of rabbits and mice or *in vitro* by *post-mortem* autolysis.

²⁹⁵ J. G. Chalmers and D. Crowfoot, *Biochem. J.*, **35**, 1270-1275 (1941).

²⁹⁶ F. Weigert and J. C. Mottram, *Biochem. J.*, **37**, 497-501 (1943).

Fig. 6. The projected intermediate compounds in the metabolism of benzpyrene.²⁹⁶

(3) *In vitro*, by *postmortem* autolysis.

(4) *In vitro*, by adsorption on alumina.

(5) *In vitro*, by heating *in vacuo* to a high temperature.

(6) *In vitro*, by sublimation *in vacuo*.

Chalmers and Kirby²⁹⁷ reported that approximately 1% of benzpyrene which had been injected subcutaneously into rats was excreted unchanged in the feces. Weil-Malherbe and Dickens²⁹⁸ reported that the rate of elimination of 3,4-benzpyrene was dependent upon the solvent employed; the faster the excretion, the greater was the incidence of sarcoma. When synthetic tricapylin in the presence of cholesterol was the medium used as the solvent, a higher elimination rate and an increased tumor incidence were noted in mice. In the presence of phospholipids, the elimination rate of the carcinogen was prolonged and the latent period of the sarcomas was extended. Not only cholesterol but also cholestanol, sitosterol, and α -tocopherol were later proved to accelerate the elimination rate of 3,4-benzpyrene,²⁹⁹ while epicholestanol, coprostanol, epicoprostanol, stigmasterol, 7-dehydrocholesterol, and ergosterol were inactive in affecting the course of excretion.

b'. 1,2,5,6-Dibenzanthracene: This compound has been proved to be a carcinogenic agent of considerable potency, capable of producing tumors in mice, rats, chickens, and rabbits.³⁰⁰

Levi and Boyland and²⁷⁵ later Dobriner *et al.*³⁰¹ isolated a dihydric phenol

²⁹⁷ J. G. Chalmers and A. H. M. Kirby, *Biochem. J.*, **34**, 1191-1195 (1940).

²⁹⁸ H. Weil-Malherbe and F. Dickens, *Cancer Research*, **6**, 171-178 (1946).

²⁹⁹ F. Dickens and H. Weil-Malherbe, *Biochem. J.*, **42**, xxxiii (1948).

³⁰⁰ K. Stern and R. Willheim, *Biochemistry of Malignant Tumors*, Chemical Publishing Co., Brooklyn, 1943, p. 174.

³⁰¹ K. Dobriner, C. P. Rhoads, and G. I. Lavin, *Proc. Soc. Exptl. Biol. Med.*, **41**, 67-69 (1939).

from the urine of rabbits after the feeding of 1,2,5,6-dibenzanthracene. Rats and mice were also shown to excrete a dihydroxy-1,2,5,6-dibenzanthracene in the urine²⁸¹ after treatment with the carcinogen, but it differed from that excreted by the rabbit. It is now known²⁸¹ that the dihydroxy compound synthesized by rats and mice has the hydroxyl groups on positions 4' and 8' as shown by the identity of this product with dihydroxybenzanthracene prepared synthetically by Cason and Fieser.³⁰² Boyland and co-workers³⁰³ suggested that the diphenol from rabbit urine was most probably the 3,7-, 4,8-, or 3,8-dihydroxy compound. Williams¹⁷⁰ is of the opinion that the compound most probably is 3,7-dihydroxy-1,2,5,6-dibenzanthracene.

The formation of 4',8'-Dihydroxydibenzanthracene in Rats and Mice after the Administration of Dibenzanthracene¹⁷⁰

The 4',8'-dihydroxy derivative of dibenzanthracene is probably not excreted as a glucuronide or as a component of the ethereal sulfate fraction, by rats, mice, or monkeys, as no increase in the amount of this compound which can be extracted results from autoclaving the urine.²⁸¹ Furthermore, Dobriner *et al.*²⁸¹ were unable to obtain any evidence of the formation of a mercapturic acid derivative.

The dihydroxy compound isolated from rabbit urine was found to have no carcinogenic activity^{281,301,303} in mice. This would indicate that the rabbit possesses the ability to convert a carcinogen into a non-carcinogen in the body. According to Abels *et al.*³⁰⁴ and also Baumann *et al.*,³⁰⁵

³⁰² J. Cason and L. F. Fieser, *J. Am. Chem. Soc.*, **62**, 2681-2687 (1940).

³⁰³ E. Boyland, A. A. Levi, E. H. Mawson, and E. Roe, *Biochem. J.*, **35**, 184-191 (1941).

³⁰⁴ J. C. Abels, A. T. Gorham, S. L. Eberlin, R. Halter, and C. P. Rhoads, *J. Exptl. Med.*, **76**, 143-161 (1942).

³⁰⁵ C. A. Baumann, E. G. Foster, and P. R. Moore, *J. Biol. Chem.*, **142**, 5976-08 (1942).

dibenzanthracene exerts its toxic action by interfering with the storage of vitamin A in the liver. Presumably it competes with vitamin A for some substance, possibly a protein, with which vitamin A is bound in the liver. On the other hand, this carcinogen does not interfere with the storage of riboflavin or of coenzyme I in the liver, as does butter yellow. For a further discussion of the absorption and metabolism of other carcinogenic and related noncarcinogenic compounds, the reader is referred to *The Lipids*, Vol. II, pages 279-282, and also to Williams.¹⁷⁰

CHAPTER VIII

THE METABOLISM OF CHOLESTEROL AND RELATED STEROLS IN THE ANIMAL BODY

1. Introduction

Because of the wide distribution of sterols in the tissues, and their important physiologic properties, these compounds constitute an especially interesting category of lipids. Although cholesterol itself cannot be regarded as the most striking member of this group on the basis of its physiologic action, this sterol must be considered as the key constituent, both because of its exceedingly wide distribution in the animal body and also because of its obvious significance as the parent substance of many of the steroid hormones. The metabolism of cholesterol will be considered in this chapter, from the standpoints of the absorption and transport of exogenous cholesterol, the biosynthesis of endogenous cholesterol, and the intermediary transformation of cholesterol, together with a consideration of the nature of the products originating from cholesterol, and finally from the viewpoint of the ultimate disposal of the sterol nucleus.

The discovery of cholesterol as an entity is one which dates back to 1769, when Poulletier de la Salle¹ first prepared it from gallstones; it was shown to be a component of the nonsaponifiable residue, by Chevreul² in 1815. However, the modern understanding of it began with the demonstration of its alcoholic nature by Berthelot.³

The actual conception of the metabolism of cholesterol and of its interrelationship with the steroid hormones has been a development of the past two decades. This advance in knowledge was impossible until the structure of cholesterol was completely elucidated. Although Reinitzer⁴ had

¹ Poulletier de la Salle, *circa* 1769; cited by de Fourcroy, *Ann. chim. phys.* [1], 3, 242-252 (1789) and by C. E. Bills, *Physiol. Revs.*, 15, 1-97 (1935).

² M. E. Chevreul, *Ann. chim. phys.* [1], 95, 5-50 (1815).

³ M. Berthelot, *Ann. chim. phys.* [3], 56, 51-98 (1859).

⁴ F. Reinitzer, *Monatsh.*, 9, 421-441 (1888).

proposed an empirical formula for cholesterol as early as 1888, the correct structural formula was not finally discovered until the work of Rosenheim and King,⁵⁻⁷ and especially that of Wieland and Dane,⁸ was completed in 1932. For a more complete exposition of the historical development of our ideas on the chemistry and structure of cholesterol and of related sterols, the reader is referred to *The Lipids*, Vol. I, pages 319 to 363. The early development in the field of sterols, from the standpoint of both chemistry and metabolism, was reviewed in 1935 by Bills.⁹ The more technical side of steroid chemistry has been summarized in the monograph of Fieser and Fieser.¹⁰ Other reviews include those of Schoenheimer¹¹ on sterol metabolism, the recent ones of Bloch,^{12,13} a symposium published by Gordon¹⁴ in 1950, the reviews of Lieberman and Dobriner,¹⁵ Samuels and Reich,¹⁶ and Klyne,¹⁷ and the most recent metabolic study of Staudinger and Stoeck¹⁸ in 1954.

2. The Absorption and Transport of Cholesterol and of Other Sterols

Of the various natural sterols, cholesterol is most readily absorbed from the gastrointestinal tract. The absorption of cholesterol may be demonstrated by the increased level of blood, lymph, and tissue cholesterol after this alcohol is fed. As early as 1906, Pribram¹⁹ reported a rise in the level of blood cholesterol of rabbits after the administration of this compound as the free alcohol or as its ester. Gardner and co-workers,²⁰⁻²² among others,

⁵ O. Rosenheim and H. King, *Chemistry & Industry*, 51, 464-466 (1932).

⁶ O. Rosenheim and H. King, *Nature*, 130, 315 (1932).

⁷ O. Rosenheim and H. King, *J. Soc. Chem. Ind.*, 52, 299-301 (1933).

⁸ H. Wieland and E. Dane, *Z. physiol. Chem.*, 210, 268-281 (1932).

⁹ C. E. Bills, *Physiol. Revs.*, 15, 1-97 (1935).

¹⁰ L. F. Fieser and M. Fieser, *Natural Products Related to Phenanthrene*, 3rd ed., Reinhold, New York, 1949.

¹¹ R. Schoenheimer, *Science*, 74, 579-584 (1931).

¹² K. Bloch, *Recent Progr. Hormone Research*, 6, 111-129 (1951).

¹³ K. Bloch, *Harvey Lectures*, 48, 68-88 (1952-1953).

¹⁴ E. S. Gordon, *A Symposium on Steroid Hormones*, Univ. Wisconsin Press, 1950.

¹⁵ S. Lieberman and K. Dobriner, *Ann. Rev. Biochem.*, 20, 227-264 (1951).

¹⁶ L. T. Samuels and H. Reich, *Ann. Rev. Biochem.*, 21, 129-178 (1952).

¹⁷ W. Klyne, *Synthesis and Metabolism of Adrenocortical Steroids*, Ciba Foundation Colloquia on Endocrinology, Vol. VII, Little, Brown, Boston, 1953.

¹⁸ H. Staudinger and G. Stoeck, *Stoffwechsel des Cholesterins und der Steroidhormone*, in B. Flaschenträger and E. Lehnartz, *Physiologische Chemie*, 10th ed., 2, Part I. *Der Stoffwechsel*, Sect. b, 869-908, Springer, Berlin, 1954.

¹⁹ H. Pribram, *Biochem. Z.*, 1, 413-424 (1906).

²⁰ C. Dorée and J. A. Gardner, *Proc. Roy. Soc. (London)*, B 81, 109-128 (1909).

²¹ M. T. Fraser and J. A. Gardner, *Proc. Roy. Soc. (London)*, B 81, 230-247 (1909).

²² M. T. Fraser and J. A. Gardner, *Proc. Roy. Soc. (London)*, B 82, 559-568 (1910).

likewise confirmed the fact that an increased level of cholesterol obtains in the blood, irrespective of whether it is fed as the alcohol or as the ester; however, cholesterol esters were shown to be hydrolyzed before being absorbed. According to Mueller,²³ the cholesterol content of the chyle is likewise increased after the administration of this sterol. The proportion of free to esterified cholesterol is similar in chyle to that of blood; moreover, it remains constant, irrespective of whether the steroid is administered in free or in esterified form. These data are in harmony with the evidence that a cholesterol esterase system is present in the intestinal wall which can mediate both hydrolysis and esterification.^{24,25}

(1) *Factors Altering Cholesterol Absorption*

A number of factors alter the absorption of cholesterol from the gastrointestinal tract. Thus, the presence of fat in the diet increases the efficiency of absorption of cholesterol from the gut, although this effect may vary with species. Some animals, as for example rabbits, absorb cholesterol readily in the absence of fat, while chickens, rats, and guinea pigs carry out this function less readily on fat-free diets.

Dam *et al.*²⁶ fed young chicks a commercial diet to which cholesterol dissolved in 10% peanut oil was added. After fourteen days of this feeding, no significant increase in the cholesterol was noted in the liver, spleen, aorta, and bile when the cholesterol content of the diet was from 0 to 1% of the ration, but a marked increase occurred in the range between 0.1 and 0.33%, and at higher levels. When the cholesterol was given without fat, the increase in the organs was much less, except in the case of the liver; in this organ the cholesterol values corresponding to 0 and 0.1% of dietary cholesterol were significantly higher on the fat-free diet than on that containing 10% peanut oil. This agrees with the findings of Alfin-Slater and co-workers,²⁷ who found higher values in the livers of rats on fat-free diets than in those of animals fed diets containing 15% of cottonseed oil.

A second important factor in stimulating the absorption of cholesterol is the presence of bile salts. Schönheimer²⁸ demonstrated that an optically visible lipemia could be produced in rabbits when they were fed a single

²³ J. H. Mueller, *J. Biol. Chem.*, **22**, 1-9 (1915); **27**, 463-480 (1916).

²⁴ E. Fröhlicher and H. Süllmann, *Biochem. Z.*, **274**, 21-33 (1934).

²⁵ M. L. Niefert and H. J. Deuel, Jr., *J. Biol. Chem.*, **177**, 143-150 (1949).

²⁶ H. Dam, I. Prange, and E. Søndergaard, *Acta Physiol. Scand.*, **34**, 141-146 (1955).

²⁷ R. B. Alfin-Slater, L. Aftergood, A. F. Wells, and H. J. Deuel, Jr., *Federation Proc.*, **13**, 174 (1954).

²⁸ R. Schönheimer, *Biochem. Z.*, **147**, 258-263 (1924).

dose of cholesterol along with bile salts. Member and co-workers²⁹ were able to induce the deposition of cholesterol in the aortas of rabbits only when it was fed with bile salts. Siperstein and co-workers³⁰ also concluded, more recently, that bile salts play an obligatory role in the passage of cholesterol from the intestinal tract to the lymph.

One of the most striking alterations in cholesterol absorption is produced when phytosterols are present in the gut together with cholesterol. Peterson and associates^{31,32} were the first to discover that, when soybean sterols were fed to chicks together with cholesterol, hypercholesterolemia was prevented. In a later report,³³ it was further demonstrated that the deposition of cholesterol in the liver was also decreased. Alfin-Slater *et al.*³⁴ reported that a similar decrease in the absorption of cholesterol, and in the deposition of this sterol in the liver, occurred in the case of rats fed cholesterol in conjunction with soy sterols.

The reduced absorption produced by the soy sterols could not be ascribed solely to the high melting point of the cholesterol and soy sterol mixture. In addition to soy sterols, mixed sitosterols, β -sitosterol, stigmasterol, and ergosterol, when fed singly, effected a similar inhibition of cholesterol absorption.³³ Cholestanol (dihydrocholesterol) produced the same effect in cockerels,³⁵ in rats,³⁶ and in mice.³⁷ Since esterification of soy sterols inhibited their ability to prevent cholesterol absorption,³⁸ it was postulated that the unesterified soy sterols inhibit an enzyme system concerned with cholesterol absorption, which may involve the esterification of cholesterol.³⁷

Swell and co-workers³⁹ ascribed the beneficial effect of bile salts on the

²⁹ S. Member, M. Bruger, and E. Oppenheim, *Arch. Pathol.*, **38**, 210-214 (1944).

³⁰ M. D. Siperstein, I. L. Chaikoff, and W. O. Reinhardt, *J. Biol. Chem.*, **198**, 111-114 (1952).

³¹ D. W. Peterson, *Proc. Soc. Exptl. Biol. Med.*, **78**, 143-147 (1951).

³² D. W. Peterson, C. W. Nichols, Jr., and E. A. Schneour, *J. Nutrition*, **47**, 57-65 (1952).

³³ D. W. Peterson, E. A. Schneour, N. F. Peek, and H. W. Gaffey, *J. Nutrition*, **50**, 191-201 (1953).

³⁴ R. B. Alfin-Slater, A. F. Wells, L. Aftergood, and H. J. Deuel, Jr., *Circulation Research*, **2**, 471-475 (1954).

³⁵ M. D. Siperstein, C. W. Nichols, Jr., and I. L. Chaikoff, *Circulation*, **7**, 37-41 (1953).

³⁶ R. H. Rosenman, S. O. Byers, and M. Friedman, *Circulation Research*, **2**, 45-47 (1954).

³⁷ W. T. Behr and W. L. Anthony, *J. Nutrition*, **52**, 519-524 (1954).

³⁸ H. H. Hernandez, D. W. Peterson, I. L. Chaikoff, and W. G. Dauben, *Proc. Soc. Exptl. Biol. Med.*, **83**, 498-499 (1953).

³⁹ L. Swell, D. F. Flick, H. Field, Jr., and C. R. Treadwell, *Proc. Soc. Exptl. Biol. Med.*, **84**, 428-431 (1953).

absorption of cholesterol, noted by many workers, to their activation of cholesterol esterase. It is believed that one molecule of bile salt and one of cholesterol combine to effect the esterification of the fatty acid.⁴⁰ These workers³⁹ noted that sodium cholate and sodium taurocholate were the most effective of the bile salts in bringing about the absorption of cholesterol, while sodium desoxycholate was less active, and sodium dehydrocholate was completely inactive. It is suggested³⁹ that bile salts function in cholesterol absorption because they provide the necessary co-factor for cholesterol esterase activity. However, all data are not unanimous in indicating that esterification is an essential prerequisite for cholesterol absorption, and it remains a moot question whether or not a change in the intestinal mucosa is required to insure absorption.

It was stated by Peterson *et al.*³³ that lecithin increases cholesterol absorption. However, Kesten and Silbowitz⁴¹ reached the opposite conclusion. The level of blood cholesterol also rises when choline or inositol is fed to chicks receiving cholesterol.⁴² This may be interpreted as analogous to the lecithin effect. Aftergood *et al.*⁴³ found that the total liver cholesterol content of rats fed a diet containing 15% of lard with 1% cholesterol was significantly decreased by the addition of high amounts of vitamin E.

Hormones also have an effect on cholesterol absorption. Thus, Coleman and co-workers⁴⁴ found that, in the case of male rats gonadectomized after sexual maturity and placed on a diet containing 15% cottonseed oil and 1% cholesterol, for six weeks, there was a marked decrease in the cholesterol concentration in the liver, as compared with values obtained for the livers of non-operated animals maintained on the same diet. In the case of gonadectomized females, the cholesterol concentration in the liver increased, as compared with that in unoperated female rats. This sex difference in absorption was also noted by Aftergood *et al.*⁴³ For a more detailed review of the absorption of cholesterol, the reader is referred to *The Lipids*, Vol. II, pages 259-268 and 270, 271.

(2) *The Absorption of Sterols Other Than Cholesterol*

In contradistinction to the relative ease with which cholesterol is absorbed from the gastrointestinal tract, it has been considered that many

⁴⁰ L. Swell, H. Field, Jr., and C. R. Treadwell, *Proc. Soc. Exptl. Biol. Med.*, **84**, 417-420 (1953).

⁴¹ H. D. Kesten and R. Silbowitz, *Proc. Soc. Exptl. Biol. Med.*, **49**, 71-73 (1942).

⁴² J. Stamler, C. Bolene, R. Harris, and L. N. Katz, *Circulation*, **2**, 714-721 (1950).

⁴³ L. Aftergood, R. B. Alfin-Slater, and H. J. Deuel, Jr., *Federation Proc.*, **15**, 541 (1956).

⁴⁴ R. D. Coleman, Yu Min Chen, R. B. Alfin-Slater, and H. J. Deuel, Jr., *Federation Proc.*, **15**, 234 (1956).

other closely related sterols do not share this property. Thus, Schönheimer and associates⁴⁵⁻⁴⁷ were the first to demonstrate that plant sterols such as sitosterol and stigmasterol were not absorbed, even when fed along with bile salts.⁴⁸ Although Fraser and Gardner²² originally believed that phytosterols were absorbed and transformed to cholesterol, Gardner and Gainsborough⁴⁹ later stated that plant sterols could not be converted to cholesterol in the normal animal.

More detailed investigation, carried out after the classical experiments of the Schönheimer group were made, reinforced the conclusion that phytosterols were incapable of absorption. Thus, a mixture of α , β , and γ -sitosterols were not absorbed by mice, rats, or rabbits.^{50,51} Moreover, Rosenheim and Webster⁵² reported that neither stigmasterol nor β -sitosterol (22-dihydrostigmasterol) was absorbed by rats. Sperry and Bergmann⁵³ noted that no increase in liver sterols resulted from the feeding of stigmasterol or sitosterol to mice. Similarly, Schönheimer *et al.*^{47,48,50} observed that, not only was sitosterol not absorbed by rabbits and dogs, but also no increase in liver sterols occurred after the feeding of stigmasterol or of brassicasterol to mice. Phytosterols do form soluble compounds with bile salts; however, in spite of the abundance of phytosterols in the human diet, the consensus of opinion has been that they cannot be converted to cholesterol by man.

However, more recent findings have cast considerable doubt on these conclusions. Gould and co-workers,^{54,55} using sitosterol labeled with C¹⁴ and tritium, in feeding experiments with human subjects and rats, found that this plant sterol was absorbed, although not as efficiently as was cholesterol. They also observed that the β -sitosterol was removed from the body considerably more rapidly than was cholesterol, and that no accumulation of this sterol occurred. In later experiments, Gould⁵⁶ determined quantitatively that the absorption of β -sitosterol in humans and

⁴⁵ R. Schönheimer, *Z. physiol. Chem.*, **180**, 1-5, 16-18, 24-32, 32-37 (1929); **185**, 119-122 (1929).

⁴⁶ R. Schönheimer and D. Yuasa, *Z. physiol. Chem.*, **180**, 5-16, 19-23 (1929).

⁴⁷ H. v. Behring and R. Schönheimer, *Z. physiol. Chem.*, **192**, 97-102 (1930).

⁴⁸ R. Schönheimer, H. v. Behring, and R. Hummel, *Z. physiol. Chem.*, **192**, 117-123 (1930).

⁴⁹ J. A. Gardner and H. Gainsborough, *Quart. J. Med.*, **23**, 465-483 (1930).

⁵⁰ R. Schönheimer, *Klin. Wochschr.*, **11**, 1793-1796 (1932).

⁵¹ F. L. Breusch, *J. Biol. Chem.*, **124**, 151-158 (1938).

⁵² O. Rosenheim and T. Webster, *Biochem. J.*, **35**, 928-931 (1941).

⁵³ W. M. Sperry and W. Bergmann, *J. Biol. Chem.*, **119**, 171-176 (1937).

⁵⁴ R. G. Gould, *Circulation*, **10**, 589 (1954).

⁵⁵ R. G. Gould, L. V. Lotz, and E. M. Lilly, *Federation Proc.*, **14**, 487 (1955).

⁵⁶ R. G. Gould, *Trans. New York Acad. Sci.*, ser. II, **18**, 129-134 (1955).

⁵⁷ R. P. Cook, Ninety-seventh Meeting Nutrition Soc. (Brit.), Aberdeen, Sept., 1955; *Proc. Nutrition Soc.*, **15**, no. 1, 41-45 (1956).

rats was 10% of that of cholesterol when the same amounts of the two sterols were fed. Ingested tritium-labeled β -sitosterol was found in the blood, both as the free sterol and in the esterified form. This led to the conclusion that enzymes capable of esterifying β -sitosterol were present in the animal body.

β -Sitosterol does not appear to have a predilection for any particular tissue.⁵⁶ However, an especially high value in the bile sterol, observed in one case of carcinoma, may indicate that the liver selectively excretes β -sitosterol in bile, and that bile contains a higher proportion of ingested sitosterol than does liver or blood.

Cook⁵⁷ has reviewed the present conclusions as to the absorption of sterols. Of the C₂₇ sterols, cholesterol is absorbed, in the presence of fat, by man, by various mammals, and by birds. Lathosterol, cholestanol, and 7-dehydrocholesterol, which accompany cholesterol, are absorbed by rabbits, whereas coprostanol is not. The C₂₃ sterols are poorly absorbed.⁵⁷ Ergosterol, from yeast and from certain fungi, is absorbed to some extent by rats and hens, chalinasterol (ostreasterol), from oysters, is absorbed by mice;⁵³ brassicasterol, from horse mussel (*Modiolus*, spp.) is absorbed to a slight degree by rabbits. The C₂₉ sterols are also poorly absorbed.⁵⁷ β -Sitosterol, from the fats of higher plants, especially cereals, is only slightly absorbed by man and by the rat, and stigmasterol, from soya bean, is not absorbed at all by rats or by mice. Considerable data are available to indicate that, in addition to the phytosterols, most cholesterol derivatives are poorly absorbed. This is also the case with β -cholestanol (dihydrocholesterol),^{53,58} and also with coprostanol.⁵⁸ Coprostenol, which is isomeric with cholesterol, is also not absorbed by mice or by dogs.^{51,58} Moreover, it does not appear in the eggs of hens after being fed to this species, which also suggests its non-absorbability.⁵⁹ Finally, Schönheimer and co-workers^{47,48,60} observed that cholestanol and its isomers are not absorbed from the gastrointestinal tract; of the C₂₇-sterols, only cholesterol has the distinction of being readily absorbed.

There is some disagreement as to whether or not the C₂₈-sterol, ergosterol (provitamin D₂) can be utilized. Thus, Schönheimer *et al.*⁶¹ failed to demonstrate any deposition of ergosterol in mice, rats, rabbits, or dogs after prolonged feeding of the sterol. On the other hand, Page and Menschick⁶² did note some absorption of ergosterol, as did also Hanahan and

⁵⁸ M. Bürger and W. Winterseel, *Z. physiol. Chem.*, **202**, 237-245 (1931).

⁵⁹ E. A. Evans, Jr., *J. Biol. Chem.*, **115**, 449-451 (1936).

⁶⁰ R. Schönheimer and H. v. Behring, *Z. physiol. Chem.*, **192**, 102-111 (1930).

⁶¹ R. Schönheimer, H. v. Behring, and V. Gottberg, *Z. physiol. Chem.*, **208**, 77-85 (1932).

⁶² I. H. Page and W. Menschick, *Biochem. Z.*, **221**, 6-10 (1930).

Wakil,⁶³ who followed the absorption of C¹⁴-labeled ergosterol. Although Bloor⁶⁴ stated that ergosterol cannot be absorbed unless it has been converted to vitamin D₂, other workers^{65,66} have convincingly demonstrated that this sterol can be utilized by laying hens when given in large amounts.

Thus, all evidence points to a selective absorption of the sterols in the gastrointestinal tract. Only cholesterol (and to a smaller extent oystersterol) can be absorbed by the several species of animals which have been tested. Exceedingly small changes in structure completely change the fate of the sterol insofar as absorption from the gastrointestinal tract is concerned. The difference in metabolism between cholesterol and the phytosterols may consist in the fact that cholesterol can be rapidly esterified when given orally. This can be deduced from the experiments with several sterols, following feeding or subcutaneous injection;⁴⁶ under these conditions, the other sterols cannot be esterified. For a more complete discussion of the absorption of sterols other than cholesterol, see *The Lipids*, Vol. II, pages 268–271.

(3) *The Transport of Cholesterol and of Other Sterols*

Because of the inability of the common sterols other than cholesterol to be absorbed from the gastrointestinal tract, information concerning the pathway of transfer from the gastrointestinal tract to the blood stream is available only for cholesterol. It was discovered by Mueller²³ as early as 1916 that cholesterol is transported *via* the lymphatics in the case of the dog; moreover, the proportion of cholesterol in ester form in the lymph was found to be the same, irrespective of whether the sterol was given in free or in esterified form. Biggs and co-workers⁶⁷ likewise confirmed the fact that cholesterol is transported by the lymphatic route; moreover, it was found impossible to demonstrate the transfer of any cholesterol by the portal system.⁶⁷ In more recent studies with C¹⁴-labeled cholesterol, Chaikoff and associates⁶⁸ were able to account quantitatively for the absorbed cholesterol in the lymph of rats. About 50% of the cholesterol in the lymph was present as the ester.^{68,69} Presumably, esterification takes place somewhere

⁶³ D. J. Hanahan and S. J. Wakil, *Arch. Biochem. Biophys.*, **44**, 150–158 (1953).

⁶⁴ W. R. Bloor, *Biochemistry of the Fatty Acids*, Reinhold, New York, 1943.

⁶⁵ R. Schönheimer and H. Dam, *Z. physiol. Chem.*, **211**, 241–245 (1932).

⁶⁶ W. Menschick and I. H. Page, *Z. physiol. Chem.*, **211**, 246–252 (1932).

⁶⁷ M. W. Biggs, M. Friedman, and S. O. Byers, *Proc. Soc. Exptl. Biol. Med.*, **78**, 641–643 (1951).

⁶⁸ I. L. Chaikoff, B. Bloom, M. D. Siperstein, J. W. Kiyasu, W. O. Reinhardt, W. G. Dauben, and J. F. Eastham, *J. Biol. Chem.*, **194**, 407–412 (1952).

⁶⁹ J. L. Bollman and E. V. Flock, *Am. J. Physiol.*, **164**, 480–485 (1951).

between the lumen of the gut and the thoracic duct from which the lymph was collected. The intestinal mucosa would seem to be the most logical spot for this transformation.

3. Changes of Sterols in the Gastrointestinal Tract

The gastrointestinal tract provides a site where changes can be effected in the cholesterol molecule. No profound alterations occur in the upper portion of the tract, other than esterification or hydrolysis; the chief activity on the cholesterol molecule is confined to the lower part of the gastrointestinal tract, where it is mediated by the bacteria normally present. These transformations may affect the cholesterol which fails to be absorbed, that which is excreted into the gut by way of the bile, or by the intestinal mucosa, and that which is synthesized in the tract.⁷⁰ According to Bürger and Winterseel,⁷¹ a considerable amount of ingested cholesterol may be excreted unchanged in the feces.

Cholesterol may also be dehydrogenated or hydrogenated in the intestine. Thus, the conversion of cholesterol to 7-dehydrocholesterol (provitamin D₃) by the removal of two hydrogen atoms can occur in the intestine of the guinea pig.⁷² A common change of cholesterol in the lower gut involves the addition of hydrogen, *i.e.*, a saturation. Thus, Windaus and Uibrig⁷³ demonstrated that cholestanol (dihydrocholesterol) can be formed from cholesterol in the lower intestinal tract.

The reaction of the cholesterol molecule which is best known and which has been recognized over the longest period is its conversion to coprostanol. As early as 1862, Austin Flint⁷⁴ demonstrated that the cholesterol-like compound in feces differed from cholesterol; it was originally called *stercorine*,⁷⁵ but the name *koprosterin* assigned to it by Bondzyński,⁷⁶ who rediscovered it in human feces in 1896, ultimately became the accepted terminology. In line with the more recent practice, the anglicized form of coprosterin, coprosterol, has been replaced by coprostanol, which indicates that it is a saturated alcohol. There is ample proof that the cholesterol → coprostanol reaction takes place in the gut, since the amount of the latter product

⁷⁰ G. Popják and M. L. Beeckmans, *Biochem. J.*, **47**, 233–238 (1950).

⁷¹ M. Bürger and W. Winterseel, *Z. physiol. Chem.*, **181**, 255–263 (1929).

⁷² M. Glover, J. Glover, and R. A. Morton, *Biochem. J.*, **51**, 1–9 (1952).

⁷³ A. Windaus and C. Uibrig, *Ber.*, **48**, 857–863 (1915).

⁷⁴ A. Flint, *Am. J. Med. Sci.*, **44**, 305–365 (1862).

⁷⁵ A. Flint, *Z. physiol. Chem.*, **23**, 363–367 (1897).

⁷⁶ S. Bondzyński, *Ber.*, **29**, 476–478 (1896).

is increased after the administration of cholesterol or of cholestenone.⁷⁷⁻⁸⁰ Apparently, the conversion of cholesterol to coprostanol is not a direct one, inasmuch as it involves a change in the steric configuration of the cyclopentenophenanthrene nucleus. The hypothesis of Rosenheim and Webster⁸¹ and of Anchel and Schoenheimer⁸² that the pathway of transformation involves cholestenone, coprostanone, and coprostanol has been adequately proved in the dog,⁷⁸ as well as in man,⁸² by the use of deuterium-labeled cholesterol and labeled cholesterol intermediates.

However, dissenting opinions are advanced by Rosenfeld *et al.*⁸³ who demonstrated by isotope studies that cholestenone is not a necessary intermediate in the hydrogenation of cholesterol. Snog-Kjaer and co-workers⁸⁴ therefore conclude that the conversion of cholesterol to coprostanol in the intestine occurs by direct bacterial hydrogenation by certain anaerobes.

For a more complete discussion of the changes in sterols in the intestine, the reader is referred to *The Lipids*, Vol. II, pages 271-274.

4. Cholesterol in the Blood

(1) *The Nature of Cholesterol in the Blood*

Cholesterol forms the chief component of the unsaponifiable fraction of blood. It is present in the blood both in the form of the free alcohol which is directly precipitable by digitonin, and in the form of the ester, which can be precipitated by digitonin only after hydrolysis. About two-thirds of the plasma cholesterol is normally esterified in the case of man. On the other hand, the cholesterol of human red blood cells occurs largely in the form of the free alcohol. The same is the case with the leucocytes. West and Todd⁸⁵ cite the normal range of esterified cholesterol in human serum as 90 to 114 mg. %, and that of free cholesterol as 60 to 70 mg. %. Boyd⁸⁶ gives the mean value (in mg. %) for cholesterol in the blood plasma of normal women as follows: total, 162; combined, 115; and free, 47.

⁷⁷ S. Bondzyński and V. Humnicki, *Z. physiol. Chem.*, **22**, 396-410 (1896-1897).

⁷⁸ R. Schoenheimer, D. Rittenberg, and M. Graff, *J. Biol. Chem.*, **111**, 183-192 (1935).

⁷⁹ G. Bischoff, *Biöchem. Z.*, **227**, 230-236 (1930).

⁸⁰ C. Dorée and J. A. Gardner, *Proc. Roy. Soc. (London)*, **B 80**, 227-239 (1908).

⁸¹ O. Rosenheim and T. A. Webster, *Nature*, **136**, 474 (1935).

⁸² M. Anchel and R. Schoenheimer, *J. Biol. Chem.*, **125**, 23-31 (1938).

⁸³ R. S. Rosenfeld, D. K. Fukushima, L. Hellman, and T. F. Gallagher, *Federation Proc.*, **13**, 284 (1954); *J. Biol. Chem.*, **211**, 301-311 (1954).

⁸⁴ A. Snog-Kjaer, I. Prange, and H. Dam, *Experientia*, **11**, 316-317 (1955).

⁸⁵ E. S. West and W. R. Todd, *Textbook of Biochemistry*, Macmillan, New York, 1951, pp. 544, 545.

⁸⁶ E. M. Boyd, *J. Biol. Chem.*, **101**, 323-336 (1933).

Cholesterol is ordinarily combined in the plasma with the most highly unsaturated fatty acids. Thus, Kelsey and Longenecker⁸⁷ reported that 62% of the ester cholesterol was combined with linoleic acid in the case of beef plasma. A considerable proportion of the cholesterol likewise appears in loose combination with protein. In the original C.A. preparation made from horse serum by Macheboeuf and Rebeyrotte,⁸⁸ as much as 17.9% was made up of cholesterol esters, and no free cholesterol was present. In later samples prepared by a similar procedure after World War II, only 4.5% of cholesterol esters were found in the C.A. preparation. A large proportion of the cholesterol in human plasma is likewise in combination with protein. Thus, the β_1 -globulin fraction of human serum was shown by Oncley *et al.*⁸⁹ to contain 8% by weight of free cholesterol and 39% of cholesterol esters. Although this lipoprotein accounts for only 5% of the total plasma protein, it was shown to carry 75% of the total serum cholesterol.⁸⁹ Gofman and associates^{90,91} likewise separated lipoproteins from rabbit and human sera by ultracentrifugation; these were found to contain cholesterol. In the rabbit, the main cholesterol-containing lipoprotein has a flotation rate (S_f) of 5 to 8 S units, while in man it is characterized by an S_f value of 3 to 8. In the case of the rabbit, a cholesterol-containing lipoprotein with an S_f index of 10 to 30 was sometimes found in the serum after cholesterol feeding. Many workers consider that the protein combinations with cholesterol represent the forms in which cholesterol can exert its atherogenic action.⁹⁰ Koehler and Hill⁹² observed that appreciable amounts of 7-dehydrocholesterol also occur in human serum. The total free 7-dehydrocholesterol was found to vary between 1.8 and 7.0 mg. %, while the range of the cholesterol present in combination was from 2.4 to 35 mg. %.⁹²

(2) Physiologic Factors Altering Blood Cholesterol

The level of blood cholesterol may be altered in a number of physiologic and pathologic conditions. However, Mayer and Schaeffer⁹³ reported in 1913 that the *lipemic constant* (blood cholesterol:blood fatty acids) remains uniform for each species. In fact, the constant is maintained during the

⁸⁷ F. E. Kelsey and H. E. Longenecker, *J. Biol. Chem.*, **139**, 727-740 (1941).

⁸⁸ M. A. Macheboeuf and P. Rebeyrotte, in *Lipoproteins*, General Discussion Faraday Soc., No. 6, 62-74, Aberdeen Univ. Press, Aberdeen, 1949.

⁸⁹ J. L. Oncley, F. R. N. Gurd, and M. Melin, *J. Am. Chem. Soc.*, **72**, 458-464 (1950).

⁹⁰ J. W. Gofman, F. T. Lindgren, H. A. Elliott, W. Mantz, J. Hewitt, B. Strisower, V. Herring, and T. P. Lyon, *Science*, **111**, 166-171, 186 (1950).

⁹¹ J. W. Gofman, F. T. Lindgren, and H. A. Elliott, *J. Biol. Chem.*, **179**, 973-979 (1949).

⁹² A. E. Koehler and E. Hill, *Federation Proc.*, **12**, 232-233 (1953).

⁹³ A. Mayer and G. Schaeffer, *J. physiol. path. gén.*, **15**, 984-998 (1913).

absorption of fat.⁹⁴ In the case of dogs the lipemic constant ($\times 100$) averaged 36, and varied only from 23 to 50 in individual determinations.⁹⁵ A constant relationship likewise exists between cholesterol and the phosphatide fraction in the blood. Bloor⁹⁶ was the first to report that the *cholesterol:lecithin* ratio is uniform in human blood, under both normal and abnormal conditions. Peters and Man⁹⁷ partially confirmed the constancy of this ratio when expressed as *cholesterol:lipid phosphorus*; the mean value was 21.6 ± 2.5 when the cholesterol level was 204.6 mg. $\%$. However, some variations were observed in the ratio, especially in the presence of higher blood cholesterol concentrations. Finally, as indicated earlier, the ratio of cholesterol ester to free cholesterol is a rather precise biologic constant under normal conditions. Sperry⁹⁸ reported an average figure of $73 \pm 1.4\%$ for this ratio as determined from tests on 91 normal adults. Confirmatory data have been supplied by Peters and Man⁹⁷ and by Brun.⁹⁹

The level of blood cholesterol varies with the species under consideration, having the lowest average in the guinea pig, and the highest in the case of man.¹⁰⁰ The ratio of cholesterol ester : total cholesterol has also been found to vary with species. Only slight variations are related to age and sex.¹⁰¹ In the case of the horse and cow, the figure for the proportion is 0.67, while in the serum of the dog, pig, and goose, it approximates a value of 0.75.

Moreover, the blood cholesterol levels of different normal animals of the same species show wide variations, so that it is impossible to state a precise value for cholesterol in any species.⁹³ Wide variations have likewise been observed in the plasma cholesterol of the same individual at different times. Thus, Bloor¹⁰² recorded an average plasma cholesterol of 80 ± 14 mg. $\%$ for one dog, with variations between 60 and 109 mg. $\%$; in a second animal, the mean value was 133 ± 14 mg. $\%$, and the range varied between 110 and 155 mg. $\%$.

Age is the most important physiologic condition which causes an altera-

⁹⁴ E. F. Terroine, *J. physiol. path. gén.*, **16**, 212-222 (1914).

⁹⁵ E. F. Terroine, *Ann. Sci. Nat. Zool.* [10], **4**, 5-397 (1920).

⁹⁶ W. R. Bloor, *J. Biol. Chem.*, **25**, 577-599 (1916).

⁹⁷ J. P. Peters and E. B. Man, *J. Clin. Invest.*, **22**, 707-714 (1943).

⁹⁸ W. M. Sperry, *J. Biol. Chem.*, **114**, 125-133 (1936).

⁹⁹ G. Brun, *Changes in the Lipide Contents of Serum in Patients with Manic-Depressive Psychosis*, H. K. Lewis, London, 1940.

¹⁰⁰ E. M. Boyd, *J. Biol. Chem.*, **143**, 131-132 (1942).

¹⁰¹ E. Darraspen, R. Florio, and P. Emangard, *Compt. rend. soc. biol.*, **143**, 1419-1420 (1949).

¹⁰² W. R. Bloor, *J. Biol. Chem.*, **103**, 699-705 (1933).

tion in blood cholesterol. According to Gordon and Cohn,¹⁰³ the level of this component was only 89 mg. % at birth in human infants; it increased to an average of 136 mg. % in the five- to twelve-month group, and leveled off at the value noted in adults by the age of five to six years. There is some disagreement in the literature as to whether or not hypercholesterolemia is associated with advanced age. According to Page and co-workers,¹⁰⁴ who examined sixty-six male subjects with ages ranging from twenty to one-hundred-one years, there was no change in the blood cholesterol value which could be ascribed to age. Sperry and Webb¹⁰⁵ were also unable to prove a direct relationship between age and the level of blood cholesterol in the case of twenty-two subjects who were kept under observation over a period of fifteen years. On the other hand, Keys and associates¹⁰⁶ concluded that a pronounced curvilinear relation exists between age and serum cholesterol in men, the maximum concentration being reached in the sixth decade. Average normal blood cholesterol levels are given as 173 mg. % for the nineteen-year-old subjects, and 252 mg. % for those who had reached the age of fifty-two years. Until further research is completed, it is impossible to say with certainty to what extent age influences the level of blood cholesterol.

Although the values for blood cholesterol reported by Bose and De¹⁰⁷ and by Boyd and Roy¹⁰⁸ for Indians do appear to be somewhat less than the corresponding values for occidentals, there is no compelling evidence that race has an appreciable effect on the level of blood cholesterol. Moreover, blood cholesterol shows no diurnal variation.¹⁰⁹

A decrease in the level of blood cholesterol has been noted in women prior to the onset of the menstrual period.^{110,111} Parhon and Parhon¹¹² noted that higher than normal blood cholesterol occurs in hens during the egg-laying period. The most striking changes in blood cholesterol have been observed in women during pregnancy; thus, the increase in chole-

¹⁰³ M. B. Gordon and D. J. Cohn, *Am. J. Diseases Children*, **35**, 193-200 (1928).

¹⁰⁴ I. H. Page, E. Kirk, W. H. Lewis, Jr., W. R. Thompson, and D. D. Van Slyke, *J. Biol. Chem.*, **111**, 613-639 (1935).

¹⁰⁵ W. M. Sperry and A. M. Webb, *J. Biol. Chem.*, **187**, 107-110 (1950).

¹⁰⁶ A. Keys, O. Mickelsen, E. O. Miller, E. R. Hayes, and R. L. Todd, *J. Clin. Invest.*, **29**, 1347-1353 (1950).

¹⁰⁷ J. P. Bose and U. N. De, *Indian J. Med. Research*, **24**, 489-508 (1936).

¹⁰⁸ T. C. Boyd and A. C. Roy, *Indian J. Med. Research*, **15**, 643-651 (1928).

¹⁰⁹ C. W. McClure and M. E. Huntsinger, *J. Biol. Chem.*, **76**, 1-18 (1928).

¹¹⁰ R. Okey and R. E. Boyden, *J. Biol. Chem.*, **72**, 261-281 (1927).

¹¹¹ C. Kaufmann and B. Mühlbock, *Arch. Gynäkol.*, **136**, 478-502 (1929).

¹¹² C. J. Parhon and M. Parhon, *Compt. rend. soc. biol.*, **89**, 349-353 (1923); **90**, 150-152 (1924).

terol begins after the second month, and continues up to the thirtieth week, after which it decreases again up to the time of delivery.¹¹³

The administration of fat itself has been reported by a number of investigators to cause a concomitant hypercholesterolemia, although there is no unanimity of opinion on this point. Conversely, Anderson and Keys¹¹⁴ reported that the serum cholesterol of patients decreased 21 mg. % over a four-week period during which the level of dietary fat was decreased from 140 to 70 g. daily; an equicaloric diet containing 0.7 g. of cholesterol was given.

In addition to the immediate effect of fat ingestion upon blood cholesterol, there is also a prolonged effect following the ingestion of this foodstuff which may persist over several days. Thus, Entenman and Chaikoff¹¹⁵ demonstrated that an increase in blood cholesterol occurred after a single fat feeding, although the serum fatty acids and phospholipids were only slightly increased. Tolstoi¹¹⁶ observed a continued hypercholesterolemia in two human subjects who lived on an exclusive meat-fat diet for over a year.

Although one might expect the ingestion of cholesterol itself to be the most potent procedure for provoking an increased level of blood cholesterol, this result cannot readily be demonstrated in man. Thus, no increase in blood cholesterol values in man, after the administration of cholesterol in doses as high as 20 g. daily, have been observed by Turner and Steiner,¹¹⁷ Gardner and Gainsborough,¹¹⁸ or by Keys.¹¹⁹ However, increased serum cholesterol values were reported in man when cholesterol was fed in the form of egg yolk.^{120,121} It is possible that the hypercholesterolemia in the latter cases may be attributed to the increased absorption resulting from the lecithin present in the egg yolk. On the other hand, the feeding of cholesterol to animals other than man is invariably followed by an increased blood cholesterol, together with the development of fatty livers. This hypercholesterolemia occurs in the case of rats,^{122,123} guinea

¹¹³ J. A. Gardner and H. Gainsborough, *Lancet*, 1929, I, 603-606.

¹¹⁴ J. T. Anderson and A. Keys, *Federation Proc.*, 12, 169 (1953).

¹¹⁵ C. Entenman and I. L. Chaikoff, *J. Biol. Chem.*, 142, 129-139 (1942).

¹¹⁶ E. Tolstoi, *J. Biol. Chem.*, 83, 753-758 (1929).

¹¹⁷ K. B. Turner and A. Steiner, *J. Clin. Invest.*, 18, 45-49 (1939).

¹¹⁸ J. A. Gardner and H. Gainsborough, *Biochem. J.*, 22, 1048-1056 (1928).

¹¹⁹ A. Keys, *Science*, 112, 79-81 (1950).

¹²⁰ R. Okey and D. Stewart, *J. Biol. Chem.*, 99, 717-727 (1933).

¹²¹ A. Steiner and B. Domanski, *Am. J. Med. Sci.*, 201, 820-824 (1941).

¹²² C. H. Best, H. J. Channon, and J. H. Ridout, *J. Physiol.*, 81, 409-421 (1934).

¹²³ R. P. Cook, *Biochem. J.*, 30, 1630-1636 (1936).

pigs,^{122,124} and dogs.¹²⁵ In the case of rabbits fed cholesterol along with bile salts, atherosclerosis^{126,127} may be observed to accompany the hypercholesterolemia.¹²⁸

Fasting may cause an alteration in the level of blood cholesterol; however, the rat¹²⁹ and the dog ordinarily do not show any change in blood cholesterol¹³⁰ or in the degree of lipemia in general,¹³¹ during inanition, unless there is a severe loss of weight due to the undernutrition.¹³⁰ Mice,¹³² rabbits,^{133,134} and man¹³⁵ usually develop higher than normal blood cholesterol levels during fasting. However, Shope¹³³ observed a hypocholesterolemia in the cat, guinea pig, swine, and, in some cases, in man under conditions of dietary restriction.

Not only fasting, but also the absence of essential fatty acids in the diet results in hypocholesterolemia in rats,¹³⁶ associated with an increase in the cholesterol content of the liver and adrenal glands. On the other hand, when the diet contained 12.5 per cent of cottonseed oil, the blood cholesterol remained constant and no excess cholesterol was found to be deposited in the liver or adrenals. It is believed that essential fatty acids must be present in the diet to insure a normal cholesterol metabolism.

(3) *Pathologic Factors Altering Blood Cholesterol*

The thyroid is the most important of the endocrine glands as regards the control of cholesterol metabolism. Hypercholesterolemia is an invariable consequence of thyroid undersecretion, while hypocholesterolemia results from hypersecretion of this gland.

Following extirpation of the thyroid gland, high serum cholesterol levels

¹²⁴ R. Okey, *Proc. Soc. Exptl. Biol. Med.*, **51**, 349-350 (1942).

¹²⁵ A. Knudson, *J. Biol. Chem.*, **45**, 255-262 (1921).

¹²⁶ K. B. Turner, *J. Exptl. Med.*, **58**, 115-125 (1933).

¹²⁷ K. B. Turner and E. H. Bidwell, *J. Exptl. Med.*, **62**, 721-732 (1935).

¹²⁸ L. Swell and D. F. Flick, *Am. J. Physiol.*, **174**, 51-53 (1953).

¹²⁹ B. Sure, M. C. Kik, and A. E. Church, *J. Biol. Chem.*, **103**, 417-424 (1933).

¹³⁰ C. Entenman, G. W. Changus, G. E. Gibbs, and I. L. Chaikoff, *J. Biol. Chem.*, **134**, 59-69 (1940).

¹³¹ B. L. Kartin, E. B. Man, A. W. Winkler, and J. P. Peters, *J. Clin. Invest.*, **23**, 824-835 (1944).

¹³² P. L. MacLachlan, *J. Biol. Chem.*, **152**, 391-394 (1944).

¹³³ R. E. Shope, *J. Biol. Chem.*, **75**, 101-113 (1927).

¹³⁴ G. W. Ellis and J. A. Gardner, *Proc. Roy. Soc. (London)*, **B 85**, 385-393 (1912).

¹³⁵ C. Fahrig and L. Wacker, *Klin. Wochschr.*, **11**, 886-891 (1932).

¹³⁶ R. B. Alfin-Slater, L. Aftergood, A. F. Wells, and H. J. Deuel, Jr., *Arch. Biochem. Biophys.*, **52**, 180-185 (1954).

have been recorded in rats,¹³⁷ rabbits,¹³⁸ and in the dog.^{139,140} In the latter case, the effect was shown to be counteracted by the administration of thyroxine¹⁴⁰ or by hypophysectomy.¹³⁹ Hypercholesterolemia has likewise been demonstrated after thyroidectomy in horses,¹⁴¹ as well as after subtotal thyroidectomy in man.¹⁴¹

When the thyroid gland is non-functional, a similar trend to increased blood cholesterol levels may be noted. Handler¹³⁷ observed a hypercholesterolemia in rats following treatment with thiouracil, although the level was somewhat decreased in choline-deficient rats. A higher than normal blood cholesterol level was observed by Westra and Kunde¹⁴² in cretin rabbits; the figure could be somewhat reduced by feeding thyroid tissue. Human cretins likewise present hypercholesterolemia.¹⁴³ Several investigators¹⁴⁴⁻¹⁴⁶ have also proved that blood cholesterol is increased in functional hypothyroidism, including myxedema.

In sharp contrast to the above results, increased thyroid activity reduces blood cholesterol. Thus, low blood cholesterol levels, as well as low blood phosphatides, have been recorded in patients having exophthalmic goiter (Basedow's disease, or Graves' disease) or other forms of hyperthyroidism.¹⁴⁷⁻¹⁵⁰

The thyroid secretion not only controls the absolute amount of blood cholesterol, but it also exerts some control over the proportion of free to esterified cholesterol present in the serum. Although the ratio of free to esterified cholesterol was found to be especially high in cases of hypofunction of the organ resulting in cretinism in children,¹⁵¹ a normal cholesterol partition has usually been noted in myxedema or other thyroid disturbances in adults.¹⁵² In hyperthyroidism, the ratio of free to total cholesterol increases; the decrease in blood cholesterol is largely in the ester fraction.¹⁵³

¹³⁷ P. Handler, *J. Biol. Chem.*, **173**, 295-303 (1948).

¹³⁸ W. Fleischmann, H. B. Shumacker, Jr., and L. Wilkins, *Am. J. Physiol.*, **131**, 317-324 (1940).

¹³⁹ K. W. Thompson and C. N. H. Long, *Endocrinology*, **28**, 715-722 (1941).

¹⁴⁰ L. H. Schmidt and H. B. Hughes, *Endocrinology*, **22**, 475-482 (1938).

¹⁴¹ H. Heckscher, *Biochem. Z.*, **158**, 417-421 (1925).

¹⁴² J. J. Westra and M. M. Kunde, *Am. J. Physiol.*, **103**, 1-4 (1933).

¹⁴³ H. Heckscher, *Biochem. Z.*, **158**, 422-427 (1925).

¹⁴⁴ L. M. Hurxthal, *Arch. Internal Med.*, **51**, 22-32 (1933); **53**, 762-781 (1934).

¹⁴⁵ L. M. Hurxthal and H. N. Simpson, *J. Clin. Endocrinol.*, **1**, 450-452 (1941).

¹⁴⁶ E. F. Gildea, E. B. Man, and J. P. Peters, *J. Clin. Invest.*, **18**, 739-755 (1939).

¹⁴⁷ H. J. Bing and H. Heckscher, *Biochem. Z.*, **158**, 403-416 (1925).

¹⁴⁸ L. M. Hurxthal, *Arch. Internal Med.*, **52**, 86-95 (1933).

¹⁴⁹ E. B. Man, E. F. Gildea, and J. P. Peters, *J. Clin. Endocrinol.*, **19**, 43-59 (1950).

¹⁵⁰ E. G. Nicholls and W. A. Perlzweig, *J. Clin. Invest.*, **5**, 195-204 (1928).

¹⁵¹ H. Schwarz and A. Topper, *J. Pediatr.*, **3**, 242-246 (1933).

¹⁵² J. P. Peters and E. B. Man, *J. Clin. Invest.*, **22**, 715-720 (1943).

¹⁵³ E. M. Boyd and W. F. Connell, *Quart. J. Med.*, n.s. **5**, 455-460 (1936).

Exceedingly high blood cholesterol values have been reported in cases of diabetes mellitus, in which condition hyperlipemia has also been noted. Blood cholesterol levels as high as 1.5% and 1.41% were recorded by Imrie¹⁵⁴ and by Herbert,¹⁵⁵ respectively; in these cases, cholesterol comprised 14.1% and 22% of the total blood lipid. Cochrane, Michaels, and Kinsell¹⁵⁶ observed that the substitution of a vegetable fat for "animal fat" in the high-protein, high-fat diabetic diets resulted in a major decline in the levels of plasma cholesterol and of phospholipids. It has been suggested that inositol is the effective agent in reducing both cholesterol and lipid P in the case of hypercholesterolemic diabetics.¹⁵⁷ Another condition, related to diabetes, known as *essential xanthomatosis* or *essential cholesterolemia*, is characterized by blood cholesterol values 400 to 500% of that of normal subjects. Arteriosclerosis is another condition which is considered by many to be related to hypercholesterolemia. For an extended discussion of the subject of blood cholesterol under normal and pathologic conditions, the reader is referred to Chapter V, Volume II, *The Lipids*. Pages 466 to 481 in the above chapter deal especially with the subject of cholesterol in relation to arteriosclerosis.

5. The Biosynthesis of Cholesterol

(1) Evidence Proving Cholesterol Synthesis

The ability of living tissue to synthesize cholesterol has long been recognized. Among the earlier experimental work, that of Beumer and Lehmann¹⁵⁸ on dogs, and the findings of Randles and Knudson¹⁵⁹ who studied rats, were particularly convincing. Gamble and Blackfan,¹⁶⁰ investigating infants, and Gardner and Fox¹⁶¹ studying adults, in both cases on a mixed diet, interpreted their data as evidence of cholesterol synthesis in man.

The experiments of Schönheimer⁴⁵ with herbivorous animals indicate that the *Herbivora* are forced to synthesize practically all of the cholesterol necessary for their tissues, since they ordinarily obtain only plant sterols in the diet; phytosterols are reported to be incapable either of absorption or

¹⁵⁴ C. G. Imrie, *J. Biol. Chem.*, **20**, 87-90 (1915).

¹⁵⁵ F. K. Herbert, *Biochem. J.*, **29**, 1887-1893 (1935).

¹⁵⁶ G. C. Cochrane, G. D. Michaels, and L. W. Kinsell, *J. Clin. Nutrition*, **1**, 295-298 (1953).

¹⁵⁷ W. C. Felch and L. B. Dotti, *Proc. Soc. Exptl. Biol. Med.*, **72**, 376-378 (1949).

¹⁵⁸ H. Beumer and F. Lehmann, *Z. ges. exptl. Med.*, **37**, 274-280 (1923).

¹⁵⁹ F. S. Randles and A. Knudson, *J. Biol. Chem.*, **66**, 459-466 (1928).

¹⁶⁰ J. L. Gamble and K. D. Blackfan, *J. Biol. Chem.*, **42**, 401-409 (1920).

¹⁶¹ J. A. Gardner and F. W. Fox, *Proc. Roy. Soc. (London)*, **B 92**, 358-367 (1921).

of conversion to cholesterol. Dam¹⁶² demonstrated that the tissues of chicks raised on cholesterol-low diets contained more of this sterol than could be accounted for in their food and in the eggs from which they had been hatched. The cholesterol unaccounted for could only have originated synthetically. Imhäuser¹⁶³ likewise proved the synthesis of cholesterol in a human subject; he was able to demonstrate more cholesterol in the feces than in the food ingested. Moreover, Schoenheimer and Breusch¹⁶⁴ reported that, when mice were fed on a bread diet, which is practically devoid of cholesterol, the animals synthesized as much cholesterol in one month as they had initially contained. More recently, cholesterol synthesis has again been demonstrated in man by the use of the so-called balance type of experiments. The extent of cholesterol synthesis in man, as judged by the amount of the negative balance on cholesterol-free diets, was calculated by Frazer¹⁶⁵ as 300 mg. per day, based upon the data of Channon,¹⁶⁶ Schoenheimer and Breusch,¹⁶⁴ and of Gardner and Gainsborough.⁴⁹

Somewhat later, the synthesis of cholesterol in the animal body was proved by the use of isotopic tracers. Thus, Rittenberg and Schoenheimer¹⁶⁷ interpreted their demonstration of the incorporation of deuterium into cholesterol in mice as evidence of the synthesis of this sterol; in fact, they suggest that it is formed "by the coupling of a large number of small molecules." It is now known that CoA functions in this reaction.

a. The Inability of Insects to Synthesize Cholesterol. In spite of the fact that the higher animals, without exception, have the capacity to synthesize cholesterol, this ability is not universal throughout the animal kingdom. Thus, a number of larvae, parasites, and insects require cholesterol or other related sterols as dietary essentials, inasmuch as they are unable to synthesize the steroid nucleus.

Fraenkel and co-workers¹⁶⁸ were the first to call attention to the fact that the larvae of the scavenger beetle (*Dermestes vulpinus*) will grow only if the food contains a sterol. The sterols which supply this requirement include purified cholesterol, cholesteryl acetate, 7-dehydrocholesterol, and 7-dehydrocholesteryl monobenzoate. The following sterols were shown

¹⁶² H. Dam, *Biochem. Z.*, **220**, 158-163 (1930).

¹⁶³ K. Imhäuser, *Klin. Wochschr.*, **9**, 71-72 (1930).

¹⁶⁴ R. Schoenheimer and F. Breusch, *J. Biol. Chem.*, **103**, 439-448 (1933).

¹⁶⁵ A. C. Frazer, *Lipid Metabolism*, in G. H. Bourne and G. W. Kidder, *Biochemistry and Physiology of Nutrition*, vol. I, Academic Press, New York, 1953, pp. 212-264.

¹⁶⁶ H. J. Channon, *Biochem. J.*, **19**, 424-432 (1925).

¹⁶⁷ D. Rittenberg and R. Schoenheimer, *J. Biol. Chem.*, **121**, 235-253 (1937).

¹⁶⁸ G. Fraenkel, J. A. Reid, and M. Blewett, *Biochem. J.*, **35**, 712-720 (1941).

to be inactive: sitosterol, ergosterol, 7-hydroxycholesteryl dibenzoate, and calciferol. The minimum quantity necessary for satisfactory growth varied with the sterol, being 1 mg./3 g. food for cholesterol and 7-dehydrocholesteryl monobenzoate, and less than 0.5 mg./3 g. food for 7-dehydrocholesterol.

The larvae of the common vinegar fly, or fruit fly (*Drosophila melanogaster*), were likewise found by van't Hoog¹⁶⁹ to require sterols, but they were less selective. According to this latter worker, the following sterols all served satisfactorily to supply the required component: cholesterol, sitosterol, stigmasterol, ergosterol, phytosterol, cholestanol (dihydrocholesterol) and cholestenone.

In a study of the larvae of the black carpet beetle (*Attagenus piceus*), McKennis¹⁷⁰ demonstrated that some larvae can use either plant or animal sterols while others, such as *A. piceus*, respond only to the animal sterols; thus, cholesterol was active (even when highly purified), while synthetic 7-hydroxycholesterol and 7-ketocholesterol did not support growth. However, cholesterol dibromide did possess activity.

Fraenkel and Blewett¹⁷¹ observed that the presence of one per cent of cholesterol in the diet improved the growth of a number of insects normally living on flour. In the absence of cholesterol, the growth was impaired somewhat in the case of *Tribolium confusum* Duv. ("confused" brown flour beetle), *Silvanus (Oryzaephilus) surinamensis* (saw-toothed grain beetle) and *Lasioderma serricorne* Fabr. (cigarette beetle), while it was appreciably or severely impaired in the case of *Ptinus tectus* Boield (brown spider beetle), *Sitodrepa panicea* L. ("drug-store" beetle) and *Ephestia kuehniella* Zell. (Mediterranean flour moth). When an aqueous yeast extract was supplied in place of yeast and cholesterol, all the insects were found to develop, although at a reduced rate. The retardation resulted from a deficiency of biotin, which is contained in the insoluble portion of the yeast. In a later report by Fraenkel and Blewett,¹⁷² it was noted that 7-dehydrocholesterol, as well as cholesterol or sitosterol with only one double bond in ring B, could be used as a source of sterol. However, when ring B was ruptured, as in vitamin D₂, the resulting compound was unsatisfactory, because the tetracyclic arrangement had been changed. The nutritional value of cholesterol was likewise destroyed by the following structural changes in the molecule: loss of the secondary alcohol group (Δ^5 -choles-

¹⁶⁹ E. G. van't Hoog, *Z. Vitaminforsch.*, **5**, 118-126 (1936).

¹⁷⁰ H. McKennis, Jr., *J. Biol. Chem.*, **167**, 645-654 (1947).

¹⁷¹ G. Fraenkel and M. Blewett, *J. Exptl. Biol.*, **20**, 28-34 (1943).

¹⁷² G. Fraenkel and M. Blewett, *Biochem. J.*, **37**, 692-695 (1943).

tene); introduction of a second alcohol group (7-hydroxycholesterol); esterification of the alcohol group (7-hydroxycholesteryl dibenzoate); and the introduction of a ketone group on C₇ (7-ketocholesteryl acetate).

Both cholesterol and ergosterol were found by Chauvin¹⁷³ to serve equally satisfactorily as sources of sterols in two species of *Orthoptera* or wingless insects. Thus, these sterols acted as growth factors for *Gryllus domesticus* (house cricket) and *Blatella germanica* L. (German cockroach, croton bug); in the case of the latter species, the dietary sterols exerted an effect on reproduction. Yeast was found to be incapable of supplying a sufficient quantity of the sterols. More recently, Noland¹⁷⁴ demonstrated that, in the German cockroach, a linear growth response to dietary cholesterol in the range 0 to 0.05 per cent of the diet may be observed. In a systematic study of the effect of structural alterations of the molecule, as exemplified in forty-one sterol derivatives, it was found that the hydroxyl group on carbon 3 was essential, either in free or in esterified form, while the double bond between carbons 5 and 6 was not required. Minor changes in the nucleus or side chain had relatively lesser effects on growth. Cholesteryl chloride exhibited an adverse effect on growth, which could be overcome by an excess of cholesterol. Cailleau¹⁷⁵ reported that cholesterol is a required dietary component for certain parasitic flagellate protozoa: *Trichomonas columbae*, which is found in birds affected with diphtheria, *T. foetus*, from the genital tract of cattle, and *Eutrichomastix colubrarum*, an intestinal parasite isolated by Cailleau from the blood of the gecko, or wall lizard (*Lacerta muralis*, of the *Geckonidae* family).

(2) Acetate as a Source of Cholesterol

Bloch and Rittenberg¹⁷⁶ were the first to demonstrate the conversion of deuterioacetate into deuteriocholesterol in mice and rats. The importance of acetate as a building stone for the cholesterol molecule is indicated by the fact that the tagged portions of the acetate were found not only in the side-chain but also in the steroid nucleus. On the basis of these data, it was postulated that acetic acid provides a fundamental unit in cholesterol synthesis in the body. In later studies by Bloch and Rittenberg,^{177, 178}

¹⁷³ R. Chauvin, *Compt. rend.*, 229, 902-903 (1949).

¹⁷⁴ J. L. Noland, *Arch. Biochem. Biophys.*, 48, 370-379 (1954).

¹⁷⁵ R. Cailleau, *Ann. inst. Pasteur*, 59, 137-172 (1937).

¹⁷⁶ K. Bloch and E. Rittenberg, *J. Biol. Chem.*, 143, 297-298 (1942); 145, 625-636 (1942).

¹⁷⁷ K. Bloch and D. Rittenberg, *J. Biol. Chem.*, 159, 45-58 (1945).

¹⁷⁸ D. Rittenberg and K. Bloch, *J. Biol. Chem.*, 160, 417-424 (1945).

in which acetate containing isotopic carbon was used, it was found that both carbons of acetic acid are used for cholesterol synthesis; at least one-half of the cholesterol was shown to be derived from acetate.

Not only has the synthesis of cholesterol been demonstrated in the intact animal, but it has been shown to occur in liver slices and in other tissues *in vitro*. Thus, Bloch, Borek, and Rittenberg¹⁷⁹ observed that slices of rat liver were able to synthesize cholesterol from isotopic acetate under aerobic but not under anaerobic conditions. Ponticorvo *et al.*¹⁸⁰ reported that, when deuterium-labeled sodium acetate was fed to growing rats, the fatty acids contained 20% and the cholesterol 45% of the carbon atoms of the acetate. In a later study to determine the rate of synthesis of fatty acids and of cholesterol from acetate in the adult rat, Pihl, Bloch, and Anker¹⁸¹ calculated the half-times of saturated and of unsaturated acids in the liver as one and two days, respectively, while that of cholesterol in this organ was estimated as six days. The corresponding half-times in the carcass were sixteen to seventeen days (saturated acids), nineteen to twenty days (unsaturated acids), and thirty-one to thirty-two days (cholesterol). Brady and Gurin¹⁸² confirmed the synthesis of cholesterol by the liver slices of rats from acetic, pyruvic, hexanoic, and octanoic acids.

Although liver homogenates were believed to be an unsatisfactory medium for cholesterol synthesis, Rabinowitz and Greenberg¹⁸³ reported that at least part of the synthesis of cholesterol from acetate can be effected by a broken cell preparation of fetal rat liver. More recently, Frantz and Bucher¹⁸⁴ proved the identity of cholesterol synthesized by homogenates of ordinary rat liver; these workers based their conclusions as to radiochemical identity and purity upon fractional crystallization, formation of the dibromide, acetylation and solubility reactions. Although C¹⁴-cholesterol obtained by perfusion of livers and of other organs of swine with carboxyl-labeled sodium acetate contains substances having a higher specific count than that of C¹⁴-cholesterol, the substance could be converted *via* 5,6-dibromo-3-cholesterol into C¹⁴-cholesterol with a constant specific count.¹⁸⁵ Schwenk and Baker¹⁸⁶ likewise demonstrated the presence in the hen of substances having a higher count than that of the C¹⁴-

¹⁷⁹ K. Bloch, E. Borek, and D. Rittenberg, *J. Biol. Chem.*, **162**, 441-449 (1946).

¹⁸⁰ L. Ponticorvo, D. Rittenberg, and K. Bloch, *J. Biol. Chem.*, **179**, 839-842 (1949).

¹⁸¹ A. Pihl, K. Bloch, and H. S. Anker, *J. Biol. Chem.*, **183**, 441-450 (1950).

¹⁸² R. O. Brady and S. Gurin, *J. Biol. Chem.*, **186**, 461-469 (1950).

¹⁸³ M. Rabinowitz and D. M. Greenberg, *Arch. Biochem. Biophys.*, **40**, 472-474 (1952).

¹⁸⁴ I. D. Frantz, Jr., and N. L. R. Bucher, *J. Biol. Chem.*, **206**, 471-481 (1954).

¹⁸⁵ E. Schwenk and N. T. Werthessen, *Arch. Biochem. Biophys.*, **40**, 334-341 (1952).

¹⁸⁶ E. Schwenk and C. F. Baker, *Arch. Biochem. Biophys.*, **45**, 341-348 (1953).

cholesterol, after subcutaneous or intraperitoneal injection of C¹⁴-labeled acetate, or after its injection into the crop gland of hens.

(3) *Primary Products Other Than Acetate
Which Are Sources of Cholesterol*

A number of substances which are convertible to acetate can serve as precursors of cholesterol, presumably after conversion to acetate. In addition to these relatively simple molecules, which presumably are converted to cholesterol *via* acetate, more complicated compounds, such as squalene, have likewise been shown to be cholesterol precursors. These latter compounds are generally considered to function as intermediates in cholesterol synthesis.

a. Ethanol. Bloch and Rittenberg¹⁸⁷ were the first to demonstrate the conversion of ethyl alcohol to cholesterol. In a later study, Curran and Rittenberg¹⁸⁸ confirmed these results; however, they concluded that ethanol is not an immediate precursor, but that it must first undergo biologic conversion into acetic acid or into an active acetyl radical before its utilization for cholesterol synthesis. The liver has been shown to be the organ most active in the metabolism of ethanol. Thus, Masoro and Abramowitch¹⁸⁹ reported that rat liver slices convert labeled ethanol to CO₂, to fatty acids, and to cholesterol. Although the kidney was shown to surpass the liver in its capacity to oxidize alcohol, this organ did not utilize significant amounts of alcohol in the synthesis of fatty acids and of cholesterol. Lung tissue also possesses the capacity to utilize small amounts of alcohol.

b. Acetone. Smedley-MacLean and Hoffert¹⁹⁰ observed that an aldehyde is an active metabolite in sterol synthesis. On the other hand, Borek and Rittenberg¹⁹¹ demonstrated that the ketone, acetone, is converted into cholesterol in the surviving rat liver. It was not certain whether the acetone molecule is transformed *in toto* into cholesterol, or whether only that portion of the molecule which is changed to acetate functions in this manner. There is some experimental evidence that the latter hypothesis is at least partially correct. A similar transformation of the isopropyl residue of leucine and of isoleucine may account for the demonstration by Bloch¹⁹²

¹⁸⁷ K. Bloch and D. Rittenberg, *J. Biol. Chem.*, **155**, 243-254 (1944).

¹⁸⁸ G. L. Curran and D. Rittenberg, *J. Biol. Chem.*, **190**, 17-20 (1951).

¹⁸⁹ E. J. Masoro and H. Abramowitch, *Federation Proc.*, **12**, 94 (1953).

¹⁹⁰ I. Smedley-MacLean and D. Hoffert, *Biochem. J.*, **20**, 343-357 (1926).

¹⁹¹ E. Borek and D. Rittenberg, *J. Biol. Chem.*, **179**, 843-845 (1949).

¹⁹² K. Bloch, *J. Biol. Chem.*, **155**, 255-263 (1944).

that these amino acids form cholesterol. Zabin and Bloch¹⁹³ were able to establish the fact that the isopropyl residue of isovaleric acid is more rapidly converted to acetone bodies by rat liver slices, and is less rapidly oxidized to CO₂, than is the two-carbon moiety. It was later reported¹⁹⁴ that the cholesterol isolated after the feeding of isovalerate-4,4'-C¹³, 1-C¹⁴ had the same isotope distribution as that synthesized from acetate. It was suggested that all the carbon atoms of the isopropyl group are utilized for cholesterol synthesis, as well as for that of the acetyl groups and fatty acids.

c. Acetoacetate. Since acetoacetate was shown to be converted to cholesterol by liver slices,¹⁹⁵ it was believed that acetoacetate might be an intermediate in cholesterol synthesis. The efficiency of pyruvate,¹⁹⁶ and the limited capacity of isovaleric acid,^{194,197} as carbon sources for cholesterol, have been attributed to the conversion of these acids to acetoacetate. Moreover, since the incorporation of C¹⁴-CaCO₃ into cholesterol is greatly increased in the presence of isovalerate, the possibility exists that acetoacetate or a similar four-carbon compound is formed *in vivo* from the isopropyl group of isovaleric acid by a C₃ + C₁ condensation; this C₄-compound is believed to be an intermediate in the transformation of isovalerate into cholesterol. Zabin and Bloch¹⁹⁸ interpreted their data obtained with butyric acid-1-C¹³, 3-C¹⁴ as supporting evidence that acetoacetate is an intermediate in the utilization of butyrate for sterol synthesis. These results were consistent with the earlier data of Little and Bloch,¹⁹⁹ which indicated that a C₄ compound is formed from C₂ units in the course of cholesterol synthesis. According to Curran,²⁰⁰ who investigated the synthesis of cholesterol from ethyl-3-C¹⁴-acetoacetate by rat liver slices, neither acetic acid nor acetone is an intermediate in this synthesis. However, the exact role of acetoacetate remains uncertain, in view of the findings of Blecher²⁰¹ that the C¹⁴ in cholesterol formed from 1-C¹⁴- or 4-C¹⁴-acetoacetate is indistinguishable from that produced by acetate. Fisher and Vars²⁰² likewise observed an increase of 20 to 25% in total cholesterol in the circulating blood six hours after the infusion of acetoacetate into the portal vein.

¹⁹³ I. Zabin and K. Bloch, *J. Biol. Chem.*, **185**, 117-129 (1950).

¹⁹⁴ I. Zabin and K. Bloch, *J. Biol. Chem.*, **192**, 267-273 (1951).

¹⁹⁵ R. O. Brady and S. Gurin, *J. Biol. Chem.*, **189**, 371-377 (1951).

¹⁹⁶ J. L. Rabinowitz and S. Gurin, *J. Biol. Chem.*, **208**, 307-313 (1954).

¹⁹⁷ I. Zabin and K. Bloch, *J. Biol. Chem.*, **185**, 131-138 (1950).

¹⁹⁸ I. Zabin and K. Bloch, *J. Biol. Chem.*, **192**, 261-266 (1951).

¹⁹⁹ H. N. Little and K. Bloch, *J. Biol. Chem.*, **183**, 33-46 (1950).

²⁰⁰ G. L. Curran, *J. Biol. Chem.*, **191**, 775-782 (1951).

²⁰¹ M. Blecher, *Federation Proc.*, **13**, 184 (1954).

²⁰² B. Fisher and H. M. Vars, *Federation Proc.*, **12**, 202-203 (1953).

d. Isovaleric Acid. This acid has proved to be an efficient carbon source for cholesterol, as determined by studies with $(\text{C}^{13}\text{H}_3)_2\text{CH}_2\cdot\text{C}^{14}\text{OOH}$ ¹⁹⁷ and C^{14} -labeled isovaleric acid $((\text{CH}_3)_2\cdot\text{C}^{14}\text{H}\cdot\text{CH}_2\cdot\text{COOH})$.²⁰³ When the doubly labeled isovalerate was tested by Zabin and Bloch,¹⁹⁷ it was found that the isopropyl carbons (C_4 and C_4') of the molecule gave rise to a higher isotope concentration than did the carboxyl carbon. This result is interpreted as evidence that isovalerate enters the synthetic process without alteration of the carbon chain. In a later report, Bloch *et al.*²⁰⁴ stated that the conversion efficiency of dimethyl acrylate $((\text{CH}_3)_2\text{CH}\cdot\text{CH}=\text{CH}\cdot\text{COOH})$ is similar to that of methyl isovalerate in cholesterol biogenesis. This suggests that β,β -dimethyl acrylate (senecioic acid) is a step in the metabolism of isovalerate. A similar hypothesis has been proposed by Bachhawat and co-workers.²⁰⁵ It is as yet uncertain whether or not this desaturation is an obligatory step in the oxidation of isovalerate. Until experiments have been completed with carboxyl-labeled dimethyl acrylate, no conclusions can be reached.

The theory that branched-chain acids are intermediates in the biosynthesis of cholesterol from acetate has gained support by the demonstration that hydroxymethylglutarate $(\text{HOOC}\cdot\text{CH}_2\cdot\text{COH}(\text{CH}_3)\cdot\text{CH}_2\cdot\text{COOH})$,^{196,206} and β,β -dimethylacrylate^{206,207} are synthesized from acetate by rat liver homogenates. The distribution of acetate carbon noted by Rudney²⁰⁶ in the two acids is identical with that predicated by Wüersch *et al.*²⁰⁸ for the isoprenoid precursor of cholesterol, which Bloch¹² considers may arise by way of squalene. Bloch and associates²⁰⁴ postulated the following possible method by which acetate is used in the synthesis of isoprenoid units:

One Possible Method for the Formation of Isoprenoid Units, from Acetate, Which Are Used in the Synthesis of Squalene and Cholesterol²⁰⁴

If the above theory is correct, then three molecules of acetate give rise to one isoprenoid unit by way of a six-carbon dicarboxylic acid. Six such

²⁰³ I. Zabin, and K. Bloch, *J. Biol. Chem.*, **192**, 267-273 (1951).

²⁰⁴ K. Bloch, L. C. Clark, and I. Harary, *J. Biol. Chem.*, **211**, 687-699 (1954).

²⁰⁵ B. K. Bachhawat, W. G. Robinson, and M. J. Coon, *J. Am. Chem. Soc.*, **76**, 3098-3099 (1954).

²⁰⁶ H. Rudney, *J. Am. Chem. Soc.*, **76**, 2595-2596 (1954).

²⁰⁷ J. L. Rabinowitz, *J. Am. Chem. Soc.*, **76**, 3037-3038 (1954).

²⁰⁸ J. Wüersch, R. L. Huang, and K. Bloch, *J. Biol. Chem.*, **195**, 439-446 (1952).

isoprenoid units are believed to condense to form squalene, which thereafter cyclizes to the steroidal ring system. The existence of this pathway is deduced from the following considerations: If the isoprene units were condensed as such, there would be only six C^{14} atoms in a cholesterol (carbons 4, 8, 10, 14, 20, and 25) formed from a tertiary-labeled isoprenoid unit. The specific activity of each of these carbons would be 4.5 times that of the whole molecule (27/6). On the other hand, if the branched-chain acids were utilized for cholesterol synthesis only after conversion to acetate,

Fig. 1. The postulated distribution of C^{14} (●) in squalene (or steroid) derived from isoprenoid precursors labeled at the tertiary carbon atom.²⁰⁴

then the labeling pattern would be similar to that noted when carboxyl-labeled acetate was the intermediate. Under these conditions, twelve carbons would be labeled, and each labeled carbon would have a specific activity 2.25 times that of the whole molecule (27/12).

The data of Bloch *et al.*²⁰⁴ support the first of these two possibilities. Thus, the specific activities of C_{25} obtained after feeding the three branched-chain acids (3- C^{14} - β -hydroxy- β -methylglutarate, 3- C^{14} - β -hydroxyisovalerate, 3- C^{14} - β -dimethylacrylate) were 3.85, 4.1, and 3.6 times greater than that of the whole molecule; this contrasts with a specific activity of 1.57 reported²⁰⁴ by Cornforth *et al.*²⁰⁹ for C_{25} when the cholesterol was formed from 1- C^{14} -acetate.¹⁹⁹ In the case of C_{10} , the specific activity of the carbon derived from methylacrylate was 444, as contrasted with a figure of 228 reported when the cholesterol was condensed from 1- C^{14} -acetate.^{204,209} These data strongly support the hypothesis that only six of the twenty-seven atoms in cholesterol are labeled when the 3- C^{14} -branched-chain acids

²⁰⁹ J. W. Cornforth, G. D. Hunter, and G. Popják, *Biochem. J.*, 54, 597-601 (1953).

are the source of the carbons of cholesterol; under these conditions, the squalene (or steroid) derived from these isoprenoid precursors would be labeled as indicated in Fig. 1.

The scheme for the utilization of the three branched-chain acids in the synthesis of squalene and cholesterol, as suggested by Bloch, Clark, and Harary,²⁰⁴ is given in the following series of equations.

Proposed Scheme for the Conversion of Branched-Chain Acids to Squalene and Cholesterol²⁰⁴

The proof of the scheme of conversion of the branched-chain acids to squalene and cholesterol rests upon the observed isotope distribution pattern. The fact that acetate may act as a source of the five-carbon (isoprenoid) units which can be converted to cholesterol has been supported by the data of Bonner and Arreguin,²¹⁰ which showed that acetate could

²¹⁰ J. Bonner and B. Arreguin, *Arch. Biochem.*, **21**, 109-124 (1949).

be used in the synthesis of natural rubber from guayule seedlings (*Parthenium argentatum*). The indication that the reactions are reversible at the 5- and 6-carbon levels is based upon the proven ability of these branched-chain acids to act as sources of carbon for the higher fatty acids, as well as upon the CO₂ fixation known to accompany the formation of acetoacetate from isovalerate²¹¹ and from β,β -dimethylacrylate.²⁰⁵

e. Miscellaneous Compounds. In an extended study of cholesterol synthesis *in vitro* in the liver, Brady and his associates²¹² noted that C¹⁴-vinylacetic acid (CH₂:CH·CH₂·COOH), crotonaldehyde (CH₃CH:·CH·CHO), acetoin (CH₃·CO·CO·CH₃), and orsellinic acid (CH₃·C₆·H₂·(OH)₂·COOH), are unimportant as precursors; the same was found to be true for formate (HCOOH) and for formaldehyde (HCHO). Only aldol (CH₃·CHOH·CH₂·CHO), which is probably partly converted to acetoacetate, appeared to have any importance as a source of cholesterol.

(4) Intermediates in Cholesterol Synthesis

a. Squalene. The most significant evidence of the course of cholesterol synthesis in the animal body was the discovery by Langdon and Bloch²¹³ that squalene is convertible to cholesterol in the mouse. Squalene is a highly unsaturated hydrocarbon with an empirical formula of $C_{30}H_{50}$, which was first isolated from shark liver oil of the genus *Zameus* by Tsujimoto²¹⁴ in 1906; it was later identified as squalene,²¹⁵ the formula of which is given below:

Squalene has been found to be widely distributed in the liver oils of the *Elasmobranchii*. Tsujimoto²¹⁶ reported the presence of this hydrocarbon in sixteen of thirty-six species of elasmobranch fishes in Japanese waters,

²¹¹ M. J. Coon, *J. Biol. Chem.*, **187**, 71-82 (1950).

²¹² R. O. Brady, J. Rabinowitz, J. van Baalen, and S. Gurin, *J. Biol. Chem.*, **193**, 137-143 (1951).

²¹³ R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 135-144 (1953).

²¹⁴ M. Tsujimoto, *J. Soc. Chem. Ind. Japan*, 9, 953 (1906); cited by M. Tsujimoto, *Ind. Eng. Chem.*, 8, 890 (1916).

²¹⁵ M. Tsujimoto, *Ind. Eng. Chem.*, 8, 889-896 (1916).

²¹⁶ M. Tsujimoto, *Ind. Eng. Chem.*, **12**, 63-73 (1920).

including the basking shark (*Cetorhinus maximus*).²¹⁷ Hilditch²¹⁸ also demonstrated the presence of squalene in the liver oils of the *Squalidae* and of other shark families. Strangely enough, squalene was discovered by Thorbjarnarson and Drummond²¹⁹ to be a component of olive oils obtained from a number of geographical sources. For a further, more complete, discussion of the distribution of squalene, the reader is referred to *The Lipids*, Vol. I, pages 400–402.

Langdon and Bloch²²⁰ found that squalene was readily absorbed from the intestine of the rat, and that 5 to 10% could be recovered from the tissues. When labeled acetate was fed together with natural squalene, the recovered squalene was radioactive.²²⁰ The rate of turnover was found to be exceedingly rapid; a maximal incorporation of acetate into squalene occurred within thirty minutes. Since only traces of squalene are normally present, it is suggested that this compound is an intermediate in a rapid metabolic process. Squalene was found to be a precursor of cholesterol, but not of fatty acids, in mice.²¹³ Although the experimental data of Langdon and Bloch^{213,220} with natural squalene would seem to be decisive, Tomkins *et al.*²²¹ failed to demonstrate the synthesis of cholesterol from deuterium-labeled synthetic squalene or from a synthetic C¹⁴-labeled squalene. Wüersch, Huang, and Bloch²⁰⁸ are of the opinion, on the basis of their extensive degradation of the cholesterol side-chain, that cholesterol is built up from isoprene units. According to Woodward and Bloch,²²² squalene, synthesized from isoprenoid groups, can be cyclized into cholesterol by a mechanism suggested earlier by Robinson,²²³ or by an alternative *trans*-formation.²²² Studies of the nature of labeled biosynthesized cholesterol, investigated by degradation reactions, by Cornforth *et al.*^{209,224} and by Dauben and co-workers,²²⁵ tend to support this concept of the cyclization of squalene into cholesterol. The distribution of the labeled acetate carbon in the quaternary methyl carbons of biosynthesized cholesterol is also believed to corroborate this theory of its synthesis.^{222,225}

²¹⁷ M. Tsujimoto, *Bull. Chem. Soc. Japan*, 10, 144–148, 149–153 (1935); *J. Chem. Soc. Japan*, 55, 702–741 (1934); *Chem. Abst.*, 28, 6184 (1934).

²¹⁸ T. P. Hilditch, *The Chemical Constitution of the Natural Fats*, Wiley, New York, 1947.

²¹⁹ T. Thorbjarnarson and J. C. Drummond, *Analyst*, 60, 23–29 (1935).

²²⁰ R. G. Langdon and K. Bloch, *J. Biol. Chem.*, 200, 129–134 (1953).

²²¹ G. M. Tomkins, I. L. Chaikoff, W. G. Dauben, H. L. Bradlow, and P. A. Sreer, *J. Am. Chem. Soc.*, 74, 6145–6146 (1952).

²²² R. B. Woodward and K. Bloch, *J. Am. Chem. Soc.*, 75, 2023–2024 (1953).

²²³ R. Robinson, *Chemistry & Industry*, 53, 1062–1063 (1934).

²²⁴ J. W. Cornforth, G. D. Hunter, and G. Popják, *Biochem. J.*, 53, xxiv–xxv (1953).

²²⁵ W. G. Dauben, S. Abraham, S. Hotta, I. L. Chaikoff, H. L. Bradlow, and A. H. Soloway, *J. Am. Chem. Soc.*, 75, 3038 (1953).

b. Cyclopentenophenanthrene Compounds. When cholesterol is synthesized in surviving livers from labeled acetate, impurities are always present which have a higher specific activity than the newly-synthesized cholesterol. These compounds, precipitable by digitonin, have been observed within one hour after the injection of carboxyl-labeled C¹⁴-acetate into rats.²²⁶ One of the impurities was shown by Schwenk *et al.*²²⁷ to be 3,5-dihydroxy-6-keto cholestane; this compound has likewise been detected as an impurity in commercial cholesterol.

The possibility that $\Delta^{4,5}$ -cholestenone may be an intermediate in cholesterol synthesis was investigated by Anker and Bloch.²²⁸ Earlier tests of Anchel and Schoenheimer⁸² with deuteriocholesterol were indecisive. Cholestenone is known to be a normal component of the feces of rats and dogs;²²⁹ its conversion to coprostanol (coprosterol) was demonstrated in dogs by Schoenheimer *et al.*⁷⁸ Obviously, cholestenone is closely related metabolically to cholesterol. However, Anker and Bloch²²⁸ concluded that, although cholestenone can be converted to cholesterol, it is not an intermediate in the total synthesis of cholesterol. It is presumably an intermediate in the cholesterol $\rightarrow$ dihydrocholesterol reaction.

(5) *The Source of the Carbons in Biosynthesized Cholesterol*

By the use of doubly-labeled acetic acid, it has been possible to trace the ultimate disposition of the methyl and carboxyl carbon of the acetate in the synthesis of cholesterol. These atoms were found to be fairly uniformly distributed in the isooctyl side-chain and in the steroid ring. Little and Bloch¹⁹⁹ showed that the methyl group was the source of carbons 18, 19, 26, 27, and presumably 17, while carbons 25 and probably 10 were derived from the carboxyl carbons. Wüersch, Huang, and Bloch²⁰⁸ adduced proof that, in the side-chain, carbons 21, 22, 24, 26, and 27 were derived from the methyl group, while carbons 20, 23, and 25 arose from the carboxyl carbon. Cornforth and associates^{209, 224, 230} concluded in 1953 that carbon atoms 2, 4, 6, and 10 in the ring originate from the carboxyl carbon of acetate, while carbons 1, 3, and 5, in the ring and 18 in the side-chain originate

²²⁶ E. Schwenk and N. T. Werthessen, *Arch. Biochem. Biophys.*, **42**, 91-93 (1953).

²²⁷ E. Schwenk, N. T. Werthessen, and H. Rosenkrantz, *Arch. Biochem. Biophys.*, **37**, 247-251 (1952).

²²⁸ H. S. Anker and K. Bloch, *J. Biol. Chem.*, **178**, 971-976 (1949).

²²⁹ O. Rosenheim and T. A. Webster, *Biochem. J.*, **37**, 513-514 (1943).

²³⁰ J. W. Cornforth, G. D. Hunter, and G. Popják, *Arch. Biochem. Biophys.*, **42**, 481-482 (1953).

from the methyl group. The identification of the source of the carbon atoms in cholesterol is indicated in the equation below:

The Conversion of Doubly Labeled Acetic Acid into Cholesterol²³¹

(6) *Factors Altering the Biosynthesis of Cholesterol*

a. Age. The synthesis of cholesterol apparently proceeds most rapidly in the young animal; a large amount of the sterol is necessary for the rapidly-developing tissues. Rosenman and Shibota,²³² using the concentration of biliary cholesterol as an index of hepatic synthesis, were able to demonstrate a considerably greater rate of synthesis in the young rat as compared with that in the adult animal.

b. Nutritional State. According to Tomkins and Chaikoff,²³³ the nutritional state has a profound effect upon the extent of cholesterol synthesis. Thus, when rats were fasted for twenty-four hours or longer, there was a marked decrease in the capacity of the liver to synthesize cholesterol from acetate. Likewise, caloric restriction for several days reduced cholesterol synthesis. It was found that the ability to synthesize cholesterol could be restored when glucose, a protein hydrolysate, or fat, was fed to the rat.

c. Cholesterol Intake. The administration of exogenous cholesterol has been shown to reduce markedly the proportion of cholesterol which is synthesized. Thus, Gould and Taylor²³⁴ observed that the rate of synthesis of cholesterol in the livers of dogs and rabbits previously on a high-cholesterol diet was only a few per cent of that which occurred in animals on a cholesterol-free diet. Moreover, the rate of conversion of labeled acetate into cholesterol was also found to be markedly reduced in the animals previously maintained on the high-cholesterol regimen. This would indicate that the rate of cholesterol synthesis is related to the amount of

²³¹ A. White, P. Handler, E. L. Smith, and De W. Stetten, Jr., *Principles of Biochemistry*, McGraw-Hill, New York, 1954, p. 494.

²³² R. H. Rosenman and E. Shibota, *Proc. Soc. Exptl. Biol. Med.*, **81**, 296-298 (1952).

²³³ G. M. Tomkins and I. L. Chaikoff, *J. Biol. Chem.*, **196**, 569-573 (1952).

²³⁴ R. G. Gould and C. B. Taylor, *Federation Proc.*, **9**, 179 (1950).

the sterol available from exogenous sources, being high when no cholesterol is present in the food, and decreased as increasing amounts of the sterol are supplied in the diet. This mechanism operates to maintain the body cholesterol at a relatively constant value.

The above observations on the control of cholesterol synthesis have been confirmed by more recent work. Tomkins and Chaikoff²³⁵ reported that the rate of cholesterol synthesis from acetate by rat liver slices is a function of the cholesterol intake of the animals from which the liver was obtained. Thus, when a diet containing 5% of cholesterol was fed to rats for eight days prior to death, the rate of cholesterol synthesis in the liver was practically 0; likewise, when the animals were fed diets containing only 0.5% of cholesterol for seven days, a marked depression in cholesterol production was noted. Even a single feeding of this alcohol was sufficient to produce an inhibitory effect. These authors postulate that the rate of cholesterol synthesis in the liver is subject to homeostatic regulation by dietary cholesterol.²³⁵ Frantz *et al.*²³⁶ presented similar data on the rat, and indicated that the inhibition of cholesterol synthesis is a function of the level of liver cholesterol; apparently it is not mediated through the thyroid gland. Langdon and Bloch²³⁷ also confirmed the inhibitory effect of dietary cholesterol on the rate of cholesterol synthesis. Alfin-Slater and associates²³⁸ observed that cholesterol synthesis was still suppressed in the livers of rats eight days after the cessation of cholesterol feeding when the subsequent diet had been a fat-free one; however, when high-fat diets were fed after the termination of cholesterol feeding, the rate of cholesterol synthesis returned to normal within eight days. Although the experimental evidence that cholesterol feeding reduces the synthesis of cholesterol in the liver is quite convincing, Gould *et al.*²³⁹ found that a similar inhibition in the rate of synthesis of cholesterol did not occur in the skin or intestinal mucosa as the result of cholesterol administration.

d. The Administration of Cholesterol-Like Compounds. Two different effects of cholesterol-like compounds have been observed, namely, that which occurs when readily absorbed sterols and related substances are fed, and that which is observed when the soy sterols and other related difficultly absorbed products are given.

²³⁵ G. M. Tomkins, H. Sheppard, and I. L. Chaikoff, *J. Biol. Chem.*, **201**, 137-141 (1953).

²³⁶ I. D. Frantz, Jr., H. S. Schneider, and B. T. Hinkelman, *J. Biol. Chem.*, **206**, 465-469 (1954).

²³⁷ R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **202**, 77-81 (1953).

²³⁸ R. B. Alfin-Slater, M. C. Schotz, F. Shimoda, and H. J. Deuel, Jr., *J. Biol. Chem.*, **195**, 311-315 (1952).

²³⁹ R. G. Gould, C. B. Taylor, J. S. Hagerman, I. Warner, and D. J. Campbell, *J. Biol. Chem.*, **201**, 519-528 (1953).

As regards the first category, Langdon and Bloch²³⁷ reported that cholesterol synthesis from acetate was inhibited in the liver of the rat, not only by cholesterol, but also as a result of the feeding of squalene, Δ^7 -cholestenol, and 7-dehydrocholesterol; the reduction in rate was accompanied in each case by a significant increase in liver cholesterol. Tomkins, Sheppard, and Chaikoff²⁴⁰ likewise noted that the administration to rats of cholestenone, dehydroisoandrosterone, 7-dehydrocholesterol and Δ^7 -cholestenol, in a single dose or in daily doses over five to seven days, markedly reduced the rate of the acetate $\rightarrow$ cholesterol reaction in the liver, without affecting the rate of $C^{14}O_2$ production. The effect of phytosterols and of related compounds, which block cholesterol absorption, is discussed in f.

e. The Effect of Fat Feeding. It has been postulated not only that animal fats, which contain cholesterol *per se*, are a source of this sterol in the animal, but also that vegetable fats provide cholesterol because the acetate molecules are available as a result of the oxidation of fatty acids. In fact, Lifschütz²⁴¹ reported that an oxidized product of oleic acid gave some of the reactions of the sterols. Minovici²⁴² noted an increase in the cholesterol content of the blood of dogs when oleic acid was given. Eckstein and Treadwell²⁴³ also observed that greater amounts of sterols were present in the livers of growing white rats when the percentages of corn or of soybean oil in their diets were increased. The latter workers are of the opinion that this result cannot be ascribed to a sterol mobilization. On the other hand, several groups of investigators^{164, 238, 244} failed to confirm a relationship between fat intake and the rate of cholesterol synthesis. Thus, Schoenheimer and Breusch¹⁶⁴ were unable to demonstrate any increased cholesterol synthesis in mice which were fed considerable amounts of lard and of other fats. Similar results were observed by Alfin-Slater *et al.*²³⁸ on rats. The latter workers found no change in the amount of cholesterol in the liver and plasma when a low-fat diet was replaced by a high-fat regimen. In the case of rats which had a high liver cholesterol level as a result of prefeeding with this sterol, it was found that the high-fat diet was more efficient in removing from the liver the cholesterol which had accumulated than was a low-fat regimen. However, Hildreth *et al.*²⁴⁵

²⁴⁰ G. M. Tomkins, H. Sheppard, and I. L. Chaikoff, *J. Biol. Chem.*, **203**, 781-786 (1953).

²⁴¹ I. Lifschütz, *Z. physiol. Chem.*, **55**, 1-7 (1908).

²⁴² S. Minovici, *Bull. soc. chim. biol.*, **17**, 369-395 (1935).

²⁴³ H. C. Eckstein and C. R. Treadwell, *J. Biol. Chem.*, **112**, 373-378 (1935-1936).

²⁴⁴ J. M. R. Beveridge and S. E. Johnson, *Brit. J. Exptl. Pathol.*, **31**, 294-305 (1950).

²⁴⁵ E. A. Hildreth, S. M. Mellinkoff, G. W. Blair, and D. M. Hildreth, *Circulation*, **3**, 641-646 (1951).

considered that the total fat content of the diet was more important in determining the serum cholesterol level than was the amount of cholesterol itself ingested.

f. The Effect of Soy Sterols. Although the effect of soy sterols in preventing the accumulation of cholesterol in the liver and tissues is largely a result of the blocking of cholesterol absorption in the intestine, it is of interest to determine whether or not a phenomenon of this nature alters the rate of cholesterol synthesis in the tissues. This question has been answered in the negative by Alfin-Slater and co-workers,²⁴⁶ who reported that the rate of synthesis of endogenous cholesterol was not affected when the doses of soy sterols were sufficient to prevent any appreciable amount of cholesterol from being absorbed.

g. The Effect of Cations and of Hydrogen-Ion Concentration. Curran²⁴⁷ reported that the optimum rate of cholesterol synthesis in surviving rat liver *in vitro* occurs between a pH of 6.2 and one of 6.8, although activity was noted in the range of 6.0 to 7.4. The effect of pH on the rate of cholesterol synthesis persists in the absence of potassium and magnesium ions, and is independent of the loss of intracellular potassium.²⁴⁸ This finding is somewhat unexpected, since sufficient K^+ and Mg^{++} must be present in the incubating medium if marked variations in the rate of cholesterol synthesis are to be avoided. The effectiveness of the acetate $\rightarrow$ cholesterol reaction at a pH of 6.5 is augmented by the presence of Mn^{++} ; this effect occurs even if Mg^{++} is present at an optimum level.²⁴⁸ Bucher *et al.*²⁴⁹ reported that the omission of $MgCl_2$ and DPN from the incubation mixture of cell-free homogenates of adult rat liver reduced the synthetic capacity for cholesterol. For a more complete discussion of the effect of cholesterol analogues on the absorption process, the reader is referred to *The Lipids*, Vol. II, pages 270–271, and page 364 of the present volume.

h. The Effect of Hormones. The synthesis of cholesterol is stimulated in hyperthyroid states, and is decreased when a hypothyroid condition obtains.^{250,251} Marx and co-workers²⁵² confirmed these findings and re-

²⁴⁶ R. B. Alfin-Slater, A. F. Wells, L. Aftergood, and H. J. Deuel, Jr., Unpublished observations, 1955.

²⁴⁷ G. L. Curran, *J. Biol. Chem.*, **200**, 17–21 (1953).

²⁴⁸ G. L. Curran and O. L. Clute, *J. Biol. Chem.*, **204**, 215–219 (1953).

²⁴⁹ N. L. R. Bucher, N. H. McGovern, R. Kingston, and M. H. Kennedy, *Federation Proc.*, **12**, 184 (1953).

²⁵⁰ R. H. Rosenman, S. O. Byers, and M. Friedman, *J. Clin. Endocrinol. and Metabolism*, **12**, 1287–1299 (1952).

²⁵¹ S. O. Byers, R. H. Rosenman, M. Friedman, and M. W. Biggs, *J. Exptl. Med.*, **96**, 513–516 (1952).

²⁵² W. Marx, S. T. Gustin, and C. Levi, *Proc. Soc. Exptl. Biol. Med.*, **83**, 143–146 (1953).

ported an increased incorporation of deuterium into the cholesterol molecule in thyroxine-injected rats as compared with normal controls. This increase was noted in the liver, intestine, kidneys, and spleen, but not in the lungs. Spirtes, Medes, and Weinhouse²⁵³ likewise recorded augmented cholesterol synthesis under these conditions, and stated that it was not directly correlated with the glycogen content of the liver; these variations were observed in spite of the fact that the oxidation of acetate was increased 30 to 60% in the hyperthyroid condition. Both groups of workers observed that the reverse situation obtained in regard to cholesterol synthesis in thyroidectomized animals.^{252,253} Stresses such as that produced by lowering the environmental temperature by 20 to 28°C. did not alter the rate of cholesterol synthesis in the athyroid rats.²⁵²

Although the pancreas is essential in the synthesis of fatty acids from acetate, this organ is not required for the acetate $\rightarrow$ cholesterol reaction. Thus, although liver slices from alloxanized rats and from pancreatectomized cats are relatively ineffective in provoking the acetate $\rightarrow$ fatty acid reaction, in contrast to liver slices from normal rats and cats, Brady and Gurin²⁵⁴ reported that the acetate $\rightarrow$ cholesterol reaction is unimpaired. However, this latter reaction may be inhibited in prolonged and severe diabetes. On the other hand, Hotta and Chaikoff²⁵⁵ have suggested that cholesterol synthesis from acetate is actually increased in the liver of diabetic rats. In a later study from their laboratory, Hotta and co-workers²⁵⁶ made the interesting observation that the rate of cholesterol synthesis from acetate in the liver of the alloxanized diabetic rat was double that of normal rats when both groups were fed a diet containing 60% glucose. However, the rate of cholesterogenesis of these rats was reduced to normal if fructose replaced glucose in the diet. It is assumed that the restoration of glycolytic activity in the diabetic liver induced by fructose was responsible for diverting the C₂ fragment from the path of cholesterol synthesis to other metabolic pathways, such as lipogenesis. Altman and collaborators²⁵⁷ observed that cortisone and insulin, when acting concomitantly, exerted a marked effect upon fatty acid synthesis, without affecting that of cholesterol. All experimental evidence indicates the independence of the lipogenic and the cholesterogenic reactions.

²⁵³ M. A. Spirtes, G. Medes, and S. Weinhouse, *XIXth Intern. Physiol. Congress, Abst.*, Montreal, Aug. 31–Sept. 3, 1953, pp. 789–790.

²⁵⁴ R. O. Brady and S. Gurin, *J. Biol. Chem.*, **187**, 589–596 (1950).

²⁵⁵ S. Hotta and I. L. Chaikoff, *J. Biol. Chem.*, **198**, 895–899 (1952).

²⁵⁶ S. Hotta, R. Hill, and I. L. Chaikoff, *J. Biol. Chem.*, **206**, 835–844 (1954).

²⁵⁷ K. I. Altman, L. L. Miller, and C. G. Bly, *Arch. Biochem. Biophys.*, **31**, 329–331 (1951).

According to Tomkins, Chaikoff, and Bennett,²⁵⁸ the rate of cholesterol synthesis from acetate in the liver is markedly reduced in hypophysectomized rats. It is suggested that the pituitary gland controls the rate of steroid production in non-endocrine as well as in endocrine target organs.

i. The Effect of Vitamin Deficiencies. (a) *Pantothenic Acid Deficiency.* Deficiencies, particularly of the B vitamins, have been found to influence cholesterol synthesis. Because pantothenic acid is a structural unit of CoA, it is only natural to expect that a deficiency of this vitamin would alter cholesterol metabolism, presumably by limiting the extent of cholesterogenesis. In support of this hypothesis, Guehring, Hurley, and Morgan²⁵⁹ noted that the increase in liver lipids and in liver and serum cholesterol was much less pronounced in pantothenic acid-deficient rats receiving 1% cholesterol than in normal animals. These effects were not related to curtailment of food intake. Winters *et al.*²⁶⁰ likewise observed the failure of rats deficient in pantothenic acid to develop fatty livers. Although the serum cholesterol was found to be normal in these animals, a marked reduction in total cholesterol was noted in the "resting state" after the deficient diet had been fed for five or six weeks. Although the level of ascorbic acid in the adrenal gland was not reduced in pantothenic acid deficiency,²⁵⁹ the cholesterol content of these endocrine glands was shown to be decreased.²⁶⁰ The latter effect is believed to be the result of a depressed synthesis of this sterol in the adrenal glands, rather than to augmented utilization. This decreased synthetic capacity is obviously the result of the reduced content of CoA available. Dumm and co-workers²⁶¹ likewise reported that adrenal cholesterol is significantly depressed in pantothenic acid-deficient rats, as compared with control rats on a normal diet. Moreover, it was demonstrated that, following unilateral adrenalectomy, the cholesterol content of the remaining adrenal gland returned to normal in a single day if the rat had been on a complete diet, but that the content was still low in this gland seven days after the operation when the rat had received a diet free from pantothenic acid. Klein and Lipmann²⁶² observed a parallelism between the level of CoA and the synthesis of cholesterol in the livers of pantothenic acid-deficient rats. When the anti-

²⁵⁸ G. M. Tomkins, I. L. Chaikoff, and L. L. Bennett, *J. Biol. Chem.*, **199**, 543-545 (1952).

²⁵⁹ R. R. Guehring, L. S. Hurley, and A. F. Morgan, *J. Biol. Chem.*, **197**, 485-493 (1952).

²⁶⁰ R. W. Winters, R. B. Schultz, and W. A. Krehl, *Proc. Soc. Exptl. Biol. Med.*, **79**, 695-696 (1952).

²⁶¹ M. E. Dumm, H. Gershberg, E. M. Beck, and E. P. Ralli, *Proc. Soc. Exptl. Biol. Med.*, **82**, 659-662 (1953).

²⁶² H. P. Klein and F. Lipmann, *J. Biol. Chem.*, **203**, 101-108 (1953).

metabolite, pantoyletauryl-*p*-anisidide, was added to normal liver slices, these workers²⁶² noted a reduction in steroid and lipid synthesis, presumably due to the relatively low content of CoA. A partial reversal could be brought about by pantethine, but not by pantothenic acid itself. Klein and Lipmann²⁶³ also pointed out that a parallelism exists in living yeast between the CoA level and the capacity to synthesize ergosterol and total lipid. Boyd²⁶⁴ subscribes to the hypothesis that the level of tissue cholesterol depends upon the CoA content, and that the latter is obviously required in the synthesis of the sterol.

In a more complete study of the mechanism of synthesis of cholesterol, Rabinowitz and Gurin¹⁹⁶ found that 1-C¹⁴-acetyl CoA was more efficient in cholesterol synthesis than was 1-C¹⁴-acetate. 2-C¹⁴-Pyruvate was also a better source of cholesterol than was free acetate, presumably because the former was more readily incorporated into the active acetyl CoA compound. It was reported that, in addition to CoA, DPN, ATP, and AMP were likewise required.¹⁹⁶

(b) *Biotin Deficiency.* Curran²⁶⁵ reported that, although the rate of fatty acid synthesis was somewhat increased in biotin-deficient rats, that of cholesterol remained normal. In fact, the increased rate of fatty acid synthesis was related to the inanition rather than to the biotin deficiency itself. On the other hand, Okey and her co-workers²⁶⁶ observed that biotin-deficient rats failed to store excess cholesterol ester in the liver when fed egg diets high in cholesterol. When an avidin-rich diet containing cholesterol was given, a marked decrease in liver cholesterol ester occurred, in spite of the high cholesterol intake. The role of biotin in cholesterol synthesis thus seems questionable.

(c) *Deficiencies in Miscellaneous Vitamins.* The role of the other B vitamins in cholesterolgenesis is not clear. Riboflavin is apparently without activity,²⁵⁹ while pyridoxine would seem to have only a limited effect. Guggenheim and Olson²⁶⁷ found that total cholesterol synthesis in rats was not appreciably reduced in nutritional disorders such as deficiencies in thiamine, pantothenic acid, biotin, and choline; the decreased production did not vary much from that of pair-fed controls. However, the specific activity of the adrenal cholesterol was found to be decreased in all types of deficiency, as well as in the pair-fed controls. Lecoq *et al.*²⁶⁸ suggested

²⁶³ H. P. Klein and F. Lipmann, *J. Biol. Chem.*, **203**, 95-99 (1953).

²⁶⁴ G. S. Boyd, *Biochem. J.*, **55**, 892-895 (1953).

²⁶⁵ G. L. Curran, *Proc. Soc. Exptl. Biol. Med.*, **75**, 496-498 (1950).

²⁶⁶ R. Okey, R. Pencharz, S. Lepkovsky, and E. R. Vernon, *J. Nutrition*, **44**, 83-99 (1951).

²⁶⁷ K. Guggenheim and R. E. Olson, *J. Nutrition*, **48**, 345-358 (1952).

²⁶⁸ R. Lecoq, P. Chauchard, and H. Mazoue, *Bull. soc. chim. biol.*, **30**, 298-305 (1948).

that, whereas nicotinamide, pantothenic acid, pyridoxine, meso-inositol, and methionine are concerned with the metabolism of both cholesterol and fats, vitamin A and choline are concerned only with that of cholesterol.

j. The Effect of Tissue Injury. Werthessen and Schwenk²⁶⁹ demonstrated that the efficiency of biosynthesis of C¹⁴-cholesterol in surviving livers perfused with C¹⁴-acetate varied inversely with the damage to the organ. When minimum damage had occurred, a minimum amount of cholesterol was formed. On the other hand, scission of the organ, multiple puncturing of its surface, the administration of bacterial toxins or of bacteria to the perfusion system, were found to increase the C¹⁴ concentrated in the newly synthesized cholesterol.

(7) *The Site of Cholesterol Synthesis*

The liver has been generally accepted as the chief site of cholesterol synthesis, but recent data have indicated that the capacity to effect this reaction is a generalized one. As indicated earlier, it is possible for liver slices to induce the synthesis of cholesterol from acetate, and broken cell preparations¹⁸³ and homogenates of the liver are also able to effect this synthesis.

Srere and co-workers²⁷⁰ demonstrated that cholesterol synthesis might be an extrahepatic function, since it proceeded in hepatectomized rats. Later they investigated the possible sites of this reaction. It was evident that the acetate → cholesterol reaction could be effected *in vitro* by surviving slices of beef adrenal cortex. In subsequent studies on hepatectomized rats, Srere and his collaborators²⁷¹ observed that, in addition to the liver and adrenals, the following tissues were able to synthesize cholesterol: kidney, testis, small intestine, and skin. The brain and skin of newborn rats also synthesized cholesterol, although this capacity was lost in brain slices of adult rats. The skin and liver were considered to be the tissues in which the most active synthesis of cholesterol takes place. Human adrenal slices were likewise shown to be active in catalyzing the acetate → cholesterol reaction.^{212,272} Gould and Taylor²³⁴ and Srere *et al.*²⁷¹ likewise demonstrated that the cholesterologenic reaction can proceed in skin at a rate comparable to that in liver. Although they also recognized the ability of adrenal cortex to function in a similar manner, they call attention to the

²⁶⁹ N. T. Werthessen and E. Schwenk, *Am. J. Physiol.*, **171**, 55-61 (1952).

²⁷⁰ P. A. Srere, I. L. Chaikoff, and W. G. Dauben, *J. Biol. Chem.*, **176**, 829-833 (1948).

²⁷¹ P. A. Srere, I. L. Chaikoff, S. S. Treitman, and L. S. Burstein, *J. Biol. Chem.*, **182**, 629-634 (1950).

²⁷² E. S. West and W. R. Todd, *Textbook of Biochemistry*, 2nd ed., Macmillan, New York, 1955, p. 928.

fact that the total quantity synthesized by the latter endocrine gland is insignificant compared with that manufactured in the liver. Gould *et al.*²³⁹ reported that the rate of synthesis of endogenous cholesterol in the skin and intestinal mucosa was not influenced by exogenous cholesterol; this is in sharp contrast to the effect of cholesterol feeding on the liver.

Popják and Beeckmans⁷⁰ proved that cholesterol synthesis occurred in the rabbit, not only in the liver but also in the intestine and ovaries. The rate of synthesis of cholesterol was found to be higher in the rabbit fetus than in maternal tissues;²⁷³ the fetal livers were especially active in cholesterol production, while the extrahepatic tissues of the fetus were more active in fatty acid synthesis. In a later study, Popják and Beeckmans²⁷⁴ demonstrated that all fetal cholesterol is obtained by synthesis within the body of the fetus. It was found that fetal extrahepatic tissues had the capacity to synthesize cholesterol, but that they were unable to degrade it. Only the fetal liver was able both to synthesize and to destroy cholesterol. The amount of cholesterol destruction amounted to only 50% of that synthesized, on the twenty-eighth day of pregnancy.

The ovarian tissues of the laying hen are likewise important sites for cholesterol synthesis. Thus, Popják and Tietz²⁷⁵ found that the membranes surrounding the growing ovum, which consist of the theca interna and theca granulosa, synthesize cholesterol as well as fatty acids *in vitro* from acetate. On the other hand, the yolk and the germinal disk possess no such capacity for synthesis. Although the theca interna and theca granulosa were inferior to the liver slices of young rats in effecting a synthesis of fatty acids from acetate, they were found to be as active as hepatic tissue in the synthesis of cholesterol. Kritchevsky and associates^{276, 277} reported that carboxyl-labeled acetate, when fed to laying hens, rapidly appeared in the cholesterol fraction and other fractions of the egg; however, the highest specific activity of the isotopic carbon was found in the cholesterol. Rice and her collaborators²⁷⁸ described the preparation of deuterium-labeled cholesterol from egg yolks by feeding deuterium oxide and/or deuterium-labeled sodium acetate to laying hens. The synthesis of cholesterol from carboxyl-labeled sodium acetate in the fertile hen's egg

²⁷³ G. Popják and M. L. Beeckmans, *Biochem. J.*, **44**, xxxvi-xxxvii (1949).

²⁷⁴ G. Popják and M. L. Beeckmans, *Biochem. J.*, **46**, 547-558 (1950).

²⁷⁵ G. Popják and A. Tietz, *Biochem. J.*, **54**, xxxv (1953).

²⁷⁶ D. Kritchevsky, C. R. Grau, B. M. Tolbert, and B. J. Krueckel, *Proc. Soc. Exptl. Biol. Med.*, **76**, 741-743 (1951).

²⁷⁷ D. Kritchevsky and M. R. Kirk, *Proc. Soc. Exptl. Biol. Med.*, **78**, 200-202 (1951).

²⁷⁸ L. I. Rice, R. B. Alfin-Slater, and H. J. Deuel, Jr., *Proc. Soc. Exptl. Biol. Med.*, **80**, 562-563 (1952).

during the latter half of incubation was demonstrated by Stokes *et al.*²⁷⁹ The largest porportion of conversion occurred in the embryo proper. Further confirmation of the remarkable degree of synthesis of cholesterol from acetate in the egg yolk was adduced by O'Donnell and Heard²⁸⁰ and by Schwenk.²⁸¹ Figure 2 indicates the relative activity of the cholesterol and of the fatty acids of neutral glycerides of egg yolk in the eggs obtained

Fig. 2. The incorporation of C^{14} into the egg yolk cholesterol and fatty acids of the natural fat of a hen given acetate- $1-C^{14}$ on the first day of the test.²⁸²

from a hen over a nine-day period following the intraperitoneal injection of acetate- $1-C^{14}$ in three doses over a period of twenty-four hours, with a total of 1.43×10^9 counts.²⁸²

²⁷⁹ W. M. Stokes, W. A. Fish, and F. C. Hickey, *J. Biol. Chem.*, **200**, 683-689 (1953).

²⁸⁰ V. J. O'Donnell and R. D. H. Heard, *Chemistry in Can.*, **4**, No. 5, 58 (1952); cited by R. D. H. Heard *et al.*, *Recent Progr. Hormone Research*, **9**, 383-40 (1954), p. 394.

²⁸¹ E. Schwenk, Personal communication cited by R. D. H. Heard *et al.*, *Recent Progr. Hormone Research*, **9**, 383-410 (1954), p. 394.

²⁸² R. D. H. Heard, R. Jacobs, V. O'Donnell, F. G. Peron, J. C. Saffran, S. S. Solomon, L. M. Thompson, H. Willoughby, and C. H. Yates, *Recent Progr. Hormone Research*, **9**, 383-410 (1954).

The mammary gland is also capable of effecting cholesterol synthesis from acetate, even when it is in the non-lactating state.²⁶⁴ The evidence of Popják and Beeckmans^{70,283} that cholesterol is not synthesized in the blood stream, but that plasma cholesterol is derived from the liver, seems quite convincing. It is well known that the liver is likewise the site of synthesis of plasma phospholipids (see *The Lipids*, Vol. II, pages 393-394).

The source of the cholesterol in the lipid accumulations which occur in atheromatosis is of great importance clinically. It was postulated by early workers^{284,285} that the plasma lipids were deposited in the wall of the aorta and of the arterial system in general. Experimental support for this hypothesis was adduced by Biggs and Kritchevsky,²⁸⁶ who demonstrated by *in vivo* tests that the atheromatous rabbit obtains the bulk of its cholesterol from the plasma, *i.e.*, that the cholesterol is exogenous. However, Siperstein *et al.*²⁸⁷ reported that the rat aorta can synthesize cholesterol from labeled acetate *in vitro*. Werthessen and co-workers²⁸⁸ likewise observed an *in vitro* synthesis of cholesterol in perfused bovine aorta. Shore, Zilversmit, and Ackerman^{289,290} reported that the major portion of plaque phospholipids in experimental rabbit atheromatosis was synthesized by the aorta rather than derived from the plasma. This is of great interest, inasmuch as it indicates that the development of cholesterol plaques in the aorta may represent a local synthesis of cholesterol from acetate rather than the deposition of preformed cholesterol.²⁹⁰ In a continuation of this study, Zilversmit and co-workers²⁹⁰ confirmed their original finding, and also demonstrated that plasma phospholipids accounted for only one-tenth or less of the total phospholipids present in the atheromatous thoracic aorta of the rabbit.

It is thus evident that cholesterol can be synthesized from acetate in a wide variety of tissues in addition to the liver. However, although Harper, Neal, and Hlavacek²⁹¹ confirmed the fact that cholesterologenesis may take place in a large number of tissues, they found that only the liver has

²⁸³ G. Popják and M. L. Beeckmans, *Biochem. J.*, **44**, xxxvii (1949).

²⁸⁴ G. L. Duff, *Arch. Pathol.*, **20**, 81-123, 259-304 (1935).

²⁸⁵ E. V. Cowdry, *Arteriosclerosis. A Survey of the Problem*, MacMillan, New York, 1933, pp. 254 ff.

²⁸⁶ M. W. Biggs and D. Kritchevsky, *Circulation*, **4**, 34-43 (1951).

²⁸⁷ M. D. Siperstein, I. L. Chaikoff, and S. S. Chernick, *Science*, **113**, 747-749 (1951).

²⁸⁸ N. T. Werthessen, L. J. Milch, R. F. Redmond, L. L. Smith, and E. C. Smith, *Am. J. Physiol.*, **178**, 23-29 (1954).

²⁸⁹ M. L. Shore, D. B. Zilversmit, and R. F. Ackerman, *Am. J. Physiol.*, **181**, 527-531 (1955).

²⁹⁰ D. B. Zilversmit, M. L. Shore, and R. F. Ackerman, *Circulation*, **9**, 581-585 (1954).

²⁹¹ P. V. Harper, Jr., W. B. Neal, Jr., and G. R. Hlavacek, *Metabolism*, **2**, 69-80 (1953).

the capacity to form cholesterol esters. No ester cholesterol was synthesized in the tissues of the dog following hepatectomy.

(8) *The Nature of the Cholesterol-Synthesizing Enzyme*

It is now recognized that the biosynthesis of cholesterol requires both an enzyme and a co-factor. It is probable that CoA is the coenzyme portion of the enzyme system. Rabinowitz and Gurin²⁹² reported the successful fractionation of a rat liver homogenate into a water-soluble enzyme system which is capable of incorporating C¹⁴-acetate into cholesterol. Bucher²⁹³ also demonstrated that the carboxyl carbon of acetate was incorporated into cholesterol when catalyzed by rat liver homogenates. It was later shown that the activity of the homogenate was confined to the "microsome-plus-soluble" fraction, while the mitochondria were apparently not essential for the synthesis.²⁹⁴

6. The Intermediary Metabolism of Cholesterol

(1) *Introduction*

The various anabolic and catabolic reactions which are related to cholesterol are exceedingly numerous and complex. According to the widespread experimental evidence available, there is no question that cholesterol can be destroyed by most tissues, as well as synthesized. Of greater importance, insofar as cholesterol is concerned, is the formation of related compounds from cholesterol which have specific physiologic effects. These include the steroid hormones. Cholesterol is the mother substance of the bile acids, and may be converted to vitamin D *via* 7-dehydrocholesterol. Finally, cholesterol may be excreted as such *via* the bile and the intestinal mucosa. There is evidence that some cholesterol may be excreted in the urine under certain conditions. Cornforth *et al.*²⁹⁵ described a new (1953) chemical method for effecting the degradation of the ring structure of cholesterol which involves the opening of ring B and the splitting off of ring A as 2-methyl-cyclohexanone, by pyrolysis. The early stages can be carried out on cholesterol ozonide or, more effectively, on cholest-5-ene.

²⁹² J. L. Rabinowitz and S. Gurin, *Biochim. et Biophys. Acta*, **10**, 345-346 (1953).

²⁹³ N. L. R. Bucher, *J. Am. Chem. Soc.*, **75**, 498 (1953).

²⁹⁴ N. L. R. Bucher, J. W. Grover, and R. Kingston, *Federation Proc.*, **13**, 19-20 (1954).

²⁹⁵ J. W. Cornforth, G. D. Hunter, and G. Popják, *Biochem. J.*, **54**, 590-597 (1953).

(2) *Experimental Evidence of
Cholesterol Destruction in the Tissues*

The earlier proof that cholesterol is destroyed by tissues was generally based upon the balance type of test. Dam²⁹⁶ reported that the disappearance of cholesterol in chicks was greater than could be accounted for by excretion. Page and Menschick²⁹⁷ observed that cholesterol fed to rabbits was deposited in all their tissues with the exception of the brain. However, after accounting for the cholesterol deposited in the tissues and that excreted, they demonstrated that more than one gram was destroyed per week. Bloch *et al.*²⁹⁸ showed that, in addition to the brain, the spinal cord also fails to take up injected cholesterol. Menschick and Page²⁹⁹ demonstrated the destruction of cholesterol by the tissues of cats. The compound was found to be stored in all the tissues except the brain. The liver content was much greater than was the case with rabbits, that of the other tissues somewhat less.²⁹⁹ Schönheimer³⁰⁰ presented evidence of cholesterol destruction in man on the basis of study of a case of hypercholesterolemia.

Dirscherl and Traut³⁰¹ reported that a negative cholesterol balance obtains in mice. This is interpreted as evidence that decomposition of cholesterol takes place. More exact proof of the synthesis and destruction of cholesterol has been adduced by Schoenheimer and Breusch,¹⁶⁴ who employed mice as the experimental animals; it was found that mice could destroy five times their total cholesterol content per month. It was postulated that a dynamic state of equilibrium exists between the synthesis and the destruction of cholesterol. The total amount present in the animal body at any one time depends upon the balance between these two processes. The rate of destruction is believed to be accelerated when the dietary cholesterol is increased.²⁹⁷

a. The Destruction of Cholesterol by Bacteria. Wainfan *et al.*³⁰² reported that destruction of cholesterol by bacteria probably occurs in the gastrointestinal tract. Thus, when feces or intestinal contents of cholesterol-fed rats were incubated with cholesterol, a significant decrease in the total cholesterol content resulted. Two species of bacteria isolated in pure culture from the feces of cholesterol-fed rats, which were active in

²⁹⁶ H. Dam, *Biochem. Z.*, **232**, 269-273 (1931).

²⁹⁷ I. H. Page and W. Menschick, *J. Biol. Chem.*, **97**, 359-368 (1932).

²⁹⁸ K. Bloch, B. N. Berg, and D. Rittenberg, *J. Biol. Chem.*, **149**, 511-517 (1943).

²⁹⁹ W. Menschick and I. H. Page, *Z. physiol. Chem.*, **218**, 95-103 (1933).

³⁰⁰ R. Schönheimer, *Z. klin. Med.*, **123**, 749-763 (1933).

³⁰¹ W. Dirscherl and H. Traut, *Z. physiol. Chem.*, **262**, 61-86 (1940).

³⁰² E. Wainfan, G. Henkin, S. C. Rittenberg, and W. Marx, *J. Biol. Chem.*, **207**, 843-849 (1954).

destroying cholesterol, were *Acrobacter (Bacterium) aerogenes* and the saprophytic flagellate, *Pseudomonas jaegeri*. 2,4-Dinitrophenol and nicotinamide significantly stimulated the oxidation of cholesterol by resting cell suspensions of *A. aerogenes*.

Stadtman and co-workers³⁰³ obtained a cholesterol dehydrogenase from the cell-free extracts of a soil *Mycobacterium* which was able to catalyze the conversion of cholesterol to Δ^4 -cholestene-3-one. It was found that carbon 4 of the steroid ring was oxidized to CO₂ about four times as rapidly by the dehydrogenase as was carbon 26 in the side-chain. The aerobic cholesterol-decomposing microorganism used in their studies was probably identical with the *M. cholesterolicum* isolated by Tak³⁰⁴ in 1942, which may be related to the *Nocardia* spp. (trichomycetes). In preliminary experiments, Tak found that *Mycobacterium phlei* ("timothy bacillus," a lymphatic pathogen from soil and grass), *M. lacticola* (a saprophyte from dairy products), *M. berolinense* (a saprophyte related to *M. lacticola*), *M. (Nocardia) salmonicolor* (soil bacterium), and *M. rubrum* (from garden soil) all developed well in a mineral cholesterol medium. The fact that the ability to decompose cholesterol is apparently common to all species of *Mycobacterium* may be useful in distinguishing between representatives of the general *Mycobacterium* and *Corynebacterium* (parasite pathogenic for warm-blooded animals).

Turfitt³⁰⁵⁻³⁰⁹ suggested that the soil organisms decomposing cholesterol belong to the genus *Proactinomyces*. These organisms are of especial interest in the breakdown of steroids, inasmuch as their oxidizing action, unlike that reported for other organisms, is not completely inhibited by the presence of the intact C₁₇ side-chain.³⁰⁷ The species represented were *Proactinomyces asteroides*, *P. farcinica*, and *P. paraffinae*, all of which belong to the genus *Nocardia*. Turfitt³⁰⁶ was unable to obtain evidence of the destruction of cholesterol by any other bacteria, by molds, or by actinomyces. The organism which destroys cholesterol *in vitro* was shown to be *Proactinomyces erythropolis* Gray and Thornton. All of the *Proactinomyces* tested brought about the oxidation of cholesterol to Δ^4 -cholestenone. Turfitt³⁰⁷ found that a number of the more common hydroxy-steroids

³⁰³ T. C. Stadtman, A. Cherkes, and C. B. Anfinsen, *J. Biol. Chem.*, **206**, 511-523 (1954).

³⁰⁴ J. D. Tak, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **8**, 32-40 (1942).

³⁰⁵ G. E. Turfitt, *Biochem. J.*, **37**, 115-117 (1943); **38**, 492-496 (1944).

³⁰⁶ G. E. Turfitt, *Biochem. J.*, **40**, 79-81 (1946).

³⁰⁷ G. E. Turfitt, *Biochem. J.*, **42**, 376-383 (1948).

³⁰⁸ G. E. Turfitt, *J. Bacteriol.*, **47**, 487-493 (1944).

³⁰⁹ G. E. Turfitt, *J. Bacteriol.*, **54**, 557-562 (1947).

could be oxidized to the corresponding keto derivatives by *Proactinomyces* spp. Apparently all hydroxysteroids are attacked, but those having shorter side-chains or those without side-chains give a lower yield of ketones. All bonds such as nuclear and side-chain double bonds remain unattacked, and ester linkages are not broken. Horváth and Krámlí³¹⁰ observed that the cholesterol $\rightarrow$ cholestenone reaction was effected much more successfully with a soil bacterium, *Azotobacter* spp. (which fixes free nitrogen) than with *Proactinomyces*. In addition to the formation of the ketone, *Azotobacter* also dehydrated the sterol ring, with the formation of sufficient 7-dehydrocholesterol to render its isolation possible.

Other organisms which have been found to effect the oxidation of hydroxy-steroids to the keto derivatives have been separated from yeast by Italian workers. Ercoli³¹¹ isolated *Micrococcus dehydrogenans* from baker's yeast; it converted androstenediol to pregneninolone. Arnaudi³¹² also described *Flavobacterium (Micrococcus) dehydrogenans*, which oxidized dehydroandrosterone to androstenedione. Ercoli and Molina,³¹³ in 1944, first described the activity of *F. androstenedionicum*, which converted androstenediol to androstenedione, and Molina and Ercoli³¹⁴ that of *F. carbonilicum*. This new microorganism from yeast was able to oxidize dehydroandrosterone to androstenedione, and also androstenediol to a mixture of testosterone and androstenedione. In general, these organisms oxidize the nuclear hydroxyl groups of the steroid compounds devoid of the C-17 side-chain. *Bacillus pseudodiphthericus (Corynebacterium hoffmanni)*, a pathogenic organism from throat cultures, oxidizes estradiol to estrone.³¹⁵ Schmidt *et al.*³¹⁶ observed that the intestinal bacterium, *Alcaligenes faecalis (Bacterium fecalis alcaligenes)*, could bring about a stepwise oxidation of the hydroxyl groups of cholic acid. Escherich's intestinal bacillus (*Escherichia coli*)³¹⁷ has been found to produce this same reaction.

In more recent work, Turfitt³⁰⁷ demonstrated that the oxidation of the

³¹⁰ J. Horváth and A. Krámlí, *Nature*, 160, 639 (1947).

³¹¹ A. Ercoli, *Biochim. e. terap. sper.*, 28, 125-130 (1941); *Chem. Abst.*, 38, 2987 (1944); *Z. physiol. Chem.*, 270, 266-270 (1941).

³¹² C. Arnaudi, *Boll. sez. ital. soc. intern. microbiol.*, 2, 208-211 (1939); *Chem. Abst.*, 34, 3301 (1940); *Zentr. Bakteriolog. Parasitenk., Abt. II*, 105, 352-366 (1942).

³¹³ A. Ercoli and L. Molina, *Boll. ist. sieroterap. milan.*, 23, 158-163, 175-180 (1944); *Chem. Abst.*, 40, 3152-3153 (1946).

³¹⁴ L. Molina and A. Ercoli, *Boll. ist. sieroterap. milan.*, 23, 164-167 (1944); *Chem. Abst.*, 40, 3152 (1946).

³¹⁵ W. Zimmermann and G. May, *Zentr. Bakteriolog. Parasitenk., Abt. I. Orig.*, 151, 462-466 (1944).

³¹⁶ L. H. Schmidt, H. B. Hughes, M. H. Green, and E. Cooper, *J. Biol. Chem.*, 145, 229-236 (1942).

³¹⁷ L. H. Schmidt and H. B. Hughes, *U. S. Patent No. 2,360,447* (Oct. 17, 1944).

cholesterol molecule could be effected beyond the ketone stage by *Proactinomyces*, resulting in a disruption of the molecule. Two types of oxidation were shown to occur; these were frequently simultaneous processes. One of these involves ring fission at the carbonyl group, while the second one consists in complete removal of the side-chain, with the result that aetiocarboxylic acid is formed. Thus, cholest-4-ene-3-one yields 3-keto-aetiochol-4-enic acid and isocaproic acid. When ring A is disrupted between carbons 3 and 4, the so-called Windaus keto acid is formed. When the fission of the A ring is prevented by blocking the free carboxyl on carbon 3 (as in cholesterol acetate), isocaproic acid is still formed by removal of the aliphatic side-chain. In the case of 3,7,12-triketocholanic acid, the side-chain is completely removed, with the formation of 3,7,12-triketoaetiocholanic acid, but there is no evidence of ring fission at any of the susceptible points.

The speed of turnover of cholesterol has been determined in several species of animals. In the rat, the half-life has been given as six days for the liver and thirty-one to thirty-two days for the carcass;¹⁸¹ in the case of the rabbit, the figure was three days for the liver and 1.5 days for the intestine.⁷⁰ London and Rittenberg³¹⁸ estimated the half-life of cholesterol in man as eight days.

(3) *Conversion to Cholic Acid and to Other Bile Acids*

Although it was long suspected that the bile acids are formed from cholesterol, only since the advent of the isotope technic has it been possible to prove the reaction unequivocally. Bloch and co-workers²⁹⁸ were the first to prove this interconversion. When deuteriocholesterol was administered intravenously to a dog after an anastomosis had been established between the gall-bladder and the pelvis of the kidney, it was found that the cholic acid isolated from the urine had the same isotope concentration as that of the injected cholesterol. It was calculated that at least two-thirds of the excreted cholic acid had arisen from the administered cholesterol.

Zabin and Barker³¹⁹ made a more comprehensive study of the cholesterol $\rightarrow$ cholic acid conversion in a bile-fistula dog, by feeding biosynthetically prepared tagged cholesterol. It was shown that the cholic acid formed originated from cholesterol by the removal of only three carbons, *i.e.*, numbers 25, 26, and 27.

³¹⁸ I. M. London and D. Rittenberg, *J. Biol. Chem.*, **184**, 687-691 (1950).

³¹⁹ I. Zabin and W. F. Barker, *J. Biol. Chem.*, **205**, 633-636 (1953).

Apparently all bile acids originate, either directly or indirectly, from cholesterol, but the preponderance of cholic acid in dog bile has made it easier to isolate this acid. In the case of rats, Bergström and Norman³²⁰ proved the origin of both cholic and chenodesoxycholic acids from cholesterol. These acids were present in bile as their taurine conjugates. Byers and Biggs³²¹ confirmed the transformation of cholesterol into cholic acid in the rat.

The excretion of cholesterol and of cholic acid in the bile varies in thyroid conditions in rats. Thus, Thompson and Vars³²² reported that the excretion of cholic acid is reduced in both hyperthyroidism and hypothyroidism, whereas the proportion of cholesterol excreted in the bile is a reflection of thyroid activity.

Robbins and co-workers³²³ demonstrated the remarkable ability of isolated rat liver to synthesize and degrade cholesterol over a prolonged period. When isolated livers were perfused with a solution containing whole rat blood diluted 1:3 with a solution of electrolytes, crystalline albumin, amino acids, and vitamins, bile was continuously formed for as long as eleven hours. The initial perfusate levels of 13.8 mg. % of cholesterol and 1.2 mg. % of cholate were changed to 18.0 and 1.4 mg. %, respectively, by the end of the perfusion. The bile formed had an average composition of 6.3 mg. % of cholesterol, and 117 mg. % of cholate. The rate of disappearance of injected cholesterol, however, could not be increased by the administration of bile salts, although there was an increase in cholesterol excretion in the bile.³²⁴

Byers and Biggs³²¹ demonstrated that the cholesterol $\rightarrow$ cholic acid reaction is not a reversible one. When tritium-labeled cholic acid, prepared by biosynthesis in the rat, was fed to rats with bile-duct obstruction, there was no evidence of tagged cholesterol in the body store. Biggs and Kritchevsky³²⁵ had previously demonstrated that tritium-labeled cholesterol is a suitable tracer compound for biologic investigations of cholesterol metabolism.

³²⁰ S. Bergström and A. Norman, *Proc. Soc. Exptl. Biol. Med.*, **83**, 71-74 (1953).

³²¹ S. O. Byers and M. W. Biggs, *Arch. Biochem. Biophys.*, **39**, 301-304 (1952).

³²² J. C. Thompson and H. M. Vars, *Proc. Soc. Exptl. Biol. Med.*, **83**, 246-248 (1953).

³²³ E. D. Robbins, S. Burton, J. Goerke, and M. Friedman, *Proc. Soc. Exptl. Biol. Med.*, **83**, 212-214 (1953).

³²⁴ M. Feldman, Jr., and T. Weinberg, *Proc. Soc. Exptl. Biol. Med.*, **73**, 619-620 (1950).

³²⁵ M. Biggs and D. Kritchevsky, *Arch. Biochem. Biophys.*, **36**, 430-433 (1952).

(4) *Conversion to Pregnanediol*

There is now good evidence that cholesterol is the mother substance, not only of cholic acid, but also of a number of the steroid hormones.³²⁶ Bloch³²⁷ demonstrated this interrelationship first in the case of pregnanediol. This investigator reported that, when deuteriocholesterol was given to a woman in the eighth month of pregnancy, pregnanediol-3 α ,20 α could be isolated from the urine as the sodium salt of pregnanediol glucuronidate. The isotope content of the pregnanediol was of the same order as that of the cholesterol circulating in the blood. It was estimated that one-half to two-thirds of the pregnanediol excreted arose by degradation of cholesterol, probably *via* progesterone. The course of the reaction is illustrated in the accompanying equation.

The Formation of Pregnanediol from Cholesterol in the Female

(5) *Conversion to Adrenocortical Hormones*

Zaffaroni, Hechter, and Pincus³²⁸ were among the first to demonstrate that both acetate and cholesterol can be transformed into the adrenocortical steroid hormones. It was observed that cholesterol was the more efficient in effecting the synthesis. Hechter *et al.*³²⁹ were later able to fractionate

³²⁶ J. A. Lovorn, *The Chemistry of Lipids of Biochemical Significance*, Methuen, London; Wiley, New York, 1955.

³²⁷ K. Bloch, *J. Biol. Chem.*, **157**, 661-666 (1945).

³²⁸ A. Zaffaroni, O. Hechter, and G. Pincus, *J. Am. Chem. Soc.*, **73**, 1390-1391 (1951).

³²⁹ O. Hechter, M. M. Solomon, A. Zaffaroni, and G. Pincus, *Arch. Biochem. Biophys.*, **46**, 201-214 (1953).

and to purify the two major corticosteroids synthesized by the adrenals. These could be synthesized either from acetate or from cholesterol itself.

In the experiments of Marx *et al.*,³³⁰ it was found that the thyrotoxicosis produced in rats by the feeding of high levels of the thyroid hormone could be markedly alleviated, the survival time prolonged, and in most cases the weight loss could be prevented by adding one per cent of cholesterol and

Fig. 3. The effect of dietary cholesterol on the body weight of rats fed toxic doses of thyroid hormone.³³⁰

0.5% of bile salts to the experimental diet. Thus, in nine-month-old rats, the average survival time was increased from 23 days to 55+ days simply by the expedient of adding the cholesterol and bile salts to the diet. In a second test, the comparative mean survival time was 86 ± 3.9 days for the control group, and greater than 111 ± 5.4 days for the cholesterol-fed group. The effect of the thyroid hormone on the body weight under these different dietary conditions is shown in Figure 3.

³³⁰ W. Marx, E. R. Meserve, and H. J. Deuel, Jr., *Proc. Soc. Exptl. Biol. Med.*, 67, 385-387 (1948).

Somewhat similar results were recorded by Anselmino and Hoffmann³³¹ and by Saegesser.³³² The conclusions were likewise confirmed independently on a different strain of rats, by Ershoff and Marx.³³³

The reason for the protective effect of the added cholesterol is problematical. One suggestion³³⁰ is that cholesterol protects the body from products resulting when a breakdown of lecithin to lysolecithin occurs, a reaction which Hoffmann and de Hoffmann³³⁴ believe is stimulated by the thyroid hormone. Since lysolecithin is supposed to affect the central nervous system, to influence cell permeability, and to exert "toxic effects in various organs" at high concentrations,³³⁵ any substance, such as cholesterol, which counteracts or neutralizes lysolecithins would have a protective action. Another possible explanation is that the concentration of cholesterol in key sites, as, for example, in the adrenocortical tissues, is reduced to a subnormal level, in the group not receiving cholesterol, by the constant stimulation of the thyroid hormone. The synthesis of adrenocortical hormones from cholesterol is decreased to such an extent that the animals die as a result of the deficiency of these hormones. When cholesterol is administered, the quantity of the sterol is maintained at a sufficiently high level in the various tissues (including the adrenal cortex) to insure an adequate amount of precursors of adrenocortical hormones at all times.

(6) *Conversion to Androgens*

According to Niwelinski,³³⁶ cholesterol also acts as a precursor of androgens. Thus, it will cause a stimulation of spermatogenesis and the secretion of ketosteroid hormones when it is implanted in the testes. However, the atrophy of the testes induced by deficiency of vitamin B₁ persists in spite of cholesterol administration, whereas the administration of sex hormones corrects the condition. It is possible that vitamin B₁ is required for the transformation of cholesterol into androgens. Brady³³⁷ demonstrated the *in vitro* synthesis of one of the androgens, testosterone, by surviving slices of testicular tissue from the hog, rabbit, and human testes; however, in this case the synthesis involved acetate, and evidence is pre-

³³¹ K. J. Anselmino and F. Hoffmann, *Klin. Wochschr.*, **12**, 99-102 (1933).

³³² M. Saegesser, *Klin. Wochschr.*, **12**, 672 (1933).

³³³ B. H. Ershoff and W. Marx, *Exptl. Med. Surg.*, **6**, 145-148 (1948).

³³⁴ F. Hoffmann and E. J. de Hoffmann, *Publ. Inst. fisiol., Univ. Chile*, 1943; cited by F. F. Foldes and A. J. Murphy, *Proc. Soc. Exptl. Biol. Med.*, **62**, 218-223 (1946), p. 220, and by B. H. Ershoff and W. Marx, *Exptl. Med. Surg.*, **6**, 145-148 (1948), p. 147.

³³⁵ F. F. Foldes and A. J. Murphy, *Proc. Soc. Biol. Med.*, **62**, 218-223 (1946).

³³⁶ J. Niwelinski, *Compt. rend. soc. biol.*, **146**, 1030-1033 (1952).

³³⁷ R. O. Brady, *J. Biol. Chem.*, **193**, 145-148 (1951).

sented which indicates that cholesterol may not be an important intermediate.

(7) Conversion to Estrogens

West and Todd³²⁷ and others^{327, 338-340} have suggested that cholesterol may be the mother substance of some of the estrogenic hormones, in addition to being the source of pregnanediol.³²⁷

(8) Conversion to 7-Dehydrocholesterol

7-Dehydrocholesterol is generally recognized as provitamin D₃. It has been an open question whether or not the animal can synthesize 7-dehydrocholesterol from cholesterol. In a study of the distribution and source of this dehydro compound, Glover, Glover, and Morton⁷² found that this unsaturated substance was concentrated in the mucosae and lamina propria of the duodenum of the guinea pig. The proportion of 7-dehydrocholesterol tends to increase as the animals increase in size, and when they exhibit liver tubercles due to *Pasteurella pseudotuberculosis*, which is pathogenic for rodents. It was demonstrated that cholesterol, when administered orally, dissolved in ethyl myristate or ethyl oleate, is dehydrogenated in the wall of the intestine by means of a dehydrogenase. It was concluded that the animal is not dependent upon provitamin D as such, but that it has the power to dehydrogenate cholesterol synthesized in its own body.⁷² Presumably 7-dehydrocholesterol can be converted into vitamin D₃ when that present in the skin is subjected to ultraviolet irradiation. Bills³⁴¹ demonstrated that irradiation of the sterol molecule may cause a break in the cyclopentenophenanthrene ring between carbons 9 and 10, resulting in the formation of products which possess vitamin D activity.

(9) Conversion to 7-Keto and 7-Hydroxy Compounds

Although cholesterol, when injected as an emulsion intravenously into rabbits, is rapidly removed from the circulation, it is not as rapidly metabolized as is 7-ketocholesterol or 7-hydroxycholesterol. Thus, although Kendall and co-workers³⁴² found that 6% of radioactive cholesterol was

³³⁸ W. H. Pearlman, *The Chemistry and Metabolism of the Estrogens*, in G. Pincus and K. V. Thimann, *The Hormones*, Vol. I, Academic Press, New York, 1948, Chapter X, 351-405 (pp. 360, 398).

³³⁹ P. Rondoni, V. Carminati, and A. Corbellini, *Z. physiol. Chem.*, **241**, 71-80 (1936).

³⁴⁰ I. Claesson and N. A. Hillarp, *Acta Physiol. Scand.*, **13**, 115-129 (1947).

³⁴¹ C. E. Bills, *J. Am. Med. Assoc.*, **110**, 2150-2155 (1938).

³⁴² F. E. Kendall, E. H. Mosbach, L. L. Abell, and W. Meyer, *Federation Proc.*, **12**, 449 (1953).

still present in the serum forty-eight hours after intravenous injection, and 32% could be recovered in the sterols isolated from the lungs, liver, spleen, adrenals, and serum after this interval, no significant radioactivity could be found in the sterols of the serum or of the organs at the end of forty-eight hours after 7-keto- or 7-hydroxycholesterol had been injected. Although the metabolic disposition of the 7-oxygenated sterols is still not understood, the possibility exists that they may be intermediates in the oxidative degradation of cholesterol in the body.³⁴² A number of years earlier, after making a careful examination of yeast fat, acorn fat, wheat germ oil, and saponified cholesterol stearate, Haslewood³⁴³ proved that no 7-hydroxy-sterols could be detected. It is suggested that, when 7-hydroxysterols are present, they represent true intermediates in cholesterol metabolism rather than substances formed during the isolation procedures.

(10) *Hydrogenation of Cholesterol
in the Gastrointestinal Tract*

In addition to dehydrogenation, hydrogenation can likewise be carried out in the gastrointestinal tract. The hydrogenation of cholesterol may involve its transformation into coprostanol (formerly called coprosterol), epicoprostanol, and cholestanol (formerly called dihydrocholesterol). The change of cholesterol $\rightarrow$ coprostanol does not merely involve a reduction to cholestanol, but requires a pathway *via* cholestenone, coprostenone, and coprostanol. The presence of cholestenone as an intermediate was proved by Rosenheim and Webster,²²⁹ who identified it by its ultraviolet absorption, and prepared cholestenone-*o*-tolylsemicarbazone from the feces of the dog and rat after feeding brain as the source of cholesterol. This pathway is further substantiated by Marker *et al.*,³⁴⁴ who succeeded in isolating epicoprostanol from the feces of a dog; this compound must have originated from cholestenone. It has been proved that labeled coprostanone is excreted as coprostanol.⁸¹ The transformation of cholesterol to coprostanol is discussed earlier in this present chapter (pages 369–370), as well as on pages 271–274 of Vol. II, *The Lipids*.

A second hydrogenation which can take place is the change, cholesterol $\rightarrow$ cholestanol. The latter compound is excreted in the feces;⁷³ it cannot be absorbed from the intestine.^{60, 345}

³⁴³ G. A. D. Haslewood, *Biochem. J.*, **36**, 389–391 (1942).

³⁴⁴ R. E. Marker, E. L. Wittbecker, R. B. Wagner, and D. L. Turner, *J. Am. Chem. Soc.*, **64**, 818–822 (1942).

³⁴⁵ R. Schönheimer and L. Hrdina, *Z. physiol. Chem.*, **212**, 161–172 (1932).

(11) *Oxidation of Cholesterol,
and the Excretion of Oxidation Products*

Until the experiments of Chaikoff and collaborators,^{346,347} little evidence was available as to whether or not cholesterol can be completely oxidized in the animal body, and how the end-products are excreted. These workers investigated the fate of cholesterol labeled at carbons 4 (in the ring) and 26 (in the side-chain). As much as 31% of the side-chain C (C 26) was eliminated in the expired air within twenty-four hours after administration, while none of the ring carbon (C4) appeared as CO₂. Intestinal bacteria were shown to play only a minor role (and possibly none whatsoever) in this oxidation. The ring carbon was found to be eliminated from the body almost entirely with the feces;³⁴⁷ none was excreted in the urine or expired from the lungs as CO₂. Of that which was excreted in the feces, at least 90% reached the intestinal tract *via* the bile, as bile acids, while a maximum of 10% was excreted through the walls of the intestine as cholesterol. Friedman *et al.*³⁴⁸ likewise reported that the removal of cholesterol from the rat after intravenous injection involves the conversion and excretion of at least 60% as bile acids. This removal of excess cholesterol requires twelve to twenty-four hours, during which a hypercholesterolemia obtains. Siperstein and Chaikoff³⁴⁷ noted that the majority of the labeled nucleus carbon was excreted in the bile and feces as a saponifiable compound; the cholesterol or cholesterol-like compound which reached the intestine through the walls of the intestine was in the form of a non-saponifiable component (presumably unchanged cholesterol, coprostanol (coprosterol), and cholestanol (dihydrocholesterol)). Actually, the non-biliary contribution of fecal cholesterol accounts for only 10% of the total C₄-labeled cholesterol excreted by the intestine.³⁴⁷

The oxidation of cholesterol by slices of different organs was studied directly by Meier, Siperstein, and Chaikoff,³⁴⁹ making use of a non-toxic, aqueous solution of cholesterol-26-C¹⁴. It was found that slices of liver, kidney, adrenal, testes, and spleen were able to oxidize the C₂₆ to CO₂; the liver was by far the most active. No C¹⁴O₂ appeared after liver slices from animals injected with cholesterol-4-C¹⁴ were incubated. Under identical conditions, as much as 1% of the C¹⁴ of cholesterol-26-C¹⁴ was re-

³⁴⁶ I. L. Chaikoff, M. D. Siperstein, W. G. Dauben, H. L. Bradlow, J. F. Eastham, G. M. Tomkins, J. R. Meier, R. W. Chen, S. Hotta, and P. A. Srere, *J. Biol. Chem.*, **194**, 413-416 (1952).

³⁴⁷ M. D. Siperstein and I. L. Chaikoff, *J. Biol. Chem.*, **198**, 93-104 (1952).

³⁴⁸ M. Friedman, S. O. Byers, and B. Gunning, *Am. J. Physiol.*, **172**, 309-316 (1953).

³⁴⁹ J. R. Meier, M. D. Siperstein, and I. L. Chaikoff, *J. Biol. Chem.*, **198**, 105-109 (1952).

covered as CO_2 in three hours. Thus, the liver appears to be not only the chief organ of synthesis of cholesterol, but also the tissue in which degradation of the side-chain of the sterol takes place. On the other hand, baby brain, previously found to be one of the most active sites of cholesterol synthesis, is unable to oxidize the side-chain carbon (C_{26}) to CO_2 . Thus, rapid synthesis and destruction do not necessarily coexist in the same tissue.

Because of the resistance of the ring-labeled cholesterol to oxidation (cholesterol-4- C^{14}) in the ring, Siperstein and co-workers³⁵⁰ were able to follow the enterohepatic circulation of the bile salts originating from it. Thus, after the intravenous injection of cholesterol-4- C^{14} , the biliary C^{14} -containing products were absorbed from the intestine, to be reëxcreted in the bile. Fifty per cent of the bile products containing C^{14} which were present in the duodenum were reëxcreted in the bile within four hours from the time that its administration was completed. Ninety per cent of the biliary products which were present in the intestine for the second time were absorbed and reëxcreted in the bile a third time. These biliary products enter the enterohepatic circulation, reaching the liver *via* the portal circulation; in sharp contrast to this, cholesterol is absorbed from the intestine by way of the lymph.

On the other hand, West and Todd⁸⁵ stated that "cholesterol is eliminated from the body chiefly in the intestinal secretions . . . and not in the bile, as previously supposed." In support of this statement, the results of a number of workers are cited.^{60,351,352} Thus, while cholesterol itself is excreted into the gut *via* the wall of the intestine, certain end-products of cholesterol metabolism, namely, the bile acids, are excreted in the bile. This concept has been repeatedly confirmed in the aforementioned works.

Another possible explanation of the fate of metabolized cholesterol was proposed by Cook,³⁵³ who suggested that the sterol was converted to fatty acids, inasmuch as a correlation apparently existed between the amount of cholesterol metabolized and the increase of fatty acids in the feces.³⁵³ In a later study, Cook *et al.*³⁵⁴ reported a loss of unsaponifiable matter (cholesterol) of the same order as the increase in the light petroleum-insoluble fecal lipids which occurred concomitantly. It is reported that an opti-

³⁵⁰ M. D. Siperstein, H. H. Hernandez, and I. L. Chaikoff, *Am. J. Physiol.*, **171**, 297-301 (1952).

³⁵¹ H. Beumer and F. Hepner, *Z. ges. expth. Med.*, **64**, 787-797 (1929).

³⁵² W. M. Sperry, *J. Biol. Chem.*, **68**, 357-383 (1926); **71**, 351-378 (1926-1927); **81**, 299-319 (1929); **85**, 455-463 (1929-1930).

³⁵³ R. P. Cook, *Biochem. J.*, **32**, 1191-1199 (1938).

³⁵⁴ R. P. Cook, N. Polgár, and R. O. Thomson, *Biochem. J.*, **47**, 600-605 (1950).

cally-active acid with a probable empirical formula of $C_{27}H_{46}O_4$ is present in this fraction. Therefore, the possibility exists that the increased acids are partly to be ascribed to the bile acids. Cook and Thomson³⁵⁵ reported that rabbits and guinea pigs destroyed a larger proportion of cholesterol than did rats; the comparative figures based upon unsaponifiable matter were 24% as contrasted with 18%. When the data for cholesterol metabolism were based upon the amount of sterol metabolized, the value for rabbits and guinea pigs was 54% as contrasted with a figure of 44% for rats. When 10 g. of cholesterol was administered to man in 50 g. of olive oil, about 20% of the cholesterol was absorbed, and an increased excretion of fecal acids was noted; however, no marked effect upon plasma cholesterol could be observed.³⁵⁶

(12) *The Excretion of Cholesterol in the Urine*

Cholesterol is not normally a component of urine. As discussed in Section (10), most of the excreted cholesterol is eliminated *via* the walls of the intestine in the feces. After oxidation of cholesterol in the liver, much of the water-soluble product is excreted into the intestine by way of the bile.

Burchell and MacLagan³⁵⁷ showed that an association exists between the supernatant urine cholesterol and a proteose fraction. Much or all of the urinary cholesterol was found in the urinary deposit or associated with heat-coagulable protein when present. It was reported by Burchell *et al.*,³⁵⁸ that the cholesterol in urine deposits from cancerous subjects varied from 0 to 0.05 mg. per twelve hr. in men and from 0 to 0.42 mg. per twelve hr. in women. The supernatant urine cholesterol varied between 0 and 0.5 mg. per twelve hr., with no sex difference. The supernatant urine cholesterol was increased in albuminuria, while hematuria and pyuria increased the urine-deposited cholesterol. Kayser and Balot³⁵⁹ stated that these forms of urinary cholesterol have their origin mostly in the mucus formed in the urogenital tract. In albuminuria the urinary cholesterol is derived from the plasma; it is present in the form of lipoprotein.

(13) *Summary of the Oxidation of Cholesterol in the Animal Body*

Under ordinary conditions, cholesterol (and presumably other sterols) are oxidized to only a minor degree. In most cases, the cyclopenteno-

³⁵⁵ R. P. Cook and R. O. Thomson, *Biochem. J.*, **49**, 72-77 (1951).

³⁵⁶ R. P. Cook and D. C. Edwards, *Biochem. J.*, **49**, xli (1951).

³⁵⁷ M. Burchell and N. F. MacLagan, *Biochem. J.*, **44**, iv (1949).

³⁵⁸ M. Burchell, J. H. O. Earle, and N. F. MacLagan, *Biochem. J.*, **44**, iv (1949).

³⁵⁹ F. Kayser and R. Balot, *Bull. soc. chim. biol.*, **34**, 806-812 (1952).

phenanthrene ring is not ruptured, and the end-product of metabolism is excreted when the side-chain has been partially or completely oxidized. There seems to be adequate proof that cholesterol can be converted to 7-dehydrocholesterol, and that the resulting product is then transformed to vitamin D₃ in the skin following irradiation, with the result that ring B is ruptured. However, the proportion of cholesterol which follows this route of degradation must be infinitesimal from a quantitative standpoint, in comparison with that which follows other metabolic pathways.

In the main, the side chain of cholesterol is only partially removed to form bile acids, which are excreted *via* the bile. Although the bile acids are reabsorbed to some extent, and undergo a cyclic absorption and re-excretion, they are eventually lost *via* the urine as urobilin and urobilinogen. Cholesterol itself is excreted mainly by way of the intestinal mucosa and the large intestine. The proportion of cholesterol which is metabolized to the bile acids constitutes about 90% of that broken down, while only 10% are excreted as such. Although it has been reported that cholesterol is excreted in the urine, the amount is small, and it does not amount to any appreciable proportion of the total, under normal conditions. Likewise, the proportion of cholesterol from which the side-chain is removed, with the consequent formation of the steroid hormones, must constitute only a very small fraction of the total metabolized.

7. The Intermediary Metabolism of Products Related to Cholesterol

(1) *Ergosterol*

Although ergosterol is not a normal component of the tissues of the animal, evidence is increasing that its synthesis in the fungi resembles that of cholesterol in the animal. Thus, Ottke, Simmonds, and Tatum,³⁶⁰ using *Neurospora crassa* mycelium (red bread mold) and a mutant which requires acetate, concluded that the acetate metabolism here resembles that of the rat in that acetate is the principal precursor of fat and sterol. In a later study, Ottke and associates³⁶¹ demonstrated that 90% of the carbon in ergosterol, in *Neurospora crassa*, as well as in the carbon of the fatty acids, was derived from acetate. It was calculated that the ergosterol was made up of fifteen methyl and thirteen carboxyl carbons (or fourteen of each) when acetate was the sole source of carbon for the sterol synthesis.

³⁶⁰ R. C. Ottke, S. Simmonds, and E. L. Tatum, *J. Biol. Chem.*, **186**, 581-589 (1950).

³⁶¹ R. C. Ottke, E. L. Tatum, I. Zabin, and K. Bloch, *J. Biol. Chem.*, **189**, 429-433 (1951).

The methyl carbon of isovaleric acid was shown to participate to a small but significant extent in the synthesis of ergosterol by *Neurospora*.³⁶¹ Hanahan and al-Wakil³⁶² proved that 74% to 84% of the carbon of ergosterol was derived from acetate when the sterol was synthesized by yeast which had previously been CoA-deficient and which was subsequently enriched. Using this procedure with labeled acetate, it was found that ergosterol of high specific activity could be prepared.³⁶² In a subsequent study of the six-carbon side-chain of ergosterol, by these workers,³⁶³ it was noted that carbons 23 and 25 were derived from the carboxyl group of acetic acid.

Although there was some evidence, based upon spectrographic data, that small amounts of ergosterol occur in animals, Schönheimer *et al.*⁶¹ demonstrated that the amount of ergosterol could not be increased in rats, mice, or rabbits by feeding ergosterol over long periods of time. It was thus shown, in opposition to the results of Page and Menschick,⁶² that ergosterol, if absorbed, was not stored. Schönheimer¹¹ later proved that, when a mixture of cholesterol and ergosterol was fed to dogs, only cholesterol appeared in the thoracic lymph, and no trace of ergosterol was present in this fluid. Schönheimer,¹¹ however, does not exclude the possibility that biologically important amounts of ergosterol may be absorbed over long periods of time. It was later demonstrated both by Schönheimer and Dam⁶⁵ and by Menschick and Page⁶⁶ that the laying hen is able to absorb ergosterol if it is given in large amounts. Ergosterol, in common with the phytosterols, has been shown to inhibit cholesterol absorption from the small intestine.³³

(2) Phytosterols

In 1908, König and Schluckebier³⁶⁴ reported that no phytosterols occur in the body fat, brain, liver, or bile of animals after prolonged feeding with vegetable fats. There is no evidence that the phytosterols are converted to cholesterol; if this were the case, Schönheimer¹¹ points out, every rabbit should exhibit a marked atherosclerosis, inasmuch as the level of plant sterols in the feed of rabbits is approximately ten times as high as the dose of cholesterol which provokes atherosclerosis in this species. After the feeding of enormous doses of sitosterol and of other phytosterols to rabbits, as well as to other animals, no demonstrable pathologic changes could be observed. Moreover, no increase in blood cholesterol occurred, nor was

³⁶² D. J. Hanahan and S. J. al-Wakil, *Arch. Biochem. Biophys.*, **37**, 167-171 (1952).

³⁶³ D. J. Hanahan and S. J. al-Wakil, *J. Am. Chem. Soc.*, **75**, 273-275 (1953).

³⁶⁴ J. König and J. Schluckebier, *Z. Untersuch. Nahr. Genussm.*, **15**, 644-661 (1908).

there any evidence of sitosterol storage.^{9,60,365} A number of phytosterols have been shown to inhibit cholesterol absorption from the small intestine of the rat.³³ According to Bisset and Cook,³⁶⁶ and to other workers,^{57,367-369} sitosterol inhibits the esterification of cholesterol in the intestine of human subjects as well, and reduces the amount of this sterol in the blood. It is suggested by Davis³⁷⁰ that ingested β -sitosterol reduces the dispersibility of cholesterol by colloidal dispersing agents in the intestine and thus reduces its absorption.

(3) β -Cholestanol (Dihydrocholesterol)

In addition to coprostanol (coprosterol), a second saturated C₂₇ steroid occurs in feces. In contradistinction to coprostanol, which originates in the gut and which does not occur in the tissues, β -cholestanol is present in the tissues, and it is apparently excreted into the gut to be eliminated in the feces.

According to Schoenheimer, as cited by Bills,⁹ β -cholestanol is present to the extent of 2% or 3% in the tissue sterols. It has been postulated that the β -cholestanol must originate by saturation of cholesterol *in situ*, since it has been proved that it cannot be absorbed. Schoenheimer¹¹ postulated that a hydrogenation-dehydrogenation reaction occurs whereby β -cholestanol is formed simultaneously with the dehydrogenation of another molecule to ergosterol. It is now recognized that this reaction is impossible, inasmuch as ergosterol is known to have twenty-eight carbons, while cholesterol has only twenty-seven carbons.

β -Cholestanol is probably not excreted *via* the bile, since gallstone cholesterol has been found to have a very small content of this sterol.¹¹ Schönheimer and Hrdina³⁴⁵ noted the absence of any sterols in the gastric and pancreatic secretions of dogs, but they did observe that the intestinal juice contained both cholesterol and β -cholestanol. The secretion of β -cholestanol by this route was verified by Schönheimer and v. Behring,⁶⁰ who isolated the lower gut of dogs and found that the sterile masses of intestinal secretion which accumulated after one or two months contained very significant amounts of β -cholestanol, but no coprosterol or cholesterol. Similar

³⁶⁵ R. Schönheimer, H. v. Behring, R. Hummel, and L. Schindel, *Z. physiol. Chem.*, **192**, 73-76 (1930).

³⁶⁶ S. K. Bisset and R. P. Cook, *350th Meeting, Biochem. Soc.*, Glasgow, April, 1956.

³⁶⁷ R. E. Shipley, *Trans. New York Acad. Sci.* [2], **18**, 111-118 (1955).

³⁶⁸ C. F. Wilkinson, Jr., R. S. Jackson, R. C. Bozian, M. R. Benjamin, A. H. Levere, G. Craft, and N. W. Davidson, *Trans. New York Acad. Sci.* [2], **18**, 119-122 (1955).

³⁶⁹ Anonymous, *Nutrition Revs.*, **14**, 39-41 (1956).

³⁷⁰ W. W. Davis, *Trans. New York Acad. Sci.* [2], **18**, 123-128 (1955).

results were reported by Schönheimer and Hrdina³⁴⁵ in the case of blind loops of the small intestine. According to these latter investigators, a similar situation had been observed in 1911 by Boehm.³⁷¹ In the case of a woman patient, several grams of β -cholestanol had accumulated in a loop of the intestine following a surgical accident. These results prove conclusively that β -cholestanol is formed in the tissues, and is excreted by way of the intestinal wall. In common with phytosterols and ergosterol, administered cholestanol has been reported to inhibit cholesterol absorption from the small intestine of cockerels,³⁵ of rats,³⁶ and of mice.³⁷

(4) Steroid Hormones

There can be no doubt that the several steroid hormones are derived directly or indirectly from cholesterol. These compounds represent a group of steroids from which the aliphatic chain at carbon 17 has been largely or completely removed; the main series of such hormones have either seventeen, nineteen, or twenty-one carbon atoms. They are all fat-soluble compounds which, however, may be excreted in the urine after conjugation with glucuronic or sulfuric acid. Small differences in composition produce marked alterations in physiologic properties. There will be no attempt in this volume to review the intermediary metabolism of these compounds, as they constitute a separate category of knowledge which is not too well defined. For a further discussion of this subject the reader is referred to the excellent treatises of Dorfman and Ungar,³⁷² Schwenk,³⁷³ Dorfman,³⁷⁴ Dobriner and Lieberman,³⁷⁵ Zander,³⁷⁶ and Heard,³⁷⁷ and also to volumes I, II, and VII of the Ciba Foundation Colloquia on Endocrinology, which deal with these hormones and adrenocortical steroids.³⁷⁸

³⁷¹ R. Boehm, *Biochem. Z.*, **33**, 474-479 (1911).

³⁷² R. I. Dorfman and F. Ungar, *Metabolism of Steroid Hormones*, Burgess, Minneapolis, 1953.

³⁷³ E. Schwenk, *Synthesis of the Steroid Hormones*, in F. R. Moulton, *The Chemistry and Physiology of Hormones*, Am. Assoc. Advancement Sci., Washington, D. C., 1944, pp. 129-143.

³⁷⁴ R. I. Dorfman, *Biochemistry of Androgens*, in G. Pincus and H. V. Thimann, *The Hormones*, vol. I, Academic Press, New York, 1948, pp. 467-548 (*Metabolism*, pp. 517-534).

³⁷⁵ K. Dobriner and S. Lieberman, *The Metabolism of Steroid Hormones in Humans*, in E. S. Gordon, *A Symposium on Steroid Hormones*, Univ. Wisconsin Press, 1950, pp. 46-88.

³⁷⁶ J. Zander, *Klin. Wochschr.*, **30**, 873-882 (1952).

³⁷⁷ R. D. H. Heard, *Chemistry and Metabolism of the Adrenal Cortical Hormones*, in G. Pincus and K. V. Thimann, *The Hormones*, vol. I, Academic Press, New York, pp. 549-629.

³⁷⁸ Ciba Foundation Colloquia on Endocrinology, vol. I, *Steroid Hormones and Tumor Growth*, and *Steroid Hormones and Enzymes*; vol. II, *Steroid Metabolism and Estimation*, Blakiston, Philadelphia, 1952; vol. VII, *Synthesis and Metabolism of Adrenocortical Steroids*, Little, Brown, Boston, 1953.

8. General Considerations Related to Cholesterol and to the Sterols in General

(1) *Functions of Cholesterol*

The widespread distribution of cholesterol in nature must be an indication of its importance in the life processes. In the extensive investigations of Dorée³⁷⁹ on the distribution of cholesterol in the animal kingdom, this sterol was present in all species except the red starfish, or "five-finger" (*Asterias rubens*), which contained a closely related complex alcohol. If it were not of primary importance, it would certainly be lacking in some animals. Although cholesterol has important functions, Shope³⁸⁰ stated, as recently as 1928, that there is a "complete absence of knowledge concerning the function of cholesterol itself." However, Bills⁹ listed a number of functions attributable to cholesterol, and to other sterols. For example, they may exert a conditioning action on skin, hair, and feathers. Cholesterol is thought to act as a conveyer in the absorption of fats. Bloor⁹⁶ reported a parallelism between the cholesterol content of blood and the fatty acid content. It is suggested by Minovici³⁸¹ and by Lovern³²⁶ that, in view of the abundance of cholesterol in brain and nerves, and its variation in mental disease, it may function as an insulating medium for the myelin sheaths. Sterols are thought to have a role in maintaining the balance between cell permeability and the membrane equilibrium of living cells. The presence of sterols in seeds, pollen, spores, ova, and sperm cells may indicate a need for these substances by the developing embryo.

According to Lovern³²⁶ a correlation exists between the lipid content of tissues and their functional activity. Higher levels of phospholipid + cholesterol appear to be associated with greater functional activity. For example, slowly-fatiguing muscle tissue such as heart muscle has the highest cholesterol content.³⁸²

The possible role of sterols as detoxicating agents has been suggested, although the evidence is still presumptive.⁹ Abderhalden and Le Count³⁸³ found that cholesterol, but not its esters, counteracted the lytic action of saponin, solanin, cobra venom, and tetanus toxin. According to Minovici,³⁸¹ numerous hemolytic agents, including venoms, saponins, bacterial toxins, bile salts, soaps, hexylresorcinol, etc., are neutralized by sterols.

³⁷⁹ C. Dorée, *Biochem. J.*, **4**, 72-106 (1909).

³⁸⁰ R. E. Shope, *J. Biol. Chem.*, **80**, 127-132 (1928).

³⁸¹ S. Minovici, *Bull. soc. chim. biol.*, **9**, 1129-1164 (1927).

³⁸² G. Embden and H. Lawaczek, *Z. physiol. Chem.*, **125**, 199-209 (1923).

³⁸³ E. Abderhalden and E. R. Le Count, *Z. expil. Pathol. Therap.*, **2**, 199-215 (1906).

Lumière and Grange³⁸⁴ noted that cholesterol, when administered in injected serum, exerted a protective action against anaphylactic shock caused by flocculation in the blood stream.

a. Relation to Physiologically Important Compounds. Although the physiologic relationship of cholesterol to the steroid hormones and to bile salts had not been proved at the time when Bills⁹ wrote his review, it was suggested that the physiologic relationship of cholesterol to other biologically important compounds which had a similar structure did present a possibility of intriguing interest. Since 1953 it has been considered, on the basis of various experiments, that cholesterol is the mother substance of the cholic acid residue of bile salts, and of sex hormones such as pregnanediol,³²⁶ the estrogens, and the androgens, and is related to the adrenocortical hormones. It has been shown that 7-dehydrocholesterol, which is an intermediate in the biosynthesis of vitamin D₃, can originate from cholesterol.³⁸⁵ It is therefore evident that the cholesterol molecule must be available if the biosynthesis of a number of physiologically important compounds is to proceed normally.

On the other hand, according to Downes,³⁸⁵ cholesterol is not an obligatory intermediate in hormone synthesis. Since both steroid hormones and cholesterol can be synthesized from acetate, there is probably at some point a branching in the synthetic pathway, so that the molecules which follow one path become cholesterol, and those which follow the other give rise to the hormones. This does not rule out the possibility that transformation of the cholesterol is an alternative method of preparing hormone molecules.³⁸⁵

³⁸⁴ A. Lumière and R. H. Grange, *Compt. rend.*, 191, 423-425 (1930).

³⁸⁵ H. R. Downes, *The Chemistry of Living Cells*, Harpers, New York, 1955.

CHAPTER IX

THE METABOLISM AND NUTRITIONAL VALUE OF THE CAROTENOIDS AND VITAMINS A

1. Introduction

The metabolism of fats and oils is intimately linked with that of the fat-soluble vitamins. This is because the fats usually serve as the solvents for these vitamins, and the two foodstuffs frequently appear in the gastrointestinal tract simultaneously for digestion and absorption. The physical nature of the fat-soluble vitamins is so similar to that of the fats that they follow the same pathway of absorption from the gastrointestinal tract, and of transport to the liver and tissues, namely by way of the lacteals and the thoracic duct, and then *via* the blood stream. In some cases fats also aid the absorption of the fat-soluble vitamins. They may likewise protect the vitamins from oxidation and destruction. On the other hand, the tocopherols (vitamins E) act to protect the fats from oxidation by virtue of their antioxidant action.

The chemical nature of the fat-soluble vitamins was described in *The Lipids*, Vol. I, while their absorption, transport, and blood levels, as well as the transformation of carotene to vitamin A, were discussed in *The Lipids*, Vol. II. The present chapter will outline the metabolism of the carotenoids and vitamins A, and will list the functions and the nutritional importance of each. Chapters X to XII will deal with the other fat-soluble vitamins in a similar manner. For a more complete discussion of the fat-soluble vitamins, the reader is referred to the monographs of Rosenberg¹ and to that of Sebrell and Harris.² The textbooks on biochemistry by West and Todd³ and of White, Handler, Smith, and Stetten⁴ likewise present

¹ H. R. Rosenberg, *Chemistry and Physiology of the Vitamins*, Interscience, New York, 1945.

² W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins, Chemistry, Physiology, Pathology*, Vols. I, II, III, Academic Press, New York, 1954.

³ E. S. West and W. R. Todd, *Textbook of Biochemistry*, 2d. ed., Macmillan, New York, 1955.

⁴ A. White, P. Handler, E. L. Smith, and DeW. Stetten, Jr., *Principles of Biochemistry*, McGraw-Hill, New York, 1954.

excellent recent reviews on the subject of the several fat-soluble vitamins. In addition to the theoretical discussion, the textbook of Hawk, Oser, and Summerson⁵ on *Practical Physiological Chemistry* includes a description of the chemical and biological methods for the determination of the vitamins. Coward⁶ has also recorded methods in detail for the bioassay of some of the vitamins, while the latest publication (1951) of The Association of Vitamin Chemists, Inc.⁷ is a contribution in this field. Finally, the series of monographs entitled *Vitamins and Hormones*⁸ furnishes a more complete discussion of specific topics concerning the vitamins in general, as well as of specific vitamins. The chronological development of the field can best be followed in the appropriate section of the *Annual Review of Biochemistry*.⁹

The carotenoids were recognized as chemical entities for almost one-hundred years before the existence of vitamin A was known. Although Wachenroder¹⁰ isolated carotene (*carotin*) from carrots as early as 1826, it was not until 1931 that Karrer, Morf, and Schöpp¹¹ prepared the first concentrate of vitamin A. The earliest experimental evidence of the occurrence of a dietary substance with the special effects which we now ascribe to vitamin A was the pioneer work of Hopkins¹² in 1912. This investigator noted that rats did not grow normally unless certain "accessory food factors" were included in their diets. One of the necessary components so classified was shown simultaneously by Osborne and Mendel¹³ and by McCollum and Davis¹⁴ to be present in some fats, and hence it was first

⁵ P. B. Hawk, B. L. Oser, and W. H. Summerson, *Practical Physiological Chemistry*, 13th ed., Blakiston, Philadelphia, 1954.

⁶ K. H. Coward, *The Biological Standardization of the Vitamins*, W. Wood, Baltimore, 1938.

⁷ Association of Vitamin Chemists, Inc., *Methods of Vitamin Assay*, 2nd ed., Interscience, New York, 1951.

⁸ *Vitamins and Hormones*, Academic Press, New York, Vol. I, published in 1943, Vol. XIII in 1955. Published annually.

⁹ *Annual Review of Biochemistry*, Vol. I published by Stanford Univ. Press, 1951; Vol. III and subsequent volumes by *Ann. Rev. Biochem., Inc.*; Vol. XXIV, 1955.

¹⁰ H. Wachenroder, *Über das Oleum Radicis Dauci Aetherum, das Carotin, den Carotinzucker, und den officinellen Suceus Dauci*, Dissertation de anthelminticis, Göttingen, *Geiger's Mag. Pharm.*, 33, 144-172 (1831); cited by L. S. Palmer, *Carotenoids and Related Pigments*, Chem. Catalog Co., New York, 1922, p. 25.

¹¹ P. Karrer, R. Morf, and K. Schöpp, *Helv. Chim. Acta*, 14, 1036-1040, 1431-1436 (1931).

¹² F. G. Hopkins, *J. Physiol.*, 44, 425-460 (1912).

¹³ T. B. Osborne and L. B. Mendel, *J. Biol. Chem.*, 15, 311-326 (1913); 17, 401-408 (1914).

¹⁴ E. V. McCollum and M. Davis, *J. Biol. Chem.*, 15, 167-175 (1913); 19, 245-250 (1914); 20, 641-658 (1915); 23, 231-246 (1915).

called the "fat-soluble accessory factor" (fat-soluble A). McCollum *et al.*¹⁵ later demonstrated that this factor consisted of two distinct vitamins; the term, *vitamin A*, was retained for the growth-promoting, antixerophthalmic principle, while the term, *vitamin D*, was assigned to the anti-rachitic substance, *i.e.*, the compound which prevents rickets.

The relationship of carotene to vitamin A was first suggested by Steenbock¹⁶ on the basis of the fact that the intensity of color due to the yellow lipochromes in butter, peas, and maize was roughly proportional to their vitamin A biopotency. However, when it was later shown that extremely potent vitamin A preparations were colorless (and that some highly-colored yellow compounds were without vitamin A activity), it was necessary to abandon the concept that vitamin A and carotene are identical. As a result of the demonstration by von Euler and co-workers¹⁷ that highly purified β -carotene, when administered in minimal amounts, could cure rats suffering from vitamin A deficiency, it was evident that both a colorless compound, having an absorption band at 328 $m\mu$, and the highly-colored β -carotene, which had no absorption band at 328 $m\mu$, had vitamin A activity. The relationship of these two compounds was explained by Moore.^{18,19} He demonstrated that vitamin A appeared in the liver fat of the vitamin A-deficient rat after the administration of either vitamin A or β -carotene; in both cases, the product had the characteristic absorption band at 328 $m\mu$, and it was also colorless. It was therefore suggested that colorless vitamin A is the active principle; β -carotene was assumed to exhibit vitamin A activity by virtue of its capacity to be converted to this vitamin in the animal body. β -Carotene is therefore a precursor of vitamin A, or a "pro-vitamin A." The relation of β -carotene to vitamin A was further elucidated by the demonstration of the structure of the two compounds. Whereas β -carotene was shown to have an empirical formula of $C_{40}H_{56}$, and to contain two β -ionone rings, vitamin A has one-half the number of carbons ($C_{22}H_{29}OH$), and contains only one β -ionone ring. Carotenes are essentially of plant origin, and may serve as precursors of vitamin A, which is found only in animals. There is no evidence that the animal can synthesize either carotene or vitamin A *de novo*.²⁰ The classic monographs which

¹⁵ E. V. McCollum, N. Simmonds, J. E. Becker, and P. G. Shipley, *J. Biol. Chem.*, **53**, 293-312 (1922).

¹⁶ H. Steenbock, *Science*, **50**, 352-353 (1919); with P. W. Boutwell, *J. Biol. Chem.*, **41**, 81-96 (1920).

¹⁷ H. von Euler, P. Karrer, and M. Rydbom, *Ber.*, **62**, 2445-2451 (1929).

¹⁸ T. Moore, *Biochem. J.*, **23**, 803-811 (1929); **24**, 692-702 (1930).

¹⁹ T. Moore, *Biochem. J.*, **25**, 275-286 (1931).

²⁰ T. W. Goodwin, *The Chemical Pathology of Carotenoids*, in R. T. Williams, *The Chemical Pathology of Animal Pigments*, Biochem. Soc. Symposia No. 12, Cambridge Univ. Press, 71-84 (1954).

deal chiefly with the chemistry and, to a lesser extent, with the physiologic action of the carotenoids are those of Palmer,²¹ of Zechmeister,²² of Lederer²³ and that of Karrer and Jucker.²⁴ An extensive treatment of the subject of the carotenoids and vitamins A, chiefly from a chemical viewpoint, is included on pages 507 to 738 of Volume I of this series of monographs.

2. The Digestion, Absorption, and Digestibility of Carotenoids and of the Vitamins A

(1) β -Carotene and Other Carotenoids

Since β -carotene is the most widely distributed and best known carotenoid, as well as that of greatest physiologic importance, more information is available concerning the digestion and absorption of this compound than can be found in the case of the other carotenoids. There is considerable evidence that β -carotene can be absorbed as such, transported in the lymph and blood, stored in the liver and other tissues, and excreted in the milk by some species of animals. It is a well-recognized fact that the color of milk and of the butterfat derived from it is chiefly determined by the quantity of carotene in the diet of the lactating animal. The same situation obtains in relation to the pigmentation of hens' eggs, although carotenoid pigments other than β -carotene are likewise involved. In the case of man, β -carotene is a normal component of the blood, but here again the amount present is related to the intake of the provitamin. Thus, when large amounts are ingested over a period of time, a condition known as carotenemia occurs in man. Abnormally high quantities of the chromogen are present in the blood; as a result considerable amounts are also deposited in the tissues. In sharp contrast to the aforementioned examples, the blood, liver, or other tissues of certain animals, such as the rat, goat, and pig, do not contain appreciable amounts of β -carotene or of carotenoids.

Goodwin²⁰ classified mammals into three groups according to the mechanism by which the carotenoids are metabolized. In Group A the tissues accumulate indiscriminantly both carotenes (hydrocarbon carotenoids)

²¹ L. S. Palmer, *Carotenoids and Related Pigments*, Chem. Catalog Co., New York, 1922.

²² L. Zechmeister, *Carotinoide*, Springer, Berlin, 1934.

²³ E. Lederer, *Récherches sur les Caroténoïdes des Animaux inférieurs, et des Cryptogames*, Lons-de-Saumier, Paris, 1937, pp. 5-93; cited by D. L. Fox, *Carotenoid and Indolic Biochromes of Animals*, *Ann. Rev. Biochem.*, 16, 443-470 (1947), pp. 448, 454, ff.

²⁴ P. Karrer and E. Jucker, *Carotinoide*, Birkhäuser, Basle, 1948; Translated and revised by E. A. Braude, Elsevier, New York, 1950.

and xanthophylls (oxycarotenoids); man belongs in this group. In Group *B*, comprising cattle and horses, mainly carotenes accumulate in the tissues. In Group *C*, in which sheep, goats, rats, mice, and rabbits are classed, neither carotenes nor oxycarotenoids accumulate. Another group of animals, namely the birds, specifically accumulate xanthophylls or oxycarotenoids. No mammals are known to fit in this category. We shall extend Goodwin's classification by referring to the birds as Group *D*.

a. Factors Altering the Absorption of β -Carotene. A number of conditions may alter the rate at which carotene can be absorbed. In the first place, this carotenoid is absorbed with relative difficulty, due to its low solubility, and because it is a hydrocarbon without functional groups in its molecule. Moreover, there is no possibility that β -carotene can yield smaller and more easily absorbed molecules, by hydrolysis or by oxidation, without permanent destruction of the carotenoid structure. It is true that it can be broken down to vitamin A, which is readily absorbable, but this ceases to represent the absorption of β -carotene as such.

Neither β -carotene nor any other of the fat-soluble vitamins can be absorbed from the stomach; they are chiefly absorbed from the small intestine. According to Shaw and Deuel,²⁵ the rate of absorption is a function of the surface area of the animal. Thus, after a massive dose of β -carotene in cottonseed oil, β -carotene was absorbed at the rate of 110 μ g. per 100 sq. cm. of body surface per hour. Bile is required for the normal utilization of β -carotene. Thus, in hepatic dysfunction, such as in obstructive jaundice, no absorption of carotene occurs.²⁶ However, Greaves and Schmidt²⁷ demonstrated that glycodesoxycholic and desoxycholic acids could effect the absorption of carotene in choledochocolostomized rats. The requirement of bile to insure the absorption of carotene in choledochocolostomized animals was confirmed by Irvin and co-workers.²⁸ The clinical observations of Clausen and McCoord,²⁹ which show a decreased carotene absorption in catarrhal jaundice (infectious hepatitis), also support this conclusion.

The nature of the solvent for β -carotene is also of considerable importance insofar as absorption is concerned. Thus, in the case of rats, cats, and chickens on fat-low diets, there is poor absorption of carotene, while it can be readily utilized when incorporated into a fat-containing regimen. The absorption of carotene is increased if it is emulsified with a water solution

²⁵ R. J. Shaw and H. J. Deuel, Jr., *J. Nutrition*, **27**, 395-401 (1944).

²⁶ J. D. Greaves and C. L. A. Schmidt, *Am. J. Physiol.*, **111**, 502-506 (1935).

²⁷ J. D. Greaves and C. L. A. Schmidt, *Am. J. Physiol.*, **111**, 492-501 (1935).

²⁸ J. L. Irvin, J. Kopala, and C. G. Johnstone, *Am. J. Physiol.*, **132**, 202-209 (1941).

²⁹ S. W. Clausen and A. B. McCoord, *J. Pediat.*, **13**, 635-650 (1938).

of the bile salts. The phospholipid, lecithin, also enhances the absorption of carotene. It has been reported that the utilization of β -carotene is augmented when it is fed as a component of margarine.³⁰ It is suggested that the lecithin, and the mono- and diglycerides present in this product, aid in absorption.

A number of workers³¹⁻³³ observed that carotene was as well absorbed, or even better, when administered in an aqueous colloidal solution solubilized by dispersing agents, for instance, "Tween 40" (polyoxyethylene sorbitan monopalmitate) than when given in an oil solution. Vavich and co-workers³⁴ found that carotene was better utilized when fed as an homogenate in non-fat milk than when it was homogenized in water.

The thyroid gland exerts an important role in the absorption of carotene. Johnson and Baumann³⁵ were the first to demonstrate in an unequivocal fashion that less vitamin A accumulated in the livers and kidneys of rats rendered hypothyroid with thiouracil than was the case with normal rats receiving the same amount of carotene. Conversely, the livers of hyperthyroid rats contained more vitamin A than did those of the normal controls. However, this phenomenon was not related to the increased oxidation caused by the thyroid, as no increase in vitamin A storage was noted in rats having increased oxygen utilization as the result of the administration of 2,4-dinitrophenol.

Goodwin²⁰ called attention to the three possible explanations of the above experimental data, namely (1) that the thyroid controls the conversion of carotene into vitamin A, (2) that it controls the stability of carotene in the lumen of the intestine, and (3) that it regulates the absorption of carotene from the lumen. Cama and Goodwin³⁶ also demonstrated that, in the case of the rat, the absorption of carotene is increased when thyroxine is given and is decreased after the thyroid antagonist, thiouracil, is administered. They are of the opinion that possibility 2 above is ruled out, since thiouracil was found to have no effect on the stability of β -carotene in the intestine. However, it was shown that the fecal excretion of carotene was increased

³⁰ H. J. Deuel, Jr., S. M. Greenberg, E. E. Savage, and D. Melnick, *J. Nutrition*, **43**, 371-388 (1951).

³¹ S. Y. Thompson, R. Braude, A. T. Cowie, J. Ganguly, and S. K. Kon, *Biochem. J.*, **44**, ix-x (1949).

³² G. H. Wise, N. L. Jacobson, R. S. Allen, and S. P. Yang, *J. Dairy Sci.*, **32**, 711 (1949).

³³ M. J. Burns, S. M. Hauge, and F. W. Quackenbush, *Arch. Biochem.*, **30**, 341-346 (1951).

³⁴ M. G. Vavich, J. W. Stull, N. Raica, and A. R. Kemmerer, *Arch. Biochem. Biophys.*, **55**, 310-314 (1955).

³⁵ R. M. Johnson and C. A. Baumann, *J. Biol. Chem.*, **171**, 513-521 (1947).

³⁶ H. R. Cama and T. W. Goodwin, *Biochem. J.*, **45**, 236-241 (1949).

after thiouracil was given; this supports suggested mechanisms 1 and 3 listed above. For a further discussion of the effect of the thyroid upon carotene utilization, see the section on transformation of carotene into vitamin A (pages 443 to 447).

Chanda and co-workers³⁷ observed a similar improved digestion of carotene in cows and goats after the injection of thyroxine, as well as a decreased utilization when thiouracil was given. It was later found³⁸ that normal goats absorb carotene more efficiently than do normal cows; this fact may be related to the observation of Schultze and Turner³⁹ that the goat thyroid is more active than the cow thyroid. Another indication of the improved absorption of carotene produced by a hyperthyroid condition is the fact that the proportion of the provitamin A excreted in milk is increased by the administration of the thyroid hormone to lactating cows.⁴⁰ Cadman⁴¹ reported that the thyroid exerts a control over carotene absorption in man similar to that observed in lower animals. Thus, in a case of myxedema (hypothyroidism), the fecal loss was decreased by thyroid therapy, whereas, in the case of patients with thyrotoxicosis (hyperthyroidism), the excretion of carotene was increased by the administration of thiouracil.

The absorption of β -carotene is sensitive to substances fed concomitantly. Thus, in contradistinction to the beneficial effects of food fats on the absorption of the provitamin, mineral oil has repeatedly been found to depress the utilization of β -carotene.^{33,42-47} Burns and co-workers³³ noted that the presence of as little as 0.08% of mineral oil, which was sufficient to prevent dustiness of lucerne leaf meal, had a deleterious effect on the utilization of carotene. On the other hand, Mahle and Patton⁴⁸ reported that certain

³⁷ R. Chanda, H. M. Clapham, M. L. McNaught, and E. C. Owen, *Biochem. J.*, **50**, 95-99 (1951).

³⁸ R. Chanda, H. M. Clapham, M. L. McNaught, and E. C. Owen, *J. Agr. Sci.*, **41**, 179-186 (1951).

³⁹ A. B. Schultze and C. W. Turner, *Univ. Missouri Coll. Agr., Agr. Expt. Sta., Research Bull. No. 392*, 1-89 (Aug., 1945).

⁴⁰ R. Chanda and E. C. Owen, *Biochem. J.*, **51**, 404-417 (1952).

⁴¹ E. F. B. Cadman, Personal communication, 1954; cited by T. W. Goodwin, *The Chemical Pathology of Carotenoids*, in R. T. Williams, *The Chemical Pathology of Animal Pigments*, Biochem. Soc. Symposia, No. 12, Cambridge Univ. Press, 71-84 (1954), p. 75.

⁴² J. I. Rowntree, *J. Nutrition*, **3**, 345-351 (1931).

⁴³ R. A. Dutcher, P. L. Harris, E. L. Hartzler, and N. B. Guerrant, *J. Nutrition*, **8**, 269-283 (1934).

⁴⁴ R. W. Jackson, *J. Nutrition*, **7**, 607-616 (1934).

⁴⁵ M. T. Burrows and W. K. Farr, *Proc. Soc. Exptl. Biol. Med.*, **24**, 719-723 (1927).

⁴⁶ R. A. Dutcher, J. O. Ely, and H. E. Honeywell, *Proc. Soc. Exptl. Biol. Med.*, **24**, 953-955 (1927).

⁴⁷ H. S. Mitchell, *Proc. Soc. Exptl. Biol. Med.*, **31**, 231-233 (1933).

⁴⁸ A. E. Mahle and H. M. Patton, *Gastroenterology*, **9**, 44-53 (1947).

hydrophilic mucilloids, which are used as purges in place of mineral oils, do not decrease the utilization of carotene. Moreover, alumina gel, which is often administered over protracted periods in the treatment of peptic ulcer, is also without a depressing action on carotene utilization.⁴⁹

The tocopherols augment the biologic activity of both carotene and vitamin A.⁵⁰ It is believed that this may be the result of a positive action of the tocopherols against an oxidative destruction of these compounds in the gastrointestinal tract. Several investigators^{51,52} have reported that the administration of the xanthophylls (lutein and zeaxanthin) reduces the utilization of β -carotene, but the results are disputed. For example, Callison and her co-workers⁵³ were unable to demonstrate any variation in the utilization of carotene for growth in rats, irrespective of whether or not lutein had been supplied in the diet.

May and McCreary⁵⁴ reported that the absorption of carotene is decreased in children suffering from celiac disease, especially during the active stage. Heymann⁵⁵ also demonstrated that a reduction in carotene utilization occurred in sepsis, pneumonia, and grippe which could not be attributed to the accompanying fever. Guggenheim⁵⁶ observed that the absorption rate of carotene is decreased after prolonged administration of the antimalarial drug, atabrine.

b. The Absorption of Carotenoids Other Than β -Carotene. There is some indication that carotenoids other than β -carotene may be absorbed. On the basis of bioassays designed to determine vitamin A biopotency, it can be stated that α - and γ -carotenes have approximately half the biopotency^{57,58} of the β -compound; pro- γ -carotene 41% of the potency of all-*trans*- β -carotene.⁵⁸ However, this evidence is not absolute, as it is possible that the provitamins A might first be split to vitamin A before absorption, and that only this simpler molecule is absorbed. On the other hand, the demonstration by Ganguly *et al.*⁵⁹ that minimal amounts of α -carotene as

⁴⁹ W. S. Hoffman and H. A. Dyniewicz, *Gastroenterology*, **5**, 512 (1945).

⁵⁰ K. C. D. Hickman and P. L. Harris, *Advances in Enzymology*, **6**, 469-524 (1946).

⁵¹ A. R. Kemmerer, G. S. Fraps, and J. DeMottier, *Arch. Biochem.*, **12**, 135-138 (1947).

⁵² B. Kelley and H. G. Day, *J. Nutrition*, **40**, 159-168 (1950).

⁵³ E. C. Callison, L. F. Hallman, W. F. Martin, and E. Orent-Keiles, *Arch. Biochem. Biophys.*, **32**, 407-413 (1951).

⁵⁴ C. D. May and J. F. McCreary, *J. Pediat.*, **18**, 200-209 (1941).

⁵⁵ W. Heymann, *Am. J. Diseases Children*, **51**, 273-283 (1936).

⁵⁶ K. Guggenheim, *J. Nutrition*, **46**, 141-149 (1952).

⁵⁷ H. J. Deuel, Jr., E. Sumner, C. Johnston, A. Polgár, and L. Zechmeister, *Arch. Biochem.*, **6**, 157-161 (1945).

⁵⁸ L. Zechmeister, J. H. Pinckard, S. M. Greenberg, E. Straub, T. Fukui, and H. J. Deuel, Jr., *Arch. Biochem.*, **23**, 242-245 (1949).

⁵⁹ J. Ganguly, J. W. Mehl, and H. J. Deuel, Jr., *J. Nutrition*, **50**, 59-72 (1953).

well as of β -carotene are present in the livers and eggs of chickens following their administration constitutes an *a priori* proof for the absorption of these compounds.

Lycopene, which has the same empirical formula as β -, α -, and γ -carotenes ($C_{40}H_{56}$), but in which the β -ionone rings have both been ruptured, is likewise absorbable, as evidenced by its presence in human fat,⁶⁰ human liver,⁶¹ human blood serum,⁶² as well as in the milk fat of the cow.⁶³ There is no evidence as to the ease and speed of absorption of lycopene as compared with β -carotene, since no experimental data are available on this point, and lycopene is not a provitamin A. The latter fact precludes a comparison of absorption rates based upon the speed of accumulation of vitamin A in the liver.

Several of the oxycarotenoids are absorbable, as determined by indirect methods. Cryptoxanthin, $C_{40}H_{55}OH$, which is a provitamin A, is not absorbed as such by the rat,⁶⁴ and chicken,⁶⁵ but is converted to vitamin A in the wall of the intestine. However, the presence of cryptoxanthin in cow's butter⁶³ and in the blood serum⁶⁶ of cattle proves that this species can absorb it unchanged. In the case of hens, the presence of lutein in egg yolk has been demonstrated,⁶⁷ this indicates that the carotenol is not entirely broken down to vitamin A in the gut wall by this species, and that some unchanged portion must escape the intestinal barrier, to gain entrance into the body.

There is likewise considerable evidence for the absorption of the two isomeric dihydroxycarotenols, lutein and zeaxanthin. Neither of these acts as a provitamin A. However, the presence of lutein has been reported in human fat,^{60,68} in the livers, obtained postmortem,⁶⁹ from presumably normal men, as well as from patients who had died from a variety of illnesses.⁶¹ The dietary origin is proved by the fact that it was found in the human skin after large amounts of winter (Hubbard) squash (*Cucurbita maxima*) had been consumed.^{70,71} Zechmeister and Tuzson⁷² reported a

⁶⁰ L. Zechmeister and P. Tuzson, *Z. physiol. Chem.*, **231**, 259-264 (1935).

⁶¹ L. Zechmeister and P. Tuzson, *Z. physiol. Chem.*, **234**, 241-244 (1935).

⁶² E. V. Dániel and G. J. Scheff, *Proc. Soc. Exptl. Biol. Med.*, **33**, 26-30 (1935).

⁶³ A. Gillam and I. M. Heilbron, *Biochem. J.*, **29**, 834-836 (1935).

⁶⁴ S. M. Patel, J. W. Mehl, and H. J. Deuel, Jr., *Arch. Biochem.*, **30**, 103-109 (1951).

⁶⁵ J. Ganguly and H. J. Deuel, Jr., Unpublished experiments, 1952.

⁶⁶ A. E. Gillam and M. S. ElRidi, *Biochem. J.*, **29**, 2465-2468 (1935).

⁶⁷ A. E. Gillam and I. M. Heilbron, *Biochem. J.*, **29**, 1064-1067 (1935).

⁶⁸ L. Zechmeister and T. Tuzson, *Z. physiol. Chem.*, **225**, 189-195 (1934); **226**, 255-257 (1934); **234**, 235-240 (1935).

⁶⁹ H. Willstaedt and T. Lindquist, *Z. physiol. Chem.*, **240**, 10-18 (1936).

⁷⁰ H. Sugimoto and K. Ueno, *Bull. Chem. Soc. Japan*, **6**, 221-228 (1931).

⁷¹ M. Ansai, *Japanese J. Med. Sci.*, **V. Pathology**, **1**, 175-190 (1931).

⁷² L. Zechmeister and P. Tuzson, *Bull. soc. chim. biol.*, **17**, 1110-1118 (1935).

high concentration of this chromogen in the fat of a woman suffering from jaundice. The presence of lutein has likewise been noted in milk and butter,⁷³ in the serum of cattle,⁶⁶ in chicken fat,⁶⁸ and in egg yolk.⁶⁷ Lutein has likewise been observed as a pigment in the feathers of the wild Madeira canary (*Serinus canarius*) and of the yellow-hammer (*Emberiza citrinella*).⁷⁴ Zechmeister and Tuzson⁷⁵ also recorded the occurrence of this carotenol in the green water-frog (*Rana esculenta*). The presence of zeaxanthin has been recorded in most of the sources in which lutein has been observed, including human fat,^{23,68} human liver,⁶⁹ hen's egg,^{24,76,77} in the egg of the Atlantic spiny dogfish (*Squalus acanthius*).⁷⁸ as well as in the feathers of the wild canary (*Serinus canarius*)⁷⁴ and in the green water-frog.⁷⁵ Zechmeister and Tuzson⁷² observed the presence of another oxy-carotenoid, capsanthin, in human fat. It is considered that the presence of this chromogen under these conditions is adventitious; presumably it occurs in the fat of Hungarian peasants, chiefly as a result of their high consumption of paprika.⁶⁵ The latter is the principal natural source of capsanthin. Although a wide variety of the *cis*- and *trans*-isomers of the several carotenoids which are provitamins A, may also serve as a source of vitamin A,⁷⁹ there is no direct evidence that these carotenoids may be absorbed as such. However, it seems quite reasonable to the author that this transformation of the molecule need not preclude the absorption of such compounds.

In the preceding discussion, evidence was adduced for the absorption of many carotenoids simply by demonstrating that these carotenoids have been isolated from animal or human tissues. The acceptance of the evidence, at least as regards the higher animals, must predicate a failure of synthesis of the carotenoids *in situ*. There is no evidence for this synthesis, especially in the case of those carotenoids which serve as provitamins A. In the absence of synthesis, the only other explanation is that they have gained entrance to the body by absorption from the gastrointestinal tract. Unfortunately, the data furnish no precise values as to the rate of absorption. For a further discussion of the absorption of carotene and of the related carotenoids, the reader is referred to *The Lipids*, Vol. II, pages 282-300.

⁷³ A. E. Gillam, I. M. Heilbron, R. A. Morton, G. Bishop, and J. C. Drummond, *Biochem. J.*, **27**, 878-888 (1933).

⁷⁴ H. Brockmann and O. Völker, *Z. physiol. Chem.*, **224**, 193-215 (1934).

⁷⁵ L. Zechmeister and P. Tuzson, *Z. physiol. Chem.*, **238**, 197-203 (1936).

⁷⁶ R. Kuhn, A. Winterstein, and E. Lederer, *Z. physiol. Chem.*, **197**, 141-160 (1931).

⁷⁷ H. H. Strain, *Chromatographic Adsorption Analysis*, Interscience, New York, London, 1945.

⁷⁸ H. v. Euler and U. Gard, *Arkiv Kemi Mineral. Geol.*, **B 10**, No. 19, 1-6 (1931).

⁷⁹ L. Zechmeister, *Vitamins and Hormones*, **7**, 57-81 (1949).

c. Factors Altering the Digestibility of β -Carotene and of Other Carotenoids. The same factors which influence the absorption of β -carotene are operative in respect to the digestibility of this provitamin. The digestibility of the carotenoids is usually not complete, even when they are given in minimal quantities, and some unabsorbed carotenoid is usually present in the feces. Because of the limited capacity for absorption, the value for the coefficient of digestibility decreases with an increased intake of the carotenoids. Thus, Kemmerer and Fraps⁸⁰ found that, when rats were fed β -carotene in dehydrated alfalfa at levels of 1, 10.5, and 20 parts per million, digestibilities of 43%, 22%, and 18 to 23%, respectively, were observed. When chickens were employed as test animals, figures of 69% and 29% were obtained for the digestibility when the diet contained, respectively, 1 and 20 parts of β -carotene per million.

A number of factors other than the quantity of β -carotene administered affect the extent of digestibility. When β -carotene was given with a fat-free diet to human subjects, Kreula⁸¹ noted a digestibility of only 10%, as contrasted with a value of 50% when the provitamin was administered in a single dose in olive oil, and one of 70% when the oil solution was given in divided doses. Kemmerer and Fraps⁸⁰ observed digestibility figures of 51% and 22%, respectively, for β -carotene when administered to rats in oil or in dried alfalfa fed with a fat-free regimen. Wilson and collaborators⁸² reported a digestibility for carotene of 90% when given in raw or cooked carrots or in cooked spinach with fat, while the value was only 50% when it was given without fat. Other values for the digestibility of β -carotene include the following: from carrots (van Eekelen and Pannevis⁸³) 1%, and from spinach, 5%; from purees of tomatoes, carrots, spinach, and carrot meal (With⁸⁴), 30 to 60%; from carrots (Accessory Food Factors Committee⁸⁵) 25%, and from spinach, 40%. According to Kreula and Virtanen,⁸⁶ 20% of carotene in finely-grated raw carrot was lost during passage through the digestive tract, 5% of that from coarsely-grated raw or cooked carrots.

⁸⁰ A. R. Kemmerer and G. S. Fraps, *J. Nutrition*, **16**, 309-315 (1938).

⁸¹ M. S. Kreula, *Biochem. J.*, **41**, 269-273 (1947).

⁸² H. E. C. Wilson, S. M. Das-Gupta, and B. Ahmad, *Indian J. Med. Research*, **24**, 807-811 (1937).

⁸³ M. van Eekelen and W. Pannevis, *Nature*, **141**, 203-204 (1938).

⁸⁴ T. K. With, *Absorption, Metabolism, and Storage of Vitamin A and Carotene*, Munksgaard, Copenhagen, 1940, and Milford, London, 1940, pp. 70, 90 ff.; also cited by M. S. Kreula, *Biochem. J.*, **41**, 269-273 (1947), p. 269.

⁸⁵ *Report of the Vitamin A Subcommittee of the Accessory Food Factors Committee*, Lister Inst., *Rep. Med. Research Council (Brit.)*, 1939-1945, pp. 107-109; H. M. Stationery Office, London, 1945.

⁸⁶ M. S. Kreula and A. I. Virtanen, *Upsala. Läkerefören, Förh.*, **45**, 355-362 (1939); *Chem. Abst.*, **34**, 5897 (1940).

The stimulating effect of thyroxine and the depressing effect of thiouracil on the carotene utilization of lactating cows and goats is even more strikingly evident in the data on digestibility than in those concerned with absorption. Thus, Chanda *et al.*⁸⁷ report the following average digestibility figures for β -carotene: *Cows*, control group, 57.4%, thyroxine-treated group, 71.5%, and thiouracil-treated group, 49.4%; *Goats*, control group, 61.5%, thyroxine-treated group, 74.0%, and thiouracil-treated group, 50.9%.

Comparatively few studies have been made on the digestibility of carotenoids other than β -carotene. Johnson and Baumann⁸⁷ did report experiments on the digestibilities of some of the more common carotenoids in rats when they were fed in cottonseed oil in amounts of 2 to 78 μ g. daily. The digestibilities were reported as 64% for cryptoxanthin, 60% for β -carotene, and 46% for α -carotene. At these low intakes, the percentage of excretion did not vary with the dose, nor was any depressing effect exerted by lutein on digestibility when the latter carotenol was fed simultaneously with the several provitamins A. These values are fairly close to those obtained earlier by Fraps and Meinke,⁸⁸ which were 57 and 64% for β -carotene and for α -carotene, respectively, when dissolved in oil. "Neo- β -carotene," which is a stereoisomeric form of β -carotene, was found to have about the same coefficient of digestibility as the natural all-trans- β -carotene.⁸⁸ The digestibility of the carotenoids is discussed on pages 300 to 303, Vol. II, of *The Lipids*.

(2) Vitamins A

a. Normal Absorption of Vitamins A. Under normal conditions, vitamin A is much more readily absorbed from the gastrointestinal tract than are its provitamins. This is because of its smaller molecular size, and probably also because it possesses a primary alcohol group which can readily form an ester with fatty acids. Moreover, in contradistinction to carotene, vitamin A is well utilized when given by the parenteral route.⁸⁹ Sobel and co-workers⁹⁰ reported that, when an aqueous dispersion of vitamin A was given to cows by the intravenous route, the increase in vitamin A in the milk was approximately fifteen times that noted when the same amount of the vitamin was administered orally, in oil.

Hydrolysis of vitamin A esters to the free alcohol is believed to be a neces-

⁸⁷ R. M. Johnson and C. A. Baumann, *Arch. Biochem.*, 19, 493-501 (1948).

⁸⁸ G. S. Fraps and W. W. Meinke, *Arch. Biochem.*, 6, 323-327 (1945).

⁸⁹ E. L. Sexton, J. W. Mehl, and H. J. Deuel, Jr., *J. Nutrition*, 31, 299-320 (1946).

⁹⁰ A. E. Sobel, A. Rosenberg, and E. Engel, *J. Nutrition*, 48, 183-192 (1952).

sary preliminary change occurring in the intestine prior to absorption.⁹¹ Gray *et al.*⁹¹ are of the opinion that an esterase in the intestinal juice is responsible for this hydrolysis. Ganguly⁹² reported the presence of an enzyme in pig pancreas capable of hydrolyzing vitamin A esters. A vitamin A esterase in rat liver homogenates was also described by McGugan and Laughland.⁹³ This observation was confirmed by Ganguly and Deuel,⁹⁴ who made the further discovery that the enzyme occurs in the microsomal fraction of the homogenate, while the nuclear, mitochondrial, and supernatant fractions contain no enzyme. On the other hand, Krause and Powell⁹⁵ reported that the activity of the liver in hydrolyzing vitamin A acetate was not concentrated in any particular fraction, but that it was distributed throughout the various fractions of rat liver homogenate. Homogenates of intestine and kidney are able to esterify vitamin A. Liver homogenates and their cellular components are unable to carry out this reaction. In an extension of his studies, Ganguly⁹⁶ again demonstrated quantitatively the presence of the esterase in the microsomal fraction of rat liver homogenates, and its absence in the other fractions. The pancreatic esterase is largely concerned with the hydrolysis of the vitamin A esters in the lumen of the gut.⁹²

A number of experiments support the theory that an intestinal hydrolysis of vitamin A esters takes place. Not only does a gradual increase in vitamin A alcohol occur in the intestinal contents as digestion proceeds, after vitamin A ester is given to rats, but a concomitant increase in vitamin A, largely in the form of the free alcohol, occurs in the intestinal wall.⁹¹ Eden and Sellers⁹⁷ confirmed the occurrence of this hydrolysis in the case of calves and sheep, and the data of Clausen⁹⁸ and of Popper and Volk⁹⁹ offer further support for this hypothesis. Lovern and co-workers^{100,101} suggest that, in the case of the fishes, the vitamin A alcohol may mediate the absorption of the fatty acids by a mechanism involving repeated ester-

⁹¹ E. L. Gray, K. Morgareidge, and J. D. Cawley, *J. Nutrition*, **20**, 67-74 (1940).

⁹² J. Ganguly, Thesis, Univ. Reading (Gt. Britain), 1949; cited by J. Ganguly and H. J. Deuel, Jr., *Nature*, **172**, 120-121 (1953), p. 120.

⁹³ W. A. McGugan and D. H. Laughland, *Arch. Biochem. Biophys.*, **35**, 428-434 (1952).

⁹⁴ J. Ganguly and H. J. Deuel, Jr., *Nature*, **172**, 120-121 (1953).

⁹⁵ R. F. Krause and L. T. Powell, *Arch. Biochem. Biophys.*, **44**, 57-62 (1953).

⁹⁶ J. Ganguly, *Arch. Biochem. Biophys.*, **52**, 186-189 (1954).

⁹⁷ E. Eden and K. C. Sellers, *Biochem. J.*, **45**, xxxiii (1945); **46**, 261-266 (1950).

⁹⁸ S. W. Clausen, *Harvey Lectures*, **38**, 199-226 (1943).

⁹⁹ H. Popper and B. W. Volk, *Arch. Pathol.*, **38**, 71-75 (1944).

¹⁰⁰ J. A. Lovern and R. A. Morton, *Biochem. J.*, **33**, 330-337 (1939).

¹⁰¹ J. A. Lovern, T. H. Mead, and R. A. Morton, *Biochem. J.*, **33**, 338-343 (1939).

fication and hydrolysis. However, Gray *et al.*⁹¹ were not able to demonstrate any such phenomenon in the rat.

b. Factors Altering the Absorption of Vitamins A. Many of the conditions which affect the absorption of the carotenoids are likewise operative in the case of the vitamins A. However, the factors which tend to have a deleterious effect upon absorption are usually quantitatively less active in relation to vitamin A than in the case of the carotenoids.

The rate of absorption of vitamin A is influenced by the age of the person receiving the vitamin. Thus, Sobel *et al.*¹⁰² observed a markedly diminished rate of utilization in the newborn baby as compared with that of children over one year of age and of adults. Young infants were found to exhibit a low vitamin A tolerance, as contrasted with that of older children.¹⁰³ On the other hand, vitamin A was absorbed at a diminished rate in the aged (69 to 89 years).¹⁰⁴

The rate of absorption of vitamin A was found by Reifman and co-workers¹⁰⁵ to be a function of the concentration fed. Thus, the amount of vitamin A absorbed, expressed in International Units per 100 sq. cm. of body surface per hour increased progressively from between 4.2 and 6.5, when the dosage was 100 I.U. per 100 sq. cm., to 10,140 at a maximum dosage of 1,000,000 I.U. On the other hand, bile plays a minor role in the absorption of vitamin A. According to Schmidt and Schmidt,¹⁰⁶ and Irvin and co-workers,²⁸ vitamin A can still be efficiently absorbed by choledochocolostomized vitamin A-deficient rats, in contrast to the failure of β -carotene to be utilized under similar conditions. Fat, likewise, is less essential as a solvent for vitamin A than for carotene, not only in rats,^{105, 107} but also in the fowl¹⁰⁸ and in man.⁸² Mineral oil, also, has a less deleterious effect upon the absorption of vitamin A than it has upon that of carotene. In fact, some workers¹⁰⁹⁻¹¹¹ failed to demonstrate that mineral oil ex-

¹⁰² A. E. Sobel, L. Besman, and B. Kramer, *Am. J. Diseases Children*, 77, 576-591 (1949).

¹⁰³ A. E. Sobel, S. P. Gottfried, B. Kramer, and L. Besman, *Abstracts, 110th Meeting, Am. Chem. Soc., Div. Biol. Chem.*, Chicago, Sept. 11, 1946, 28 B-29 B.

¹⁰⁴ H. A. Rafsky and B. Newman, *Gastroenterology*, 10, 1001-1006 (1948).

¹⁰⁵ A. G. Reifman, L. F. Hallman, and H. J. Deuel, Jr., *J. Nutrition*, 26, 33-42 (1943).

¹⁰⁶ W. Schmidt and C. L. A. Schmidt, *Univ. Calif. (Berkeley) Pub. Physiol.*, 7, 211-221 (1930).

¹⁰⁷ N. K. De, *Indian J. Med. Research*, 24, 751-766 (1937).

¹⁰⁸ W. C. Russell, M. W. Taylor, H. A. Walker, and L. J. Polskin, *J. Nutrition*, 24, 199-211 (1942).

¹⁰⁹ E. Moness and W. G. Christiansen, *J. Am. Pharm. Assoc.*, 18, 997-998 (1929).

¹¹⁰ A. C. Curtis and P. B. Horton, *Am. J. Med. Sci.*, 200, 102-107 (1940).

¹¹¹ B. Alexander, E. Lorenzen, R. Hoffman, and A. Garfinkel, *Proc. Soc. Exptl. Biol. Med.*, 65, 275-278 (1947).

erted any harmful effect whatsoever upon the utilization of vitamin A. Others^{5,112-115} reported some decrease in absorption of vitamin A when this dietary adjunct was consumed, although it was quantitatively of a lower order than that exerted upon β -carotene.

One interesting factor which may influence the speed of absorption of vitamin A is its form of administration, *i.e.*, whether it is given as the ester or as the free alcohol. This factor cannot apply to the utilization of carotene and of other carotenoids, although it might also be operative in the case of the carotenols. If one accepts the hypothesis that hydrolysis of the vitamin A esters is a prerequisite to absorption, then any conditions which retard or inhibit hydrolysis will likewise decrease the rate of absorption of this vitamin. Thus, Week and Sevigne¹¹⁶ noted that the vitamin A storage in the liver of rats following given doses of vitamin A in the form of the free alcohol, the acetate, and the natural ester, was fairly uniform when corn oil or castor oil (which is completely utilized by the rat) was used as the diluent; the alcohol and acetate were stored to a slightly greater degree than the natural ester. However, when the several forms of vitamin A were dissolved in a poorly utilized vehicle such as jojoba oil, the proportion of vitamin A deposited was one-third less when it was fed as the natural ester than when it was given as the free alcohol or acetate. The variation in vitamin A storage, as influenced by the form in which it was given and the vehicle in which it was dissolved, was even more strikingly illustrated in the case of the chicken.¹¹⁷ Man exhibits a similar pattern for utilization of the several forms¹¹⁸; thus, after 134,000 μ g. of vitamin A had been given in 50 g. of margarine, the best absorption, as determined by the vitamin A tolerance test, was observed in the case of the free alcohol. This was followed by the acetate, and the poorest showing was noted in the case of the natural ester. March, English, and Biely¹¹⁹ confirmed the fact that vitamin A alcohol and acetate are better utilized than the natural ester when given in cottonseed oil. This difference was also shown by the variation in the amount of vitamin A present in the intestinal wall when the several forms of vitamin A were employed. These workers attribute the poorer effect

¹¹² O. Andersen, *Klin. Wochschr.*, 18, 499-502 (1939).

¹¹³ T. K. With, *Nord. Med.*, 3, 2468-2470 (1939).

¹¹⁴ O. Andersen, *Acta Paediat.*, 24, 422-427 (1939).

¹¹⁵ M. C. Smith and H. S. Spector, *Univ. Arizona, Coll. Agr., Ariz. Agr. Expt. Sta., Tech. Bull. No. 84*, 373-395 (1940).

¹¹⁶ E. F. Week and F. J. Sevigne, *J. Nutrition*, 39, 251-257 (1949).

¹¹⁷ E. F. Week and F. J. Sevigne, *J. Nutrition*, 39, 233-250 (1949).

¹¹⁸ E. F. Week and F. J. Sevigne, *J. Nutrition*, 40, 563-576 (1950).

¹¹⁹ B. E. March, E. English, and J. Biely, *Arch. Biochem. Biophys.*, 36, 259-268 (1952).

of the palmitate, as compared to the free alcohol and acetate, to interference by the oil with the absorption of the natural ester through the intestinal wall, rather than to differences in stability of vitamin A. However, when vitamin A was administered in an aqueous carrier, the reverse situation was obtained, namely the natural ester and the acetate were better utilized than the free vitamin A alcohol.¹¹⁹

Emulsifying agents such as lecithin enhance the absorption of vitamin A, not only in the case of rats,¹²⁰ but also in the case of cattle;¹²¹ lecithin also improves the absorption of vitamin A by human subjects suffering from sprue, but not in patients with liver disease.¹²² It has likewise been reported by a number of workers,^{102,103,123-125} on the basis of higher blood levels and greater storage of vitamin A in rats, guinea pigs, and children, that aqueous solutions of vitamin A are more effective than are oil solutions of the vitamin. The variations are apparently not related to the form of vitamin A administered, as the improved vitamin utilization when aqueous suspensions were used was equally striking irrespective of whether the free alcohol or the palmitate was employed.¹²⁶ When PSM (Tween 80, polyoxyethylene sorbitan monooleate) was employed as the dispersing agent, Krantz¹²⁷ observed maximum blood values, in normal and in diseased patients, 400 to 500% greater than in the tests in which the vitamin A was administered in oil solution. In 1952, Sobel¹²⁸ completed a comprehensive review of vitamin A absorption.

The thyroid gland also exerts an effect upon vitamin A utilization. Conversely, vitamin A, when given in large doses, may exhibit an anti-thyroid action.¹²⁹ The storage of vitamin A was found to be highest in thyroidectomized rats, intermediate in those treated with thyroxine, and least in normal control animals.¹³⁰ The unexpected result in the normal

¹²⁰ G. C. Esh and T. S. Sutton, *J. Nutrition*, **36**, 391-404 (1948).

¹²¹ G. C. Esh, T. S. Sutton, J. W. Hibbs, and W. E. Krauss, *J. Dairy Sci.*, **31**, 461-478 (1948).

¹²² D. Adlersberg, S. Kann, A. P. Maurer, K. Newerly, W. Winternitz, and H. Sobotka, *Gastroenterology*, **10**, 822-830 (1948).

¹²³ J. M. Lewis, O. Bodansky, J. Birmingham, and S. Q. Cohlan, *J. Pediat.*, **31**, 496-508 (1947).

¹²⁴ B. Kramer, A. E. Sobel, and S. P. Gottfried, *Am. J. Diseases, Children*, **73**, 543-553 (1947).

¹²⁵ A. E. Sobel, M. Sherman, J. Lichtblau, S. Snow, and B. Kramer, *J. Nutrition*, **35**, 225-238 (1948).

¹²⁶ B. M. Kagan, D. A. Jordan, and D. S. Gerald, *J. Nutrition*, **40**, 275-279 (1950).

¹²⁷ J. C. Krantz, Unpublished observations; cited in *Nutrition Revs.*, **7**, 205-207 (1949).

¹²⁸ H. E. Sobel, *Vitamins and Hormones*, **10**, 47-67 (1952).

¹²⁹ D. P. Sadhu and S. Brody, *Am. J. Physiol.*, **149**, 400-403 (1947).

¹³⁰ C. B. Heimer, H. L. Maslow, and A. E. Sobel, *J. Nutrition*, **38**, 345-352 (1949).

group is probably to be ascribed to the fact that these animals grew normally, as compared with those in the other two groups, and used a considerably large portion of the vitamin A for the growth process, so that less remained for storage.

Guggenheim⁵⁶ reported that the rate of absorption, not only of carotene but also of vitamin A, decreased following the prolonged administration of atabrine. For an extended discussion of the absorption of vitamin A, the reader is referred to *The Lipids*, Vol. II, pages 310 to 320.

3. Changes in the Carotenoids and Vitamin A in the Intestinal Wall

(1) *The Transformation of β -Carotene and of Other Provitamins A into Vitamin A*

a. β -Carotene. Until recently, on the basis of the classic experiments of Moore,¹⁹ it had been generally accepted that the liver is the main site, if indeed not the only one, where carotene can be changed to vitamin A in the animal body. However, a number of recent observations have led to a revision of this concept. In the first place, Sexton and co-workers⁸⁹ reported experimental data which led them to postulate that, in rats, the intestinal wall is a probable site for the transformation of carotene to vitamin A. A number of facts support this hypothesis. Thus, no appreciable quantity of carotene appears in the livers of rats, irrespective of the amount of this provitamin in the diet. Moreover, when β -carotene was administered in oil solution parenterally by the intrasplenic route, the provitamin was deposited in the liver as such. Apparently, it could not be changed to vitamin A under these conditions, since it could not counteract the symptoms of avitaminosis, and the rats receiving the vitamin A-deficient diet died, displaying typical symptoms of vitamin A-deficiency, in spite of the fact that their livers were found to contain sufficient β -carotene to supply the necessary vitamin A requirement for a year, had the liver been able to transform the β -carotene into vitamin A. No intrinsic impairment of the carotene utilization occurred in these animals; the avitaminosis symptoms promptly cleared up when the provitamin A was given orally. Lease *et al.*¹³¹ recorded a similar absence of utilization of carotene when given parenterally. A third experimental observation which argues for the conversion of carotene to vitamin A in the gut wall is the fact that vitamin A accumulates in the intestinal wall after carotene feeding, before any increase can be noted in the liver. Higher levels of vitamin A were found

¹³¹ J. G. Lease, E. J. Lease, H. Steenbock, and C. A. Baumann, *J. Lab. Clin. Med.*, 27, 502-510 (1941-1942).

in the intestinal wall than in the liver for four hours after the carotene feeding.¹³² Mattson¹³³ proved that the material accumulating in the intestinal wall under these conditions was actually vitamin A. The intestinal wall was shown to be a site of conversion of β -carotene to vitamin A not only in the case of the rat,^{132,134-136} but also in the guinea pig,¹³⁷ the pig,^{134,138} and in rabbits, goats, and sheep,¹³⁹ all of which belong to Group C. Stallcup and Herman¹⁴⁰ proved that the intestine is one of the sites for the conversion of carotene to vitamin A in dairy calves (which belong to Group B). Thompson *et al.*¹⁴¹ and Cheng and Deuel¹⁴² also noted that the intestine is the site for the conversion of carotene in the chicken, which we have assigned to Group D.

Further support for the thesis that the intestinal wall is the site of the conversion of carotene into vitamin A can be derived from *in vitro* studies. Thus, Wiese and her collaborators¹⁴³ demonstrated the synthesis of vitamin A from carotene when suspensions of the chromogen in Tween 80 (PSM) were allowed to incubate in rat intestine. Rosenberg and Sobel¹⁴⁴ also confirmed these results with rat intestine, while McGillivray¹⁴⁵ demonstrated that highly significant increases occurred in sections of sheep intestine which had been incubated with β -carotene. The fact that thoracic lymph of a wide variety of animals belonging to Group C contains no appreciable carotene, but only increased proportions of vitamin A, after the provitamin A is introduced orally, affords additional excellent proof of the synthesis of vitamin A from carotene at some stage between the lumen of the gut and the lacteals of the villi. The intestinal mucosa would obviously be the most probable site for this change. The reader is referred to the section on transport (see page 455) for a further discussion of the relation of vitamin A in the lymph and blood to administered carotene.

¹³² F. H. Mattson, J. W. Mehl, and H. J. Deuel, Jr., *Arch. Biochem.*, **15**, 65-73 (1947).

¹³³ F. H. Mattson, *J. Biol. Chem.*, **176**, 1467-1468 (1948).

¹³⁴ S. Y. Thompson, J. Ganguly, and S. K. Kon, *Brit. J. Nutrition*, **1**, v (1947); **3**, 50-78 (1949).

¹³⁵ J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **41**, xlv (1947); **43**, 512-518 (1948).

¹³⁶ A. B. McCoord and S. W. Clausen, *Abst. 114th Meeting, Am. Chem. Soc., Div. Biol. Chem.*, Washington, Aug. 30, 1946, p. 16C.

¹³⁷ L. Woytkiw and N. C. Esselbaugh, *J. Nutrition*, **43**, 451-458 (1951).

¹³⁸ M. E. Coates, S. W. Thompson, and S. K. Kon, *Biochem. J.*, **46**, xxx-xxxi (1950).

¹³⁹ T. W. Goodwin and R. A. Gregory, *Biochem. J.*, **43**, 505-512 (1948).

¹⁴⁰ O. T. Stallcup and H. A. Herman, *J. Dairy Sci.*, **33**, 237-242 (1950).

¹⁴¹ S. Y. Thompson, M. E. Coates, and S. K. Kon, *Biochem. J.*, **46**, xxx (1950).

¹⁴² A. L. S. Cheng and H. J. Deuel, Jr., *J. Nutrition*, **41**, 619-628 (1950).

¹⁴³ C. E. Wiese, J. W. Mehl, and H. J. Deuel, Jr., *Arch. Biochem.*, **15**, 75-79 (1947).

¹⁴⁴ A. Rosenberg and A. E. Sobel, *Arch. Biochem. Biophys.*, **44**, 320-325 (1953).

¹⁴⁵ W. A. McGillivray, *Australian J. Sci. Research, B4*, 370-376 (1951).

b. Provitamins A Other Than β -Carotene. Provitamins A other than β -carotene must also be broken down to vitamin A in the gut wall; otherwise they would appear in the lymph and blood of the rat and other animals having a similar carotenoid metabolism. Since α - and γ -carotenes do not appear as such in the liver of rats after they have been fed, but only in the form of increased levels of vitamin A, it is natural to postulate a metabolic fate similar to that of β -carotene. Ganguly *et al.*¹⁴⁶ proved that rats have the ability to transform α -carotene to vitamin A in the gut wall. Moreover, since practically no chromogen is deposited in the liver of hens after the ingestion of pro- α -carotene, which is a potent provitamin A,¹⁴⁷ one may conclude that this carotenoid can also be metabolized in the intestinal wall. Cryptoxanthin, also, was found by Patel *et al.*⁶⁴ to undergo transformation to vitamin A in the small intestine of rats, while Ganguly and Deuel⁶⁵ observed a similar phenomenon in the case of chickens.

c. Carotenoids Other Than Provitamins A. Little is known about the metabolic fate of carotenoids other than the provitamins A, since no end-products comparable to vitamin A have been identified. One can differentiate between the behavior of the carotenoids in animals which have β -carotene in the blood and their action in animals whose blood does not contain this component. In the case of man and cattle, which represent the first class of animals in respect to their carotenoid metabolism, the presence of characteristic provitamins A, including α - and γ -carotenes, pro- γ -carotene, and cryptoxanthin, in the tissues and in metabolic products such as milk and eggs, speaks for the absorption of these compounds in unchanged form. Moreover, the widespread distribution of carotenoids not convertible to vitamin A, as for instance zeaxanthin, lutein, lycopene, and capsanthin, in the tissues of man and of cattle and, in some cases, in those of chickens, indicates that these carotenoids, also, are readily absorbed unchanged.

Since β -carotene can be converted to vitamin A in the intestinal wall not only by animals in Group C but also by those in Group A and even by chickens (Group D), some explanation for the variations in metabolism other than the capacity for conversion in the intestinal wall is necessary. Goodwin²⁰ suggested that the differences can be explained on the basis of differences in efficiency of the enzyme systems in the several species. Thus, Group C animals would have the most efficient carotenase system, "because no carotene overflows into the general circulation." In this group,

¹⁴⁶ J. Ganguly, N. Krinsky, and H. J. Deuel, Jr., Unpublished experiments, 1955.

¹⁴⁷ H. J. Deuel, Jr., J. Ganguly, B. K. Koe, and L. Zechmeister, *Arch. Biochem. Biophys.*, 33, 143-149 (1951).

the xanthophylls are oxidized completely or to colorless products which are not as yet known, and this oxidation presumably occurs in the intestinal wall. Group *B* would thus have a less efficient carotenase system than exists in Group *C*, but the "xanthophyll oxidase" continues to be as effective as in Group *C*. Finally, in Group *A*, both the carotenase and the xanthophyll oxidase systems are relatively inefficient. In the case of the fowl (referred to as Group *D*), the carotenase enzyme system works rather well, but no "xanthophyll oxidase" occurs. It is possible that another transformation may occur in the gut wall of Group *D*, namely the transformation of carotene to the carotenols (oxycarotenoids). For a further discussion of the distribution of the carotenoids, the reader is referred to another section of this chapter (see pages 476 to 520).

d. Sites Other Than the Intestinal Wall for the Conversion of Carotenoids into Vitamin A. Considerable variations in the results of experiments designed to demonstrate the conversion of carotene to vitamin A in the liver have been noted. Some of these discrepancies may well be attributed to the species variation in carotenoid metabolism to which reference has already been made. Zechmeister¹⁴⁸ recognized these variations in the metabolism of the carotenoids. On the basis of an extensive investigation of the carotene content of the livers of thirty-three mammals comprising twenty-one species, forty-one birds comprising thirty-six species, four reptiles belonging to two species, and of eight different human samples, Jensen and With¹⁴⁹ called attention to the fact that carotenoids were completely absent from many of the livers examined, or present only in small amounts. The variation in the amount of vitamin A and of carotenoids in the livers of the various species was considerable. No simple proportionality could be noted between the amount of vitamin A and carotene in the food ingested and the concentration in the liver. Considerable amounts of carotenoids were found in the livers of beasts of prey, birds, and herbivora. They also occurred in the case of chickens, horses, and humans.¹⁴⁹ On the other hand, the carotenoids have not been detected in appreciable amounts in the internal organs or blood of mice, rats, guinea pigs, pigs, sheep, or goats, although the wild roe deer (*Capreolus capraca*) stores a considerable amount. Of the wild birds examined, the shaggy-footed buzzard (*Archibuteo lapogus*) and the garganey (*Anas querquedula* or *Querquedula circia*) presented the highest vitamin A content, the Amazon parrot (*Amazona (Chrysotis) aestiva*) the lowest. Among the reptiles, no appreciable decrease in vitamin A stores was noted during hibernation or inani-

¹⁴⁸ L. Zechmeister, *Ergeb. Physiol.*, **39**, 117-191 (1937).

¹⁴⁹ H. B. Jensen and T. K. With, *Biochem. J.*, **33**, 1771-1786 (1939).

tion. The liver of a monitor lizard (*Varanus comodensis*), which had eaten almost nothing during a six-month voyage from Commodo Island to Denmark, contained large amounts of vitamin A. A cetacean, the grampus or "Risso's dolphin" (*Grampus griseus*) stores insignificant amounts of vitamin A. The mole (*Talpa europaea*) and the bat (*Vespertilio*, spp.) store a large amount. They subsist mainly on insects and worms; apparently insects are a rich source of vitamin A. Dogs and cats store a considerable amount, but not as much as do the wild fox (*Canis vulpes*) and fitchet or fitchew (*Mustela putorius*), which is a European polecat.

There is some evidence that cattle, which normally have high levels of blood and liver carotene, and which likewise may secrete large amounts of this provitamin into the milk, also have the capacity to convert carotene to vitamin A in the intestinal wall.¹⁴⁰ It would thus appear that the provitamins A occur in the blood and tissues of some species, not because the intestinal wall lacks the capacity for splitting carotene, but because a considerable proportion escapes the gut wall before it has been transformed into vitamin A.

It is generally agreed that some sites for changing carotene to vitamin A, other than the walls of the small intestine, must exist for species whose tissues and blood contain considerable proportions of carotenoids. The liver and reticuloendothelial (RE) systems have been cited as the most probable sites for this reaction.

Moore¹⁹ believes that he had demonstrated the presence of an enzyme, called "carotenase," in the liver of dogs; this enzyme catalyzed the carotene $\rightarrow$ vitamin A reaction. Positive results on vitamin A synthesis from carotene were also demonstrated by Pariente and Ralli,¹⁵⁰ who used the dog-liver carotenase, by v. Euler and Klusmann¹⁵¹ in cow's liver, and by Wilson *et al.*¹⁵² in the liver of rabbits. On the other hand, the results were completely negative in the case of the Atlantic spiny dogfish (*Squalus acanthius*) and black goby (*Gobius niger*), as cited by v. Euler and v. Euler,¹⁵³ and in the cat, according to Rea and Drummond,¹⁵⁴ or rabbit, reported by Drummond and MacWalter.¹⁵⁵ Olcott and McCann¹⁵⁶ did report positive evidence of the change in rat liver slices. However, these

¹⁵⁰ A. C. Pariente and E. F. Ralli, *Proc. Soc. Exptl. Biol. Med.*, **29**, 1209-1210 (1931-1932).

¹⁵¹ H. v. Euler and E. Klusmann, *Svensk Kem. Tidskr.*, **44**, 223-224 (1932).

¹⁵² H. E. C. Wilson, B. Ahmad, and B. M. Mazumdar, *Indian J. Med. Research*, **25**, 85-88 (1937).

¹⁵³ B. v. Euler and H. v. Euler, *Svensk Kem. Tidskr.*, **43**, 174-178 (1931).

¹⁵⁴ J. L. Rea and J. C. Drummond, *Z. Vitaminforsch.*, **41**, 177-183 (1932).

¹⁵⁵ J. C. Drummond and R. J. MacWalter, *Biochem. J.*, **27**, 1342-1347 (1933).

¹⁵⁶ H. S. Olcott and D. C. McCann, *J. Biol. Chem.*, **94**, 185-193 (1931).

latter investigators were able to demonstrate only a slight inflection in the absorption curve at 328 m μ , and they did not succeed in establishing any other proof of the presence of vitamin A. It would appear to the author that the liver affords an additional site, other than the gut wall, for the transformation of β -carotene into vitamin A in species whose tissues contain carotene; the possibility of this change in the livers of the animals not normally containing carotene in the tissues and blood must still be considered questionable.

Samaras and Hingerty¹⁵⁷ suggested that, in addition to the liver, the reticuloendothelial (R.E.) system plays a role in the conversion of carotene to vitamin A. When carotene was given parenterally, in aqueous solution, to normal rats, it was found to be converted to vitamin A within two hours. The rate of change was increased eighteen hours after the R.E. system in the normal rat had been blocked by the administration of trypan blue. It was believed that the dye stimulated the activity of the R.E. system. On the other hand, in the case of the vitamin A-deficient rat, the injection of the dye markedly reduced the ability of the rat to form vitamin A from carotene.

There is now increasing evidence that the conversion of carotene to vitamin A can occur in tissues other than the small intestine, not only in the case of the group of animals whose blood and tissues contain appreciable amounts of carotene, but also in the case of the types which normally do not have the carotenoids in the blood or tissues. Thus, Bieri and Sandman¹⁵⁸ observed growth in vitamin A-deficient rats when β -carotene, solubilized in water by means of Tween 40, was given by the intramuscular route in amounts as small as 1.6 μ g. daily. However, four to six times as much carotene was required to produce maximum growth when it was administered by the parenteral route as when it was given orally. These workers were able to obtain their results with aqueous suspensions of β -carotene, but not when the carotene was given in oil solution. This confirms the results of Sexton *et al.*⁸⁹ Bieri and Schultze¹⁵⁹ noted small amounts of vitamin A in the blood and kidneys of normal and of hypothyroid, vitamin A-depleted rats after the intramuscular injection of carotene solubilized with Tween 80, although no significant increase in liver vitamin A was observed. Under these conditions, the administration of thiouracil had little or no effect upon the vitamin A level. Bieri and Pollard¹⁶⁰ later reported that an aqueous suspension of β -carotene in Tween 40 pro-

¹⁵⁷ S. C. Samaras and D. J. Hingerty, *Am. J. Physiol.*, 159, 588-589 (1949).

¹⁵⁸ J. G. Bieri and R. P. Sandman, *Proc. Soc. Exptl. Biol. Med.*, 77, 617-619 (1951).

¹⁵⁹ J. G. Bieri and M. O. Schultze, *Arch. Biochem. Biophys.*, 34, 280-284 (1951).

¹⁶⁰ J. G. Bieri and C. J. Pollard, *Federation Proc.*, 12, 409 (1953).

vided better growth and longer survival when the carotene was given in a single dose intravenously than when the same amount of the provitamin was administered orally. The intestinal wall is not required for the transformation of intravenously-injected carotene in Tween 40 into vitamin A, as is indicated by the fact that the plasma vitamin A of vitamin A-deficient rats was markedly increased four to six hours after the administration of the provitamin, even when the rats had previously been subjected to the surgical removal of the small intestine.^{160,161} Kirschman and MacVicar¹⁶² later obtained a similar result with ilectomized sheep. Other positive reports of increased vitamin A in the plasma after parenterally-introduced carotene include those of Eaton and co-workers¹⁶³ and of Warner and Maynard¹⁶⁴ with calves, and of Kowalewski *et al.*¹⁶⁵ with dogs. Although Eaton *et al.*¹⁶³ could detect no concomitant increase in liver vitamin A, Kowalewski and associates¹⁶⁵ did note an increase of liver vitamin A in dogs two hours after the provitamin had been injected. Kon¹⁶⁶ also confirmed the fact that parenterally-introduced β -carotene can be converted to vitamin A. On the other hand, Church and co-workers¹⁶⁷ were unable to demonstrate any increase in the vitamin A content of the plasma or liver of Hereford calves which had received aqueous suspensions of carotene. However, in the case of sheep, these workers reported that an increase in plasma vitamin A followed intravenous injection of carotene. The vitamin A level in the blood rose from a control value of 21.4 $\mu\text{g. \%}$ to 34 $\mu\text{g. \%}$ one hour later, and to 47.9 $\mu\text{g. \%}$ two hours after the carotene injection. One can only conclude from these data that extra-intestinal sites exist for the conversion of carotene into vitamin A, not only in the species whose blood and tissues normally contain appreciable amounts of carotene, but also in those animals which do not ordinarily have appreciable quantities of the provitamin A in these sites.

e. Factors Affecting the Conversion of Carotene into Vitamin A. (a) *The Effect of the Thyroid Gland.* The effect of thyroxine and of thyroid preparations in stimulating the absorption of carotene, as determined by a

¹⁶¹ J. G. Bieri and C. J. Pollard, *Biochem. J.*, 55, xxxi-xxxii (1953).

¹⁶² J. C. Kirschman and R. MacVicar, *Federation Proc.*, 14, 439 (1955).

¹⁶³ H. D. Eaton, L. D. Matterson, L. Decker, C. F. Helmboldt, and E. L. Jungherr, *J. Dairy Sci.*, 34, 1073-1080 (1951).

¹⁶⁴ R. G. Warner and L. A. Maynard, *J. Animal Sci.*, 11, 780 (1952).

¹⁶⁵ K. Kowalewski, E. Henrotin, and J. van Geertruyder, *Acta gastro-enterol. belg.*, 14, 607-616 (1951); *Nutrition Revs.*, 13, 124 (1955).

¹⁶⁶ S. K. Kon, 1954. Private communication; cited by T. W. Goodwin, *The Chemical Pathology of Carotenoids*, in R. T. Williams, *The Chemical Pathology of Animal Pigments*, Biochem. Soc. Symposia, No. 12, Cambridge Univ. Press, 71-84 (1954), p. 72.

¹⁶⁷ D. C. Church, R. Mac Vicar, J. G. Bieri, F. H. Baker, and L. S. Pope, *J. Animal Sci.*, 13, 677-683 (1954).

decrease in the fecal excretion of carotene, was attributed by Goodwin²⁰ to a direct effect upon the carotene $\rightarrow$ vitamin A reaction, as well as to the possibility that it might exert a direct effect upon the absorption of the provitamin. It has been demonstrated that the thyroid does not exert any effect upon the stability of carotene in the intestine.

The interpretations of the experimental data have largely been formulated in terms of the first hypothesis. Thus, since von Noorden,¹⁶⁸ in 1907, suggested that carotenemia may be associated with a metabolic disorder, which was later ascribed by Anderson and Soley¹⁶⁹ to an alteration in thyroid function, a number of workers¹⁷⁰⁻¹⁷³ have also subscribed to this theory. Several other experiments have afforded confirmation of the commanding role which the thyroid plays on the carotene $\rightarrow$ vitamin A conversion. Thus, in the case of rabbits, xerophthalmia was noted eight to twenty months after thyroidectomy.¹⁷⁴ In the case of the goat, the carotene content of the milk increased, concomitantly with a decrease in vitamin A concentration, following the removal of the thyroid gland.¹⁷⁵ On the other hand, in the case of the cow, increased carotene was likewise found in thyroid-treated animals. This may be interpreted as due to a better absorption of dietary carotene.⁴⁰ Contrary to most opinions, von Euler and Klusmann¹⁷⁶ claimed that thyroxine exerted an inhibitory effect upon the growth promoting action of carotene. Drill and Truant¹⁷⁷ were unable to cure ocular symptoms of hypovitaminosis A in rats thyroidectomized by the administration of as much as 10 μ g. of β -carotene per day. However, these results are open to question, since Remington and co-workers¹⁷⁸ were able to effect a cure under these conditions within seven to nine days, with a daily dosage of only 0.6 μ g. of the provitamin. Goodwin¹⁷⁹ demonstrated the converse of the above, namely that carotene is more effectively utilized in the hyperthyroid rat than in the normal animal.

Further confirmation of a depression of the carotene $\rightarrow$ vitamin A reac-

¹⁶⁸ C. von Noorden, *Die Zuckerkrankheit*, 4th ed., Hirschwald, Berlin, 1907; cited by V. A. Drill, *Physiol. Revs.*, **23**, 255-379 (1943), p. 359.

¹⁶⁹ H. H. Anderson and M. H. Soley, *Am. J. Med. Sci.*, **195**, 313-318 (1938).

¹⁷⁰ M. G. Wohn and J. B. Feldman, *Endocrinology*, **24**, 389-396 (1939).

¹⁷¹ R. F. Escamilla, *J. Clin. Endocrinol.*, **2**, 33-35 (1942).

¹⁷² T. Mandelbaum, S. Candel, and S. Millman, *J. Clin. Endocrinol.*, **2**, 465-467 (1942).

¹⁷³ H. Wendt, *Klin. Wochschr.*, **14**, 9-14 (1935).

¹⁷⁴ M. M. Kunde, *Proc. Soc. Exptl. Biol. Med.*, **23**, 812 (1926).

¹⁷⁵ H. Fasold and E. R. Heidemann, *Z. ges. exptl. Med.*, **92**, 53-56 (1933).

¹⁷⁶ H. von Euler and E. Klusmann, *Z. physiol. Chem.*, **213**, 21-34 (1932).

¹⁷⁷ V. A. Drill and A. P. Truant, *Endocrinology*, **40**, 259-264 (1947).

¹⁷⁸ R. E. Remington, P. L. Harris, and C. L. Smith, *J. Nutrition*, **21**, 597-606 (1942).

¹⁷⁹ T. W. Goodwin, *Biochem. J.*, **43**, xliii-xliv (1948).

tion in hypothyroidism was obtained when the condition was produced by the use of the thyroid antagonist, thiouracil, rather than by thyroidectomy. Thus, following treatment with this drug, rats were found to have a lower than normal content of vitamin A in the liver after the administration of carotene.^{35,180} Moreover, carotene was found to be ineffective in removing the ocular symptoms caused by avitaminosis A under these conditions.¹⁸¹ The administration of desiccated thyroid powder corrected these abnormalities in carotene metabolism.

The level of plasma vitamin A ester has been used as a measure of the effect of the thyroid hormone upon the conversion of carotene to vitamin A. In normal blood, the vitamin A alcohol is believed to originate in the liver, and it usually has a fairly constant value;¹⁵² in contradistinction to this, the ester fraction is believed to represent the small amount of vitamin A *en route* from the intestinal wall to the liver, where it is stored as such until liberated into the blood stream as free vitamin A alcohol.^{182,183} Thus, the observation of Chanda and Owen¹⁸⁴ that the amount of vitamin A ester in the blood was increased in cows fed carotene when they were treated with either thyroxine or a thyrotropin preparation has been interpreted as evidence that the extra vitamin A originates from the carotene in the intestine.

A number of reports have failed to confirm the thyroid effect upon the carotene $\rightarrow$ vitamin A reaction. Thus, Wiese and her collaborators^{185,186} were unable to demonstrate variations in carotene metabolism in rats rendered hypothyroid by means of thiouracil. Morgan and Arnrich¹⁸⁷ are of the opinion that the normally functioning thyroid gland is not essential for carotene utilization in rats and dogs. Finally, a number of investigators¹⁸⁸⁻¹⁹⁰ have been unable to confirm the results of Kaplanskiĭ

¹⁸⁰ B. Kelley and H. G. Day, *J. Biol. Chem.*, **175**, 863-866 (1948).

¹⁸¹ J. M. Canadell and F. G. Valdecasas, *Experientia*, **3**, 35-36 (1947).

¹⁸² J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **43**, 109-114 (1948).

¹⁸³ R. Chanda, H. M. Clapham, and E. C. Owen, *Biochem. J.*, **60**, 391-399 (1955).

¹⁸⁴ R. Chanda and E. C. Owen, Unpublished results, 1954; cited by T. W. Goodwin, *The Chemical Pathology of Carotenoids*, in R. T. Williams, *The Chemical Pathology of Animal Pigments*, Biochem. Soc. Symposia, No. 12, Cambridge Univ. Press, 71-84, (1954), p. 76.

¹⁸⁵ C. E. Wiese, H. J. Deuel, Jr., and J. W. Mehl, *Proc. Soc. Exptl. Biol. Med.*, **66**, 213-214 (1947).

¹⁸⁶ C. E. Wiese, J. W. Mehl, and H. J. Deuel, Jr., *J. Biol. Chem.*, **175**, 21-28 (1948).

¹⁸⁷ A. F. Morgan and L. Arnrich, *Federation Proc.*, **12**, 424-425 (1953).

¹⁸⁸ H. R. Cama and T. W. Goodwin, *Biochem. J.*, **43**, xlv (1948).

¹⁸⁹ C. W. Lowry and J. R. Lowry, *Arch. Biochem.*, **26**, 287-290 (1950).

¹⁹⁰ C. E. Wiese, *The Site of Conversion of Carotene to Vitamin A in the Rat*, Thesis, Univ. So. Calif., Dept. Biochem. Nutrit., June, 1948.

and Balaba,¹⁹¹ who noted an *in vitro* conversion of carotene into vitamin A when thyroglobulin or iodinated casein was added to the incubation mixture. In spite of these negative reports, which may simply indicate that the conditions were unfavorable for the functioning of the thyroid hormone, one must conclude that the thyroid gland is active in increasing the availability of dietary carotene. The question still unanswered is whether this effect occurs as a result of an increased absorption of carotene or whether it should be ascribed to a more efficient conversion of carotene to vitamin A.

Goodwin²⁰ is inclined to believe that the beneficial effect of the thyroid hormone in reducing the loss of carotene in the feces is due to a better absorption of carotene. This would explain the increased milk carotene occurring in thyroid-treated cows⁴⁰ as due to a greater "overspill" from the gut wall. Such a condition would not be expected if the increased removal of carotene from the intestine resulted from a reinforced activity of carotenase. On the other hand, if the effect of the thyroid hormone were on the carotenase, one might assume that a "carotene-saturated" intestinal wall in a normal animal is rendered "unsaturated" after thyroid treatment because of an accelerated removal of carotene due to conversion to vitamin A.²⁰ This situation would allow for a greater absorption of carotene, but it would not be expected to result in an increased carotene content in the milk; in fact, the opposite situation should obtain.

One fact which has been cited³⁷ against the increased absorption theory and in favor of the carotenase activity hypothesis as an explanation for the effect of the thyroid on carotene metabolism is the finding of Johnson and Baumann³⁵ that the level of thyroid activity has no effect upon the amount of vitamin A laid down in the liver and kidney following vitamin A feeding. It should be recalled that the absorption of carotene and vitamin A poses two different questions. Under usual conditions, only 50% of carotene is absorbed,¹⁹² whereas vitamin A is almost completely utilized under similar circumstances. One would not expect to improve the conditions for absorption for a substance which is normally practically 100% absorbed, but it would be reasonable to expect improvement in the absorption of a poorly-utilized substance like carotene.

Another fact which is difficult to reconcile with the carotene absorption theory is that the thyroid has been known to stimulate absorption only in the case of substances, such as sugars, which can be phosphorylated. On

¹⁹¹ S. Kaplanskii and T. J. Balaba, *Biokhimiya*, 11, 327-331 (1946); *Chem. Abst.*, 41, 507 (1947).

¹⁹² T. W. Goodwin, *The Comparative Biochemistry of Carotenoids*, Chapman & Hall, London, 1952; *Carotenoids, Their Comparative Biochemistry*, Chem. Pub. Co., New York, 1954.

the other hand, amino acids, which are probably not phosphorylated during absorption, are unaffected by the thyroid hormone. On the basis of this reasoning, it is difficult to see how the thyroid could enhance the absorption of a hydrocarbon like carotene which cannot possibly be phosphorylated. Goodwin²⁰ concludes that, although the bulk of evidence suggests that the thyroid functions, in carotene metabolism, by increasing the absorption of the provitamin, one cannot completely rule out the possibility that this effect is to be ascribed to the influence of the hormone on the carotenase activity. Drill¹⁹³ reviewed the subject of the thyroid in relation to carotene metabolism up to 1943. He concluded that "all the evidence to date (1943) indicates that, in the absence of the thyroid gland, carotene is not metabolized to vitamin A."

(b) *The Effect of Hypervitaminosis A.* In addition to hyperthyroidism, hypervitaminosis A has been found to have a profound effect on the metabolism of carotene. In this case there is no evidence to challenge the hypothesis that the carotene $\rightarrow$ vitamin A reaction proceeds at an augmented rate. Thus, Deuel and co-workers^{194,195} reported that, when massive doses of vitamin A (70,000 to 1,690,000 μ g. per day) were given to cows, the plasma carotene and also that present in the milk gradually decreased to minimum values, coincident with a marked increase of vitamin A in the milk, in spite of the fact that the cows were maintained on a uniform diet. The depressing action of large doses of vitamin A on the carotene content of the blood of calves and milk of cows was confirmed by Jensen *et al.*,¹⁹⁶ by Fountaine and Bolin,¹⁹⁷ by Blaxter, Kon, and Thompson,¹⁹⁸ and by Eaton *et al.*¹⁹⁹

A similar physiologic response to excessive doses of vitamin A was observed in the case of chickens by the Deuel group.^{200,201} Thus, when the vitamin A supplement present in the food was increased from 0 to 60,000

¹⁹³ V. A. Drill, *Physiol. Revs.*, **23**, 355-379 (1943).

¹⁹⁴ H. J. Deuel, Jr., N. Halliday, L. Hallman, C. Johnston, and A. J. Miller, *J. Nutrition*, **22**, 303-313 (1941).

¹⁹⁵ H. J. Deuel, Jr., L. F. Hallman, C. Johnston, and F. Mattson, *J. Nutrition*, **23**, 567-579 (1942).

¹⁹⁶ C. Jensen, P. D. Boyer, P. H. Phillips, I. W. Rupel, and N. S. Lundquist, *J. Dairy Sci.*, **25**, 931-937 (1942).

¹⁹⁷ F. C. Fountaine and D. W. Bolin, *J. Dairy Sci.*, **27**, 155-158 (1944).

¹⁹⁸ K. L. Blaxter, S. K. Kon, and S. Y. Thompson, *J. Dairy Research*, **14**, 225-230 (1946); *Chem. Abst.*, **41**, 3845 (1947).

¹⁹⁹ H. D. Eaton, A. A. Spielman, J. K. Loosli, J. W. Thomas, C. L. Norton, and K. L. Turk, *J. Dairy Sci.*, **30**, 795-802 (1947).

²⁰⁰ H. J. Deuel, Jr., M. C. Hrubetz, F. H. Mattson, M. G. Morehouse, and A. Richardson, *J. Nutrition*, **26**, 673-685 (1943).

²⁰¹ F. H. Mattson and H. J. Deuel, Jr., *J. Nutrition*, **25**, 103-112 (1943).

$\mu\text{g.}$ per pound, serum carotene decreased progressively from 143.2 to 37 $\mu\text{g. \%}$, that present in the yolks declined from 32.3 ± 2.0 to $8.4 \pm 0.5 \mu\text{g./g.}$, liver carotene dropped from 7.8 to 2.7 $\mu\text{g./g.}$, while the carotenoid content of the body fat was usually, but not always depressed. Marked increases in the vitamin A content of the serum, yolks, liver, and body fat occurred concomitantly with the decreased carotenoid content. The extent of the augmentation of vitamin A under these conditions far exceeded that which would have occurred solely as the result of the increased carotene breakdown. These data are of interest in connection with those reported by Hammon and Harshaw,²⁰² in which it was demonstrated that fortified codliver oil possesses a factor which prevents the deposition of xanthophyll in the shanks and skins of chicks. Proof that this phenomenon is related to the increased vitamin A intake was furnished by Rubin and Bird,²⁰³ who reported that the pigment-depressing factor was, in fact, vitamin A. Moreover, a similar carotenoid-depressing effect of massive doses of vitamin A was observed in the case of turkeys, by Gurcay *et al.*,²⁰⁴ and also in ducks, by Rigdon and co-workers.²⁰⁵ On the other hand, Hrubetz, Deuel, and Hanley²⁰⁶ failed to note a similar depression in the carotene content of human milk when massive doses of vitamin A were given to lactating mothers, although an increased vitamin A content in the milk was observed.

No satisfactory explanation for the carotene-depressing effect of massive doses of vitamin A has been propounded. Because of the slowness of the rate at which the hypocarotenoid condition develops (three to four weeks), it has been suggested that it occurs concomitantly with the development or increased action of an enzyme system which destroys vitamin A. Because of the similarity of the molecular structures of provitamins A and of vitamin A, the former would probably also be attacked by this enzymatic mechanism. It is also possible that the change of β -carotene $\rightarrow$ vitamin A may be stimulated in this condition. It has not been determined whether or not vitamin A exerts its effect on the carotene metabolism in conjunction with, or because of its action on the thyroid gland.

(c) *Miscellaneous Factors.* Shaw *et al.*²⁰⁷ observed a decrease in the plasma carotene of cows when ground soybeans were added to the diet in

²⁰² J. C. Hammon and H. M. Harshaw, *Poultry Sci.*, **20**, 437-444 (1941).

²⁰³ M. Rubin and H. R. Bird, *Science*, **103**, 584-586 (1946).

²⁰⁴ R. Gurcay, R. V. Boucher, and E. W. Callenbach, *J. Nutrition*, **41**, 565-582 (1950).

²⁰⁵ R. H. Rigdon, J. C. Rude, and J. G. Bieri, *Arch. Pathol.*, **52**, 299-314 (1951).

²⁰⁶ M. C. Hrubetz, H. J. Deuel, Jr., and B. J. Hanley, *J. Nutrition*, **29**, 245-254 (1945).

the amount of 30%; the vitamin A in both liver and plasma was even more markedly decreased. Goodwin²⁰ suggests that this effect may be due to an active factor in the lipid fraction.²⁰⁸

In addition to thyroxine, the cortical hormones may influence the carotene $\rightarrow$ vitamin A change.²⁰⁹ It is suggested that this effect is mediated by the adrenal gland, since it does not occur when cortisone is injected into adrenalectomized rats

Almquist and Maurer²¹⁰ observed that certain antibiotics (a mixture of chlortetracycline and diamine penicillin, and chlortetracycline alone) increased the extent of the carotene $\rightarrow$ vitamin A reaction in chicks, as judged by the level of liver vitamin A. Burgess *et al.*²¹¹ also noted an increased level of plasma vitamin A and serum carotenoids in twenty-six-day-old chicks which received penicillin G in the diet, but it was uncertain whether the results were related to an increased transformation of carotene to vitamin A or to an improvement in the absorption of carotene. Aureomycin was shown to increase the vitamin A stores in the livers of rats, but the reason for the effect is not clear. High²¹² ascribed the increased vitamin A content of the liver when aureomycin was given to an improvement in the carotene $\rightarrow$ vitamin A reaction, since the group receiving carotene with the antibiotic had higher levels of vitamin A than did the control group, while the deposition of vitamin A in the liver in the group receiving pre-formed vitamin was not affected by the presence or absence of aureomycin in the diet. On the other hand, Murray and Campbell²¹³ observed that aureomycin exerted a beneficial effect in the case of vitamin A-depleted rats receiving vitamin A acetate, as compared with those not receiving the antibiotic; the difference was determined by the vaginal smear assay. The survival time of the female rats, but not of the males, was increased by aureomycin.

Vitamin B₁₂, also, was reported by High and Wilson²¹⁴ to increase the deposition of vitamin A from carotene. Because vitamin B₁₂ was ineffective in producing this beneficial effect in the presence of penicillin, while it

²⁰⁷ J. C. Shaw, L. A. Moore, and J. F. Sykes, *J. Dairy Sci.*, **34**, 176-180 (1951).

²⁰⁸ R. L. Squibb, C. Y. Cannon, and R. S. Allen, *J. Dairy Sci.*, **31**, 421-427 (1948); **32**, 565-569 (1949).

²⁰⁹ I. Clark and R. W. Colburn, *Federation Proc.*, **12**, 190 (1953).

²¹⁰ H. J. Almquist and S. Maurer, *Arch. Biochem. Biophys.*, **55**, 297-298 (1955).

²¹¹ R. C. Burgess, M. Gluck, G. Brisson, and D. H. Laughland, *Arch. Biochem. Biophys.*, **33**, 339-340 (1951).

²¹² E. G. High, *Federation Proc.*, **14**, 437 (1955).

²¹³ T. K. Murray and J. A. Campbell, *Federation Proc.*, **14**, 446 (1955).

²¹⁴ E. G. High and S. S. Wilson, *J. Nutrition*, **50**, 203-212 (1953).

was active in the case of aureomycin, it was concluded that the two antibiotics have different effects.²¹²

Vitamin E (α -tocopheryl acetate) and certain fat-soluble antioxidants related to hydroquinone were found by High *et al.*²¹⁵ to depress the conversion of carotene to vitamin A in rats. However, Almquist and Maurer²¹⁰ were unable to show that the antioxidant DPPD (*N,N'*-diphenyl-*p*-phenylenediamine) exerted any effect upon the conversion of carotene to vitamin A. Herbert and Morgan²¹⁶ found that the addition of 0.5 or 1.0 mg. of α -tocopherol daily to the diet of rats fed on diets containing generous amounts of vitamin A or carotene consistently failed to augment the storage of vitamin A in the liver to any great extent.

Octachloronaphthalene (OCN), which is a widely used insecticide, markedly decreased the storage of vitamin A in the liver.²¹⁷ Thus, a progressive decrease in the liver vitamin A of rats was found with diets containing 0, 0.05, 0.2, and 0.5% of OCN, namely 37.6, 13.9, 8.4, and 4.6 μ g./g., respectively. When β -carotene was employed as the source of vitamin A, no difference in liver vitamin A was observed after the provitamin had been injected intravenously in aqueous solution, whether or not OCN was included in the diet. However, when carotene was given orally, the drug apparently decreased vitamin A storage.

Rosenberg and Sobel²¹⁸ demonstrated that the amount of hepatic stores of vitamin A originating after a standard dose of β -carotene was much less in rats suffering from alloxan diabetes than in normal controls. It is believed that this phenomenon obtains because alloxan depresses the conversion of carotene to vitamin A.

(2) The Esterification of Vitamin A

It is generally agreed that an enzyme exists in the intestinal wall which functions in the esterification of vitamin A. This hypothesis is supported by the fact that the vitamin A present in the chyle of the rat and pig appears to a considerable extent in the form of vitamin A ester, irrespective of whether its source was ingested vitamin A alcohol, vitamin A ester, or one of the provitamins A. Thompson *et al.*¹³⁴ observed that about 50% of the vitamin A in the intestinal wall was in the form of its ester when it

²¹⁵ E. G. High, H. C. Smith, Jr., H. H. Taylor, and S. S. Wilson, *J. Biol. Chem.*, **210**, 681-686 (1954).

²¹⁶ J. W. Herbert and A. F. Morgan, *J. Nutrition*, **50**, 175-190 (1953).

²¹⁷ J. G. Bieri and R. J. Edwards, *Federation Proc.*, **14**, 428 (1955).

²¹⁸ A. Rosenberg and A. E. Sobel, *Arch. Biochem. Biophys.*, **44**, 326-329 (1953).

arose from any one of the three sources noted above. In later studies,²¹⁹ a figure of 75% was indicated as the approximate proportion of vitamin A ester in the total vitamin A of the intestinal wall. Glover and co-workers¹⁸² found evidence of active esterification of vitamin A alcohol, both in the intestinal mucosa and also in the subcutaneous tissue. In sharp contrast to the occurrence of vitamin A in the intestinal wall largely in the form of the ester, that in the plasma of fasting animals is almost completely in the form of the free alcohol.^{118,220} Vitamin A in the liver occurs mainly in the esterified form.²²¹ However, following the administration of large doses of vitamin A, the major proportion of the elevated plasma vitamin A level is in the form of the ester.¹¹⁸ Krause and Alberghini²²² demonstrated the presence of a vitamin A esterase in the serum and plasma, but not in the cellular elements of man, rats, and rabbits.

(3) *The Reduction of the Retinenes to Vitamins A*

An important reaction which occurs in the wall of the intestine involves the reduction of retinene, and retinene₁ to the corresponding vitamins A. Ball, Goodwin, and Morton²²³ were the first to prove that vitamin A aldehyde and the purple-colored pigment, retinene, present in the retina, are similar compounds. Although it is realized that the reduction of retinenes to the vitamins A may occur in places other than the intestinal wall (including the retina itself), this reaction may serve an important purpose in the gut wall, particularly if vitamin A aldehyde is an intermediate product in the conversion of carotene to vitamin A.

Most of the available information concerning the vitamin A alcohol $\rightleftharpoons$ vitamin A aldehyde (retinene) reaction was developed in relation to the visual systems. Wald^{224,225} is responsible for the clarification of our understanding of the nature of the light-sensitive carotenoid proteins, which are proteins bearing prosthetic groups to which they owe their color and their sensitivity to light. Three types of compounds have been described, namely rhodopsin and porphyropsin which occur in the rods, and iodopsin which is present in the cones. When exposed to light, all three carotenoid-

²¹⁹ S. Y. Thompson, R. Braude, M. E. Coates, A. T. Cowie, J. Ganguly, and S. K. Kon, *Brit. J. Nutrition*, **4**, 398-421 (1950).

²²⁰ J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **41**, 94-96 (1947).

²²¹ E. LeB. Gray, K. C. D. Hickman, and E. F. Brown, *J. Nutrition*, **19**, 39-46 (1940).

²²² R. F. Krause and C. Alberghini, *Arch. Biochem.*, **25**, 396-400 (1950).

²²³ S. Ball, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **42**, 516-523 (1948).

²²⁴ G. Wald, *Vitamins and Hormones*, **1**, 195-227 (1943).

²²⁵ G. Wald, *Harvey Lectures*, **41**, 117-160 (1945-1946).

proteins break down to the protein and retinene pigment. According to Wald *et al.*,²²⁴⁻²²⁶ these compounds differ from each other as follows:

Retinene₁ was identified as ordinary vitamin A aldehyde (vitamin A₁ aldehyde), while retinene₂ was later shown to be vitamin A₂ aldehyde.²²⁷ For a more complete discussion of the chemical nature and the functioning of these systems, the reader is referred to a later section of this chapter (see page 569).

a. The Identity of Retinene Reductase and Alcohol Dehydrogenase. "Retinene reductase" is the name which Wald and Hubbard²²⁸ first applied to the enzyme system in the retina which transfers two hydrogen atoms to the aldehyde group of retinene, transforming it to the primary alcohol group of vitamin A. The enzyme was shown to consist of an apoprotein, which works together with the coenzyme, cozymase (coenzyme I, or DPN). The reaction, with DPN as the coenzyme, would be as follows:

The Transformation of Retinene₁ to Vitamin A₁

Retinene reductase was early recognized to be similar to *alcohol dehydrogenase*. The latter enzyme is known to occur in a wide variety of tissues including liver, kidney, and intestine. Lutwak-Mann²²⁹ pointed out that this enzyme, together with cozymase as a coenzyme, catalyzes the equilibrium between various alcohols and their aldehydes.

Considerable evidence now exists which proves that retinene reductase and alcohol dehydrogenase are the same. For example, Bliss²³⁰ reported that crude preparations of liver dehydrogenase catalyze the equilibrium between retinene and vitamin A. Hubbard and Wald²³¹ have confirmed this observation, using a crystalline alcohol dehydrogenase prepared from

²²⁶ G. Wald, P. K. Brown, and P. H. Smith, *Federation Proc.*, **11**, 304-305 (1952).

²²⁷ K. P. Farrar, J. C. Hamlet, H. B. Henbest, and E. R. H. Jones, *J. Chem. Soc.*, **1952**, 2657-2668.

²²⁸ G. Wald and R. Hubbard, *J. Gen. Physiol.*, **32**, 367-389 (1948-1949).

²²⁹ C. Lutwak-Mann, *Biochem. J.*, **32**, 1364-1374 (1938).

²³⁰ A. F. Bliss, *Biol. Bull.*, **97**, 221-222 (1949); *Arch. Biochem. Biophys.*, **31**, 197-204 (1951).

²³¹ R. Hubbard and G. Wald, *Proc. Nat. Acad. Sci., U.S.*, **37**, 69-79 (1951).

horse liver. Yudkin²³² made the converse observation, *i.e.*, that the retinal enzyme of the frog oxidizes ethyl alcohol to acetaldehyde. Wald²³³ is now also of the opinion that alcohol dehydrogenase and retinene reductase are identical; he suggests that retinene reductase should be referred to by the more general designation. The same enzyme functions with both the rhodopsin and the porphyropsin system, in one case reducing retinene₁ to vitamin A₁ and in the second instance changing retinene₂ to vitamin A₂. Wald^{234,235} showed that both systems are interchangeable and that both have the same coenzyme.

Two alcohol dehydrogenase systems are known, both of which catalyze the equilibrium between ethyl alcohol and acetaldehyde. One of the dehydrogenase systems is present in animal tissues, while the second occurs in yeast. Bonnichsen²³⁶ crystallized the first type from horse liver, while Negelein and Wulff²³⁷ and Racker²³⁸ prepared the second variety in crystalline form from yeast. According to Bonnichsen²³⁹ and Bliss,²⁴⁰ the yeast enzyme is inactive in catalyzing the retinene $\rightarrow$ vitamin A reaction. For a further discussion of the nature of these enzyme systems, the reader is referred to the article by Wald, in Sebrell and Harris, *The Vitamins*, Vol. I.²³³

b. The Role of Alcohol Dehydrogenase in Carotene Utilization. The alcohol dehydrogenase system in the gut wall is believed to play a part in the conversion of carotene to vitamin A. Glover and co-workers¹⁸² suggested that vitamin A aldehyde is an intermediate in the carotene $\rightarrow$ vitamin A reaction.

The formation of vitamin A aldehyde has been repeatedly demonstrated in the *in vitro* oxidation of β -carotene. Thus, Hunter and Williams²⁴¹ noted that small amounts (0.4 to 0.5%) of this aldehyde were formed when β -carotene was oxidized with hydrogen peroxide in a glacial acetic acid solvent. A yield of as much as 30% of vitamin A aldehyde was obtained

²³² W. Yudkin, Unpublished experiments; cited by G. Wald, *Biochemical Systems*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 1954, p. 67.

²³³ G. Wald, *Biochemical Systems*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 1954, pp. 59-87.

²³⁴ G. Wald, *Science*, 109, 482-483 (1949).

²³⁵ G. Wald, *Biochim. et Biophys. Acta*, 4, 215-228 (1950).

²³⁶ R. K. Bonnichsen, *Acta Chem. Scand.*, 4, 715-716 (1950).

²³⁷ E. Negelein and H. J. Wulff, *Biochem. Z.*, 293, 351-389 (1937).

²³⁸ E. Racker, *J. Biol. Chem.*, 184, 313-319 (1950).

²³⁹ R. K. Bonnichsen, personal communication; cited by R. Hubbard and G. Wald, *Proc. Nat. Acad. Sci., U.S.*, 37, 69-79 (1951), p. 75.

²⁴⁰ A. F. Bliss, *Arch. Biochem. Biophys.*, 31, 197-204 (1951).

²⁴¹ R. F. Hunter and N. E. Williams, *J. Chem. Soc.*, 1945, 554-556.

when β -carotene was oxidized with hydrogen peroxide-osmium tetroxide.²⁴² Meunier and associates^{243,244} recorded a 60% yield of vitamin A aldehyde on oxidizing β -carotene on solid manganese dioxide. Ball and co-workers²²³ and Wald²⁴⁵ likewise employed the latter method for transforming vitamin A to retinene. This type of oxidation is referred to as a "chromatographic oxidation," in which a solid acts both as the adsorbent and as the reagent. It is suggested that this may mimic the specificity and the directness of an enzymatic reaction. All of these results obtained *in vitro* tend to support the hypothesis that vitamin A aldehyde is an intermediate in the conversion of carotene to vitamin A.

Since β -carotene is insoluble in aqueous media, while the enzyme systems are water-soluble, a question arises as to how a reaction can be effected between the enzyme and the substrate. It is suggested by Wald²³³ that the carotenoid becomes water-soluble by combining with a plasma protein, as is evident from the pioneer study of Palmer and Eckles,²⁴⁶ who prepared "carotoalbumin" from cattle serum; this was shown to be a protein- β -carotene complex. The relationship between species and the ability of a carotenoid to be absorbed may be related to the possibility that such a combination exists.^{148,233} This has also been suggested more recently by Ganguly *et al.*²⁴⁷ Ball *et al.*²⁴⁸ reported that retinene combines spontaneously with a variety of proteins, while Wald²³³ noted that retinene in the retina and in tissues is combined with alcohol dehydrogenase and with opsin. Finally, in the further metabolism of β -carotene in the intestinal wall, the vitamin A alcohol formed may be esterified in the gut wall as described in the previous section.

Thus, it is suggested by Wald²³³ that β -carotene, probably in a water-soluble complex with protein, is oxidized by carotene-oxidase to retinene. This chromogen is reduced by the alcohol-dehydrogenase system to vitamin A.

²⁴² N. L. Wendler, C. Rosenblum, and M. Tishler, *J. Am. Chem. Soc.*, **72**, 234-239 (1950).

²⁴³ P. Meunier, J. Jouannetau, and G. Zwingelstein, *Compt. rend.*, **231**, 1170-1171 (1950).

²⁴⁴ P. Meunier, *Rev. intern. vitaminol. (Intern. Z. Vitaminforsch.)*, **23**, 21-36 (1951).

²⁴⁵ G. Wald, *J. Gen. Physiol.*, **31**, 489-504 (1947-1948).

²⁴⁶ L. S. Palmer and C. H. Eckles, *J. Biol. Chem.*, **17**, 223-236 (1914).

²⁴⁷ J. Ganguly, N. I. Krinsky, J. W. Mehl, and H. J. Deuel, Jr., *Arch. Biochem. Biophys.*, **38**, 275-282 (1952).

²⁴⁸ S. Ball, F. D. Collins, R. A. Morton, and A. L. Stubbs, *Nature*, **161**, 424-426 (1948).

4. The Transport of Carotenoids and of Vitamins A from the Intestine

Two routes are possible for the transport of nutrients from the intestine to the internal organs; the first of these is *via* the mesenteric blood vessels and the portal vein to the liver, while the second route involves the lacteals in the intestinal wall, the mesenteric lymphatics, and the thoracic duct, from which the contents are poured into the subclavian vein to gain access to the general circulation. In the case of the triglycerides, it has been proved many times that the lymphatics are the chief routes of transport, if not, in fact, the only path of travel. There seems to be some evidence that the triglycerides composed of the short-chain acids, and these short-chain acids themselves, may employ the portal vein as the route of transport.

(1) *Transport in the Lymph*

It has been definitely proved that both β -carotene (in those species in which the unchanged carotenoid is present in the blood) and vitamin A are transported exclusively by way of the lymphatic system. Drummond, Bell, and Palmer,²⁴⁹ who studied this problem in a case of chylothorax, were able to recover administered vitamin A quantitatively in the chyle of the patient, while carotene was recovered only to the extent of 20% after this provitamin A had been ingested. The failure to account more quantitatively for the provitamin A may well have been due to the fact that a large portion of the provitamin A was converted to vitamin A in the wall of the gut, and was transported from the gut wall as such.

An investigation of the composition of lymph after the feeding of β -carotene has provided cogent proof that the carotenoid must be broken down to vitamin A in the intestinal wall in many species. Thus, it was demonstrated by Thompson and co-workers³¹ that no carotene was to be found in the systemic or portal blood of pigs or of normal or vitamin A-deficient rats after the feeding of carotene, although a concomitant increase in vitamin A in the lymph occurred under such conditions. An increase in vitamin A has been noted in the mesenteric lymph nodes of rats³¹ and of pigs¹³⁴ after the feeding of carotene. Woytkiw and Esselbaugh¹³⁷ reported an increase in the vitamin A content of the lymph of guinea pigs when either carotene or vitamin A was fed. No carotene could be detected in the lymph of any of these species. A high content of vitamin A has likewise been reported in the intestinal lymph of sheep¹⁴⁵ after carotene feeding, as well as in the intestines of bullocks under similar dietary conditions.

²⁴⁹ J. C. Drummond, M. E. Bell, and E. T. Palmer, *Brit. Med. J.*, 1935, I, 1208-1210.

The latter result is of particular interest, since the blood of the bullock normally contains carotene. This corroborates the opinion that the conversion of carotene into vitamin A occurs to some extent even in the case of species which absorb carotene in unchanged form.

On the other hand, no carotene or vitamin A is transported from the intestine *via* the portal route after carotene feeding in the case of guinea pigs¹³⁷ or in that of sheep and goats.²⁵⁰ Moreover, Thompson *et al.*²¹⁹ reported that the level of vitamin A in systemic and in portal blood of pigs increased at approximately the same rate after carotene or vitamin A had been administered in peanut oil. In fact, Eden and Sellers²⁵¹ recorded a higher content of vitamin A in systemic than in the portal blood of bullocks immediately after preformed vitamin A had been given. Another proof of the fact that transport of vitamin A occurs solely by way of the lymphatics and not by the portal vein is that no increase occurs in the level of vitamin A in the blood or liver after the feeding of carotene when the lymph is diverted and prevented from emptying into the blood stream.²¹⁹ The vitamin A present in lymph after the administration of β -carotene is present almost exclusively in ester form.^{134,219}

Both the pathway of transport and the extent of esterification of vitamin A are the same whether vitamin A is given as the alcohol or as the ester, and are similar to those observed after the administration of carotene. As already noted, the fact that a quantitative recovery of administered vitamin A could be demonstrated in the lymph of the patient suffering from chylothorax²⁴⁹ constitutes proof that the lymphatics are the sole route of transport from the intestine to the liver. Moreover, a number of investigators^{134,139,246,249,251,252} proved that the portal vein does not transport preformed vitamin A from the intestine to the liver and other internal organs. Radice and Herraiz²⁵³ presented the only evidence for transport of vitamin A by the portal route; they observed a fluorescence of portal blood similar to that of lymph, which was attributed to the presence of vitamin A. However, the bulk of evidence is against the hypothesis that the portal vein serves as an avenue for the transport of vitamin A after the administration of carotene or vitamin A.

5. Carotenoids and Vitamins A in the Blood

Although the portal circulation does not serve as the route of transfer of the carotenoids or of vitamins A from the gut wall to the internal organs,

²⁵⁰ T. W. Goodwin, A. D. Dewar, and R. A. Gregory, *Biochem. J.*, **40**, lx-lxi (1946).

²⁵¹ E. Eden and K. C. Sellers, *Biochem. J.*, **42**, xlix (1948).

²⁵² E. Eden and K. C. Sellers, *Biochem. J.*, **44**, 264-267 (1949).

²⁵³ J. G. Radice and M. L. Herraiz, *Rev. asoc. méd. argentina*, **287-292** (1947).

the blood stream in general appears to be the pathway of transfer of the provitamins A and of the vitamins A in the animal after absorption has been completed. The postprandial levels of carotene and of vitamin A reflect to some extent the reserve of these components stored in the tissues.

(1) *β -Carotene and Other Carotenoids in the Blood*

β -Carotene occurs in the blood of the species of animals which do not convert this provitamin A completely to vitamin A in the walls of the gut. However, β -carotene occurs in the blood stream in these species only when it is obtained from exogenous sources. If none of the pigment is present in the diet over an extended period, the amount stored in the tissues is gradually depleted, so that eventually the proportion in the blood decreases or entirely disappears. A number of investigators have demonstrated the presence of β -carotene in human blood^{69, 254, 255} and in that of cattle.^{21, 246, 256, 257}

Carotenoids other than β -carotene have been found in greater or lesser amounts in the blood. The presence of carotenoids must be considered as adventitious, since they reflect the composition of the diet. However, a more important consideration in relation to the presence of the several carotenoid components is species. In the case of man (Group A), all of the dietary carotenoids are present in the blood. On the other hand, in the case of cattle (Group B), carotene forms the major component, and only minor amounts of the xanthophylls are present. In the animals in Group C, no carotenoids appear in the blood, irrespective of their presence in the diet, inasmuch as they cannot be absorbed unchanged. Thus, no carotenoids occur in the blood of pigs, rats, and mice, even when they are present in the diet. In the case of chickens and birds, which have been referred to earlier as Group D, only carotenols are present in the blood.

In the case of man, in addition to β -carotene, lycopene^{62, 258} appears in the blood. Small amounts of xanthophylls are found in the blood of cows,^{66, 246} but not in that of newborn calves.²⁵⁹ Although Palmer and Eckles^{246, 259} considered that the latter components were free, more recent work has established the fact that they occur in human blood in esterified

²⁵⁴ H. Willstaedt and T. K. With, *Z. physiol. Chem.*, **253**, 40-46 (1938).

²⁵⁵ J. W. Mehl, Personal communication, 1944; also cited by F. R. N. Gurd, J. L. Oncley, J. T. Edsall, and E. J. Cohn, *Lipoproteins*, General discussion Faraday Soc., No. 6, 70-74, Aberdeen Univ. Press, 1949, p. 73.

²⁵⁶ B. v. Euler, H. v. Euler, and H. Hellström, *Biochem. Z.*, **203**, 370-384 (1928).

²⁵⁷ J. Ganguly, J. W. Mehl, and H. J. Deuel, Jr., *J. Nutrition*, **50**, 73-84 (1953).

²⁵⁸ E. V. Dániel and T. Béres, *Z. physiol. Chem.*, **238**, 160-162 (1936).

²⁵⁹ L. S. Palmer and C. H. Eckles, *J. Biol. Chem.*, **17**, 211-222, 237-243 (1914).

form.^{260,261} The provitamin A, cryptoxanthin, has also been noted under some conditions.⁶⁶ The level of plasma carotenoid is considerably higher in cows than in bulls.^{66,262} Carotene is the predominating carotenoid in the blood plasma of cows and horses.^{263,264} even after the feeding of large amounts of xanthophylls. Although Palmer and Eckles ascribed the comparative absence of the xanthophyll fraction in cow plasma to its destruction in the gut wall,^{246,259} Zechmeister and Tuzson⁶⁸ are of the opinion that its non-appearance is due to a selectivity in absorption whereby the carotenes but not the xanthophylls gain entrance to the blood stream. This hypothesis is based upon the fact that a higher proportion of xanthophylls occurs in horse dung than was present in the fodder.⁶³

Peirce²⁶⁵ recorded levels of 2 $\mu\text{g.}/100$ ml. for carotene in the blood of sheep in Australia during the dry season, and values as high as 16 $\mu\text{g. } \%$ after green fodder, while Paulsen *et al.*²⁶⁶ found values in the range of 0 to 17 $\mu\text{g.}/100$ ml. for sheep in South Dakota. However, in striking contrast to these results, a number of workers have been unable to demonstrate any appreciable amount of carotene in the plasma of sheep.^{20,139,257,267} Carotenemia did not occur in the case of rabbits and guinea pigs given intravenous injections of this substance,²⁶⁸ and only traces of carotene are normally present in the plasma of dogs.²⁶⁹ In the case of hedgehogs (*Erinaceus europaeus*), only 5% of the plasma carotenoids are xanthophylls.²⁷⁰

In the case of animals which do not accumulate carotenoids, the blood contains neither carotenes nor xanthophylls. Thus, carotene has been found to be absent from the portal and systemic blood of goats.¹³⁹ Even after high doses of carotene, this pigment did not appear in the blood of rats,^{271,272} swine,^{273,274} or rabbits.¹³⁹ The fate of ingested xanthophylls in

²⁶⁰ H. Süllmann and A. Vischer, *Biochem. Z.*, **274**, 16-20 (1934).

²⁶¹ F. Verzár, H. Süllmann, and A. Vischer, *Biochem. Z.*, **274**, 7-15 (1934).

²⁶² T. S. Sutton and P. A. Soldner, *J. Dairy Sci.*, **28**, 859-867 (1945).

²⁶³ L. S. Palmer, *J. Biol. Chem.*, **23**, 261-279 (1915).

²⁶⁴ L. S. Palmer, *J. Biol. Chem.*, **27**, 27-32 (1916).

²⁶⁵ A. W. Peirce, *Australian J. Exptl. Biol. Med. Sci.*, **23**, 295-303 (1945); **24**, 231-240 (1946); **25**, 112-117 (1947).

²⁶⁶ T. M. Paulsen, R. J. Hilmoe, and A. L. Moxon, *Proc. S. Dakota Acad. Sci.*, **24**, 81-84 (1944).

²⁶⁷ A. L. Pope, P. H. Phillips, and G. Bohstedt, *J. Animal Sci.*, **8**, 57-66 (1949).

²⁶⁸ J. V. Duhig, *Med. J. Australia*, **1931**, Part I, 260-261.

²⁶⁹ K. W. Keane, F. I. Nakamura, and M. L. Morris, *North Am. Veterinarian*, **28**, 587-590 (1947).

²⁷⁰ P. Soumalainen, *Skand. Arch. Physiol.*, **83**, 94-103; 104-112 (1939-1940).

²⁷¹ R. F. Krause and H. B. Pierce, *Arch. Biochem.*, **19**, 145-148 (1948).

²⁷² L. S. Palmer and C. Kennedy, *J. Biol. Chem.*, **46**, 559-577 (1921).

²⁷³ R. H. Grummer, C. K. Whitehair, G. Bohstedt, and P. H. Phillips, *J. Animal Sci.*, **7**, 222-227 (1948).

²⁷⁴ H. van den Bergh, P. Muller, and J. Broekmeyer, *Biochem. Z.*, **108**, 279-303 (1920).

this group is less certain. Ganguly and co-workers²⁵⁷ reported that the plasma value of rats contains no carotenes or xanthophylls. Goodwin¹⁹² reported that xanthophylls are never found in the portal or systemic blood of rabbits.

a. The Nature of the Carotenoids in the Blood. Considerable evidence has accumulated in support of the concept that, when β -carotene and other carotenoids occur in the blood, they do so in combination with protein. In 1915-1916, Palmer^{263, 264} called attention to the fact that the carotene was not directly extractable from cow serum by means of ethanol-free ether. Later Palmer and Eckles²⁴⁶ prepared the so-called "caroto-albumin" from cow serum which was believed to be the combination normally occurring in blood. Dzialoszynski *et al.*²⁷⁵ were of the opinion that carotene was combined with an albumin fraction in human blood, but later workers²⁷⁶⁻²⁸⁰ believed that the carotene-containing protein was a β -globulin. In the recent report of Oneley *et al.*²⁸¹ and that of Cohn and co-workers,²⁸² it was noted that carotenoids were present in human blood in the β -lipoprotein fraction. Ganguly *et al.*²⁴⁷ reported that vitamin A ester occurred in the least soluble fraction in chicken plasma, and vitamin A alcohol and lutein in the more soluble fractions. In beef blood, β -carotene occurred along with lutein and vitamin A alcohol in the more soluble (presumably albumin) fraction. In pig plasma, vitamin A alcohol was associated with the less soluble protein.

Additional evidence, which supports the concept that β -carotene and carotenoids in blood are combined with protein, is to be gleaned from the number of protein complexes of carotenoids which occur in nature. These include the lycopene-protein complex in tomato,²⁸³ rhodopsin, porphyrop-

²⁷⁵ L. M. Dzialoszynski, E. M. Mystkowski, and C. P. Stewart, *Biochem. J.*, **39**, 63-69 (1945).

²⁷⁶ H. Bennhold, E. Kylin, and S. Ruzsinyák, *Die Eiweisskörper des Blutplasmas*, Steinkopff, Dresden-Leipzig, 1938; Chap. 7, H. Bennhold, *Die Vehikelfunktion der Bluteiweisskörper*, pp. 220-303.

²⁷⁷ H. Bennhold, *Verhandl. deut. Ges. inn. Med.*, **45th Congress**, Wiesbaden, April, 1933, 357-359.

²⁷⁸ W. M. Bendien and I. Snapper, *Acta Brevia Neerland. Physiol., Pharmacol. Microbiol.*, **1**, 69-71 (1931).

²⁷⁹ J. W. Mehl, Personal communication, 1952.

²⁸⁰ L. B. Pett and G. A. LePage, *J. Biol. Chem.*, **132**, 585-593 (1940).

²⁸¹ J. L. Oneley, F. R. N. Gurd, and M. Melin, *J. Am. Chem. Soc.*, **72**, 458-464 (1950).

²⁸² E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, D. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid, and E. Uroma, *J. Am. Chem. Soc.*, **72**, 465-474 (1950).

²⁸³ R. Kuhn and H. J. Bielig, *Ber.*, **73**, 1080-1091 (1940).

sin and iodopsin in the retina,²²⁶ and the astaxanthin-protein complex, known as overdin, in lobsters.^{284,285}

In spite of the widespread experimental evidence that β -carotene and the carotenoids occur in combination with protein, several investigators have indicated the possibility that they are present in simple aqueous colloidal solutions. Van den Bergh and Muller²⁸⁶ observed that carotene could not be readily extracted from simple aqueous colloidal extracts with ether, even though no protein was present. Drummond and McWalter²⁸⁷ reported similar results. Although colloidal carotene solutions became unextractable when added to plasma, Dziaszowski *et al.*²⁷⁵ noted that they could be extracted by ether if the plasma protein had been denatured previously. These results do not necessarily preclude the concept that β -carotene and other carotenoids occur in plasma as protein complexes. In fact, when one considers the positive evidence for such a protein-carotenoid combination together with the indications of a similar phenomenon in the case of vitamin A, which will be given later, this would seem to be the most readily acceptable hypothesis.

b. Normal Values for Blood Carotene. Blood carotene in normal human subjects has been reported at levels varying between 50 and 500 $\mu\text{g. \%}$, with an average value of 200 $\mu\text{g. \%}$. Some average figures (in $\mu\text{g. \%}$) reported include the following: Kimble,²⁸⁸ 166 (30 normal men) and 187 (34 normal women); Murrill *et al.*,²⁸⁹ 213 ± 72 ; Ralli *et al.*,²⁹⁰ 123; Yarbrough and Dann,²⁹¹ 183 ± 18.4 ; Caveness and co-workers,²⁹² 138 ± 62 ; Hsu,²⁹³ 140 (33 young Chinese adults) and Abels *et al.*,²⁹⁴ 210 (men) and 180 (women). Harris and associates²⁹⁵ reported a figure of 210 ± 31 $\mu\text{g. \%}$ as the average value for seventy normal subjects. Finally, Sin-

²⁸⁴ R. Kuhn and N. A. Sørensen, *Ber.*, 71, 1879-1888 (1938).

²⁸⁵ K. Stern and K. Salomon, *J. Biol. Chem.*, 122, 461-475 (1938).

²⁸⁶ H. H. Van den Bergh and P. Müller, *Proc. Acad. Sci., Amsterdam*, 22, 748-757 (1920).

²⁸⁷ J. C. Drummond and R. J. MacWalter, *J. Physiol.*, 83, 236-242 (1935).

²⁸⁸ M. S. Kimble, *J. Lab. Clin. Med.*, 24, 1055-1065 (1939).

²⁸⁹ W. A. Murrill, P. B. Horton, E. Leiberman, and L. H. Newburgh, *J. Clin. Invest.*, 20, 395-400 (1941).

²⁹⁰ E. P. Ralli, E. Bauman, and L. B. Roberts, *J. Clin. Invest.*, 20, 709-713 (1941).

²⁹¹ M. E. Yarbrough and W. J. Dann, *J. Nutrition*, 22, 597-607 (1941).

²⁹² H. L. Caveness, G. H. Satterfield, and W. J. Dann, *Arch. Ophthalmol.*, 25, 827-832 (1941).

²⁹³ H.-C. Hsu, *Chinese Med. J.*, 61, 238-243 (1943).

²⁹⁴ J. C. Abels, A. T. Gorham, G. T. Pack, and C. P. Rhoads, *J. Clin. Invest.*, 20, 749-764 (1941).

²⁹⁵ P. L. Harris, K. C. D. Hickman, J. L. Jensen, and T. D. Spies, *Am. J. Pub. Health*, 36, 155-160 (1946).

clair²⁹⁶ cited as the arbitrary standard of the Oxford Nutrition Survey a value above 100 $\mu\text{g. \%}$ as normal; a figure below 50 $\mu\text{g. \%}$ represents extreme abnormality.

In the case of the cow, the blood carotene values vary with the nature of the feed. Averages as high as 1520 $\mu\text{g. \%}$ were recorded by Braun,²⁹⁷ although levels as low as 140 $\mu\text{g. \%}$ have also been noted on low carotene intakes. Moore²⁹⁸ stated that the sera of Jersey cows and of other Channel Island breeds frequently contain as much as 2000 $\mu\text{g. \%}$; 200 $\mu\text{g. \%}$ may be considered to be an average level for plasma carotene during the summer months, when the intake of carotene is high. In contrast to this, the figure for the blood carotene level of the horse is given by Rasmussen *et al.*²⁹⁹ as only $97 \pm 78 \mu\text{g. \%}$.

In a study by Ganguly and collaborators,²⁵⁷ the following values were noted for carotenes and xanthophylls, respectively (expressed in $\mu\text{g. \%}$), for animals belonging to the several classes, as related to carotenoid metabolism: Group A (high carotene, high carotenol), frogs, 0 and not determined; Group B (high carotene, low carotenol), cow, 435, 16.9; Group D (low carotene, high carotenol), chicken, 4.2, 162; and Group C (low carotene, low carotenol), rat, 0, 0 and ewe, 0, 0.

c. Factors Altering the Level of Blood Carotene. (a) *Diet.* As noted earlier, the most important factor responsible for variations in blood carotene is the effect of diet. Getz³⁰⁰ was able to increase the blood carotene in patients to maximum values of 900 to 1100 $\mu\text{g. \%}$ by the simple expedient of feeding large doses of carotene (120 mg.). As the dosage of β -carotene was further increased, the rise in the blood carotene level did not keep pace with the increased intake. Presumably, a larger proportion was lost in the feces.

After moderate doses of carotene, the maximum level is reached in the blood after seven to ten hours.³⁰¹ Murrill *et al.*²⁸⁹ reported a similar result after a large dose of this provitamin A (80 mg.), although a second maximum level was obtained twenty-four to forty-eight hours after its adminis-

²⁹⁶ H. M. Sinclair, *Vitamins and Hormones*, 6, 101-162 (1948).

²⁹⁷ W. Braun, *J. Nutrition*, 29, 61-71 (1945).

²⁹⁸ T. Moore, *The Fat-Soluble Vitamins*, in G. H. Bourne and G. W. Kidder, *Biochemistry and Physiology of Nutrition*, Vol. I, Academic Press, New York, 265-290, 1953, p. 269.

²⁹⁹ R. A. Rasmussen, C. L. Cole, and M. J. Miller, *J. Animal Sci.*, 3, 346-350 (1944).

³⁰⁰ H. R. Getz, *Induction of Vitamin A Deficiency in Man*, presented at Vitamin Conference, A.A.A.S., Gibson Island, July, 1944; cited by J. C. Fritz, *Ann. Rev. Biochem.*, 14, 525-560 (1945), p. 533.

³⁰¹ E. P. Ralli, H. Brandaleone, and T. Mandelbaum, *J. Lab. Clin. Med.*, 20, 1266-1275 (1935).

tration. Following massive doses in oil (600 mg.), a hypercarotenemia obtained for seventy-two hours before the peak value was reached.³⁰¹

According to Szymanski and Longwell,³⁰² it is possible to predict the blood carotene levels in man from the quantities present in the diet. The formula for expected plasma carotene in $\mu\text{g. \%}$ (X_0) is as follows:

$$X_0 = 0.46 I_0 + 0.06 I_3 + 86$$

I_0 is the average daily intake of vitamin A (carotene) from plant sources in I.U. per kilogram body weight over the preceding three months, while I_3 is the same value during the period three to six months before the date of the prediction.

In the case of cattle, Davis and Madsen³⁰³ obtained the following averages for plasma carotene ($\mu\text{g./100 ml.}$) when the carotene intake was expressed in $\mu\text{g./kg.}$ of body weight: *Low intake*, 14 $\mu\text{g./100 ml.}$; 30 $\mu\text{g.}$, 46; 45 $\mu\text{g.}$, 41; 60 $\mu\text{g.}$, 81; 120 $\mu\text{g.}$, 103; *high* (pasture), 813.

When massive doses of carotene are ingested over a prolonged period, especially by individuals who have hepatic disease, diabetes mellitus, or myxedema (which conditions tend to prevent a normal utilization of carotene), and also by entirely normal individuals, excessively high blood carotene values result, with the concomitant deposition of the pigment in the subcutaneous tissue. This produces a condition known as "carotenemia," or "carotinosis," which outwardly simulates jaundice. Normal children may develop carotenemia after an excessive intake of carrots,³⁰⁴ Hubbard squash (*Cucurbita maxima*),³⁰⁵ "mikan," or Japanese orange (*Citrus nobilis*), Lour., var. *microcarpa*),³⁰⁵ as well as after other foods rich in carotene.³⁰⁶ Carotenemia is an apparently harmless metabolic condition which does not have the unfavorable prognosis of hypervitaminosis A. The carotenemia and skin coloration rapidly disappear when the carotene intake is reduced. Although plasma vitamin A is usually normal in cases of carotenemia, indicating that the accumulation of the provitamin is not the result of its failure to be converted to vitamin A, there is one report³⁰⁷ of this type of condition in which carotene passed into the blood in such quantities that the normal carotene:xanthophyll ratio was reversed.

When massive doses of vitamin A are administered to cows, a marked depression in the carotene levels of blood obtains, even though the carotene

³⁰² B. B. Szymanski and B. B. Longwell, *J. Nutrition*, 45, 431-442 (1951).

³⁰³ R. E. Davis and L. A. Madsen, *J. Nutrition*, 21, 135-146 (1941).

³⁰⁴ A. F. Hess and V. C. Myers, *J. Am. Med. Assoc.*, 73, 1753-1745 (1919).

³⁰⁵ H. Hashimoto, *J. Am. Med. Assoc.*, 78, 1111-1112 (1922).

³⁰⁶ H. Koch, *Biochem. J.*, 37, 430-433 (1943).

³⁰⁷ H. Cohen, *Quart. J. Med.*, 18, 397-398 (1949).

intake remains constant; this effect occurs concomitantly with a decreased level of carotene in the butterfat. Similarly, the administration of large doses of vitamin A to hens resulted in a marked depression in the carotene levels of the blood serum, egg yolks, liver and body fat, with a concomitant increase in the vitamin A values in these sites.²⁰⁰ For a more complete discussion, see pages 447 to 448.

(b) *Physiologic Factors.* Age is an important factor in controlling the level of blood carotene. Minimum levels of blood carotene have been reported in the newborn (34 $\mu\text{g. \%}$ at two days); according to Szymanski and Longwell,³⁰² there is a gradual increase until a maximum figure of 328 $\mu\text{g.}$ is obtained at the age of twelve months. Carotene levels during youth have been reported as 126 $\mu\text{g. \%}$ (13–16 years),³⁰² 140 $\mu\text{g. \%}$ (2–12 years),³⁰⁸ $116 \pm 45.7 \mu\text{g. \%}$ (179 school children),³⁰⁹ and 100 to 168 $\mu\text{g. \%}$ (Michigan children, 6–16 years).³¹⁰ The figures for blood carotene are apparently lower in the aged than in the case of middle-aged adults. Thus, Rafsky, Newman, and Jolliffe³¹¹ reported mean values of 80 $\mu\text{g. \%}$ (males, 74.7; females, 85) for subjects varying from sixty-nine to eighty-three years. Kirk and Chieffi³¹² observed a progressive decrease in the level of plasma α - and β -carotenes with increasing age. Gillum, Morgan, and Sailer³¹³ also noted a slight decline in the plasma carotene level in older individuals.

Sex is another factor which appears to have some influence upon the proportion of both plasma carotene and plasma vitamin A. Thus, Kimble,²⁸⁸ Popper and Steigmann,³¹⁴ and Hoffmann *et al.*³¹⁵ recorded higher plasma carotene levels in women than in men. A similar sex variation has been noted in infants and young children,³⁰² although the situation becomes reversed at the start of adolescence. Rafsky *et al.*³¹¹ reported sex variations in the plasma carotene of aged subjects. On the other hand, Abels *et al.*²⁹⁴ and Haig and Patek³¹⁶ found that males had a higher plasma carotene value than did females. Although there is thus some disagreement as to the effect of sex on blood carotene, the majority of data indicate that females

³⁰⁸ C. D. May, K. D. Blackfan, J. F. McCreary, and F. H. Allen, *Am. J. Diseases Children*, 59, 1167–1184 (1940).

³⁰⁹ R. F. Krause and H. B. Pierce, *J. Nutrition*, 33, 633–640 (1947).

³¹⁰ A. Robinson, M. Leshner, A. P. Harrison, E. Z. Moyer, M. C. Gresock, and C. Saunders, *J. Am. Dietet. Assoc.*, 24, 410–416 (1948).

³¹¹ H. A. Rafsky, B. Newman, and N. Jolliffe, *Gastroenterology*, 8, 612–615 (1947).

³¹² E. Kirk and M. Chieffi, *J. Nutrition*, 36, 315–322 (1948).

³¹³ H. L. Gillum, A. F. Morgan, and F. Sailer, *J. Nutrition*, 55, 655–670 (1955).

³¹⁴ H. Popper and F. Steigmann, *J. Am. Med. Assoc.*, 123, 1108–1114 (1943).

³¹⁵ R. Hoffmann, A. Schneider, and Y. Quamo, *J. Invest. Dermatol.*, 15, 409–419 (1950).

³¹⁶ C. Haig and A. J. Patek, Jr., *J. Clin. Invest.*, 21, 309–317 (1942).

have higher blood levels. This sex variation is corroborated by the extensive data on the basis of which a sex variation in plasma vitamin A has also been established. In the latter case, the situation is reversed, *i.e.*, higher plasma vitamin A values are noted in men than in women.

Parturition is a phenomenon which is associated with marked variations in blood carotene in cows. Sutton *et al.*³¹⁷ reported a sharp decrease in blood carotenoids immediately after the birth of the calf, with a slow recovery occurring over the subsequent fourteen to twenty-one days. Goodwin and Wilson³¹⁸ have confirmed these results, although they related these changes to the general alterations in the blood during this period.

Season is another factor which can be correlated with changes in the carotene content of the blood of cattle. The effect in this case is apparently indirect, due to the fact that the quantity of available carotenoid-containing foods varies at different times of the year. Thus, Braun²⁹⁷ reported a maximum level of 1350 $\mu\text{g. \%}$ of plasma carotenoids in the blood of Holsteins when they were on green pasture in March, while the minimum figure of 280 $\mu\text{g. \%}$ was observed in the October samples when green feed was not available. Similar variations were noted in Lord³¹⁹ for Ayrshire cows. Seasonal variation in the availability of green foods may result in variations in blood carotene not only in the case of cattle but also in that of children; this is indicated by the results of Szymanski and Longwell,³⁰² who reported average figures of 152 $\mu\text{g. \%}$ for the summer period (June to November) and 132 $\mu\text{g. \%}$ for the winter months (December to May).

Certain miscellaneous dietary constituents may also cause variations in blood carotene. Thus, the administration of mineral oil concomitantly with carotene causes a decrease in the level of blood carotene,^{48,111,320} presumably as a result of the reduced absorption of the provitamin A under these conditions. Sulfonamide therapy was found by Ronning and Knodt³²¹ to be without effect on the level of plasma carotene. Dietary penicillin was reported by Burgess *et al.*²¹¹ to cause a significant rise in plasma carotenoids in the chick.

(c) *Pathologic Factors.* In the case of human subjects, pronounced variations in the values of blood carotene may obtain which are not associated with a corresponding alteration in intake or with other physiologic factors.

Diabetes mellitus is the classical disease in which carotenemia has fre-

³¹⁷ T. S. Sutton, H. E. Kaeser, and P. A. Soldner, *J. Dairy Sci.*, **28**, 933-939 (1945).

³¹⁸ T. W. Goodwin and A. A. Wilson, *Biochem. J.*, **49**, 499-503 (1951).

³¹⁹ J. W. Lord, *Biochem. J.*, **39**, 373-374 (1945).

³²⁰ A. C. Curtis and E. M. Kline, *Arch. Internal Med.*, **63**, 54-63 (1939).

³²¹ M. Ronning and C. B. Knodt, *J. Dairy Sci.*, **33**, 424-429 (1950).

quently been noted. In fact, the association of the two conditions is indicated by the term "xanthosis diabetica," which has been applied to pigmentation of the skin under these conditions. This has led many workers to the general conclusion that the conversion of carotene to vitamin A is interfered with in diabetes mellitus, and that this fact accounts for the high level of plasma carotene.³²²⁻³²⁹ Galeone³²⁹ suggests a possible hormonal effect upon vitamin A and metabolism in this disease. Ralli and associates^{301,322} were able to demonstrate a fasting carotenemia in their diabetic patients, and also reported that the carotene tolerance curve was elevated in these patients. The blood carotene reached higher values and remained elevated longer in the case of diabetics than in normal subjects. Moreover, it was found that the carotene content of the liver was higher in patients with diabetes mellitus than in normal subjects.³⁰¹ Rabinowitch³²⁶ is of the opinion that the extent of carotenemia can be directly correlated with the severity of the diabetic condition. The cause of the disordered carotene metabolism is not known.

However, there has recently been an increasing tendency to consider the attendant carotenemia in diabetes mellitus as an independent phenomenon. Goodwin²⁹ is of the opinion that the high plasma carotene values are merely a reflection of the modification in dietary regimen recommended in diabetes mellitus, *i.e.*, one which is rich in vegetables containing carotene. Moreover, Kimble, Germak, and Sevringhaus³³⁰ reported that no statistically significant increase in the plasma carotene level could be demonstrated in diabetics. Similar conclusions as to the absence of a causative relationship between diabetes mellitus and carotenemia were propounded by Mosenthal and Loughlin,³³¹ Lambrechts *et al.*,³³² and Escudero and co-workers.³³³ The latter³³³ found the plasma carotene increased, but at-

³²² E. P. Ralli, A. C. Pariente, H. Brandaleone, and S. Davidson, *J. Am. Med. Assoc.*, **106**, 1975-1978 (1936).

³²³ W. Heymann, *J. Am. Med. Assoc.*, **106**, 2050-2052 (1936).

³²⁴ G. H. Stueck, G. Flaum, and E. P. Ralli, *J. Am. Med. Assoc.*, **109**, 343-344 (1937).

³²⁵ G. Oliva and M. Pescarmona, *Arch. sci. med.*, **68**, 537-546 (1939).

³²⁶ I. M. Rabinowitch, *Arch. Internal Med.*, **45**, 586-592 (1930).

³²⁷ H. Brandaleone and E. P. Ralli, *Proc. Soc. Exptl. Biol. Med.*, **32**, 200-201 (1934).

³²⁸ J. G. Brazer and A. C. Curtis, *Arch. Internal Med.*, **65**, 90-105 (1940).

³²⁹ A. Galeone, *Arch. sci. med.*, **75**, 560-576 (1943); *Chem. Zentr.*, Vol. **114**, 1943, II; 1727; *Chem. Abst.*, **39**, 119 (1945).

³³⁰ M. S. Kimble, O. A. Germak, and E. L. Sevringhaus, *Am. J. Med. Sci.*, **212**, 574-585 (1946).

³³¹ H. O. Mosenthal and W. C. Loughlin, *J. Mt. Sinai Hosp.*, **12**, 523-526 (1945).

³³² A. Lambrechts, C. Leleux, and A. Thomas, *Acta med. Scand.*, **116**, 11-19 (1943).

³³³ P. Escudero, P. M. Cereijo, M. L. Herraiz, A. M. Benedetto, and E. Musmanno, *Rev. asoc. argentina dietol.*, **5**, 261-266 (1947); *Chem. Abst.*, **42**, 5986 (1948).

tributed this increase to the large amounts of vitamin A and carotene in the diet of the diabetic patients. Peters and Van Slyke³³⁴ also questioned whether or not carotenemia is a direct result of diabetes mellitus. There is no experimental evidence that the destruction of carotene is associated with the utilization of carbohydrate.³³⁴ Although the reason why carotenemia occurs in diabetes mellitus must still be regarded as open to question, one must incline to the explanation based upon dietary changes as the more probable one.

Alloxan diabetes produces an abnormally low level of vitamin A in the liver. Thus, Sobel and co-workers³³⁵ observed that rats with severe alloxan diabetes (blood sugar over 150 mg./100 ml.) stored a much smaller amount of vitamin A in their livers after the administration of 2000 μ g. of carotene (dissolved in 0.2 ml. of cottonseed oil) than did normal rats; the comparative vitamin A stores were 22 ± 20 μ g. and 88 ± 25 μ g. per liver, respectively. When vitamin A itself was fed, the variations in hepatic vitamin A were much less marked (90 ± 41 μ g. as compared with 123 ± 30 μ g.). The most probable explanation for the failure of the alloxan-diabetic rats to store more vitamin A after the administration of carotene is that the transformation of carotene to vitamin A is depressed under these conditions. That this is the case is indicated by the later results of Rosenberg and Sobel,²¹⁸ who reported a decreased *in vitro* conversion of β -carotene in the gut of the alloxan-treated rat. Thus, when the excised intestine of rats, killed immediately after carotene feeding, according to the procedure of these investigators,¹⁴⁴ was incubated at 45°C. for two hours, an average of only 0.76 μ g. of vitamin A was found to be present per entire intestinal wall, as contrasted with a mean value of 4.25 μ g. in normal rats under similar conditions.

Mastitis is another condition which, it was formerly believed, involved an abnormal carotene metabolism. Miller, Lease, and Anderson³³⁶ reported that the milk carotenoid levels were considerably increased in experimentally-induced mastitis in the cow, but that the total daily output of carotenoid was markedly lower than normal as a result of the decreased yield of milk. Chanda³³⁷ confirmed the fact that a concentration of carotene occurs in the milk of cows infected with mastitis (*Streptococcus agalactiae contagiosae*), but he noted that nitrogen, chloride, and phosphatase

³³⁴ J. P. Peters and D. D. Van Slyke, *Quantitative Clinical Chemistry*, Vol. I, *Interpretations*, 2nd ed. Williams & Wilkins, Baltimore, 1946, p. 514.

³³⁵ A. E. Sobel, A. Rosenberg, and H. Adelson, *Arch. Biochem. Biophys.*, **44**, 176-180 (1953).

³³⁶ P. G. Miller, E. J. Lease, and G. W. Anderson, *J. Dairy Sci.*, **23**, 573-574 (1940).

³³⁷ R. Chanda, *Biochem. J.*, **54**, 68-77 (1953).

were also increased. However, the proportion of total fat, total phosphorus, ester phosphorus, thiamine, and vitamin A was decreased. The proportion of β -carotene in the total carotenoids remained constant in the mastitic milk, and the blood carotenoid level remained essentially unchanged. Goodwin²⁰ suggests that a possible explanation for the high carotene values in milk in mastitis is its specific leakage across the damaged glandular epithelium of the mammary gland.

Acetonemia in cows has been reported to be associated with a high level of blood carotenoids and a relatively low content of plasma vitamin A;³³⁸ one suggestion is that the conversion of carotene into vitamin A is depressed in this condition. Massive doses of vitamin A have been found to reduce the blood carotene to normal, and simultaneously to cause an increase in the level of plasma vitamin A to normal values.³³⁸⁻³⁴¹ However, it should be recalled that plasma carotene is depressed in normal animals, as well, by massive doses of vitamin A. It remains undecided whether or not any abnormality in the synthesis of vitamin A from carotene is associated with acetonemia in cows.

Miscellaneous conditions in which lower than normal carotene figures have been reported in blood include fever³⁴² (in which the level of many other blood components is also depressed), cancer of the gastrointestinal tract,²⁹⁴ leprosy,³⁴³ and the active phase of pneumonia.³⁴⁴ The provitamin A has been found to remain unchanged in folliculosis in children³⁰⁹ and in nephrosis.³⁴⁵ The entire subject of blood carotene is more completely discussed in *The Lipids*, Vol. II, pages 365 to 369 and 486 to 509.

(2) *Vitamins A in the Blood*

a. The Nature of Vitamins A in Blood. (a) *Vitamin A-Protein Combinations.* Both vitamin A alcohol and vitamin A ester occur in the blood in combination with protein,^{275,280} just as is the case with β -carotene and with other carotenoids. According to Ganguly *et al.*,²⁴⁷ vitamin A alcohol and vitamin A ester are associated with different plasma proteins in chicken blood. The ester combines with the least soluble fraction (globulin),

³³⁸ J. W. Patton, *Vet. Med.*, 39, 271-278 (1944).

³³⁹ J. W. Patton, *Vet. Med.*, 39, 150-153 (1944).

³⁴⁰ J. Mackay, *Vet. Record*, 55, 455 (1943).

³⁴¹ A. C. Burt, *Can. J. Comp. Med. Vet. Sci.*, 8, 187-188 (1944).

³⁴² H. C. S. Aron, R. M. Craig, C. J. Farmer, H. W. Kendell, and G. X. Schwemlein, *Proc. Soc. Exptl. Biol. Med.*, 61, 271-276 (1946).

³⁴³ I. Ikegaki, *Z. Vitaminforsch.*, 6, 206-209 (1937).

³⁴⁴ H. W. Josephs, *Am. J. Diseases Children*, 65, 712-747 (1943).

³⁴⁵ B. M. Kagan, E. M. Thomas, D. A. Jordan, and A. F. Abt, *J. Clin. Invest.*, 29, 141-145 (1950).

while the alcohol is associated with the most soluble fraction (albumin). It was also demonstrated that vitamin A alcohol in pig blood is linked with the more soluble protein fraction. Lovern *et al.*³⁴⁶ called attention to the fact that, even in the case of fishes, the liver vitamin A is in part associated with protein. Vitamin A aldehyde (retinene) was found by Rajagopal and Datta³⁴⁷ to combine with protein. The vitamin A aldehyde content of the protein-retinene complex was 0.003%; in the case of the vitamin A-protein complex, 120 to 200 I.U. of vitamin A was associated with 6 to 8 g. of protein.³⁴⁷

(b) *The Presence of Vitamin A Alcohol and of Vitamin A Esters.* Vitamin A in the plasma occurs both in the form of the free alcohol and as the ester. Vitamin A alcohol is the predominant form in the fasting individual.¹¹⁸ However, when vitamin A is administered orally, the ester form alone is increased, irrespective of whether the vitamin A is given as the alcohol or as the ester.^{220,348,349} Hoch also noted some increase in the vitamin A alcohol in the plasma as a result of the mobilization of endogenous vitamin A, for example, following parturition, or the ingestion of ethanol.^{348,349}

The view is widespread that no definite relationship exists between the level of vitamin A in the blood and that in the liver.^{290,350-354} Thus, Lewis *et al.*³⁵⁰ suggest that the plasma tends to resist any changes in the vitamin A concentration, in spite of wide variations in the proportion of vitamin A present in the liver. However, Glover and co-workers^{355,356} interpreted their data as an indication that the level of vitamin A alcohol in the plasma is proportional to that of the free vitamin A in the liver, although they recognize that the level of plasma vitamin A does not correspond to that of the total hepatic stores of vitamin A, which are largely in the form of the ester. Ganguly and Krinsky³⁵⁷ reached conclusions opposite to those of

³⁴⁶ J. A. Lovern, J. R. Edisbury, and R. A. Morton, *Nature*, **140**, 276 (1937).

³⁴⁷ K. Rajagopal and P. K. Datta, *Nature*, **170**, 370-371 (1952).

³⁴⁸ H. Hoch, *Nature*, **158**, 59 (1946).

³⁴⁹ H. Hoch and R. Hoch, *Brit. J. Exptl. Pathol.*, **27**, 316-328 (1946).

³⁵⁰ J. M. Lewis, O. Bodansky, K. G. Falk, and G. McGuire, *J. Nutrition*, **23**, 351-363 (1942).

³⁵¹ J. M. Lewis, O. Bodansky, K. G. Falk, and G. McGuire, *Proc. Soc. Exptl. Biol. Med.*, **46**, 248-250 (1941).

³⁵² H. W. Josephs, *Bull. Johns Hopkins Univ.*, **71**, 253-264 (1942).

³⁵³ P. B. Horton, W. A. Murrill, and A. C. Curtis, *J. Clin. Invest.*, **20**, 387-393 (1941).

³⁵⁴ S. Brenner, M. C. H. Brookes, and L. J. Roberts, *J. Nutrition*, **23**, 459-471 (1942).

³⁵⁵ J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **40**, lvii (1946); **41**, 97-100 (1947).

³⁵⁶ J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **41**, 97-100 (1947).

³⁵⁷ J. Ganguly and N. I. Krinsky, *Biochem. J.*, **54**, 177-181 (1953).

Glover *et al.*,^{355,356} they were unable to find any relationship between the level of vitamin A alcohol in the liver and that in the plasma in normal male rats, or in those depleted of vitamin A. In spite of wide variations in concentration of vitamin A alcohol in the liver, that in the plasma remained fairly constant.

b. Normal Values for Vitamin A. The values of plasma vitamin A more or less coincide with those of β -carotene, the figures normally varying between 20 and 60 $\mu\text{g. \%}$ (67 to 200 I.U.). One International Unit is equivalent to 0.3 $\mu\text{g.}$ The following averages for plasma vitamin A (expressed as $\mu\text{g. \%}$ and as I.U.) have been recorded by various workers: Kimble,²⁸⁸ 38 and 27 for men and for women (127 and 91 I.U.); Murrill *et al.*,²⁸⁹ 27.9 (93 ± 15 I.U.); Sobotka,³⁵⁸ 18 to 24 (60 to 80 I.U.); Ralli and co-workers²⁹⁰ 47.4 (158 I.U.); Yarbrough and Dann,²⁹¹ 21.3 (71 ± 33 I.U.); Caveness *et al.*,²⁹² 78.9 (263 ± 96 I.U.); Abels and co-workers,²⁹⁴ 51 and 44.7 (170 and 149 I.U.); and Harris, Hickman, Jensen and Spies,²⁹⁵ 61 ± 13 (203 I.U.). Other values reported for special groups of subjects include a figure of 16.8 (56 I.U.) for healthy young Chinese adults, cited by Hsu,²⁹³ one of 35.4 (118 I.U.) given by Harris and Moore³⁵⁹ for normal individuals in Great Britain, and 44.7 (149 I.U.) by Van Bruggen and Straumfjord³⁶⁰ for hospital patients. Youmans and associates³⁶¹ consider any figure in excess of 21 $\mu\text{g. \%}$ (70 I.U.) as normal. However, Sinclair²⁹⁶ considers that this figure is the lowest normal value, and that any level below 9 $\mu\text{g. \%}$ (30 I.U.) is abnormally low. Popper and Steigmann³¹⁴ reported average values for 2673 determinations on 454 hospital patients as 58 $\mu\text{g. \%}$ for men and 47 $\mu\text{g. \%}$ for women (193 and 157 I.U., respectively).

So-called "normal" values have also been predicated for a number of animals. Figures as high as 1520 $\mu\text{g. \%}$ were recorded for cows when large amounts of carotene were present in the food, or as low as 140 $\mu\text{g. \%}$ when no green feeds were available.²⁹⁷ In the case of the horse, Rasmussen *et al.*,²⁹⁹ recorded the low average value of 12.5 ± 3.5 $\mu\text{g. \%}$ (41.6 I.U.), which is in line with the relatively low figure reported by them for blood carotene. On the other hand, Rudra³⁶² cited a mean value of 28 I.U./100 ml. (8.4/ $\mu\text{g.}$) for the serum vitamin A of English horses; the val-

³⁵⁸ H. H. Sobotka, Personal communication; cited by M. E. Yarbrough and W. J. Dann, *J. Nutrition*, 22, 597-607 (1947), p. 601.

³⁵⁹ A. D. Harris and T. Moore, *Brit. Med. J.*, 1947, I, 553-559.

³⁶⁰ J. T. Van Bruggen and J. V. Straumfjord, *J. Lab. Clin. Med.*, 33, 67-74 (1948).

³⁶¹ J. B. Youmans, E. W. Patton, W. R. Sutton, R. Kern, and R. Steinkamp, *Am. J. Pub. Health*, 34, 368-378 (1944).

³⁶² M. N. Rudra, *Biochem. J.*, 40, 500-501 (1945).

ues ranged between 6 and 52 I.U. Maddock and co-workers³⁶³ recorded relatively high values for plasma vitamin A for dogs. The figures ranged between 173 and 355 $\mu\text{g.}\%$ (577 and 1183 I.U., respectively); these are higher than those observed under normal conditions in other species. The vitamin A content of the plasma of chickens on a farm diet was reported by Ganguly *et al.*⁵⁹ as 82 I.U./100 ml., while the levels on several diets varied from 55 to 151 I.U. Lewis and collaborators³⁵⁰ gave 10 to 30 $\mu\text{g.}/100$ ml. (33 to 100 I.U.) as the average range for the vitamin A content of the blood of rats when the vitamin A intake was maintained for six weeks within what is considered to be a normal range.

c. Factors Altering the Level of Vitamin A. (a) *The Effect of Diet.* Although the amount of carotene in the ordinary diet has little or no influence upon the level of plasma carotene and of vitamin A,²⁸⁸ Getz³⁰⁰ did find that plasma vitamin A was increased somewhat, along with plasma carotene, when large doses of the provitamin A were given to men. Kimble²⁸⁸ and Murrill *et al.*²⁸⁹ also demonstrated some relationship between the increase of plasma vitamin A and plasma carotene after the administration of β -carotene. Davis and Madsen³⁰³ cited the following mean values (in italics) and range (in parentheses) for plasma vitamin A (expressed in $\mu\text{g.}/100$ ml.) in cows which had received different amounts of carotene in the diet: "Low diet," 10 (3-16); 30 $\mu\text{g.}/\text{kg.}$, of body weight (14-21); 45 $\mu\text{g.}/\text{kg.}$, 19 (16-23); 60 $\mu\text{g.}/\text{kg.}$, 26 (20-31); 120 $\mu\text{g.}/\text{kg.}$, 35 (33-36); and "High" (pasture), 49 (40-65).

The administration of vitamin A, either as the ester or as the alcohol, causes a prompt rise in the level of blood vitamin A, which returns to normal shortly after absorption is completed. Ralli and co-workers²⁹⁰ recorded higher plasma values for vitamin A in male subjects receiving 30,000 $\mu\text{g.}$ of vitamin A (100,000 I.U.) than in those receiving 6000 $\mu\text{g.}$ (20,000 I.U.) during the first twenty-four hours after feeding, but the differences disappeared thereafter.

In the case of rats, Lewis and collaborators³⁵⁰ did report a progressive rise in plasma vitamin A which, to some extent, paralleled the intake. Thus, after the following amounts of vitamin A (expressed in $\mu\text{g.}$ per day) were given to rats for six weeks, the corresponding levels of vitamin A ($\mu\text{g.}/100$ ml.) were noted in the plasma: 0, 0; 0.3, 2.1; 0.6, 4.2; 3.0, 10.5; 7.5, 20.7; 15, 30.0; 30, 33.6; 300, 33.0; and 30,000, 143.4. The constant level of vitamin A in the blood was not obtained until 15 to 30 $\mu\text{g.}$ were given daily, and this plasma value was maintained when the in-

³⁶³ C. L. Maddock, S. B. Wolbach, and S. Maddock, *J. Nutrition*, **39**, 117-137 (1949).

take reached as high as 300 $\mu\text{g.}$ per day.³⁵⁰ When a massive dose was given (30,000 $\mu\text{g.}$ daily), a considerable increase in the plasma vitamin A was noted; however, this last condition cannot be considered to be normal, since the rats suffered from hypervitaminosis, and many of them (30%) died during the six-week feeding period.

When vitamin A was given to chickens in widely varied doses, no marked variations in the plasma vitamin A levels were noted, in spite of the carotene-depressing action of the vitamin, and the increased vitamin A levels in the liver, body fat, and egg yolks.²⁰⁰ The following are the average figures for plasma vitamin A (in $\mu\text{g. } \%$) at several levels of vitamin A intake ($\mu\text{g.}$ of vitamin A/lb. food): 0 (in food), 139; 333, 166; 9,000, 134; 18,000, 174; 30,000, 133; and 60,000, 207. Josephs³⁵² observed that, when carotene-free and vitamin A-free diets were fed to rats, the plasma was free from detectable vitamin A within twenty days, and definite signs of avitaminosis appeared within twenty-six days. Hartzler³⁶⁴ reported a progressive decrease both in plasma carotene and in vitamin A in human subjects on a completely vitamin A-free diet. The period required for depletion is much greater in man, and, after 140 days on the vitamin A-deficient diet, plasma carotene had dropped from 149 to 15 $\mu\text{g. } \%$, while blood vitamin A had decreased only from 39.9 to 22.8 $\mu\text{g. } \%$ (133 to 76 I.U., respectively). It is thus evident that, in man, plasma vitamin A is much more resistant to change than is plasma carotene. Hartzler³⁶⁴ showed that it was possible to render the human plasma almost completely free from carotene without evoking any vitamin A-deficiency symptoms. Wald *et al.*³⁶⁵ were also unable to produce a vitamin A-deficiency in any of five men who were maintained on a diet low in vitamin A for six months.

a'. Vitamin A Tolerance Curves: When standard dosages of vitamin A are given, it is possible to obtain characteristic vitamin A "tolerance" curves which are considered to be of value in assessing the vitamin A status of the subject.

The nature of the vitamin A tolerance curve varies with the dosage of vitamin A employed, and with other conditions of the test. In the procedure used by Krause and Pierce³⁰⁹ in testing school children, 7500 or 15,000 $\mu\text{g.}$ (25,000 or 50,000 I.U.) were given in a single dose; maximum blood levels of vitamin A, which were higher when the higher dose of vitamin A was administered, were obtained in five hours, and the blood picture had returned to practically normal within twenty-four hours. Week and Seigne¹¹⁸ obtained comparable results, as regards the time of the oc-

³⁶⁴ E. Hartzler, *J. Nutrition*, 36, 381-390 (1948).

³⁶⁵ G. Wald, L. Brouha, and R. E. Johnson, *Am. J. Physiol.*, 137, 551-556 (1942).

currence of the maximum blood level, in the case of normal human subjects; 134,000 $\mu\text{g.}$ of vitamin A were given as the free alcohol, acetate or natural ester in 50 g. margarine fat, to adult subjects. The highest plasma levels were recorded after vitamin A alcohol had been fed, while somewhat lower figures were noted when vitamin A acetate was the source of vitamin A. The lowest vitamin A tolerance curves were noted after the natural vitamin A esters were fed. These results are interpreted as indicative of variations in the absorption of vitamin A as related to the form in which it is administered. As noted earlier, although vitamin A alcohol constitutes 80 to 90% of the total plasma vitamin A of the fasting samples, it was found to account for only 10% of the sample at the apex of the plasma vitamin A curve.

(b) *Physiologic Factors Altering Blood Vitamin A.* Age is an important factor with which both plasma vitamin A and blood carotene can be correlated. Thus, a number of investigators have reported low values in the newborn which increase to levels comparable with those in the adult during the first year of life. Henley *et al.*³⁶⁶ observed a value of only 20.4 $\mu\text{g. \%}$ (68 I.U.) in three-week-old premature babies, while Szymanski and Longwell³⁰² noted the minimum plasma vitamin A in the case of infants two days old (23 $\mu\text{g. \%}$ or 77 I.U.). Lewis *et al.*³⁵⁰ reported a precipitous drop of plasma vitamin A to 11.1 $\mu\text{g. \%}$ (37 I.U.) within forty-eight hours after birth. Progressively increasing levels have been noted during the first year of life. Thus a maximum value of 56 $\mu\text{g. \%}$ (168 I.U.) was observed in the nine-month-old group by Szymanski and Longwell.³⁰² Lewis *et al.*³⁵⁰ recorded vitamin A values in the blood of older infants as 13.5 to 42.3 $\mu\text{g. \%}$ (45 to 141 I.U.). Robinson and co-workers³¹⁰ reported values between 30 and 36 $\mu\text{g. \%}$ (100 and 120 I.U.) for children between six and sixteen years of age.

Slightly lower values than normal have been noted in the aged. Rafsky *et al.*³¹¹ reported plasma vitamin A levels of 29.3 and 25 $\mu\text{g. \%}$ for aged males and females (98 and 83 I.U.), respectively. Kirk and Chieffi³¹² cited somewhat lower figures for the plasma vitamin A in the oldest groups.

Fetal levels of plasma and liver vitamin A are consistently considerably lower than those of the mother.³⁶⁷⁻³⁷² It has been suggested that the

³⁶⁶ T. H. Henley, M. Dann, and W. R. C. Golden, *Am. J. Diseases Children*, 68, 257-264 (1944).

³⁶⁷ W. J. Dann, *Biochem. J.*, 26, 1072-1080 (1932); 28, 634-637 (1934).

³⁶⁸ W. Neuwiler, *Z. Vitaminforsch.*, 13, 275-280 (1943).

³⁶⁹ G. H. Wise, M. J. Caldwell, and J. S. Hughes, *Science*, 103, 616-618 (1946).

³⁷⁰ C. J. Lund and M. S. Kimble, *Am. J. Obstet. Gynecol.*, 46, 207-221 (1943).

³⁷¹ J. Portes and J. Varangot, *Compt. rend. soc. biol.*, 136, 166-168 (1942).

³⁷² J. N. Byrn and N. J. Eastman, *Bull. Johns Hopkins Hosp.*, 73, 132-137 (1943).

placenta limits the access of vitamin A to the fetus. Williamson³⁷³ demonstrated that this does not result from a general inhibition of the transfer of lipids to the fetus, but is specific for vitamin A. When pregnant rats were fed high-cholesterol diets, the lipid content of the placentae was found to be about twice that of the controls; however, the placentae of the experimental rats had significantly less vitamin A than in the case of the control placentae.

Sex is another important factor which one must consider in relation to blood vitamin A. The level of plasma vitamin A in the male usually exceeds that for the female by a significant amount. On the other hand, the sex differences for plasma carotene are reversed. In the case of infants and of young children, the plasma vitamin A has been found to be higher in girls than in boys.³⁰² However, in studies of the adult, Getz and Koerner,³⁷⁴ Kimble,²⁸⁸ Popper and Steigmann,³¹⁴ Abels *et al.*²⁹⁴ and Week and Sevigne¹¹⁸ consistently found higher values for plasma vitamin A in men than in women. Moreover, Week and Sevigne¹¹⁸ observed that the plasma vitamin A was consistently lower in females than in males after they were subjected to a vitamin A tolerance test by the administration of 134,000 μ g. of vitamin A in 50 g. margarine. However, Kirk and Chieffi³¹² demonstrated that there are no definite sex differences in plasma vitamin A values in the case of aged men and women, respectively.

Hormones, particularly those associated with sex changes, influence plasma vitamin A levels. Changes in the estrogenic hormones are presumably responsible for the sharp decrease in plasma vitamin A in cows following parturition.³¹⁷ A decreased plasma vitamin A level was observed when large doses of estradiol benzoate were injected into thyroidectomized rabbits, although no effect was noted in the case of unoperated animals.³⁷⁵ On the other hand, this hormone evoked an increase in the level of vitamin A esters in the plasma when injected into pullets.³⁷⁶ Testosterone, one of the male hormones, was found to be without effect on the amount of blood vitamin A in pullets,³⁷⁶ although it has been reported to increase plasma vitamin A levels in the case of man.³⁷⁷

Young and Wald³⁷⁸ stated that an increase in the amount of vitamin A occurred in the blood of rabbits following the injection of adrenalin, but

³⁷³ M. B. Williamson, *J. Biol. Chem.*, **174**, 631-636 (1948).

³⁷⁴ H. R. Getz and T. A. Koerner, *Am. J. Med. Sci.*, **202**, 831-847 (1941).

³⁷⁵ M. B. Williamson, *Proc. Soc. Exptl. Biol. Med.*, **68**, 621-623 (1947).

³⁷⁶ V. E. Gardiner, W. E. Phillips, W. A. Maw, and R. H. Common, *Nature*, **170**, 80-81 (1952).

³⁷⁷ A. Danish and T. C. Klopp, *Cancer Research*, **10**, 211 (1950).

³⁷⁸ G. Young and G. Wald, *Am. J. Physiol.*, **131**, 210-215 (1940).

Goodwin and Wilson³⁷⁹ were unable to verify this finding. Bodansky and Markardt³⁸⁰ found that 3- β -acetoxy-17- α -hydroxyallopregnan-20-one ("Compound L") which, they state,³⁸⁰ was isolated from the adrenal gland by Reichstein,³⁸¹ decreases plasma vitamin A when injected into male rats. Apparently the decrease in the plasma vitamin A level occurs concomitantly with an increased deposition in the liver.³⁸⁰

Season is a factor which must be taken into consideration with respect to the level of vitamin A and of carotene in the plasma, particularly in the case of cattle. Braun²⁹⁷ recorded a maximum average of 43.5 $\mu\text{g. \%}$ (145 I.U.) in March for the plasma vitamin A of Holsteins, while the minimum level of September and October samples was 10 $\mu\text{g. \%}$ (33 I.U.). Lord³¹⁹ reported similar variations in Ayrshire cows, with a maximum figure of 45 $\mu\text{g. \%}$ (150 I.U.) when on summer pasturage, and a minimum average of 9.3 $\mu\text{g. \%}$ (31 I.U.) during stall feeding in the winter. No seasonal variations in plasma vitamin A levels have been observed in school children.³⁰²

(c) *Pathologic Factors Altering Blood Vitamin A.* *Diabetes mellitus* is believed to be associated with impairment of the conversion of carotene to vitamin A. According to a number of workers,³²²⁻³²⁸ the carotenemia is associated with a low plasma vitamin A, although Kimble *et al.*³³⁰ were unable to demonstrate any reduction of vitamin A in 116 unselected cases of diabetes, prior to hospitalization.

Acetonemia in cattle has been found to be associated with an extremely low plasma vitamin A level (1.2 $\mu\text{g. \%}$ or 4 I.U.) and an unusually high level of plasma carotene.³³⁸ It is suggested that inadequate transformation of carotene to vitamin A may be the cause of the low level of vitamin A in the plasma. The acetonemia has been successfully treated by a number of investigators,³³⁸⁻³⁴¹ by the administration of vitamin A.

Liver disease may likewise result in abnormally low levels of plasma vitamin A. Thus, Harris and Moore³⁵⁹ reported a level of 16.4 $\mu\text{g. \%}$ (54.7 I.U.) in hepatitis, which increased to 35.4 $\mu\text{g. \%}$ (118 I.U.) when a cure was effected. Low vitamin A values were reported in cases of liver disease in man.^{316,382-384} A decrease in plasma vitamin A was noted in pneumonia.³⁴⁴ No changes could be demonstrated in this value in children

³⁷⁹ T. W. Goodwin and A. A. Wilson, *Biochem. J.*, **45**, 370-372 (1949).

³⁸⁰ O. Bodansky and B. Markardt, *J. Biol. Chem.*, **190**, 83-93 (1951).

³⁸¹ T. Reichstein, *Helv. Chim. Acta*, **19**, 29-63, 402-412 (1936).

³⁸² N. Fiessinger and H. Torres, *Compt. rend. soc. biol.*, **135**, 636-637 (1941).

³⁸³ N. Fiessinger, H. Torres, and A. Gasnier, *Compt. rend. soc. biol.*, **135**, 697-698 (1941).

³⁸⁴ H. Popper, F. Steigmann, and Z. Zevin, *J. Clin. Invest.*, **22**, 775-783 (1943).

suffering from folliculosis, even after high dosages of vitamin A.³⁰⁹ Chieffi and Kirk³³⁵ found that hyperkeratosis of the skin follicles, and local conjunctival thickening occurred with greater frequency in subjects having a low plasma vitamin A (1 to 15 $\mu\text{g. \%}$ or 3 to 50 I.U.) than in those having a higher plasma vitamin A level (25 to 60 $\mu\text{g. \%}$ or 83 to 200 I.U.). However, no correlation could be demonstrated between the plasma vitamin A level and the number of epithelial cells excreted daily in the urine, and no relationship was observed between plasma vitamin A and the proportion of keratinized cells in the urinary sediment. Blood vitamin A, and the factors which influence it, are more completely treated in *The Lipids*, Vol. II, pages 366 to 369 and 486 to 509.

6. The Storage of Carotenoids and of Vitamins A

Carotenoids are widely distributed in the plant kingdom, both in the higher forms and in the grass-green varieties of algae (*Chlorophyta*),³³⁶ and in the parasitic "red-snow" ascomycetous fungus (*Chlamydomonas nivalis*).³³⁶ They are present to a lesser extent in animals. Larger amounts are found in the lower forms of animal life than occur in the tissues and fat depots of the higher forms. On the other hand, vitamin A is exclusively an animal product, which has an exceedingly wide distribution in practically all species of animals. For a discussion of the distribution of carotenoids in plant and animal sources, the reader is referred to *The Lipids*, Vol. I, pages 507 to 666, while the distribution of vitamin A in animals is included on pages 675 to 713 of that treatise. Reference is especially made to the monographs of Williams,³³⁷ of Goodwin,^{192,338} and of Fox³⁸⁹ for the most complete information on carotenoids in plants, invertebrates and vertebrates. Other reviews on this subject include the treatises of Rosenberg,¹ With,⁸⁴ Lederer,³⁹⁰ and Fox.³⁹¹ Goodwin³⁹² reviewed the biogenesis of carotenoids.

³³⁵ M. Chieffi and E. Kirk, *J. Nutrition*, **37**, 67-79 (1949.)

³³⁶ G. M. Smith, *The Fresh-Water Algae of the United States*, 2nd ed., McGraw-Hill, New York, 1950, pp. 19-23, 39-41, 110-111.

³³⁷ R. J. Williams, *The Significance of the Vitamin Content of Tissues, Vitamins and Hormones*, **1**, 229-247 (1943).

³³⁸ T. W. Goodwin, *Biol. Revs. Cambridge Phil. Soc.*, **25**, 391-413 (1950).

³⁸⁹ D. L. Fox, *Animal Biochromes and Structural Colours*, Cambridge Univ. Press, 1953.

³⁹⁰ E. Lederer, *Les Carotenoides des Animaux*, Hermann, Paris, 1935.

³⁹¹ D. L. Fox, *Ann. Rev. Biochem.*, **16**, 443-470 (1947).

³⁹² T. W. Goodwin, *J. Sci. Food Agr.*, **4**, 209-220 (1953).

(1) *Comparative Distribution in Different Animals*

a. In Invertebrates. Goodwin¹⁹² emphasized the widespread distribution of carotenoids throughout all groups of invertebrates and vertebrates. In the case of the marine invertebrates, the carotenoids are of two types, *viz.*, (a) those derived directly from plants eaten as food, and (b) those characteristic of the animal species, which are produced either by altering the alimentary carotenoids or by *de novo* synthesis. However, Goodwin¹⁹² is of the opinion that it is unlikely that carotenoids originate *de novo* in the invertebrates. Several types of carotenoids have been identified in the same organism. Thus, Goodwin alone³⁹³ and with Taha³⁹⁴ noted the presence of four carotenoids in the sex organs of the limpets (*Patella vulgata* and *P. depressa*, British Isles), namely β -carotene, echinenone, cryptoxanthin, and zeaxanthin. Traces of α -carotene were also occasionally found. The male gonads were found to be pink, due to carotenoids not attached to protein. The green-brown color of the ovaries was attributed to an unidentified pigment, "Y," attached to a protein.³⁹⁴ Astaxanthin, which is 3,3'-dihydroxy-4,4-diketo- β -carotene, is widely distributed in the crustaceans,²⁹⁵ for example in the planktonic copepod (*Calanus finmarchicus*).³⁹⁵

(a) *Carotenoids in Protozoa.* According to Fox,³⁹⁰ carotenoids in certain species of protozoa give rise to the conspicuous green, yellow, orange, and red colors frequently observed in rain ponds, lakes, salt ponds, the sea, and even in snow.³⁹⁶⁻³⁹⁹ As examples various authors cite the green euglena (*Mostigophora (Euglena) viridis*) and the red euglena (*M. sanguinea*), which are freshwater flagellate protozoa, and a sub-genus of the *Euglenidae* a brown euglena (*Trachelomonas* spp.)³⁹⁸ and also the green and red varieties of the brine flagellate protozoon (*Dunaliella salina*)^{396,397} found in salt ponds; both the green and the red varieties contain considerable amounts of β -carotene. The red, freshwater flagellate protozoon (*Haematococcus pluvialis*)^{396,399} contains a carotene which colors rain water red. Chatton and co-workers⁴⁰⁰ observed that certain parasitic infusoria acquire the carotenoid pigments by consuming the eyes of *Crustacea*, such as the free-swim-

³⁹³ T. W. Goodwin, *Biochem. J.*, **47**, 249-251 (1950).

³⁹⁴ T. W. Goodwin and M. M. Taha, *Biochem. J.*, **47**, 244-249 (1950).

³⁹⁵ H. v. Euler, H. Hellström, and E. Klusmann, *Z. physiol. Chem.*, **228**, 77-89 (1934).

³⁹⁶ H. F. Blum and D. L. Fox, *Univ. Calif. (Berkeley), Publs. Physiol.*, **8**, 21-30 (1933).

³⁹⁷ D. L. Fox and M. C. Sargent, *Chemistry & Industry*, **16**, (57), 1111 (1938).

³⁹⁸ F. E. Fritsch, *The Structure and Reproduction of the Algae*, Cambridge Univ. Press, London, 1935, Vol. I, pp. 32-35, 725-729.

³⁹⁹ M. Lwoff and A. Lwoff, *Compt. rend. soc. biol.*, **105**, 454-456 (1930).

⁴⁰⁰ E. Chatton, A. Lwoff, and M. Parat, *Compt. rend. soc. biol.*, **94**, 567-570 (1926).

ming deep-sea copepod (*Idya furcata*). It is interesting that *M. sanguinea* is red in the sunlight and green in the shade.

(b) *Carotenoids in Metazoa*. a'. *Porifera* (Sponges): A number of members of this group of Metazoa exhibit brilliant pigments, which were first examined in 1882 by Krukenberg.⁴⁰¹ Lönnberg⁴⁰² demonstrated the carotenoid character of the pigments in the "crumb of bread" sponge (*Halichondria panicea*), in the fig-shaped sponge (*Suberites ficus*), in the brittle red Mediterranean sponge (*Dysidea fragilis*), and in the red sponge (*Axinella rugosa*). The nature of the carotenoids in sponges is unique, in that they consist chiefly of carotenes, while most other invertebrates store xanthophyll pigments.⁴⁰³ Lederer²³ reported the presence of a complex mixture of hydrocarbons containing α -, β -, and γ -carotenes, lycopene and torulene in the clavuline sponge (*Suberites domuncula*) and in the fig-shaped sponge (*Ficulina ficus*). Astaxanthin (astacene) was isolated by Karrer and Solmssen⁴⁰⁴ from the Atlantic red sponge (*Axinella cristagalli*). Drumm *et al.*^{405,406} observed the presence of α -, β -, and γ -carotenes, as well as of a pigment believed to be echinenone, in the red-rock sponge (*Hymeniacidon sanguinea*); however, no astaxanthin was found.

b'. *Cnidaria*: Of all the animal kingdom, this group, which includes the jelly-fishes, sea-anemones, corals, and related species, exhibits the greatest variety and degree of pigmentation.⁴⁰⁷ Carotenoids were noted in a hydroid gymnoblast (*Clava squamata*).¹⁹² The most complete reviews on the distribution of carotenoids in this group, and in other invertebrates, are those of Lederer,⁴⁰⁸ and of Fox and Pantin.⁴⁰⁹

The carotenoids have been shown to be conjugated with proteins in some cases. Thus, the blue pigmentation of the siphonophore, the blue jelly-fish, "by-the-wind sailor" (*Verella spirans*) is acquired through conjugation with protein.^{410,411}

⁴⁰¹ C. F. W. Krukenberg, *Die Lipochrome der Spongien, Vergleichendphysiologische Studien* [2], Part 3, 108-115 (1882); cited by D. L. Fox, *Ann. Rev. Biochem.*, **16**, 443-470 (1947), p. 444.

⁴⁰² E. Lönnberg, *Arkiv Zool.*, **A 22**, No. 14, 1-49 (1931); **A 25**, No. 1, 1-17 (1932); **A 26**, No. 7, 1-36 (1933).

⁴⁰³ D. L. Fox, D. M. Updegraff, and G. D. Novelli, *Arch. Biochem.*, **5**, 1-23 (1944).

⁴⁰⁴ P. Karrer and U. Solmssen, *Helv. Chim. Acta*, **18**, 915-921 (1935).

⁴⁰⁵ P. J. Drumm and W. F. O'Connor, *Nature*, **145**, 425 (1940).

⁴⁰⁶ P. J. Drumm, W. F. O'Connor, and L. P. Renouf, *Biochem. J.*, **39**, 208-210 (1945).

⁴⁰⁷ E. Lönnberg and H. Hellström, *Arkiv Zool.*, **A 23**, No. 15, 1-74 (1931).

⁴⁰⁸ E. Lederer, *Biol. Rev. Cambridge Phil. Soc.*, **15**, No. 3, 273-306 (1940).

⁴⁰⁹ D. L. Fox and C. F. A. Pantin, *Biol. Rev. Cambridge Phil. Soc.*, **19**, 121-134 (1944).

⁴¹⁰ D. Haurowitz and H. Waelsch, *Z. physiol. Chem.*, **161**, 300-317, 318 (1926).

⁴¹¹ B. Krepp, *Biol. Bull.*, **60**, 120-123 (1931).

Although Palmer,²¹ in 1922, stated that there was no evidence of the presence of carotenoids in the coelenterates, a number of investigators, particularly Lönnberg,^{402,407,412} demonstrated their widespread distribution in these *Metazoa*.

Several factors are responsible for the particular color developed. For example, Schultze⁴¹³ reported that, in the case of the freshwater polyps (*Hydra* spp.), the nutritional condition influenced the color; whereas well-nourished animals were yellow, red, or black, they became colorless when fasted. This is also true of two sea-mollusks, the nudibranch (*Fiona marina* Forsk.) and the blue sea-snail (*Janthina communis* Lam.), which feed on the blue jelly-fish (*Vellela spirans*), and become colorless when deprived of this food.⁴¹³

The type of color developed may also vary within the species. Thus, three varieties of the purple-red sea-anemone (*Actinia (equina) mesembryanthemum*) have different colors, red, brown, and green. All of them derive their carotenoids originally from the diet.⁴¹⁴ It is evident either that a striking specificity in primary selective absorption obtains, or that there is a variation in the metabolism of a common compound absorbed by all three varieties. The red animals were found by Lederer⁴¹⁵ and by Fabre and Lederer⁴¹⁶ to have, in addition to α - and β -carotenes, a red carotenoid ester combined with protein, which was called actinioerythrin. This finding was confirmed by Heilbron et al.⁴¹⁷ The parent carotenoid portion was later isolated and given the name *violerythrin*.⁴¹⁷ The yellow pigment was found by Heilbron and co-workers⁴¹⁷ to be carotene, or the taraxanthin ester. It is believed that the brown variety is intermediate between the green and the red, and that it contains both actinioerythrin and the taraxanthin ester.¹⁹²

A number of specific xanthophylls have been identified in coelenterates, including sulcatoxanthin, from the sea-anemone (*Anemonia sulcata*);⁴¹⁷ a similar if not identical pigment was reported in the Pacific coast rough sea-anemone (*Anthopleura xanthogrammica*),⁴¹⁸ and in the snowy white or red, yellow, or brown plumose sea-anemone (*Metridium senile*).⁴¹⁹ However, the sulcatoxanthin appears to be identical with piridin in obtained from the

⁴¹² E. Lönnberg, *Arkiv Zool.*, A 30, No. 6, 1-10 (1938).

⁴¹³ P. Schultze, *Arch. Biontol. (Berlin)*, 4, No. 2, 33-119 (1917).

⁴¹⁴ M. Abeloos-Parize and R. Abeloos-Parize, *Compt. rend. soc. biol.*, 94, 560-562 (1926).

⁴¹⁵ E. Lederer, *Compt. rend. soc. biol.*, 113, 1391-1392 (1933).

⁴¹⁶ R. Fabre and E. Lederer, *Bull. soc. chim. biol.*, 16, 105-118 (1934).

⁴¹⁷ I. M. Heilbron, H. Jackson, and R. N. Jones, *Biochem. J.*, 29, 1384-1388 (1935).

⁴¹⁸ H. H. Strain, W. M. Manning, and G. Hardin, *Biol. Bull.*, 86, 169-191 (1944).

⁴¹⁹ D. L. Fox and C. F. A. Pantin, *Phil. Trans. Roy. Soc. London*, B 230, 415-450 (1941).

microplanktonic dinoflagellate (*Peridinium cinctum*), and it is probable that the sulcatoxanthin found in the tentacles of the sea-anemones originated in the algae which inhabit them. It was concluded that the astacin-like pigment in *Metridium senile* was not astacin but a new compound, which was called metridin.⁴¹⁹ The white variety contains small amounts of astacin esters; the red anemone has larger amounts of carotenoids, consisting of metridin esters, carotene, xanthophyll and xanthophyll esters; the yellow-orange variety contains considerable carotene and xanthophyll, and some xanthophyll esters. The brown anemone contains only small amounts of carotene, xanthophyll and its esters, and astacene esters. Tetronerythrin was found in the verrucose, sessile coral or "sea-fan" (*Gorgonia verrucosa*),⁴²⁰ and a pigment spectroscopically resembling lutein was noted in the purple-red sea-anemone (*Actinia mesembryanthemum*).⁴²¹

(c) *Carotenoids in Echinodermata.* a'. *Asteroidea*: The tissues of this group have bright carotenoid colors, especially the integument, digestive glands, and eggs.¹⁹² The so-called "zoöerythrine rouge"¹⁹² or tetronerythrin identified by Merejkowski⁴²⁰ in fourteen species of echinoderms is probably identical with astaxanthin.¹⁹² Euler and Hellström⁴²² isolated a blue chromoprotein from the red, "five-finger" starfish (*Asterias rubens*) which, on hydrolysis, gave rise to a carotenoid called asteric acid. This is most probably astaxanthin.²⁴ A number of other *Asteroidea* have been shown to contain astaxanthin, including the sun-star (*Echinaster sepositus*),⁴⁰⁴ the reddish-violet, short-spined starfish (*Solaster endica*),⁴²² the reddish-purple, cushion-shaped starfish (*Porania pulvillus*),⁴²² and the rose-star, a cream and pink starfish (*Crossaster (Solaster) papposus*).⁴²² The dorsal skin of the last species contains a chromoprotein which, on denaturation, yields astaxanthin. Fox and Scheer⁴²³ also identified astaxanthin in a number of echinoderms found on the Pacific coast, including the paxillate starfish (*Astropecten californicus*), the broad-disk star (*Patiria miniata*), the purple or ochre starfish (*Pisaster ochraceus*), and the giant star (*P. giganteus*). In addition to astaxanthin, zeaxanthin or a pigment similar to it was found in all of these species, except *Astropecten californicus*, which contained pectenoxanthin. Mytiloxanthin was detected in *Pisaster ochraceus*, and metridin in *Pisaster giganteus*. In *P. ochraceus*, 49% of the pigment was in the skin, and 47% in the pyloric cecum, while in *P. giganteus* the values were 7.2% and 22%, respectively.

⁴²⁰ C. De Merejkowski, *Compt. rend.*, 93, 1029-1032 (1881).

⁴²¹ C. A. MacMunn, *Phil. Trans. Roy. Soc. London*, 176, 641-663 (1880).

⁴²² H. v. Euler and H. Hellström, *Z. physiol. Chem.*, 223, 89-97 (1934).

⁴²³ D. L. Fox and B. T. Scheer, *Biol. Bull.*, 80, 441-455 (1941).

Although it would seem that astaxanthin is characteristic of the *Asteroidea*, there is equally good reason for considering it to be characteristic of the *Crustacea*.

b'. *Ophiuroidea*: Lönnberg⁴²⁴ demonstrated the presence of carotenoids in a number of species of brittle-stars. According to Goodwin,¹⁹² they were found in the epidermis of these stars. Fox and Scheer⁴²³ demonstrated that the predominant pigment in one brittle-star (*Ophiopteris papillosa*), in the red-black, spiny brittle-star (*O. spiculata*) and in the rough serpent-star (*Ophiothrix rudis*), is a xanthophyll resembling taraxanthin. A previously undescribed pigment which is very unstable also occurs. Pectenoxanthin is also found in *O. papillosa*. One interesting finding is that β -carotene was completely absent from all of these species. Karrer and Benz⁴²⁵ identified astaxanthin as the pigment in the dark red brittle sea-star (*Ophidiaster ophidianus*).

c'. *Holothuroidea*: The red-yellow gonads of the Northern sea-cucumber (*Mesothuria (Holothuria) intestinalis*), and the blue gonads of the tentacled sea-cucumber (*Cucumaria lactea*), were shown^{192,402} to contain carotenoids; 48% of the carotenoid in *C. lactea* is carotene, while some echinenone is also present.⁴²³

d'. *Crinoidea*: Although no measurable amounts of carotenoids were detected in the sea-lily (*Antedon rosacca*),⁴⁰⁴ some have been observed qualitatively in a related erinoid (*A. petasus*).⁴⁰²

e'. *Echinoidea*: The major portion of the carotenoids stored by the echinoids is present in the gonadal and intestinal tissues. Echinenone is a prominent pigment in this group, and was originally believed to be characteristic. Lederer⁴²⁶ first obtained echinenone from the sex glands of the brownish-purple Atlantic sea-urchin (*Strongylocentrotus lividus*), and it has since been isolated from the gonads of the edible sea-urchin (*Tripneustes esculentus*), from those of the Pacific pale sea-urchin (*Lytechinus pictus*), by Fox and Scheer,⁴²³ and from those of the straw-colored, burrowing heart-urchin (*Echinocardium cordatum*) by Goodwin and Srisukh.⁴²⁷ β -Carotene and lutein were also present in the latter organisms. However, echinenone is also widely distributed among other invertebrates. Goodwin and Taha⁴²⁸ found that it is probably identical with myxoxanthin, isolated from the purple plankton, freshwater alga (*Oscillatoria rubescens*) by Heil-

⁴²⁴ E. Lönnberg, *Arkiv Zool.*, A 28, No. 6, 1-4 (1936).

⁴²⁵ P. Karrer and F. Benz, *Helv. Chim. Acta*, 17, 412-416 (1934).

⁴²⁶ E. Lederer, *Compt. rend.*, 201, 300-302 (1935).

⁴²⁷ T. W. Goodwin and S. Srisukh, *Nature*, 167, 358-359 (1951).

⁴²⁸ T. W. Goodwin and M. M. Taha, *Biochem. J.*, 48, 513-514 (1951).

bron and Lythgoe,⁴²⁹ and with aphanin, separated from the "water-bloom" plankton (*Aphanizomenon flos-aquae*) by Tischer.^{430,431} Echinenone likewise occurs in the gonads of two gastropods, the limpets (*Patella vulgata*, *P. depressa*),³⁹⁴ and in the red-rock sponge (*Hymeniacidon sanguinea*).^{405,406}

In the case of *Strongylocentrotus lividus*, Lederer^{23,426} reported that α - and β -carotenes, pentaxanthin, and other xanthophylls were present along with echinenone. In two other species, the purple urchin (*Strongylocentrotus purpuratus*) and the pale sea-urchin (*Lytechinus pictus*), Fox and Scheer⁴²³ observed large stores of carotenoids in the intestine and gonads of both sexes. In *Strongylocentrotus lividus*, the concentration of carotenoids in the female gonads was three times that in the male gonads. In both species of *Strongylocentrotus* examined, the gonad carotenoids consisted exclusively of carotenes. On the other hand, in the case of *Lytechinus pictus*, the male sex glands yielded about four times as much carotene and twice as much xanthophyll as did the ovaries.

Echinenone was also observed in the perivisceral fluid and gonads of the heart-urchin (*Echinocardium cordatum*), and in the urchin (*Tripneustes esculentus*).⁴²⁷

(d) *Carotenoids and Vitamins A in Arthropoda.* a'. *Crustacea:* Lwoff^{432,433} was the first to emphasize that the carotenoid in the deep-sea copepod (*Idya furcata*) was present as a protein complex. Carotenoids were likewise noted in the luminous organs of small bioluminescent, shrimp-like crustaceans (*Euphausiidae*) and in the retinae of the shrimp-like crustaceans (*Nebalia*) and of the hermit crab (*Eupagurus prideauxii*). Lwoff^{432,433} alone and with co-workers,⁴⁰⁰ and Verne,⁴³⁴⁻⁴³⁶ almost simultaneously reached the conclusion that carotenoids are attached to proteins in marine invertebrates.

Astaxanthin is considered to be one of the principal carotenoids in the crustaceans. Thus, Euler, Hellström, and Klussmann³⁹⁵ reported large amounts of this carotenoid in a subarctic plankton copepod (*Calanus finmarchicus*), together with a small proportion of α - and β -carotene. Lederer³⁹⁰ was able to prepare crystalline astaxanthin from the hermit crab

⁴²⁹ I. M. Heilbron and B. Lythgoe, *J. Chem. Soc.*, 1936, 1376-1380.

⁴³⁰ J. Tischer, *Z. physiol. Chem.*, 251, 109-128 (1938).

⁴³¹ J. Tischer, *Z. physiol. Chem.*, 260, 257-271 (1939).

⁴³² A. Lwoff, *Compt. rend. soc. biol.*, 93, 1602-1604 (1925).

⁴³³ A. Lwoff, *Bull. biol. France Belg.*, 61, 193-240 (1927).

⁴³⁴ J. Verne, *Compt. rend. soc. biol.*, 94, 1349-1350 (1926).

⁴³⁵ J. Verne, *Les Pigments dans l'Organisme animale*, Doine, Paris, 1926, pp. 1-608; cited by T. W. Goodwin, *The Comparative Biochemistry of the Carotenoids*, Chapman & Hall, London, 1952, p. 167.

⁴³⁶ J. Verne, *Compt. rend. soc. biol.*, 97, 1290-1292 (1927).

(*Eupagurus prideauxii*); Goodwin and Srisukh⁴³⁷ demonstrated that the red pigmentation of the small red copepod (*Tigriopus fulvus*) is due to the presence of free and esterified astaxanthin. Other sources of astaxanthin include the eggs of the cirripeds, the goose-barnacles (*Lepas fascicularis* and *L. anatifera*).⁴³⁸ The skin of the amphipods, the European sand-hopper shrimp (*Orchestia gammarellus*), the coastal freshwater shrimp (*Gammarus marinus*) and of the freshwater flea shrimp (*Gammarus pulex*) was found to contain carotenoid pigments and an acid resembling astaxanthin. The most interesting occurrence of astaxanthin, however, is as a component of the green chromoprotein, ovoverdin, which occurs in the carapace and eggs of the European lobster (*Homarus vulgaris*).^{285, 439} Although the product first prepared from ovoverdin differed somewhat from astaxanthin, and was called astacin, Kuhn and Sørensen²⁸⁴ showed that astacin is an oxidative artefact of astaxanthin. Stern and Salomon^{285, 440} proved that ovoverdin and the blue pigment in lobster carapaces are astaxanthin-protein complexes. Burkhardt *et al.*⁴⁴¹ demonstrated the presence of astaxanthin in the Norway lobster (*Nephrops norvegicus*). The pigment in the hypodermis was found to be an esterified astaxanthin, while that in the eggs and carapace was unesterified.⁴³⁷ Ball⁴³⁸ stated that the blue pigment in the ovaries and developing embryo of the goose barnacles (*Lepas anatifera* and *L. fascicularis*), is a carotenoprotein with astaxanthin as the prosthetic group, and euglobulin as the protein. The sources of astaxanthin known up to 1939 are listed by Kuhn *et al.*⁴⁴²

β -Carotene appears to be only a minor component of the crustaceans. Thus, Kon and co-workers⁴⁴³⁻⁴⁴⁵ noted the almost complete absence of the carotenes from the "krill" forming the food of whales, and from other *Crustacea*, such as the schizopods and euphausiids (*Meganctiphanes norvegica*, *Thysanoessa inermis* and *T. raschii*), the northern prawn (*Pandalus bonnierii*), the spiny shrimp (*Spirontocarus spinus*), the nipper prawn (*Crangon allmani*), the European prawn (*C. vulgaris*), and planktonic, phosphorescent shrimp (*Euphausia superba* Dana), found in the food ("krill") of

⁴³⁷ T. W. Goodwin and S. Srisukh, *Biochem. J.*, **45**, 268-270 (1949).

⁴³⁸ E. G. Ball, *J. Biol. Chem.*, **152**, 627-634 (1944).

⁴³⁹ R. Kuhn and E. Lederer, *Ber.*, **66**, 488-495 (1933).

⁴⁴⁰ K. G. Stern and K. Salomon, *Science*, **86**, 310-311 (1937).

⁴⁴¹ G. N. Burkhardt, I. M. Heilbron, H. Jackson, E. G. Parry, and J. A. Lovern, *Biochem. J.*, **28**, 1698-1701 (1934).

⁴⁴² R. Kuhn, J. Stene, and N. A. Sørensen, *Ber.*, **72**, 1688-1701 (1939).

⁴⁴³ S. K. Kon and S. Y. Thompson, *Arch. Biochem.*, **24**, 233-234 (1949).

⁴⁴⁴ S. K. Kon and S. Y. Thompson, *Biochem. J.*, **45**, xxxi-xxxii (1949).

⁴⁴⁵ E. Batham, L. R. Fisher, K. M. Henry, S. K. Kon, and S. Y. Thompson, *Biochem. J.*, **48**, x-xi (1951).

antarctic whales. However, astaxanthin was found to occur in large amounts in all of these crustaceans.^{444,446} Fisher *et al.*⁴⁴⁶ likewise found that β -carotene was absent from the first two crustaceans listed above.

No carotenoids could be demonstrated in the skin of the cavernicolous hypogean white well-shrimps (*Niphargus (Stylodytes) balcanicus* Absolom, *Niphargus (Antroplates) herculeanus* Absolom), or in the hypogean freshwater isopod crustacean (*Asellus aquaticus cavernicolus*), although cryptoxanthin and β -carotene were present in the body tissues of *Asellus*. Carotenoid pigmentation is considered to be characteristic of amphipods, and would therefore be the ancestral pigment of the cave forms. A lack of surface pigment in the latter may be due to absence of light; it is not attributable to lack of carotenoids in the food.¹⁹²

In spite of the almost complete absence of β -carotene, preformed vitamin A has been found in many species of crustaceans.⁴⁴⁶ Thus, the *Euphausiidae*, *Meganyctiphanes norvegica* and *Thysanoessa raschii* (M. Sars), which make up the main food supply of blue and fin whales in northern waters, were found by Kon and Thompson⁴⁴⁴ to contain fairly large amounts of preformed vitamin A. In the case of *M. norvegica*, the vitamin A concentration was augmented as the animal grew in size,⁴⁴⁶ but did not increase in the case of *T. raschii*. However, the total astaxanthin content, but not its concentration, was higher in the larger animals of both species.⁴⁴⁶ The eyes of the euphausiids were found by Fisher *et al.*^{446,447} to contain 90% of the total vitamin A, while the balance of the vitamin was present in the exoskeleton and in the contents of the cephalothorax. Fisher and co-workers⁴⁴⁸ found a *cis*-isomer of vitamin A, with an absorption peak at 315 m μ in *n*-hexane, in the eyes of the euphausiid crustacean (*M. norvegica*). A large proportion of astaxanthin likewise occurs in the eyes.⁴⁴⁶ Batham *et al.*⁴⁴⁵ confirmed the fact that two-thirds of the total vitamin A in *Euphausia superba* Dana occurs in the eyes.

Opinions differ concerning the presence of vitamin A in these and in other related invertebrates. Thus, Drummond and Hilditch⁴⁴⁹ reported that the Norway lobster (*Nephrops norvegicus*) and the shrimp-like crustacean (*Meganyctiphanes norvegica*) contained little or no vitamin A. It

⁴⁴⁶ L. R. Fisher, S. K. Kon, and S. Y. Thompson, *J. Marine Biol. Assoc. United Kingdom*, **31**, 229-258 (1952).

⁴⁴⁷ L. R. Fisher, S. K. Kon, and S. Y. Thompson, *Biochem. J.*, **49**, xv-xvi (1951).

⁴⁴⁸ L. R. Fisher, K. M. Henry, S. K. Kon, P. A. Plack, and S. Y. Thompson, *Biochem. J.*, **61**, vi (1955).

⁴⁴⁹ J. C. Drummond and T. P. Hilditch, Empire Marketing Board, *Publ. No. 35*, 1-129 (1930); cited by L. R. Fisher, S. K. Kon, and S. Y. Thompson, *J. Marine Biol. Assoc. United Kingdom*, **31**, 229-258 (1952), p. 230.

will be recalled that Kon and Thompson⁴⁴⁴ had reported that the latter species contained a considerable proportion of vitamin A. Drummond and Gunther⁴⁵⁰ concluded that vitamin A as such is apparently present in only small amounts in phytoplankton, and is entirely absent from zoöplankton. The phytoplanktons studied were a diatom (*Chaetoceras* spp.) and a spring diatom from the Irish Sea (*Lauderia borealis*), while the types of zoöplanktons investigated included mixed copepods and a subarctic pelagic copepod (*Calanus finmarchicus*). Three reports state the absence of vitamin A in the last species.^{446, 451, 452} On the other hand, Gillam and co-workers⁴⁵³ did find vitamin A in these invertebrates; the maximum amounts of vitamin A coincided with the period of maximum phytoplankton occurrence, and with the zoöplankton breeding season. Fisher and colleagues⁴⁴⁶ recorded the presence of small amounts of vitamin A in the pelagic plankton copepod (*Euchaeta norvegica*).

Vitamin A has also been demonstrated in some of the higher members of the crustaceans and cephalopods. Thus, this vitamin was found in the amount of 600 I.U./g. in the oil extracted from the viscera of the edible Pacific crab (*Cancer magister*) by Pugsley.⁴⁵⁴ Wald⁴⁵⁵ reported that 1 to 2 µg. of vitamin A was found in the retina of the squid (*Loligo pealii*). Approximately three times as much retinene was present in this tissue. Vitamin A was likewise shown to occur in the eyes of the fiddler crab (*Uca pugnax*) and the green-shore crab (*Carcinides maenas*), as well as in those of an unspecified variety of lobster, but no retinene was found. Both vitamin A and retinene, as well as astaxanthin, were found in the eyes of the freshwater crayfish (*Cambarus virilis*).⁴⁵⁵ On the other hand, Fisher *et al.*⁴⁴⁶ could find no vitamin A in the amphipods, isopods, or water-fleas (*Cladocera*).

According to Neilands⁴⁵⁶ the American lobster (*Homarus americanus*), is able to convert carotene to vitamin A; thus on a carotene-free and on a carotene-containing diet, the concentration of vitamin A in the hepatopancreas was 36 and 53 I.U./g., respectively, while that in the eyes was 100 and 183 I.U./g., respectively. Vitamin A was found to be present in lower concentration in the *Decapoda* than in the *Euphausidae*, since the

⁴⁵⁰ J. C. Drummond and E. R. Gunther, *Nature*, 126, 398 (1930); *J. Exptl. Biol.*, 11, 203-209 (1934).

⁴⁵¹ G. Collin, J. C. Drummond, and T. P. Hilditch, *J. Exptl. Biol.*, 11, 198-202 (1934).

⁴⁵² E. Lederer, *Bull. soc. chim. biol.*, 20, 567-610 (1938).

⁴⁵³ E. Gillam, M. S. El Ridi, and R. S. Wimpenny, *J. Exptl. Biol.*, 16, 71-88 (1939).

⁴⁵⁴ L. I. Pugsley, *J. Fisheries, Research Board, Can.*, 5, 344-346 (1941).

⁴⁵⁵ G. Wald, *Am. J. Physiol.*, 133, 479-480 (1941).

⁴⁵⁶ J. B. Neilands, *Arch. Biochem.*, 13, 415-419 (1947).

vitamin was absent from the eyes; the bodies of these two groups contained comparable amounts of vitamin A.⁴⁴⁶ Morton and Creed,⁴⁵⁷ in 1939, also noted the conversion of β -carotene into vitamin A in two freshwater species, the dace (*Leuciscus*, spp.) and the European freshwater perch (*Perca fluviatilis*).

a". Possible Sources of Vitamin A for the Whale. The source of the vitamin A in the livers of species which have a large store of this component has been widely discussed. Since the main sources of food for fishes of this type are the zoöplankton, it is only natural to assume that they are the sources of vitamin A. Copepods are the "permanent" members of the zoöplankton, while the eggs and larvae of fishes and of invertebrates comprise the so-called "transitory" sources.⁴⁵⁸ Phytoplankton are utilized to a smaller extent.

The vitamin A content of copepods is relatively low. They live on diatoms which have a small content of β -carotene and of fucoxanthin, which is not a provitamin A. The copepod apparently converts these carotenoids mainly to astaxanthin, and stores only a minute amount as carotene. Goodwin¹⁹² indicates that a similar carotenoid distribution obtains in the case of eggs and of larvae of invertebrates in the zoöplankton. The β -carotene content of the phytoplankton is apparently similar to that of the zoöplankton.

The amount of β -carotene present in zoöplankton is believed to be inadequate to account for the large accumulation of vitamin A in plankton-eating fishes. It is claimed⁴⁵⁹ that the astaxanthin isolated from the fringed decapod shrimp (*Aristeomorpha* (*Penaeus*) *foliaceae*) is active as a source of vitamin A. However, Goodwin¹⁹² considers it highly improbable, as do most other investigators. If one discounts astaxanthin as a possible provitamin A, then, according to Goodwin,¹⁹² the possible sources of the vitamin A for the marine forms which consume the plankton are: (a) pre-formed vitamin A in the zoöplankton, (b) synthesis of vitamin A *de novo*, or (c) synthesis of vitamin A from provitamins A. The first of these possibilities is considered to be the most probable.

Some confusion has arisen since the report of Wagner⁴⁶⁰ that the plankton organisms, which were identified as *Euphausia superba* Dana, obtained

⁴⁵⁷ R. A. Morton and R. H. Creed, *Biochem. J.*, **33**, 318-324 (1939).

⁴⁵⁸ J. Johnstone, A. Scott, and H. C. Chadwick, *The Marine Plankton*, Univ. Liverpool Press, 1934.

⁴⁵⁹ R. Grangaud and R. Massonet, *Compt. rend.*, **230**, 1319-1321 (1950).

⁴⁶⁰ K. H. Wagner, *Vitamin A und β -Carotin des Finn-, Blau-, und Spermwals*, J. A. Barth, Leipzig, 1939, pp. 1-70; cited by L. R. Fisher, S. K. Kon, and S. Y. Thompson, *J. Marine Biol. Assoc. United Kingdom*, **31**, 229-258 (1952), p. 229.

from the intestine of blue and fin whales caught near the Faroë Islands in the arctic area, contained 14.5 mg./kg. of β -carotene. It was believed that this might be the source of the liver vitamin A in these whales. However, Fisher *et al.*⁴⁴⁶ question this finding, since *E. superba* Dana is not usual in the arctic areas, but only in the Antarctica. Thompson *et al.*¹³⁴ demonstrated that the "krill" from whales from the antarctic regions did consist of *E. superba* Dana. Although these euphausiids were found to contain large amounts of astaxanthin, only small quantities of β -carotene were present. Fisher *et al.*⁴⁴⁶ are of the opinion that the organisms which Wagner⁴⁶⁰ reported to be *Euphausia superba* Dana were actually *Meganyetiphanes norvegica* (M. Sars). As indicated earlier, Kon and Thompson⁴⁴⁴ demonstrated the presence of preformed vitamin A in *Meganyetiphanes norvegica* as well as in *Thysanoessa rasehii* (which is closely related to *T. inermis*, a form more commonly present). Fisher and co-workers⁴⁴⁶ calculated that the baleen whales (*Mystacoceti*), which have a stomach capacity of roughly 1200 liters, would therefore have a capacity of approximately 2000 lb. of the organisms in the food. Since the latter would have an average vitamin A content of 20 I.U./g., the whale could obtain 20,000,000 I.U. of vitamin A from one feeding. This figure was originally calculated incorrectly as 2,000,000 I.U. It would thus appear that the preformed vitamin A in these euphausiids is sufficient to account for the vitamin A stored in the livers and tissues of these whales.

b'. *Mollusca*: Lönnberg^{402,412} reported the presence of carotenoids in almost eighty species of mollusks. In most cases the pigments were present in the ripe eggs and digestive glands, but in some instances they were also found in the skin and eyes.

a". *Lamellibranchiata*. Several unique carotenoid pigments have been demonstrated in this group. One of these is *glycymerin*, which was first prepared from the sex glands of the round arc clam (*Glycymeris glycymeris*) by Lederer^{23,461} and by Fabre and Lederer;⁴¹⁶ it is a neutral xanthophyllic pigment. Lederer^{23,415} prepared another carotenoid pigment, *pectenoxanthin*, from the red gonads and mantle of the St. Jacques scallop (*Pecten maximus*).⁴⁶² In addition to this new xanthophyll, other esterified xanthophylls and β -carotene were present. A carotenoid somewhat similar to pectenoxanthin was isolated by Euler *et al.*³⁹⁵ from the horse mussel (*Volsella modiolus*). A third pigment, which is widely distributed, namely *astaxanthin* (astacene), is likewise found in some molluscs, including the

⁴⁶¹ E. Lederer, *Compt. rend. soc. biol.*, 113, 1015-1016 (1933).

⁴⁶² E. Lederer, *Compt. rend. soc. biol.*, 116, 150-153 (1934).

red scallop (*Pecten jacobaeus*),⁴⁰⁴ and astacene in the feet of the scallop-like bivalve, the tentacled mussel (*Lima excavata*).⁴⁶³

Scheer⁴⁶⁴ isolated another carotenoid, called *mytiloxanthin*, from the gonads, digestive diverticulum, and integument of the California plankton-feeding mussel (*Mytilus californianus*). In addition to the above acidic carotenoid, zeaxanthin was found. Almost no carotenes occurred in *Mytilus*.

In the case of the edible Portuguese oyster (*Gryphca angulata*) and the European flat oyster (*Ostrea edulis*), Brooks and Paulais⁴⁶⁵ reported the presence of both carotenes and xanthophylls; the viscera contained about twenty times the concentration of the pigments found in the rest of the animal. Jones and Murphy⁴⁶⁶ found that oysters are relatively rich in preformed vitamin A.

b". *Cephalopoda*. In general, the carotenoids are largely absent from this group. Lutein was reported in the eyes of three species by Lönnberg,⁴²⁴ namely the cuttlefish (*Sepiolo scandica*), the octopus cuttlefish (*Rossia macrosoma*), and the Atlantic octopus, devil-fish (*Eledone cirrosa*). Carotenoids were detected in the liver of *E. cirrosa*, but none were present in the other decapods, and they were absent even from the eggs of *Rossia macrosoma* and *Sepiolo scandica*. Wagner and Vermeulen⁴⁶⁷ likewise reported the absence of carotenoids from cuttlefish, and Leong⁴⁶⁸ found only traces.

The above investigations, which indicate the scarcity of the carotenoids in the *Cephalopoda*, are in line with other studies. Thus, Wald⁴⁶⁵ found no carotenoids in the retina or other tissues of the squid (*Loligo pealii*), although vitamin A and retinene were found in the retina. Fox and Crane⁴⁶⁹ observed the presence of traces of carotenoids in the eyes of the opalescent squid (*Loligo opalescens*), but none, or only questionable traces, could be located in other tissues. However, in the case of the liver of the two-spotted Pacific octopus (*Octopus bimaculatus*), lutein or a carotenol resembling it, other unfamiliar xanthophylls and β -carotene, were identified. All except the carotenes were found to be excreted in the ink.

c". *Gastropoda*. Although carotenoids have been detected in a number of gastropods,¹⁹² few detailed data are available. The gonads of the Euro-

⁴⁶³ N. A. Sørensen, *Kgl. Norske Videnskab. Selskabs Skrifter*, 1936-1937, No. 1, 1-14.

⁴⁶⁴ B. T. Scheer, *J. Biol. Chem.*, 136, 275-299 (1940).

⁴⁶⁵ G. Brooks and R. Paulais, *Bull. soc. chim. biol.*, 22, 304-310 (1940).

⁴⁶⁶ D. B. Jones and J. C. Murphy, *Proc. Soc. Exptl. Biol. Med.*, 23, 519-520 (1926).

⁴⁶⁷ K. H. Wagner and M. Vermeulen, *Ernährung*, 4, 193-199 (1939).

⁴⁶⁸ P. C. Leong, *J. Malaya Branch Brit. Med. Assoc.*, 2, 219-228 (1939).

⁴⁶⁹ D. L. Fox and S. C. Crane, *Biol. Bull.*, 82, 284-291 (1942).

pean limpets (*Patella vulgata* and *P. depressa*) have been shown to contain at least five different carotenoids, viz., α - and β -carotenes, echinenone, cryptoxanthin, and zeaxanthin. A compound resembling astacene was found in one of the *Tectibranchiata*, the mantled sea-slug (*Pleurobranchus elegans*).⁴⁷⁰ Strain⁴⁷⁰ reported the presence of another, hitherto undescribed rose-pink carotenoid, hopkinsiaxanthin, in the nudibranch mollusc (*Hopkinsia rosacea*).

c'. *Insecta*: The carotenoids are widely distributed in insects, although the data on their distribution and metabolism are quite limited. The most comprehensive review of this subject is that of Goodwin.¹⁹²

a". *Coloptera*. A so-called "linacarotene" was reported as early as 1893 by Zopf⁴⁷¹ in the reddish-brown elytras (thickened fore-wings), body wall, and eggs of a large iridescent poplar beetle (*Lina populi*), and of the small poplar beetle (*L. tremulae*); it was likewise reported in the yellow juice which they excrete. The same substance was identified by Zopf⁴⁷¹ in the corresponding tissues of the seven-spotted ladybird (*Coccinella septempunctata*) and in a ladybird with five spots (*C. quinquepunctata*). Although Wall⁴⁷² identified carotene in a nine-spotted variety (*C. novempunctata*), Lederer⁴⁷³ proved that the elytral "linacarotene" from *C. septempunctata* was actually a mixture of α - and β -carotenes and lycopene. No free xanthophylls were present, but some esterified xanthophylls occurred in the body.

The early report of Palmer and Knight⁴⁷⁴ indicates a similar carotenoid pattern in the Colorado potato beetle (*Leptinotarsa (Doryphora) decemlineata*), i.e., carotene but no xanthophylls. However, Manunta⁴⁷⁵ believes that this beetle carotenoid is similar to that extracted from the carmine flamingo (*Phoenicopterus roseus*), which has been called "phoenicoxanthin." Sweetman and Palmer⁴⁷⁶ reported that the confused flour beetle or weevil (*Tribolium confusum*) can exist on carotenoid-free diets; furthermore, it does not synthesize these compounds *de novo*. Crowell and McCay⁴⁷⁷ found that the webbing clothes moth (*Tineola bisselliella* Hum.) required neither fat nor fat-soluble vitamins for growth.

b". *Lepidoptera*. Carotenoids were first identified in the hemolymph,

⁴⁷⁰ H. H. Strain, *Biol. Bull.*, 97, 206-209 (1949).

⁴⁷¹ W. Zopf, *Beitr. Physiol. Morphol. niederer Organ.*, 1, 30-56 (1892); 2, 3-35 (1892); 3, 26-47 (1893).

⁴⁷² R. E. Wall, *Ann. Entomol. Soc. Amer.*, 26, 425-463 (1933).

⁴⁷³ E. Lederer, *Compt. rend. soc. biol.*, 117, 413-416 (1934).

⁴⁷⁴ L. S. Palmer and H. H. Knight, *J. Biol. Chem.*, 59, 443-449 (1924).

⁴⁷⁵ C. Manunta, *Nature*, 162, 298 (1948).

⁴⁷⁶ M. D. Sweetman and L. S. Palmer, *J. Biol. Chem.*, 77, 33-52 (1928).

⁴⁷⁷ M. F. Crowell and C. M. McCay, *Physiol. Zool.*, 10, 368-372 (1937).

larvae, and in the chrysalids of *Lepidoptera* in 1885, by Poulton,⁴⁷⁸ while Meyer⁴⁷⁹ reported carotenes in the hemolymph of nine families of *Lepidoptera*. β -Carotene alone was found to exist in the hemolymph and fat bodies of a grayish-yellow, spotted, leaf-eating butterfly (*Caradrina quadripunctata*), as well as in the intestine and pupae of the white cabbage butterfly (*Pieris brassicae* (*rapae*)) and of the nettle tortoise-shell butterfly (*Vanessa urticae*). Manunta⁴⁸⁰ stated that the hemolymph and the hypodermis of the lemon, rose, and orange strains of the silk-worm contained both carotenes and xanthophylls. The carotenoids of *P. brassicae* were identified as α -carotene and taraxanthin.⁴⁸¹

The clothes moth (*Tineola bisselliella*) belongs in the same category as the flour beetle or meal worm (*Tenebrio molitor*), since it also does not require carotenoids, nor does it synthesize any on a carotenoid-free diet. Poulton⁴⁷⁸ observed a definite relationship between the coloration of this and of other species and the diet. For example, the aromatic butterfly (*Sphinx* (*Hyloicus*) *ligustri*) larvae fed on ash leaves were grayish green; those raised on privet had a bright yellow ground color with red-purple stripes, and those fed on lilac were vivid purple to brownish. Similar color mutations were observed in blue-green caterpillars of the alfalfa butterfly, or orange-sulphur (*Colias* (*Eurymus*) *philodice*) at different times of the year, corresponding to changes in the food available.⁴⁸² Manunta⁴⁸³ found that the hemolymph of the yellow-blood species of the Chinese silk-worm (*Bombyx mori*) contained 100 to 150 times as much carotene as did the white species. The xanthophylls were present in larger proportions than were the carotenes. At the time of metamorphosis the pigments pass from the blood to the silk glands, and are found in the cocoon. The blood of the Japanese green silkworm (*Theophila mandarina*) contains traces of carotenoids.⁴⁸³ However, on some yellow and green hybrid races of silkworm, Manunta found no carotenes but only xanthophylls.⁴⁸³

c". *Hemiptera*. Two members of this group obtain their carotenoids second-hand from other insects. Palmer and Knight⁴⁷⁴ reported that a bi-oculate predaceous bug (*Perillus bioculatus* Fabr.) obtains its carotenoid, which is probably β -carotene, by sucking the hemolymph of the larval and

⁴⁷⁸ E. B. Poulton, *Proc. Roy. Soc. London*, 38, 269-315 (1885).

⁴⁷⁹ P. F. Meyer, *Z. vergleich. Physiol.*, 11, 173-209 (1930).

⁴⁸⁰ C. Manunta, *Atti soc. nat. e mat. Modena*, 65, 88-93 (1934).

⁴⁸¹ C. Manunta, *Atti accad. Italia (Rome), Rend. classe sci. fis. e. nat.*, 3, 151-153 (1941).

⁴⁸² J. H. Gerould, *J. Exptl. Zool.*, 34, 385-415 (1921).

⁴⁸³ C. Manunta, *Atti accad. naz. Lincei*, [6], 18, No. 11, 513-515 (1933); *Boll. soc. ital. sper.*, 8, 1278-1282 (1933); *Mem. accad. naz. Lincei*, [6], 6, 75-161 (1935).

adult Colorado potato beetle (*Leptinotarsa decemlineata*) which, in turn, obtained it from the leaves of potato plants. Although β -carotene was found by Wall⁴⁷² to be the only carotenoid in the cotton aphid, melon or plant louse (*Aphis gossypii* Glover), Lederer²³ reported that another member of the Hemiptera, the red and black fire-bug (*Pyrrhocoris apterus*) contained only lycopene, and no β -carotene.

d". *Hymenoptera*. This order includes the ichneumon flies, ants, and bees. A black, hymenopterous parasite (*Apanteles flaviconchae*) normally feeds on the larvae of a blue-green caterpillar of the clouded sulfur butterfly (*Colias (Eurymus) philodice*); the xanthophylls which it obtains in this manner are excreted in the yellow silk with which it makes its cocoons. When it feeds on the green mutant of *C. philodice*, which contains no xanthophyll, the silk produced is colorless.⁴⁸² Von Euler and co-workers³⁹⁵ reported the presence of carotene in the eggs of certain ants. The parasitic braconid fly (*Microgaster (Apanteles) glomeratus*) contains the same carotenoids as does its host, which is the caterpillar of the cabbage butterfly (*Pieris brassicae*), viz., α -carotene and taraxanthin.⁴⁸¹

Carotene has been demonstrated in honey and beeswax,^{192,484} as well as in propolis (bee glue).⁴⁸⁵ In addition to β -carotene in honey, which Schuette and Bott⁴⁸⁴ believed was its only carotenoid, Tischer⁴⁸⁵ demonstrated the presence of lutein; it is, of course, probable that the constituent pigments vary with the source of the pollen.

e". *Orthoptera*. The carotenoids in the *Orthoptera*, which include the locusts and similar winged insects, have recently been extensively examined. Podiapolsky,⁴⁸⁶ in 1907, was the first to call attention to the xanthophylls in one member of this order, namely the great long-horned green grasshopper or leaf-locust (*Locusta (Tettigonia) viridissima*); Panu and Verrier⁴⁸⁷ also reported that large amounts of carotenoids were produced in some red dry-leaf insects (*Phyllium siccifolium*), by raising them on the oil of green oak leaves. The green variety, nourished on ordinary oak leaves, contained no carotenoids or xanthophyll.⁴⁸⁷

The most comprehensive investigation of the *Orthoptera* was made by Przibram and Lederer.⁴⁸⁸ Carotenes have been universally found to be the most common carotenoid in this group of insects. For example, Przibram and Lederer⁴⁸⁸ reported that α - and β -carotenes, "chlorophyll," and a blue water-soluble pigment were responsible for the green coloration of the East Indian walking-stick insect (*Dixippus morosus*). It is now believed that the blue pigment in *D. morosus* is not "chlorophyll" but a

⁴⁸⁴ H. A. Schuette and P. A. Bott, *J. Am. Chem. Soc.*, 50, 1998-2000 (1928).

⁴⁸⁵ J. Tischer, *Z. physiol. Chem.*, 267, 14-22 (1940).

combination of a bile pigment, mesobiliverdin, and protein. This chromoprotein pigment, called "insectoverdin,"¹⁹² or carotenoid-albumin, was investigated by Junge,⁴⁵⁹ and is believed to be widely distributed in insects.^{490,491} Goodwin and Srisukh⁴⁹¹ isolated it from the solitary locust (*Locusta solitaria*), and Junge⁴⁸⁹ found it in the skin of the larvae of the aromatic butterfly (*Sphinx (Hyloicus) ligustri*). Przibram and Lederer⁴⁸⁸ also found that the red femoral swellings in *Dixippus morosus* consisted largely of α -carotene. Carotenes, but no xanthophylls, were found at every stage of the metamorphosis of the walking-leaf insect (*Phyllium pulchrifolium*).⁴⁸⁸ Grayson alone⁴⁹² and with Tauber⁴⁹³ reported that carotene is the main pigment in the two-striped locust (*Melanoplus bivittatus*), which ranges in color from reddish brown to dark green, with yellow stripes. Small amounts of xanthophyll are undoubtedly also present. The carotene was found mainly in the body cavity, where it was concentrated in the gonads. Although β -carotene was present in both the light and the dark phase of this locust, it was deposited directly below the cuticle only in the light phase.

Considerable data indicate that, in addition to carotenes, a xanthophyll-protein complex is quite widespread in this group. Thus, Lederer²³ called attention to the presence of an unidentified hydroxycarotenoid in the band-winged locust (*Oedipoda miniata*), in addition to β -carotene; Okay⁴⁹⁰ verified the results of Lederer,²³ and proved that the carotenoid was present in the wing as a water-soluble protein complex. Orthopteroerythrin, obtained by Okay⁴⁹⁴ from the bright red hind legs of *Oedipoda miniata*, was shown to be a carotenoid-protein complex instead of a bile pigment-protein complex as suggested earlier. A similar chromoprotein was found in other *Orthoptera*, including the blue-winged locusts (*Oedipoda schochii* and *O. caerulescens*), the red-winged locust (*Acrotylus insubricus*), the Italian red-winged migratory locust (*Caloptenus italicus*), and the yellow-winged locust (*O. aurea*).⁴⁹⁴⁻⁴⁹⁶ Okay^{494,495} reported carotenoproteins in a num-

⁴⁸⁶ P. Podiapolsky, *Zool. Anz.*, **31**, 362-366 (1907).

⁴⁸⁷ A. Panu and M. L. Verrier, *Compt. rend. soc. biol.*, **100**, 1118-1120 (1929).

⁴⁸⁸ H. Przibram and E. Lederer, *Anz. Akad. Wiss. Wien, Math. Naturw. Kl.*, **1933**, No. 17, 163-165.

⁴⁸⁹ H. Junge, *Z. physiol. Chem.*, **268**, 179-186 (1941).

⁴⁹⁰ S. Okay, *Rev. faculté sci. univ. Istanbul, B* **12**, 1-8 (1947); *Chem. Abst.*, **42**, 2683-2684 (1948).

⁴⁹¹ T. W. Goodwin and S. Srisukh, *Biochem. J.*, **48**, 199-203 (1950).

⁴⁹² J. M. Grayson, *Iowa State Coll. J. Sci.*, **17**, 69-70 (1942).

⁴⁹³ J. M. Grayson and O. E. Tauber, *Iowa State Coll. J. Sci.*, **17**, 191-196 (1943).

⁴⁹⁴ S. Okay, *Nature*, **155**, 635 (1945).

⁴⁹⁵ S. Okay, *Rev. faculté sci. univ. Istanbul, B* **12**, 89-106 (1947); *Chem. Abst.*, **42**, 2684 (1948).

⁴⁹⁶ S. Okay, *Bull. Soc. Zool. France*, **74**, 11-15 (1949).

ber of other *Orthoptera*, for example, in the carnivorous green praying mantis (*Mantis religiosa*), and in the phytophagic short-horned grasshopper (*Acrida turrila*), the narrow-winged bush katydid (*Phaneroptera quadripunctata*), and the phytophagic European locust (*Isophya kraussi*). According to Okay,⁴⁹⁶ the carotenoid in the protein complex of these *Orthoptera* is astaxanthin; both the free pigment and the protein conjugate appear in the red wings, while the blue and yellow wings contain only the free carotenoid. Junge⁴⁹⁹ identified lutein as the xanthophyll component in the protein-carotenoid complex present in the skin of the green locust (*Locusta (Tettigonia) viridissima*), of the twittering locust (*Locusta cantans*), and of the parasitic oak locust (*Meconema varium*), in which it forms part of the insectoverdin complex.

The locusts have recently been the subject of considerable investigation. According to Goodwin,¹⁹² Volkonsky and Lederer⁴⁹⁷ could not identify any carotenoids in the red integumental pigment of young North African desert locusts (*Schistocerca gregaria* Forsk.); however, they did find these compounds in the mature insect. In the latter case, Chauvin⁴⁹⁸ identified the pigment as a mixture of α - and β -carotenes, while those in the immature locusts were reported to be unknown carotenoids, acridioxanthin (yellow-brown pigment) and acridioerythrin (red pigment). Further studies were carried out by Goodwin and Srisukh.^{499,500} They identified the rose-colored pigment in the immature desert locust (*Schistocerca gregaria* Forsk.), and in the African migratory locust (*Locusta migratoria migratorioides* R and F) as astaxanthin; β -carotene also occurs. Astaxanthin is present chiefly in the integument, wings, and eyes, while β -carotene occurs in fatty tissues, hemolymph, eggs, and eyes; some is present in the cuticle of male insects. The astaxanthin apparently occurs as the protein complex, not only in the two species listed above, but also in the red-banded locust (*Nomadacris septemfasciata*).⁵⁰⁰

Some of the *Orthoptera* apparently do not need carotenoids, or they are able to synthesize them. Thus, Przibram and Lederer⁴⁹⁸ reported that the predatory green mantis (*Sphodromantis bioculata*) synthesizes carotenoids, since it contained these pigments even when raised on a carotene-free regimen from colorless eggs. On the other hand, the Mediterranean brown

⁴⁹⁷ M. Volkonsky and E. Lederer, unpublished work cited by E. Lederer, *Les Carotenoides des Animaux*, Hermann, Paris, 1935.

⁴⁹⁸ R. Chauvin, *Ann. Soc. Entomol. France*, 10, 133-272 (1941). *Compt. rend. soc. biol.*, 135, 334-335 (1941).

⁴⁹⁹ T. W. Goodwin and S. Srisukh, *Nature*, 161, 525-526 (1948).

⁵⁰⁰ T. W. Goodwin and S. Srisukh, *Biochem. J.*, 45, 263-268 (1949).

variant of the mantis could not synthesize carotenoids, as it did not contain them under any circumstances. It is uncertain whether the German cockroach or croton bug (*Blatella germanica*), which can be raised on a diet completely devoid of carotenoids or of vitamin A,^{23,192} does not require these substances because it is able to synthesize them, or because it can carry out its metabolic processes without vitamin A. Bowers and McCay⁵⁰¹ were unable to demonstrate the presence of any preformed vitamin A in an extract obtained from a large number of cockroaches.

Goodwin⁵⁰² estimated the proportion of carotene in the African and desert locusts as 10 to 15 $\mu\text{g./g.}$ of fresh weight. This is a considerably lower figure than that (2.5% of dry weight) quoted by Brodskis and Rungs^{503,504} for the desert locust (*Schistocerca gregaria* Fors.). Although it has been reported that preformed vitamin A occurs in locusts,^{503,504} this claim could not be verified by Goodwin and Srisukh⁵⁰⁰ either in the case of *Locusta* or of *Schistocerca*. Goodwin^{192,388} is of the opinion that vitamin A is probably absent in these insects, first because it has not been found in other insects examined, and secondly because some species have been shown to exist on diets completely devoid of carotenoids and vitamin A. However, this last argument loses some weight in view of the recent demonstration of preformed vitamin A in the case of some crustaceans, such as the North Sea prawn (*Palaemon serratus*),⁴³³ and the Euphausiacean shrimps (*Meganycitaphanes norvegica* and *Thysanoessa raschii*).⁴⁴⁴

Many interesting variations in carotenoids occur in this group under certain conditions. Thus, although the amount of astaxanthin exceeds that of β -carotene in the locust throughout the first several stages of development, it begins to decline after the third stage, and β -carotene becomes the principal carotenoid.⁵⁰² On the other hand, the concentration of β -carotene, which is high in the eggs, decreases during incubation, concomitantly with an increase in the astaxanthin content.⁵⁰² It is also known that, when locusts swarm, *i.e.*, change from the solitary to the gregarious phase, they undergo marked color changes. The green pigmentation characteristic of the solitary phase is replaced by a much darker coloration, due to melanin.^{505,506} The green pigment in the locusts during the solitary phase has been shown by Junge⁴⁸⁹ to be insectoverdin; this consists of a yellow component (made up of a carotenoprotein complex containing both β -

⁵⁰¹ R. E. Bowers and C. M. McCay, *Science*, **92**, 291 (1940).

⁵⁰² T. W. Goodwin, *Biochem. J.*, **45**, 472-479 (1950).

⁵⁰³ B. Brodskis and C. Rungs, *Bull. soc. sci. nat. Maroc*, **24**, 7-9 (1944).

⁵⁰⁴ B. Brodskis, *Bull. soc. sci. nat. Maroc*, **24**, 9-10 (1944).

⁵⁰⁵ T. W. Goodwin, *Biochem. J.*, **47**, 554-562 (1950).

⁵⁰⁶ T. W. Goodwin, *Biol. Revs.*, **27**, 439-460 (1952).

carotene and astaxanthin) and a blue component, which is a chromoprotein having mesobilirubin as the prosthetic group. In the gregarious period of this insect's life, the insectoverdin disappears, while the astaxanthin and β -carotene are masked by either melanin or insectorubin.⁵⁰⁵ Goodwin⁵⁰⁷ was unable to demonstrate a variation in pigment metabolism associated with the breeding temperature. A certain degree of genetic control of carotenoids was reported in *Locusta migratoria migratorioides*.⁵⁰²

f". Résumé of the Occurrence and Function of Carotenoids in *Insecta*. β -Carotene is the most widely distributed carotenoid pigment in insects, while α -carotene is second in importance as far as occurrence is concerned. Insects accumulate their carotenoids in three ways, namely (a) by indiscriminate storage, (b) by preferential storage of one or two dietary carotenoids, or (c) by alteration of the absorbed dietary carotenoids before storage. In the case of locusts, (b) and (c) occur simultaneously. In addition to β -carotene, astaxanthin is an important carotenoid component in the insects.

Insofar as (c) is concerned, newly formed carotenoids usually arise by oxidation of their precursors. This is the case with astaxanthin, which appears in the developing egg coincident with the disappearance of β -carotene. The fact that astaxanthin occurs in the integument but not in the hemolymph, while β -carotene is present only in the latter fluid, further supports the concept that β -carotene is the mother substance of astaxanthin. On the other hand, oxidation is not the only transformation possible, since the ladybird (*Coccinella* spp.) accumulates lycopene; the source of this hydrocarbon, however, is uncertain.

No clear-cut function has been assigned to the carotenoids as far as insects are concerned. The presence of astaxanthin in the eyes of *Schistocerca* and *Locusta* suggests the possibility of its function in vision. In fact, Wald²²⁴ is of the opinion that this is the chief reason for the occurrence of astaxanthin in invertebrates in general. Another possible function of carotenoids is in reproduction. The insect eggs usually contain β -carotene, which decreases as development proceeds. Thus, the carotenes and the xanthophylls have been found in the eggs of the Chinese mulberry silkworm (*Bombyx mori*).⁵⁰⁸

A generic factor must also be considered. A number of investigators, including Manunta,^{483,508} Uda,⁵⁰⁹ and Gerould,⁴⁸² found that the Chinese

⁵⁰⁷ T. W. Goodwin, *Biochem. J.*, **49**, 86-87 (1951).

⁵⁰⁸ C. Manunta, *Boll. soc. ital. biol. sper.*, **8**, 1278-1284 (1933); **12**, 31-32, 32-33, 33-34, 626-628 (1927).

⁵⁰⁹ H. Uda, *Genetics*, **4**, 395-416 (1919).

silkworm (*Bombyx mori*) requires one gene to produce carotenoid pigmentation in the blood and serigenous gland from alimentary sources, and a second gene to secrete silk containing the yellow (carotenoid) factor.

The intestinal permeability for carotenes and for xanthophylls, and that of the serigenous gland, differ in the various species.⁵¹⁰ The lemon strain presents more permeability than do other races.⁵¹⁰ Carotene alone occurs in the eggs of *Melanoplus bivittatus*,^{492,493} *Locusta migratoria migratoriorides*,⁵⁰⁰ *Schistocerca gregaria*,⁵⁰⁰ and also in the larvae of *Lepidoptera* in general.⁴⁷⁹ A sex difference is noted in *Bombyx mori*, in that the hemolymph of the male adults and larvae is colorless, and that of the females is bright yellow. The pigment (carotene) in the hemolymph is transmitted to the eggs, and acts as a protective coloring.⁵¹¹

b. In Vertebrates. Vitamin A appears to be present in all vertebrates. In some cases, particularly in the case of freshwater fishes, the usual form of the vitamin (vitamin A₁) may be in part or entirely replaced by vitamin A₂. Although β -carotene and other carotenoids are sometimes stored in the vertebrates, their presence is nowhere near as universal as is the case with the lower forms. In contradistinction to the invertebrates, in which almost all of the vitamin A stores may be localized in the eyes, the vertebrates usually concentrate practically all of the vitamin A in the liver; however, this vitamin is also present in the retina, as well as in other organs such as the kidney and the lungs.¹⁹ In some cases, as, for example, the chicken, considerable amounts of vitamin A may be concentrated in the body fat, especially when the intake of the vitamin has been high. On the other hand, the body fat of many animals (pig, goat, rat) contains no detectable amounts of carotene or vitamin A.

(a) *Carotenoids and Vitamins A in Fishes.* Although the carotenoid content of most fishes is overshadowed by their tremendous capacity to store vitamin A, the carotenoids are of considerable importance in many species, especially in those with conspicuous skin colors. Astaxanthin and similar pigments account for the color in the numerous red fishes; it is believed that this carotenoid is derived from the invertebrates which the fishes consume. According to Goodwin,⁵¹² the xanthophylls in fishes are restricted to lutein and a taraxanthin-like carotenoid, in addition to astaxanthin.

Astaxanthin is found in the muscle of the North American salmon

⁵¹⁰ C. Manunta, *Atti soc. nat. e mat. Modena*, 66, 104-113 (1936).

⁵¹¹ K. Geyer, *Z. wiss. Zool.*, 105, 349-499 (1913).

⁵¹² T. W. Goodwin, *Biochem. J.*, 47, liv (1950).

(*Salmo salar*),^{513,514} and of the Pacific sockeye salmon (*Oncorhynchus nerka*).⁵¹⁵ β -Carotene has been demonstrated in the muscle of the salmon (*Salmo salar*) by Euler and co-workers.⁵¹⁴ The oarfish or scythe fish (*Regalecus glesne*) deposits astaxanthin in the liver. The lump-sucker (*Cyclopterus lumpus*) yields a red liver oil containing astaxanthin, and appears to mobilize this carotenoid in the tissues and skin during the spawning season.⁵¹⁶ The red skin, the mucus of the gills and mouth, the iris, and the sclera of the marine dorado or gold mackerel (*Beryx decadactylus*) contain rich stores of astaxanthin.^{23,389} Other fishes which contain astaxanthin include the common goldfish (*Carassias auratus*),²³ some but not all varieties of European freshwater perch (*Perca fluviatilis*),²³ the rosefish (*Sebastes marinus*),²³ the seadevil fish, angler, or goosefish (*Lophius piscatorius*), where it occurs with taraxanthin in the liver-oil.⁴⁴¹ It is found in the flesh of the mountain and the sea races of the freshwater brown trout (*Salmo trutta*),⁵¹⁷ and, with α -carotene, in the orange liver oil of the sunfish (*Orthogoriscus mola*).⁵¹⁸ The taraxanthin in the liver of *L. piscatorius* is in combination with a protein.^{441,519} Although the eyes of most of the fishes contain some carotenoid, in only one, namely the marine dorado (*Beryx decadactylus*), was astaxanthin found in this organ; in this species it occurs in the iris and sclera, but not in the retina.⁵²⁰

Taraxanthin, or a taraxanthin-like compound is the second carotenoid which has been found to have a limited distribution in fishes. The California killifish (*Fundulus parvipinnis* Girard) contains appreciable concentrations of this carotenoid.^{521,522} This fish was fed on the following: opaleye or greenfish (*Girella nigricans*); California halibut (*Paralichthys maculosus californicus*); "Garibaldi," orange-red marine goldfish (*Hypsypops rubicundus*); and the shiner, or sea-perch (*Cymatogaster aggregata*).

⁵¹³ A. Emmerie, M. van Eekelen, B. Josephy, and L. Wolff, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **4**, 139-141 (1934).

⁵¹⁴ H. v. Euler, H. Hellström, and M. Malmberg, *Svensk. Kem. Tidskr.*, **45**, 151-152 (1933).

⁵¹⁵ B. E. Bailey, *J. Biol. Board Can.*, **3**, 469-472 (1937).

⁵¹⁶ S. Schmidt-Nielsen, N. A. Sørensen, and B. Trumpy, *Kgl. Norske Videnskab. Selskabs. Förh.*, **5**, No. 29, 114-117; **30**, 118-121 (1932).

⁵¹⁷ N. A. Sørensen and J. Stene, *Kgl. Norske Videnskab. Selskabs. Skrifter*, 1938-1939, No. 8, 1-14, No. 9, 1-9.

⁵¹⁸ N. A. Sørensen, *Kgl. Norske Videnskab. Selskabs. Förh.*, **6**, No. 40, 154-156 (1933).

⁵¹⁹ N. A. Sørensen, *Kgl. Norske Videnskab. Selskabs. Skrifter*, 1934, No. 1, 1-14.

⁵²⁰ E. Lederer, *Compt. rend. soc. biol.*, **118**, 542-543 (1935).

⁵²¹ F. B. Sumner and D. L. Fox, *J. Exptl. Zool.*, **66**, 263-299 (1933); **71**, 101-123 (1935).

⁵²² F. B. Sumner and D. L. Fox, *Proc. Nat. Acad. Sci., U.S.*, **21**, 330-340 (1935); D. L. Fox, *Ibid.*, **22**, 50-54 (1936).

These species contained little xanthophyll, but did contain carotene, which was converted to the xanthophyll by the *Fundulus*. A xanthophyll resembling taraxanthin was reported, by Wald,⁵²³ in combined pigment-epithelia and in the choroid layers of the eyes of the red-brown sea robin or gurnard (*Prionotus carlinus*), the black sea-bass (*Centropristis striatus*), and the scup or goby (*Stenotomus chrysops*). Lönnberg^{402,524} also noted the presence of the taraxanthin-like pigment and lutein in a number of species of marine fishes. Fontaine and Busnel⁵²⁵ reported the occurrence of carotenoids in the skin of eels. Lönnberg^{526,527} observed that, of a large number of species examined, the eyes of only two were devoid of carotenoids, *i.e.*, a cod (*Gadus esmarkii*) and the Atlantic spiny dogfish (*Squalus acanthius*).

β -Carotene is an almost universal constituent of the ovaries and ova of a wide variety of fishes. Euler *et al.*⁵¹⁴ reported that β -carotene is present in the roe of many species of fish. Although it is a minor constituent in the egg, it has seldom been observed in other organs of the fishes.^{192,388} In addition to β -carotene, unesterified xanthophylls are usually found in the ova and ovaries. The complete absence of the carotenoids from the milt of two fishes, the sand-eel or sand lance (*Ammodytes tobianus*),^{192,528} and the herring (*Clupea harengus*),^{192,529} is in strong contrast to their relative abundance in eggs.

According to Sumner and Fox,⁵²¹ carotenoids may play a role in the photoresponses of some fishes. Wald⁵²³ does not consider the presence of carotenoids in the retinae to be of overwhelming significance, since they are widely distributed in other tissues as well. It is possible that they increase visual acuity by reducing chromatic aberration and glare.⁵³⁰ The mobilization of the carotenoids in the gonads indicates that they may exert some specific function, but its nature has not as yet been established. Free xanthophylls have been noted in the ova of *Ammodytes tobianus*,⁵²⁸ but not in those of *Salmo salar*, which contained only free astaxanthin.⁵³¹ In the case of *Fundulus parvipinnis*,⁵²¹ free xanthophylls are transferred

⁵²³ G. Wald, *J. Gen. Physiol.*, **20**, 45-56 (1936).

⁵²⁴ E. Lönnberg, *Arkiv Zool.*, **B 33**, No. 1, 1-7 (1941-1942).

⁵²⁵ M. Fontaine and R. G. Busnel, *Compt. rend.*, **204**, 1591-1593 (1937).

⁵²⁶ E. Lönnberg, *Arkiv Zool.*, **A 28**, No. 4, 1-14 (1935-1936).

⁵²⁷ E. Lönnberg, *Arkiv Zool.*, **A 31**, No. 1, 1-14 (1939).

⁵²⁸ T. W. Goodwin, 1949; unpublished experiments cited by T. W. Goodwin, *The Comparative Biochemistry of the Carotenoids*, Chem. Publ. Co., New York, 1954.

⁵²⁹ G. N. Festenstein, 1951; unpublished experiments cited by T. W. Goodwin, *The Comparative Biochemistry of the Carotenoids*, Chem. Publ. Co., New York, 1954.

⁵³⁰ G. L. Walls and H. D. Judd, *Brit. J. Ophthalmol.*, **17**, 641-675, 705-725 (1933).

⁵³¹ M. Glover, R. A. Morton, and G. D. Rosen, *Biochem. J.*, **50**, 425-429 (1952).

to the ripening eggs. Both males and females of *Cyclopterus lumpus* mobilize astaxanthin from the liver to the flesh and skin during the summer spawn.⁵¹⁶

The fishes as a class have the ability to store the largest proportion of vitamin A per unit of liver of any of the vertebrates. It is known that the Atlantic cod (*Gadus callarius*) can convert carotene to vitamin A, but that it can also absorb substantial amounts of the carotenoids unchanged.⁴⁵⁶ It seems reasonable to postulate that much of the preformed vitamin A in fish livers does originate from carotenes.

Exceedingly wide variations exist in the amount of vitamin A found in different species. Thus, Rosenberg¹ listed values for the common fishes ranging from a low value of 65 I.U./g. for the haddock (*Gadus aeglefinus*) to a high level of 600,000 I.U./g. for black sea-bass (*Stereolepis gigas*). The liver oil of the cod (*Gadus morrhua*) is recorded as having a mean of 600 I.U./g. while at least eleven species have levels between 25,000 and 80,000 I.U./g., and four others levels between 120,000 and 300,000 I.U./g.

In the case of freshwater fishes, a variable proportion of the vitamin A₁ is replaced by vitamin A₂. Heilbron, Gillam, and Morton⁵³² were the first to call attention to the aberrant reaction of certain fish liver oils to antimony trichloride, although they did not realize that it occurred because another form of vitamin A was present. The credit for this concept should go to Edisbury, Morton, and Simpkins,⁵³³ they made the suggestion that the compound be called vitamin A₂. Almost simultaneously, Lederer and Rosanova⁵³⁴ discovered the presence of a large proportion of vitamin A₂ in liver oils from Russian freshwater fishes, by making use of the same aberrant antimony trichloride reaction. Although the occurrence of vitamin A₂ in freshwater fishes is universal, the proportion of vitamin A₁ contained along with it is subject to wide variations. Thus, the highest proportion of vitamin A₂:vitamin A₁ is found in the carnivorous fishes such as the pike (*Esox lucius*), the pike perch or sander (*Lucioperca sandra*), the European freshwater perch (*Perca fluviatilis*), and the wels or European catfish (*Silurus glanis*).⁵³⁵ A considerably lower vitamin A₂:vitamin A₁ ratio was found to obtain in omnivorous fishes such as the bream (*Abramis brama*), the carp (*Cyprinus carpio*) and the tench (*Tinca tinca*). Lower

⁵³² I. M. Heilbron, A. E. Gillam, and R. A. Morton, *Biochem. J.*, **25**, 1352-1366 (1931).

⁵³³ J. R. Edisbury, R. A. Morton, and G. W. Simpkins, *Nature*, **140**, 234 (1937).

⁵³⁴ E. Lederer and V. A. Rosanova, *Biokhimiya*, **2**, 293-303 (1937); *Chem. Abst.*, **31**, 5105 (1937).

⁵³⁵ R. A. Morton, *The Application of Absorption Spectra to the Study of Vitamins, Hormones, and Coenzymes*, 2nd. ed., Jarell, Ash, Boston, 1942.

ratios were likewise observed in migratory fishes as, for example, the North Atlantic salmon (*Salmo salar*), the rainbow trout (*Salmo irideus*), and others. In no cases were the levels of liver vitamin A₂ comparable in amount to those of vitamin A₁ found in many of the saltwater fishes. For a more extended discussion of the subject of vitamin A storage in fishes, as well as of that of vitamins A₁ and A₂ as related to species, the reader is referred to *The Lipids*, Vol. I, pages 675 to 683.

(b) *Carotenoids and Vitamins A in Aquatic Mammals*. Although one might expect the presence of carotenoids in species of whales such as *Odontoceti* (toothed whales) which subsist largely on small crustaceans ("krill"), Drummond and MacWalter⁵³⁶ are of the opinion that the carotenoids in the food remain largely unabsorbed; they demonstrated that the feces of these mammals contained a large amount of astaxanthin. On the other hand, Wagner⁴⁶⁰ found considerable β -carotene in the food in the intestines of blue and fin whales caught near the Farøe Islands. He is of the opinion that this provitamin is the source of the vitamin A in these mammals (see page 486). Astaxanthin is rarely found in the body oils.⁴⁴¹ In fact, Barua and Morton^{192,537} state that they have never encountered astaxanthin in whale liver oils. Schmidt-Nielsen *et al.*⁵¹⁶ obtained red oils from the livers of the great blue whale (*Balaenoptera musculus*), but Burkhardt and co-workers⁴⁴¹ consider that this occurs only under pathologic conditions. Wagner and Vermeulen⁴⁶⁷ reported the absence of carotene from the flesh, liver, and milk of blue, sperm, and fin-backed whales, although vitamin A was present in considerable amounts, especially in the sperm whale.⁴⁶⁷ Morton and Rosen, cited by Goodwin,¹⁹² were unable to find carotenoids in whale ovaries. In the case of Risso's dolphin, or grampus (*Grampus griseus*), which subsists on such prey as seals and smaller whales, and which would therefore consume no carotenoids in the diet, the liver oils are devoid of carotenoids, as would be expected.¹⁴⁹

The vitamin A in the liver oils of whales is largely esterified. Kascher and Baxter⁵³⁸ stated that 100% of the liver oil of *Cetaceae* spp. is in the form of the ester, while Morton and Stubbs⁵³⁹ reported that the ester fraction of whale liver oil based upon chromatographic separation was 88.7%, while that present as the free alcohol amounted to 9.6%.

⁵³⁶ J. C. Drummond and R. J. MacWalter, *J. Exptl. Biol.*, 12, 105-107 (1935).

⁵³⁷ R. K. Barua and R. A. Morton, unpublished observations, 1949, cited by T. W. Goodwin, *The Comparative Biochemistry of the Carotenoids*, Chapman & Hall, London, 1952, p. 197.

⁵³⁸ H. M. Kascher and J. G. Baxter, *Ind. Eng. Chem., Anal. Ed.*, 17, 499-503 (1945), p. 499.

⁵³⁹ R. A. Morton and A. L. Stubbs, *Biochem. J.*, 40, lviii (1946).

Although the vitamin A content of whale liver oil (6000 I.U./g.) is reported to be considerably lower than that of many fish liver oils, large amounts of kitol are present in whale liver oil, and this is convertible to vitamin A on distillation. Embree and Shantz⁵⁴⁰ were the first to discover kitol in whale liver oil, and it has since been found to occur in smaller amounts in fish liver oils,^{540,541} as well as in the liver oil of oxen and sheep.^{540,542} According to Barua and Morton⁵⁴³ kitol appears to be a di-vitamin A which cannot be depolymerized biologically. Barua and Morton⁵⁴³ established the fact that kitol has a chain (or two chains) of four conjugated double bonds instead of the five conjugated double bonds of vitamin A. Rats were found to be unable to convert kitol esters to vitamin A, or to store kitol as such in the liver.⁵⁴³ Kitol also occurs in fin-whale milk. Hickman⁵⁴² believes that kitol is a detoxication product of vitamin A. It should therefore be referred to as a post-vitamin A rather than as a pro-vitamin A. For a further discussion of kitol, the reader is referred to *The Lipids*, Vol. I, pages 710 to 713.

Rodahl and Davies⁵⁴⁴ have made an exhaustive study of the composition of the liver oils of hooded seals (*Cystophora cristata*), Greenland seals (*Phoca groenlandica*), harbor seals (*Phoca vitulina*), and of an Atlantic gray seal (*Halichoerus grypus*). Very high levels of vitamin A (up to 24,000 I.U./g.) were observed in the liver during the breeding season. On the other hand, a low value of only 388 I.U./g. was noted in four specimens of the liver of *Cystophora cristata* obtained after the breeding season (in July). Most of the vitamin A was present in the liver, and only relatively small amounts were found in other tissues. Porpoise liver (*Phocaena communis*) was found to contain approximately 200 µg./g. of carotene, but none was observed in the livers of two embryos.⁵⁴⁵

(c) *Carotenoids and Vitamins A in Amphibia*. The carotenoids appear to be widely distributed in the Amphibia. Hydroxy carotenoids were reported^{192,390} in the fire-bellied toad (*Bombinator igneus*). Van Eekelen⁵⁴⁶ and Lönnberg⁵⁴⁷ proved the presence of carotenes and of xanthophylls in

⁵⁴⁰ N. D. Embree and E. M. Shantz, *J. Am. Chem. Soc.*, **65**, 910-913 (1943).

⁵⁴¹ F. B. Clough, H. M. Kascher, C. D. Robeson, and J. G. Baxter, *Science*, **105**, 436 (1947).

⁵⁴² K. Hickman, *Ann. Rev. Biochem.*, **12**, 353-396 (1943).

⁵⁴³ R. K. Barua and R. A. Morton, *Biochem. J.*, **45**, 308-317 (1949).

⁵⁴⁴ K. Rodahl and A. W. Davies, *Biochem. J.*, **45**, 408-412 (1949).

⁵⁴⁵ E. Marcussen, *Dansk. Tids. Farm.*, **17**, 73-78 (1943); *Chem. Zentr.*, vol. **114**, 1943, Part II, 932; *Chem. Abst.*, **38**, 5995 (1944).

⁵⁴⁶ M. van Eekelen, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **4**, 65-66 (1934).

⁵⁴⁷ E. Lönnberg, *Arkiv Zool.*, **21 B**, No. 3, 1-4 (1929).

the skin of the European, brown, grass frog (*Rana temporaria*), the edible green water-frog (*R. esculenta*), and the leaf-frog (*Hyla arborea*);⁵⁴⁷ Rand⁵⁴⁸ confirmed these results and reported that carotenoids also occur in the liver, kidneys, lungs, ovaries and eggs, testes, and fat bodies of both summer and winter frogs. The highest carotenoid content occurs in the liver.⁵⁴⁹ Zechmeister and Tuzson⁷⁵ were able to prepare pure β -carotene, a small amount of α -carotene, zeaxanthin and lutein from the livers of *R. esculenta*. Ganguly and co-workers²⁵⁷ demonstrated the presence of both carotenes and carotenols in the ovaries, as well as in the livers of *R. esculenta*; however, carotenoids were absent from the blood. Zechmeister¹⁴⁸ is of the opinion that the frog is in the same class as man (Group A) insofar as carotenoid metabolism is concerned, since it exercises little or no selection between hydrocarbon and alcoholic types of carotenoids. According to Morton and Rosen,⁵⁵⁰ the carotenoids in *R. temporaria* are dispersed throughout the various tissues, but quite unevenly; the storage of carotenoids in the ovaries is so large that it results in a markedly higher storage of these pigments in the females as a whole than in the males. A marked seasonal variation in tissue carotenoid concentration was likewise found in the frogs; however, the carotenoid reserves were retained in the liver during hibernation and inanition.

Frogs also have considerable stores of vitamin A. Morton and Rosen⁵⁵⁰ reported the presence of vitamin A in the eyes of tadpoles of the species, *R. temporaria*. In the adult frog, about 85% of the vitamin A was present as the ester in the liver, while the remaining 15% was equally divided between the kidneys and the eyes. A higher proportion of vitamin A was found in the kidneys of frogs than is present in mammalian kidneys. The vitamin A was not ingested as such, but originated from the breakdown of carotene. Wald²²⁵ reported that tadpoles of the North American bullfrog (*R. catesbiana*) contain vitamin A₂, while only vitamin A₁ occurs in this species after metamorphosis. However, Collins and co-workers⁵⁵¹ reported that vitamin A₁ is the sole type of vitamin A in the tadpoles of *R. esculenta* and *R. temporaria*. Both carotenoids and vitamin A have been isolated in salamanders. Thus Beatty⁵⁵² identified β -carotene and unesterified xanthophylls in the body proper (without viscera) of the olm, a pale or white, blind, aquatic, tailed eel-like salamander, with blood-red gills (*Proteus anguinus*), obtained from dark underground caves. β -carotene

⁵⁴⁸ C. Rand, *Biochem. Z.*, **281**, 200-205 (1935).

⁵⁴⁹ O. Brunner and R. Stein, *Biochem. Z.*, **282**, 47-50 (1935).

⁵⁵⁰ R. A. Morton and D. G. Rosen, *Biochem. J.*, **45**, 612-627 (1949).

⁵⁵¹ F. D. Collins, R. M. Love, and R. A. Morton, *Biochem. J.*, **53**, 632-636 (1953).

⁵⁵² R. A. Beatty, *J. Exptl. Biol.*, **18**, 144-152 (1941).

was the only carotenoid present in the liver. Karrer and co-workers⁵⁵³ failed to find vitamin A in the liver of the axlotl salamander (*Ambystoma* spp.). However, Collins and associates⁵⁵⁴ reported the presence of carotenoids and also of both vitamin A₁ and vitamin A₂ in the lipids obtained from the crested newt (*Triturus* (*Triton*) *cristata*) and from an aquatic salamander (*Triturus carnifex*).

(d) *Carotenoids and Vitamins A in Reptiles*. According to Lederer,³⁹⁰ Krukenberg and MacMunn, in their investigations of the distribution of carotenoids,¹⁹² concluded that these compounds were absent from a number of snakes and alligators. Villela and Prado⁵⁵⁵ reported that no carotenoids were present in the plasma of the Mexican rattlesnake (*Crotalus terrificus*) or of the "boipeva" viper (*Xenodon merremi*). However, they did observe xanthophylls (but no carotenes) in the blood of two Brazilian snakes, the poisonous pit-viper (*Bothrops jararaca*), which is brown, with red and black spots, and a colubrine snake (*Eudryas bifossatus*). Karrer and co-workers⁵⁵³ failed to find vitamin A in the liver of the leopard adder (*Coluber quadri-lineatus*).

The hydroxy carotenoids were reported by Krukenberg, as cited by Lederer³⁹⁰ and by Goodwin,¹⁹² as important chromogens in a number of lizards, including the European wall lizard (*Lacerta muralis*), the English sand lizard (*L. agilis*), and the common chameleon (*Chameleon vulgaris*). Manunta⁵⁰⁸ reported the presence of xanthophyll esters, of unesterified xanthophylls, and of traces of carotene in the skin of chameleons. Lutein and traces of carotene were found in the eggs. Comparatively large amounts of carotenoids are stored in the liver of the monitor lizard (*Varanus comodensis*), but only about 10 to 20% consist of carotenes.¹⁴⁹ Lacertofulvin, β -carotene, and lutein occur in the skin of the Italian green land lizard (*Lacerta sicula*).^{556,557} Lacertofulvin was recognized as a lipochrome in 1882, and was given its name by Krukenberg.⁵⁵⁸ Fox³⁹¹ observed that the spiny-tailed iguana (*Ctenosaura acanthura*) secretes a taraxanthin-like ester, which appears in the yellow-brown kernels from the femoral pores.

⁵⁵³ P. Karrer, H. v. Euler, and K. Schöpp, *Helv. Chim. Acta*, **15**, 493-495 (1932).

⁵⁵⁴ F. D. Collins, R. M. Love, and R. A. Morton, *Biochem. J.*, **53**, 629-632 (1953).

⁵⁵⁵ G. G. Villela and J. L. Prado, *J. Biol. Chem.*, **157**, 693-697 (1945).

⁵⁵⁶ H. K. Loevenich, G. v. Studnitz, and H. Wigger, *Naturwissenschaften*, **31**, 568-569 (1943).

⁵⁵⁷ K. Wallenfels and H. J. Bielig, *Z. physiol. Chem.*, **270**, 220-222 (1941).

⁵⁵⁸ C. F. W. Krukenberg, *Die Farbstoffe der Reptilienhaut, I., Vergleichend-physiologische Studien, ser. 2, Part 2*, 50-54 (1882); *Die Pigmente der Fischhaut, II, Ibid., Part 3*, 138-143 (1882); cited by K. Wallenfels and H. J. Bielig, *Z. physiol. Chem.*, **270**, 220-222 (1941), p. 220.

Although Halliburton,⁵⁵⁹ in 1885, noted the presence of lipochromes (carotenoids) in the serum and body fat of turtles, as well as in *Crustacea* and in the pigeon, hen, and dove, the first modern work on turtles was carried out in 1938, when Lederer^{23,560} reported a study of the Japanese green water-turtle (*Chrysemys scripta elegans*). This reptile was found to have γ -carotene in the red spots under the eyes, α -carotene in the yellow dorsal carapace, and a mixture of α -carotene and lutein in the gut. The occurrence of a pigment similar to lutein in the skin of the slider terrapin (*Chrysemys* spp.) has been reported.¹⁹² Astacene was identified in the retinae of the wood terrapin (*Clemmys insculpta*).⁵⁶¹ The liver of the Greek tortoise (*Testudo graeca*) contains a considerable quantity of carotenoids.⁵⁶²

(c) *Carotenoids and Vitamins A in Birds (Aves)*. Birds belong to the group of higher animals (Group D) which store the carotenols, while only small amounts of carotenes occur in the tissues. This latter condition may be related to the fact that birds are able to convert provitamins A into vitamin A in the wall of the intestine.¹⁴² Although the carotenol, cryptoxanthin, does not ordinarily occur in chicken blood, it may be present after it has been administered.⁵⁹ However, there is no evidence that the hen is able to change other carotenoid pigments to cryptoxanthin, as suggested by Grimbleby and Black.⁵⁶³ Lycopene and zeaxanthin are also present in the blood of chickens after these carotenoids have been fed. On the usual farm diet, lutein normally occurs in the blood of chickens. Ganguly and co-workers⁵⁹ reported the presence of lutein to the extent of 162 $\mu\text{g.}/100\text{ ml.}$ plasma when the birds had received the ordinary "farm" diet. However, when these chickens were fed a lutein-free diet for thirty or one-hundred sixty days, the value for this carotenoid in the plasma dropped to zero, and remained at this level in animals continuing on the same diet. Small amounts of β -carotene (4.2 $\mu\text{g.}/100\text{ ml.}$) were likewise observed in the sera of chickens on the farm diet, but this value promptly fell to zero in a number of groups which had been given carotene-free diets; periods as short as seven days were able to effect this change. No β -carotene could be demonstrated in the blood when 14 mg. of pure β -carotene were administered over a seven-day period. The adventitious origin of the carotenoids in the blood is illustrated by the fact that 7.2 $\mu\text{g.}$ of lycopene, 213 $\mu\text{g.}$ of cryptoxanthin and 526 $\mu\text{g.}$ of zeaxanthin were noted in the blood after these

⁵⁵⁹ W. D. Halliburton, *J. Physiol.*, **6**, 300-335 (1885); **7**, 324-326 (1886).

⁵⁶⁰ E. Lederer, *Bull. soc. chim. biol.*, **20**, 554-566 (1938).

⁵⁶¹ G. Wald and H. Zussman, *J. Biol. Chem.*, **122**, 449-460 (1938).

⁵⁶² R. M. Love (1951), unpublished work; cited by T. W. Goodwin, *The Comparative Biochemistry of the Carotenoids*, Chem. Pub. Co., New York, 1954.

⁵⁶³ F. H. Grimbleby and D. J. Black, *Brit. J. Nutrition*, **6**, 393-397 (1952).

several carotenoids had been administered separately to chickens, although none of the carotenoids were present when normal diets were fed.

Carotene was found in the liver oil of the California gull (*Larus fuscus*) and of the black-backed sea-gull (*L. marinus*), but not in the fat extracted from the serum of the latter.¹⁵³ The carotenoid pigments, usually the xanthophylls, are stored not only in the blood, liver, and retina, as one might expect, but also in the adipose tissue, skin, feet, bill, and face, and even in the plumage. They are likewise present in the yolk of the egg. Vitamin A is present in the blood, liver, adipose tissue, and egg yolk.

The classical observations on the role of the carotenoids in the coloring of chick tissues are those of Palmer,²¹ who recognized that the yellow fat, skin, and egg yolk of white Leghorns are colored by dietary xanthophylls, but contain only small amounts of carotenes. During the laying season, xanthophylls are deposited in the egg yolk instead of in the skin of the legs and in the other areas. Lutein is the principal carotenol to be deposited in the skin; it is esterified.⁷⁴ Its occurrence in the tissues and egg yolk must be considered as adventitious, since it completely disappears when the birds are fed on a carotenoid-free diet.⁵⁹ In addition to lutein, various other dietary xanthophylls such as capsanthin from pimiento and chili peppers, and zeaxanthin from yellow corn, may be stored in the skin and egg yolk.⁵⁶⁴⁻⁵⁷¹ Astaxanthin was observed in the red wattles of the Korean pheasant (*Phasianus colchicus*), and in the eggs of the black-headed gull (*Larus ridibundus*) and of the European white stork (*Ciconia ciconia*).⁷⁴ An astaxanthin-like carotenoid has also been found in fat from the carmine Indian flamingo (*Phoenicopterus roseus*), but it is not certain whether or not it actually is astaxanthin. The pigment has been called *phoenicopterin* or *phoenicoxanthin*.⁵⁷² These are the only reports of the occurrence of astaxanthin in the fowl in tissues other than in the retina. Guilbert and Hinshaw⁵⁷³ observed storage of xanthophylls in the livers of hens and of

⁵⁶⁴ W. L. Brown, *Georgia Agr. Expt. Sta. Bull.*, 160, 3-11 (1930); *Chem. Abstr.*, 24, 5800 (1930).

⁵⁶⁵ W. L. Brown, *Georgia Agr. Expt. Sta. Bull.*, 183, 1-8 (1934); *Chem. Abst.*, 28, 6783 (1934).

⁵⁶⁶ W. L. Brown, *J. Biol. Chem.*, 110, 91-94 (1935).

⁵⁶⁷ W. L. Brown, *J. Biol. Chem.*, 122, 655-659 (1938).

⁵⁶⁸ V. Heiman and L. A. Wilhelm, *Poultry Sci.*, 16, 400-403 (1937).

⁵⁶⁹ J. S. Hughes and L. F. Payne, *Poultry Sci.*, 16, 135-138 (1937).

⁵⁷⁰ W. A. Morgan and J. G. Woodroof, *Georgia Agr. Expt. Sta. Bull.*, 147, 210-215 (1927); *Chem. Abst.*, 22, 465 (1928).

⁵⁷¹ H. W. Titus, J. C. Fritz, and W. R. Kauffman, *Poultry Sci.*, 17, 38-45 (1938).

⁵⁷² C. Manunta, *Helv. Chim. Acta*, 22, 1151-1153 (1939).

⁵⁷³ H. R. Guilbert and W. R. Hinshaw, *J. Nutrition*, 8, 45-56 (1934).

turkeys, while Lönnberg⁵²⁶ reported their occurrence in the skin, fat, face, and bill of fifteen species of wild fowl.

Xanthophyllic pigments occur extensively in the eyes of birds. Lönnberg⁵⁷⁴ noted the presence of carotenoids in some species of wild birds, while Hollander and Owen⁵⁷⁵ found both carotene and xanthophyll in the iris of numerous species of domestic chickens, but they were not present in this tissue in the case of pigeons. Nelson⁵⁷⁶ states that the carotenoids in the iris are so labile that the color of the eyes can be changed by an alteration in diet.

Considerable work has been completed on the nature of the carotenoid in the retinas of birds. As early as 1877, Capranica⁵⁷⁷ reported that three types of oil droplets could be obtained from hens' retinas. These were characterized by Kühne and Ayres,⁵⁷⁸ in 1878, as three differently colored pigments which they called "chlorophane" (greenish), "xanthophane" (yellowish) and "rhodophane" (reddish). Sixty years later, Wald and Zussman^{581,579} found that the greenish pigment resembled the bacterial carotenoid *sarcinene*, in a micrococcus from human skin (*Sarcina lutea*). The yellowish substance consisted of a mixture of lutein and zeaxanthin in the same ratio as they occur in egg, and the reddish pigment was found to be astaxanthin. Von Studnitz *et al.*^{556,580} are of the opinion that the greenish yellow pigment is lacertofulvin, the red substance is astaxanthin, and the bright yellow pigment is lutein. *Galloxanthin* is another carotenoid which Wald⁵⁸¹ isolated from chicken retina; the composition is not known, but Goodwin¹⁹² suggests that it may be an epoxide.

Feathers constitute a unique site in which carotenoids may be deposited; the feather carotenoids can be considered in the same category as those stored in the skin and external structures of bright-colored sea animals. The component carotenoids are xanthophylls, which may be synthesized from the alimentary carotenoids, and are characteristic of the species. The early work on plumage carotenoids was summarized by Palmer²¹ and by Karrer and Jucker.²⁴ Lönnberg⁵⁷⁴ reported the presence of carotenoids in the feathers of a wide variety of birds. Brockmann and Völker⁷⁴ discovered a pigment in the feathers of the wild canary (*Serinus canarius*)

⁵⁷⁴ E. Lönnberg, *Arkiv Zool.*, A 21, No. 11, 1-12 (1930).

⁵⁷⁵ W. F. Hollander and R. D. Owen, *Poultry Sci.*, 18, 385-387 (1939).

⁵⁷⁶ N. M. Nelson, *Poultry Sci.*, 23, 541-542 (1944).

⁵⁷⁷ S. Capranica, *Arch. Physiol.*, 1877, 283-295.

⁵⁷⁸ W. Kühne and W. C. Ayres, *J. Physiol.*, 1, 109-130 (1878-1879).

⁵⁷⁹ G. Wald and H. Zussman, *Nature*, 140, 197 (1937).

⁵⁸⁰ G. von Studnitz, H. J. Neumann, and H. K. Loevenich, *Arch. ges. Physiol.* (Pflüger's), 246, 652-663 (1943).

⁵⁸¹ G. Wald, *J. Gen. Physiol.*, 31, 377-383 (1948).

resembling violaxanthin and taraxanthin, but distinct in properties from either of these; it was named *canaryxanthophyll*. Lutein was found to be a second carotenoid widely distributed in feathers. According to Brockmann and Völker,⁷⁴ the following classification of feather carotenoids encompasses the various types:

- (1) Contains mostly lutein and little canaryxanthophyll.
- (2) Contains only a little lutein.
- (3) Contains considerable canaryxanthophyll.

Although Brockmann and Völker⁷⁴ noted that the canary could store lutein in the fat, in the liver, and in the egg yolk, it changed the carotenoid into a new taraxanthin-like xanthophyll before it was secreted into the new yellow feathers. In addition to the above three groups, two other pigments are occasionally found, namely a green one in the gray-headed woodpecker (*Picus canus*), and the green woodpecker, yaffle, or rain-bird (*P. viridis*), and a red pigment in the fire-finch or weaver (*Pyromelana franciscana*).

Test⁵⁸² noted the presence of three pigments in the feathers of the yellow-shafted woodpecker or Northern flicker (*Colaptes auratus luteus*), i.e., (a) a taraxanthin-like substance, (b) an unidentified red neutral carotenoid, suggesting astacene, occurring both in the free form and as an ester, and (c) α -carotene. Although α -carotene comprises a minor part of the color in *C. auratus luteus*, in the West Indian gilded woodpecker (*C. chrysocaulosis*), and in the southwestern gilded woodpecker (*C. chrysoides*), it plays a major role in the closely related red-shafted flicker (*C. cafer collaris*).

Kritzler^{583,584} identified three carotenoid pigments in the plumage of the African bishop birds, the wild-fire weaver (*Euplectes franciscanus*), the grenadier weaver (*Euplectes orix* or *Pyromelana oryx*), and the black-bellied weaver (*E. nigroventris*), and in the head plumes of the red-headed woodpecker (*Melanerpes erythrocephalus*). These are characterized according to their adsorption on alumina as R₁, lutein, and R₂. Carotenoids of unknown composition have been found in the yellow and red wings of a number of parrots.^{585,586} These include the Australian grass parrakeet or budgerigar (*Melopsittacus undulatus* Shaw), the Roselle parrakeet (*Platyercus eximius*), the red lory (*Eos cyanogenys*), the red and black lory

⁵⁸² F. H. Test, *Univ. Calif. Pub. Zool.*, **46**, 371-389 (1942).

⁵⁸³ H. Kritzler, *Physiol. Zool.*, **16**, 241-255 (1943).

⁵⁸⁴ H. Kritzler, *Anat. Record*, **84**, 528-529 (1942).

⁵⁸⁵ O. Völker, *Biol. Zentr.*, **62**, 7-13 (1942).

⁵⁸⁶ O. Völker, *J. f. Ornithol.*, **84**, 618-630 (1936); **85**, 136-146 (1937); **87**, 639-643 (1939).

(*Lorius salvadorii*), the blue and yellow macaw (*Ara ararauna*), the green and yellow parrot of Guiana (*Caica leucogaster*), the Australian lory (*Domicella garrula*), and kindred species. These birds store lutein in the body fat and egg yolk but not in the feathers.

Völker⁵⁸⁷ noted the occurrence of astaxanthin in the red feathers of the African shrike, or red-bellied butcher-bird (*Lanarius atrococcineus*). Inasmuch as this bird feeds mainly on insects, which have not been found to contain astaxanthin, it may be assumed tentatively that the shrike can transform other carotenoids into astaxanthin.

Frank⁵⁸⁸ found unidentified "lipochrome" in a large number of birds, and Lönnberg⁵⁸⁹ described the occurrence and importance of carotenoid substances in the plumage, bill, feet, and skin of various avian species. Völker⁵⁹⁰ attributed the lipochrome in feathers mainly to the carotenoids in the diet. Lutein alone was found in the yellow bunting (*Emberiza icterica*), the gray wagtail (*Motacilla cinerea*), the yellow wagtail (*M. flava*), the wood-warbler (*Phylloscopus sibilatrix* juv.), the blackhooded weaver (*Ploceus cucullatus*), the yellow-green warbler, wood-thrush, "kinglet" (*Regulus regulus*), and the canary (*Serinus canarius*). Lutein, mainly and some canaryxanthophyll, occurred in the yellowhammer (*Emberiza citrinella*), the Old World oriole (*Oriolus oriolus*), the British blue titmouse (*Parus caeruleus*) and the great titmouse (*P. major*). Canaryxanthophyll predominated over the lutein in the siskin, olive-green finch (*Carduelis spinus*), and the green finch (*Chloris chloris*), while canary-xanthin alone occurred in the European goldfinch (*Carduelis carduelis*), and in the wild canary (*Serinus canarius*). Lutein was associated with small amounts of canary-xanthin and with a red decomposition product in the red feathers of the fire-finch, or weaver (*Pyromelana franciscana*), and of the red crossbill (*Loxia curvirostra pusilla*). Only red decomposition products were observed in the bullfinch (*Pyrrhula pyrrhula*). Picofulvin predominated in the green feathers of the Central American woodpecker (*Chloronerpes yucatanensis*), of the gray-headed woodpecker (*Picus canus*), and of the green woodpecker (*Picus viridis*). Picofulvin was also found in the yellow feathers of a woodpecker (*Hypoxanthus rivoli*), and in the black feathers of the great black-spotted woodpecker (*Dryobates major*).

Vitamin A is widely distributed in the birds. Jensen and With¹⁴⁹ recorded the presence of vitamin A in thirty-six species of birds. The highest

⁵⁸⁷ O. Völker, *Naturwissenschaften*, 37, 309 (1950).

⁵⁸⁸ F. Frank, *J. f. Ornithol.*, 87, 426-523 (1939).

⁵⁸⁹ E. Lönnberg, *The Occurrence and Importance of Carotenoid Substances in Birds*, Trans. Eighth Intern. Ornithol. Congress, Oxford, July, 1934, pp. 410-424.

⁵⁹⁰ O. Völker, *J. f. Ornithol.*, 82, 439-450 (1934).

concentrations of vitamin A occur in the liver. Appreciable amounts are also found in the body fat of chickens, and, in the case of hens, in the ovaries and in the eggs.²⁰⁰ Bieri⁵⁹¹ observed that aqueous β -carotene suspensions injected intravenously into chicks deficient in vitamin A was converted to the vitamin, and appeared as such in the liver and serum, whereas injected cryptoxanthin did not result in significant amounts of vitamin A. No vitamin A was found in the liver of the cormorant (*Phalacrocorax carbo*).

The vitamin A content of the liver oil of chickens which have been fed a normal diet is given as 20,000 I.U./g., while that of the liver oil from the goose on a normal dietary regimen is 14,000 I.U./g.⁵⁹² These values compare with those of Mattson and Deuel,²⁰¹ who cited liver contents of 7.9 ± 0.9 I.U./g. (carotene-free, vitamin A-low diet), 117.0 I.U./g. (high carotene-free, vitamin A-low diet), 117.0 I.U./g. (high carotene, vitamin A-low diet), 5327 ± 630 and 4660 ± 1025 I.U./g. (high vitamin A with carotene-free or carotene-high diet, respectively). In later tests²⁰⁰ on chickens, the liver contained the following amounts of vitamin A (I.U./g.) corresponding to the various levels of vitamin A in the diet (I.U./lb. of food): 0 (diet), 370 (liver); 1,000, 665; 30,000, 4592; 60,000, 6940; 100,000, 4654; and 200,000, 4626. It is evident that up to a certain point there is a proportionality between the vitamin A intake and the amount stored in the liver; however, when the optimum level is reached (60,000 I.U./lb.), further increases in vitamin A in the food do not result in further increase in liver vitamin A, but actually in a decrease. Ganguly and co-workers⁵⁹ recorded average figures for liver vitamin A of 136 to 348 I.U./g. in chickens fed on experimental diets low in carotenoids; in one group a value as low as 14.0 I.U./g. was recorded.

The amount of vitamin A stored in the body fat of chickens continued to increase progressively to the highest levels of vitamin A intake, in the tests of Deuel *et al.*²⁰⁰ cited above. The chicken body fat had the following vitamin A content (I.U./g.) at different levels of intake (I.U./lb. food): 0 (diet), 32.3 (fat); 1,000, 23.4; 30,000, 42.2; 60,000, 59.3; 100,000, 92.7; and 200,000, 222.6. Thus, the concentration of vitamin A in the body fat of the birds receiving the food containing 200,000 I.U. of vitamin A per pound was more than twice that observed in chickens which received the diet containing 100,000 I.U. of vitamin A/lb. of food. These values are much higher than those recorded by Ganguly *et al.*,⁵⁹ which varied between 1.8 and 11.0 I.U./g. for the experimental diets without an especially high

⁵⁹¹ J. G. Bieri, *Arch. Biochem. Biophys.*, 56, 90-96 (1955).

⁵⁹² H. Vogel and H. Knobloch, *Chemie und Technik der Vitamine*, 3rd ed., F. Enke, Stuttgart, 1950, p. 41; cited by H. H. Inhoffen and H. Pommer in W. H. Sebrell, Jr. and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 1954, pp. 100-106.

vitamin A content. The vitamin A content of the ovary varies considerably. Values between 0.7 and 27.0 I.U./g. have been recorded for normal chickens.⁵⁹ For a discussion of the vitamin A and carotenoid content of eggs, the reader is referred to page 562.

(f) *Carotenoids and Vitamins A in Higher Vertebrates.* The higher vertebrates store variable amounts of carotenoids, but these stored pigments do not ordinarily constitute an important source of vitamin A. As described earlier, it is only in animals which store both carotene and carotenols (xanthophylls) (Group A), such as man, or carotene but not the carotenols (Group B), *i.e.*, cattle and horses, that the stored provitamin A may constitute an important source of vitamin A. On the other hand, Group C, which stores neither the carotenoid hydrocarbons nor the carotenols (sheep, goats, and rats), and Group D which stores only carotenols (such as chickens), do not usually have any source of vitamin A in their tissues other than preformed vitamin A. However, it is possible, in the case of Group D, that vitamin A might arise from cryptoxanthin if the animals had previously received a diet rich in the carotenol, since it is an excellent provitamin A.

In the higher vertebrate forms, as in the case of the fishes and birds, the liver contains the greatest proportion of carotenoids and of vitamin A. However, exceedingly wide variations are found in the proportion of vitamin A stored in the liver by different species. It is uncertain whether or not the discrepancies observed can be ascribed to variations in diet or to differences in the ability of the several species to store vitamin A. Karrer and co-workers⁵⁵³ reported that no vitamin A was present in the livers of a number of mammals, including the following: Bengal tiger (*Felis tigris*), adult and young male lion (*Felis leo*), crab-eating raccoon (*Procyon cancrivorus*), and harbor seal (*Phoca vitulina*). On the other hand, Jensen and With¹⁴⁹ demonstrated the presence of vitamin A in the livers of a number of species of animals including man. The vitamin A concentration is the highest in the livers of the fishes, followed next by the livers of reptiles and birds, while the lowest values are noted in the livers of the mammals.¹⁴⁹ Moore²⁹³ listed the vitamin A content (in I.U./g.) of the livers of mammals as follows: guinea pig, 10; pig, 100; cow, 150; rabbit, 170; rat (wild), 250; sheep, 600; sperm whale, 4,400; bearded seal, 13,000; and polar bear, 20,000. The low value in the guinea pig is ascribed to an inefficient transformation of carotene to vitamin A. The rabbit, which together with the guinea pig consumes chiefly green food as a source of vitamin A, appears to be more efficient in converting carotene to vitamin A than is the guinea pig.²⁹³ Bieri,⁵⁹¹ also, found appreciable amounts of

vitamin A in the liver and serum of rabbits injected with carotene. The guinea pig is able to accumulate moderate stores of vitamin A when it is given as the preformed vitamin. The stores are rapidly depleted when the intake of vitamin A is reduced.⁵⁹³

Rodahl and Moore⁵⁹⁴ recorded extremely high values for vitamin A in the case of polar bears, and sometimes in seals. It is believed that the seal obtains its vitamin A from the cod and other fishes upon which it preys, while the polar bear is believed to derive its high store of vitamin A from the seal, which constitutes its main article of diet. Presumably because of the high concentration of vitamin A in the livers of polar bears, these organs are toxic; this toxicity has been repeatedly demonstrated among Arctic explorers since Elizabethan times.²⁹⁸ The high vitamin A content of whale liver was accounted for by Kon and Thompson⁴⁴⁴ on the basis of the preformed vitamin A present in the ocean plankton shrimps upon which the animals subsist. It is recognized by Moore²⁹⁸ that the values recorded for the several species "can be considered to be typical only when they are related to a characteristic nutritional background."

The tissues of several other species of animals contain a minimum amount of carotene. Thus, the flesh of the carabao or Philippine water buffalo (*Bubalus kerabau*) has 0.2 $\mu\text{g.}/\text{g.}$ of carotene, in spite of the fact that the body fat is colorless.³⁸⁹ β -Carotene is the only carotenoid present in the corpus luteum of the Nubian wild ass (*Equus asinus (africanus)*).¹⁹² Only traces of carotene have been reported in the liver of the dog.⁵⁹⁴

a'. Man: Man absorbs and stores the carotenoids unselectively. The blood carotenoids, those stored in the liver and depots, as well as those secreted into the milk, usually reflect the type of pigments which are present in the diet. In addition to β -carotene,⁷² human fat may contain α -carotene,⁵⁹⁵⁻⁵⁹⁷ lutein,^{24,72} zeaxanthin,²⁴ capsanthin,^{24,72} and lycopene.^{24,72} The only carotenoids which have been considered to be non-absorbable are the epoxides.⁵⁹⁸

Carotenoids likewise occur in many tissues other than fat depots, and the proportion of the several carotenoids corresponds to that in the diet. This relationship has been established in human subjects and in other

⁵⁹³ L. S. Bentley and A. F. Morgan, *J. Nutrition*, **30**, 159-169 (1945).

⁵⁹⁴ K. Rodahl and T. Moore, *Biochem. J.*, **37**, 166-168 (1943).

⁵⁹⁵ E. V. Rouir, *Bull. soc. chim. biol.*, **29**, 754-755 (1947).

⁵⁹⁶ L. Zechmeister, *Bull. soc. chim. biol.*, **16**, 993-1008 (1934).

⁵⁹⁷ J. G. Thomson, *Z. ges. exp. Med.*, **92**, 692-710 (1934).

⁵⁹⁸ P. Karrer and E. Krause-Voith, *Helv. Chim. Acta*, **31**, 802-803 (1948).

animals for liver,^{69,599} adrenals,⁶⁰⁰ heart,⁶⁰¹ pancreas,⁹⁸ nerves,⁶⁰² bone-marrow,²⁵⁶ and placenta.⁵⁹⁹ Maas isolated an unidentified lipochrome in the seminal vesicles.⁶⁰³ β -Carotene is the preponderant pigment in the corpora lutea, in association with traces of xanthophyll,²¹ while lutein may be the only carotenoid present in the macular region of the retina.^{24,192}

Carotenoids accumulate in human fat throughout life. This presumably accounts for the fact, observed by Thomson,⁵⁹⁷ that the adult fat is yellow while that of the infant is white. When fat is mobilized from the fat depots, the carotenoids remain behind. This accounts for the fact that the fat from emaciated humans is yellower than that from well-nourished subjects. Fat from the abdomen and chest is believed to have a higher carotenoid content than does that from the thighs and arms.⁵⁹⁷ Aschoff⁶⁰⁴ reported that the carotenoid concentration in fat from the adrenal gland is twenty times, and that from atheromas is ten times, as high as that in subcutaneous fat. No carotenoids or vitamin A have been reported in human sweat,⁶⁰⁵ in spermatozoa,⁶⁰⁵ or in cerebrospinal fluid.⁶⁰⁵ Menken⁶⁰⁶ found that the blood serum of human mothers contained six times as much carotene as did that of the infants, indicating that the placenta is impermeable to carotene. However, carotene may be converted to vitamin A in the placenta to some extent. The amount of vitamin A in the mother's serum was found to be approximately the same as that in the infant's blood. Colostrum contains large amounts of both carotene and vitamin A.⁶⁰⁶ It is a matter of dispute whether or not there are carotenoids in human bile; von Drigalski⁶⁰⁵ stated that they were absent. Willstaedt and Lindquist⁶⁹ believe that they have isolated two new carotenoids from liver extract.

The average vitamin A content was calculated by Williams³⁸⁷ to be 5,500 and 4,680 I.U./kg. of carcass in the case of two men; 69 and 85%, respectively, of the total was in the liver. He lists the following values (in I.U./g.) for several human tissues: liver, 156; adrenal, 6.0; kidney, 2.7; heart, 1.4; skin, 1.3; stomach, 1.3; ileum, 1.3; lung, 1.2; spleen, 1.0; colon, 0.8; skeletal muscle, 0.5; and brain, 0.5. The concentration of vitamin A in the liver of normal humans has been reported as 123 I.U./g.

⁵⁹⁹ R. Kuhn and H. Brockmann, *Z. physiol. Chem.*, **206**, 41-64 (1932).

⁶⁰⁰ G. M. Findlay, *J. Pathol. Bacteriol.*, **23**, 482-489 (1920).

⁶⁰¹ D. H. Dolley and F. V. Guthrie, *J. Med. Research*, **42**, 289-301 (1921).

⁶⁰² D. H. Dolley and F. V. Guthrie, *J. Med. Research*, **40**, 295-309 (1919).

⁶⁰³ F. Maas, *Arch. Mikr. Anat.*, **34**, 452-510 (1889).

⁶⁰⁴ L. Aschoff, *Verhandl. deut. pathol. Ges.*, **27**, 145-152 (1934).

⁶⁰⁵ W. von Drigalski, *Z. Vitaminforsch.*, **3**, 37-74 (1934).

⁶⁰⁶ J. G. Menken, *Maandschr. Kindergeneesk.*, **4**, 22-35 (1934).

for Egypt,⁶⁰⁷ 300 I.U./g. for England,³⁵⁹ 504 I.U./g. in Scotland,⁶⁰⁸ and 820 I.U./g. in Africa.⁶⁰⁹

b'. Cattle: Palmer and Eckles^{246,259} called attention to the fact that the chief carotenoid in the tissues of cattle was β -carotene. According to Parrish *et al.*⁶¹⁰ and Goodwin,¹⁹² from 92 to 97% of the total carotenoids in the blood serum consist of carotenes. β -Carotene is the main component of the carotene fraction in the tissues of cows, but a number of investigators have demonstrated the occurrence of α -carotene in butter.^{63,66,611,612} Gillam and Heilbron⁶³ reported that the principal carotenoids in butterfat are α -carotene, β -carotene, and lycopene. In the carotenol fraction, which comprises only 5 to 8% of the total carotenoids, lutein is the chief component, but cryptoxanthin has also been noted.^{63,613} The relative amounts of carotene and of xanthophylls in milk and butter depend upon the forage.^{614,615}

Carotenoids, preferentially β -carotene, accumulate in many tissues of cattle and of other animals. They are present not only in blood, but also in the liver,^{18,19,616} adrenal glands,⁶¹⁶⁻⁶¹⁹ retina,^{580,620-622} the pigment epithelium and iris,⁶²² pituitary gland,^{151,623} ovaries,^{619,624,625} corpus luteum,^{151,246,259,623,624,626-630} and corpus rubrum,⁵⁹⁹ placenta,^{151,616,619,623} umbilical cord blood,⁶³¹ colostrum,⁶³² bone-marrow,⁶¹⁷ testes,^{143,619,625} thy-

⁶⁰⁷ G. Nor El Din, *J. Roy. Egypt. Med. Assoc.*, **27**, 251-286 (1944).

⁶⁰⁸ L. Dzialiszyński and W. Tomaszewski, *Edinburgh Med. J.*, **54**, 252-259 (1947).

⁶⁰⁹ C. Auffret and F. Tanguy, *Algérie méd.*, **51**, 186 (1948).

⁶¹⁰ D. B. Parrish, G. H. Wise, and J. S. Hughes, *J. Biol. Chem.*, **172**, 355-365 (1948).

⁶¹¹ H. H. Strain, *J. Biol. Chem.*, **111**, 85-93 (1935).

⁶¹² H. H. Strain, *J. Biol. Chem.*, **127**, 191-201 (1939).

⁶¹³ F. W. Quackenbush, H. Steenbock, and W. H. Peterson, *J. Am. Chem. Soc.*, **60**, 2937-2941 (1938).

⁶¹⁴ J. H. Mitchell and G. H. Wise, *J. Dairy Sci.*, **27**, 189-196 (1944).

⁶¹⁵ B. C. Johnson, W. H. Peterson, and H. Steenbock, *J. Dairy Sci.*, **24**, 813-817 (1941).

⁶¹⁶ H. v. Euler and E. Klusmann, *Biochem. Z.*, **256**, 11-17 (1932).

⁶¹⁷ H. v. Euler, U. Gard, and H. Hellström, *Svensk. Kem. Tidskr.*, **44**, 191-198 (1932).

⁶¹⁸ O. Bailly and R. Netter, *Compt. rend.*, **193**, 961-963 (1931).

⁶¹⁹ O. Bailly, *Bull. acad. med. (Paris)*, [3], **108**, 932-935 (1932).

⁶²⁰ O. Brunner, E. Baroni, and W. Kleinau, *Z. physiol. Chem.*, **236**, 257-262 (1935).

⁶²¹ H. v. Euler and E. Adler, *Arkiv Kemi, Mineral., Geol.*, **11 B**, No. 20, 1-6 (1933).

⁶²² H. J. Bielg and L. Busch, *Z. physiol. Chem.*, **280**, 56-63 (1944).

⁶²³ H. v. Euler, B. Zonek, and E. Klusmann, *Arkiv Kemi, Mineral., Geol.*, **11 B**, No. 2, 1-5 (1932).

⁶²⁴ R. Kuhn and E. Lederer, *Z. physiol. Chem.*, **200**, 246-254 (1931).

⁶²⁵ R. Netter, *Bull. soc. chim. biol.*, **14**, 1555-1559 (1932).

⁶²⁶ J. L. W. Thudichum, *Proc. Roy. Soc.*, **17**, 253-256 (1869).

⁶²⁷ G. Piccolo and A. Lieben, *Giorn. sci. nat. econ. Palermo*, **2**, 258-272 (1866).

⁶²⁸ H. v. Euler and E. Klusmann, *Biochem. Z.*, **250**, 1-6 (1932).

⁶²⁹ H. H. Escher, *Z. physiol. Chem.*, **83**, 198-211 (1913).

⁶³⁰ R. Kuhn and W. Schlientz, *Helv. Chim. Acta*, **17**, 7-8 (1934).

⁶³¹ G. Gaetgens, *Klin. Wochschr.*, **16**, 894-895 (1937).

⁶³² J. Truka-Tuzson, *Z. Geburtshilfe u. Gynakol.*, **120**, 86-90 (1939).

mus,⁶¹⁷ muscle,⁶³³ and bile.^{528,634} Cabrera and De Jesus⁶³³ found that the meat from cattle contained more carotene than did carabao meat. Both species have the same ability to absorb carotene from feeds, but the liver of the carabao is more efficient in converting the pigment into colorless vitamin A. Piccolo and Lieben⁶²⁷ gave the name "hemolutein" to the "new" substance which they isolated, in 1866, from the corpus luteum of cattle.

One of the most interesting sites in which carotenoids are deposited is beef fat.^{616,635,636} According to Poulsson,⁶³⁵ the provitamin A deposits are greater in cows during the reproductive period than in steers. Since xanthophylls are present in much greater proportion in cow and sheep feces than is carotene,⁶³⁷ it is postulated that the preferential accumulation of carotenes in the tissues and milk results from the failure to absorb xanthophylls from the gastrointestinal tract. However, the absorption of carotene from the gastrointestinal tract of cows on green pasturage is also not complete. Moore⁶³⁸ cited Whitnah *et al.*,⁶³⁹ who reported a carotene content of over 100 $\mu\text{g./g.}$ in the feces of the cow. Moore attributed this high figure to the adventitious presence of yellow chromogens which were not carotene. When these chromogens were removed from feces of cattle on summer pasture by filtration of the petroleum ether extract through a column of dicalcium phosphate, the figures for carotene ranged from 72.5 to 96.8 $\mu\text{g./g.}$ of material.

Carotene accumulates in the body fat with increasing age. As much as 2 mg. of α - and β -carotene were obtained by Zechmeister and Tuzson⁶³⁶ from 1 kg. of cow fat. The highest concentration of β -carotene in the cow occurs in the corpus luteum and corpus rubrum, where figures as high as 6 mg. and 120 mg./100 g., respectively, have been reported.¹⁹² Ganguly and co-workers²⁵⁷ observed values for carotenes as high as 28.5 mg./100 g. in the case of cow ovaries, while the corresponding maximum carotenol level was 0.7 mg./100 g. Piccolo and Lieben⁶²⁷ were able to obtain carotene in crystalline form from the corpora lutea.

Carotene is present in the muscle in a concentration of 25 $\mu\text{g./100 g.}$ ⁶³³ Astaxanthin⁵⁵⁰ (or a pigment resembling astaxanthin), in addition to β -carotene,⁶²² has been found in the retina of cattle. However, β -carotene alone was detected in the retina by Brunner *et al.*⁶²⁰ Moreover, only β -

⁶³³ D. J. Cabrera and Z. De Jesus, *Philippine J. Animal Ind.*, **6**, 5-42 (1939).

⁶³⁴ H. Fischer and R. Hess, *Z. physiol. Chem.*, **187**, 133-136 (1930).

⁶³⁵ E. Poulsson, *Deut. Med. Wochschr.*, **56**, 1688-1689 (1930).

⁶³⁶ L. Zechmeister and P. Tuzson, *Ber.*, **67**, 165-155 (1934).

⁶³⁷ H. Fischer, *Z. physiol. Chem.*, **96**, 292-295 (1916).

⁶³⁸ L. A. Moore, *Ind. Eng. Chem., Anal. Ed.*, **12**, 726-729 (1940).

⁶³⁹ C. H. Whitnah, W. J. Peterson, F. W. Atkeson, and H. W. Cava, *J. Agr. Research*, **58**, 343-355 (1939).

carotene occurs in the iris of cattle.⁶²² The amount of carotene in the liver of cows was found by Ka⁶⁴⁰ to vary with the seasonal intake of carotenoids. In the tests of Ganguly *et al.*,²⁵⁷ the average value for liver carotene was 20.6 $\mu\text{g./g.}$, with a range between 12.0 and 32.6 $\mu\text{g./g.}$, while the mean xanthophyll value was 1.2 $\mu\text{g./g.}$, with a range between 0.8 and 1.3 $\mu\text{g./g.}$

Preformed vitamin A is likewise present in the blood and liver of cows. Moore²⁹⁸ recorded an average figure of 150 I.U./g. liver for cows which presumably had received large amounts of carotene in the form of grass over long periods. Moore and Payne⁶⁴¹ cited a value of 144 I.U./g. for beef liver and of only 39 I.U./g. for calf liver.

c'. Horses: Zechmeister and Tuzson⁶⁸ reported that β -carotene is the predominant carotenoid deposited in the fat of horses; this confirms the early work of Palmer,²⁶⁴ who showed that the same situation obtains in the blood. It is also suggested that, in horses as in cattle, the preponderance of the carotenes in the liver and other tissues, and the low levels of the xanthophylls in these tissues, may be ascribed to selective absorption, since relatively larger amounts of xanthophylls, as compared with carotene, were found in horse dung after the feeding of green fodder.^{68,642} Palmer and Eckles^{246,259} suggested that the absence of xanthophylls from the body carotenoids might be due to their destruction in the gastrointestinal tract.

In addition to the carotenoids, vitamin A is also a component of the blood and liver of horses. Rudra³⁶² reported an average vitamin A level in the livers of English horses of 628 I.U./g., with variations between 32 and 2000 I.U./g. This compares with a figure of 1173 I.U./g. for horse liver reported by Andersen and Hart,⁶⁴³ and one of 500 I.U./g recorded by Jensen and With.¹⁴⁹ Andersen and Hart⁶⁴³ found that the vitamin A in the liver of the horse was reduced to zero when the animals were maintained on a vitamin A-free diet for 152 days.

d'. Sheep: According to Peirce,²⁶⁵ the livers of Australian sheep contain only traces of carotene (1 to 3 $\mu\text{g./g.}$). The amount of the carotene was not influenced by a diet which completely depleted the liver of vitamin A. The vitamin A content of normal sheep liver was reported as 459 I.U./g., by Moore and Payne.⁶⁴¹ The vitamin A level remained uniform in winter and summer, in contrast to the values in the livers of cattle and pigs, which were higher in the summer.

e'. Miscellaneous Animals Which Do Not Accumulate Carotenoids:

⁶⁴⁰ H. Ka, *Rept. Inst. Sci. Research, Manchoukuo*, 3, 371-385, *trans.* 2 pp. (1939).

⁶⁴¹ T. Moore and J. E. Payne, *Biochem. J.*, 36, 34-36 (1942).

⁶⁴² L. Zechmeister, P. Tuzson, and E. Ernst, *Nature*, 135, 1039 (1935).

⁶⁴³ A. C. Andersen and G. H. Hart, *Am. J. Vet. Research*, 4, 307-317 (1943).

In the group of higher vertebrates, the animals which do not accumulate carotenoids in the liver and other tissues are far more common than are those which store carotenoids. According to Jensen and With,¹⁴⁹ the vast majority of animals examined contained only traces, or no carotenoids whatsoever. The failure to accumulate these pigments has been attributed to the very efficient conversion of the provitamins A in the wall of the intestine to vitamin A. It is not known what the fate of the xanthophylls may be, but it is believed that they may be broken down in a manner resembling that of the carotenes. Goodwin⁵²³ obtained evidence that lutein (xanthophyll) is absorbed by the rat; as he was able to recover small amounts of the mono-esters and di-esters from the intestinal wall. No trace of the absorbed pigment could be detected in other tissues.

The blood and liver of the animals which belong in this category contain no carotenoid, or only traces. These include goats,^{139,644,645} swine,^{273,274,646,647} rats,^{18,271,272,648} rabbits,^{139,649} as assumed¹⁹² on the basis of indirect evidence furnished by the staining experiments of Connor,⁶⁴⁹ hares,⁶⁵⁰ and guinea pigs.¹³⁷ Sheep apparently are on the border line, since their livers contain only traces of carotenoids.²⁶⁵ Ganguly *et al.*²⁵⁷ were unable to demonstrate the presence of carotenes or of carotenols in the blood or ovaries of a sheep presumably from the Southern California area. Only traces appeared in the liver. According to Coombes *et al.*,⁶⁵¹ foxes might also be considered to be in a borderline group, since they have a low plasma carotenoid content, and no carotenoids are stored in the liver. Even after the feeding of high doses of β -carotene, no accumulation occurs in the blood of rats,^{271,272} pigs,^{272,273} guinea pigs,¹³⁷ or rabbits.¹³⁹ Only small amounts of carotene were found in the blood of sheep following the administration of massive doses.⁶⁵² Zechmeister and Tuzson⁶⁴⁷ reported that less than 0.1 mg./kg. of carotenol appeared in the fat of pigs which had been fed on a diet rich in these pigments for an extended period. The liver was found to contain only one-fifth to one-tenth of the above amount.

⁶⁴⁴ B. N. Banerjee and N. C. Datta, *Agr. Livestock, India*, 8, 563-567 (1938); *Chem. Abst.*, 33, 2564 (1939).

⁶⁴⁵ V. R. Smith, R. P. Niedermeier, and L. A. Schultz, *J. Animal Sci.*, 7, 544 (1948).

⁶⁴⁶ L. A. Palmer, *Am. J. Physiol.*, 87, 553-557 (1929).

⁶⁴⁷ L. Zechmeister and P. Tuzson, *Z. physiol. Chem.*, 239, 147-148 (1936).

⁶⁴⁸ R. L. Shub, *Akusherstvo i. Ginekol.*, 1940, No. 10, 6-8; *Chem. Abst.*, 37, 1170-1171 (1943).

⁶⁴⁹ C. L. Connor, *Am. J. Pathol.*, 4, 235-244 (1928).

⁶⁵⁰ R. A. Morton and G. D. Rosen, 1949, unpublished data cited by T. W. Goodwin, *The Comparative Biochemistry of Carotenoids*, Chem. Pub. Co., New York, 1954, p. 247.

⁶⁵¹ A. I. Coombes, G. L. Ott, and W. Wisnicky, *North Am. Veterinarian*, 21, 601-606 (1940).

⁶⁵² E. R. Barrick, F. N. Andrews, and J. F. Bullard, *J. Animal Sci.*, 7, 539 (1948).

Although there is no question that animals in the above group can be readily separated from the other group because of the minimum quantities of β -carotene stored, there is still some evidence that the small amount of chromogenic material in the liver consists of carotenoids. Beadle *et al.*⁶⁵³ did prove that the yellow fat sometimes encountered in pigs did not contain carotenoids. However, Ganguly *et al.*²⁵⁷ were able to prove by unequivocal methods that the small amount of chromogenic material present in the livers of rats after the administration of carotene was, in fact, all-*trans*- β -carotene. Thus, after saponification of the livers, followed by extraction with solvent and chromatography on an alumina column, it was found that a 4% acetone eluate was colored. The fact that this was β -carotene was proved by comparing the extinction with pure β -carotene in carbon disulfide, benzene, and hexane, as well as by the demonstration that a mixed chromatogram with pure β -carotene gave a single bond with an unchanged extinction curve.²⁵⁷ The amount of all-*trans*- β -carotene in rat livers was found to average 2.1 $\mu\text{g.}/\text{g.}$ liver.

In view of the above results, the author is forced to conclude that the difference between the carotene metabolism in the carotenoid accumulators and in those which do not store these substances is quantitative rather than qualitative. Actually, small amounts of carotene do escape the intestinal barrier to gain entrance to the body, but the "carotenase" mechanism is sufficient to limit the quantity of carotene which is not broken down. If this concept is accepted, it would provide a possible explanation for the β -carotene in the retina of the pig, when no trace of the carotenoid could be demonstrated in the blood or liver of this species.

Moore and Payne⁶⁴¹ reported a vitamin A value of 45 I.U./g. of liver in the pig. The livers of pigs had more vitamin A in the summer than in the winter. This was found to be similar to the situation in cows, but did not apply to sheep. Williams³⁸⁷ furnished the following data on the vitamin A content in the carcasses of several species (I.U./kg.) and the per cent of the total vitamin A in the liver: monkey, 6,150, 67%; guinea pig, 29,500 and 2,040, 92% and 10%; rabbit, 1,840 and 2,500, 35% and 21%; gopher, 11,800, 67%; rat, 24,000, 97%; cat, 2,100 and 56,400, 24% and 92%; and dog, 7,925, 21,000 and 27,500, 19%, 16% and 43%. One cannot make many deductions from the above data except that wide variations obtain in the amount of vitamin A present, and in the proportion in the liver. Since the liver is the organ in which variable amounts of vitamin A are stored, one would expect that the proportion in this organ would be highest

⁶⁵³ B. W. Beadle, O. H. M. Wilder, and H. R. Kraybill, *J. Biol. Chem.*, 175, 221-229 (1948).

when the total vitamin A storage is greatest; this relationship is, in fact, borne out by the above results. The wide variations noted must be a reflection of widely varying nutritional conditions insofar as vitamin A is concerned.

The distribution of vitamin A in the several tissues of the normal rat (reported in I.U./g. moist tissue) is reported by Williams³⁸⁷ as follows: adrenal, 49; liver, 7.6; lung, 3.3; kidney, 2.7; muscle, 0.7; heart, 0.2; and brain, 0.0. Clausen and McCoord, in unpublished studies cited by Williams,³⁸⁷ found the following tissues devoid of vitamin A: lung (dog, guinea pig); small intestine (rabbit); large intestine (cat); stomach (cat, guinea pig); spleen (guinea pig, rabbit); pancreas (rabbit); ovaries (rabbit); thyroid gland (rabbit) and adrenal gland (gopher).

(2) *Comparative Distribution of Carotenoids
and of Vitamins A in Different Tissues*

The distribution of carotenoids in the invertebrates has a certain similarity to that in the higher forms. The gonads are among the most common sites for the storage of carotenoids in these forms. Thus, the eggs of *Asteroidea* contain carotenoids, and their presence in the gonads of the *Holothuroidea*, *Echinoidea*, *Mollusca*, and *Gastropoda* has been demonstrated. The integument provides another storage site for carotenoids in the lower forms (*Asteroidea*, *Crustacea*, *Mollusca*). The coloring matter in the skin of tropical fishes and of other highly colored specimens of this group consists mainly of carotenoids. The pigmentation of the plumage of birds is also attributable to carotenoids. An accumulation of carotenoids has been noted in the digestive glands of the *Asteroidea* and *Echinoidea*, as well as in the digestive diverticula of some *Mollusca*. Lovern and co-workers³⁴⁶ demonstrated that substantial amounts of vitamin A were present in the gastric and intestinal tissues of halibut. Later, Lovern and Morton¹⁰⁰ found that the pyloric caeca in the halibut (*Hippoglossus hippoglossus*) was very rich in vitamin A; the main concentration was in the tunica propria. In an extension of this work, Glover and Morton⁶⁵⁴ corroborated the finding that the vitamin A was largely concentrated in the tunica propria and mucosa. A larger proportion of the vitamin A in the mucosa was in the form of the free alcohol than was the case with that present in the tunica propria. It is believed that a lipase effects alternate synthesis and hydrolysis of the esters during absorption; this condition would account for variations in the proportion of free alcohol. Grangaud and co-

⁶⁵⁴ J. Glover and R. A. Morton, *Biochem. J.*, 42, lxiii-lxiv (1948).

workers⁶⁵⁵ also reported that, although the liver is usually the storehouse for vitamin A, in the case of the hake (*Merluccius merluccius*) the intestine carries out this function. Lovern, Morton, and Ireland⁶⁵⁶ likewise observed that the vitamin A concentration in the oil from the intestines of the sturgeon (*Acipenser sturio*) was ten times as high as it was in the liver oil. Since this is a freshwater fish, part of the vitamin A was in the form of vitamin A₂. It was found that the ratio of vitamin A to vitamin A₂ was much higher in the intestine than in the liver. It is concluded that vitamin A and vitamin A₂ do not replace each other with equal facility in all functions. Lovern *et al.*⁶⁵⁶ noted that sea birds had a higher proportion of vitamin A in the intestines than did animals such as the rabbit. In the blue-gray American and European herring gull (*Larus argentatus smithsonianus*) and the great red-brown skua gull or bonxie (*Megalestris catarrhactes*), the liver contained more vitamin A than did the intestine. No vitamin A₂ was found. In the white gannet or Solan goose (*Sula bassana*) the lungs contained relatively large amounts of vitamin A, and the liver and intestines very little. On the other hand, a high proportion of the vitamin A was stored in the body fat of the eel (*Anguilla vulgaris*).

The main sites in which vitamin A is stored in the vertebrates are the liver and eyes. Although vitamin A is ordinarily not present in many of the invertebrates, when it does occur, it is almost entirely concentrated in the eyes. Thus it was found that practically the entire vitamin A content of the whole organism in the northern euphausiid shrimps (*Meganyctiphanes norvegica* and *Thysanoessa raschii*) is present in the eyes.⁴⁴⁶ A large proportion of the total astaxanthin in these species is present in the eyes. Although as high an actual amount of vitamin A is to be found in the eyes of the vertebrates, it constitutes only an insignificant proportion of the total vitamin A stored, because a much larger total amount of vitamin A is present in the higher animals than in lower forms.

The liver constitutes the main storage site in the birds and in the higher vertebrates, including the rat⁶⁵⁷⁻⁶⁵⁹ and pig.⁶⁶⁰ Smaller concentrations are found in the kidney, the lungs, the adrenals, the adipose tissues and in the blood plasma. While the liver is the principal organ in which the excess vitamin A is removed from the blood for storage, apparently the kidneys

⁶⁵⁵ R. Grangaud, R. Massonet, and H. Larroque, *Compt. rend. soc. biol.*, 143, 1181-1183 (1949).

⁶⁵⁶ J. A. Lovern, R. A. Morton, and J. Ireland, *Biochem. J.*, 33, 325-329 (1939).

⁶⁵⁷ A. B. McCoord and E. M. Luce-Clausen, *J. Nutrition*, 7, 557-572 (1934).

⁶⁵⁸ H. C. Sherman and L. C. Boynton, *J. Am. Chem. Soc.*, 47, 1646-1653 (1925).

⁶⁵⁹ C. A. Baumann, B. M. Riising, and H. Steenbock, *J. Biol. Chem.*, 107, 705-715 (1934).

⁶⁶⁰ T. B. Osborne and L. B. Mendel, *J. Biol. Chem.*, 34, 17-27 (1918).

require a small amount of vitamin A for normal functioning, and they will remove this quantity from the blood and store it, to the exclusion of the liver. Thus, Johnson and Baumann⁶⁶¹ reported that, when rats were receiving minimum quantities of carotene or of vitamin A, more vitamin A was accumulated in the kidneys than in the livers. When the dosage of vitamin A was increased, the opposite condition obtained. They also reported⁶⁶² a greater storage of vitamin A in the kidney than in the liver when small doses of α -carotene, β -carotene, neo- β -carotene B and U were fed to depleted rats. Eden and Moore⁶⁶³ confirmed the fact that, in young rats, more vitamin A acetate was stored in the kidney than in the liver, except when large doses were given. A greater storage of vitamin A occurred in the kidneys of male rats than in those of female rats.

(3) *The Distribution of Carotenoids and of Vitamins A Within the Cell*

Little information is available concerning the part of the cell in which carotenoids are stored, except for the report of Berger.⁶⁶⁴ This investigator made use of a specific reagent for the identification of carotenoids, which allowed him to detect the presence of nuclei of various colors in the retinae of mammals. Since the coloration disappeared in vitamin A deficiency, it was concluded that the materials identified were carotenoids. It is also postulated that vitamin A may constitute a portion of these nuclei.

The site of deposition of vitamin A within the liver cell is a matter of controversy. This may be due to the employment of different technics for the separation of the fractions, and also to the fact that storage of vitamin A may vary according to whether it is in the form of the ester of free alcohol. Joyet-Lavergne⁶⁶⁵ reported that vitamin A was present in the mitochondria of liver cells. These findings were confirmed by Goerner⁶⁶⁶ and by Collins.⁶⁶⁷ However, Powell and Krause⁶⁶⁸ reported that, in the liver cell of the normal rat, fraction "X" (the cytoplasm remaining after removal of the nuclei and mitochondria) contained the most vitamin A, while the next highest concentration was in the mitochondria, and the

⁶⁶¹ R. M. Johnson and C. A. Baumann, *Federation Proc.*, **6**, 265 (1947); *J. Nutrition*, **35**, 703-715 (1948).

⁶⁶² R. M. Johnson and C. A. Baumann, *Arch. Biochem.*, **14**, 361-367 (1947).

⁶⁶³ E. Eden and T. Moore, *Biochem. J.*, **47**, vi-vii (1950).

⁶⁶⁴ P. Berger, *Compt. rend. soc. biol.*, **144**, 606-607 (1950).

⁶⁶⁵ P. Joyet-Lavergne, *Bull. soc. chim. biol.*, **18**, 1041-1054 (1936).

⁶⁶⁶ A. Goerner, *J. Biol. Chem.*, **122**, 529-538 (1938).

⁶⁶⁷ F. D. Collins, *Biochem. J.*, **51**, xxxviii (1952).

⁶⁶⁸ L. T. Powell and R. F. Krause, *Arch. Biochem. Biophys.*, **44**, 102-106 (1953).

least occurred in the nuclei. When vitamin A was administered to rats in sufficient amounts to increase the quantity in the liver, a shift in distribution occurred. Fraction "X" still contained the largest proportion of vitamin A, followed by the nuclei; the mitochondria had the lowest concentration. On the other hand, Krinsky and Ganguly⁶⁶⁹ reported that vitamin A ester was present solely in the centripetally migrating fraction ("cream" fraction), while vitamin A alcohol occurred not only in the centripetally-migrating material but also, in appreciable amounts, in the supernatant and microsomal fractions. The mitochondrial and nuclear fractions were found to be essentially free from vitamin A. It is believed that vitamin A ester and vitamin A alcohol are stored in combination with different proteins within the liver cell. Several years ago, Glover and Morton⁶⁷⁰ called attention to the fact that two types of vitamin A storage occur in the liver. It is believed that the one site in which the ester is stored contains no lipase, while this enzyme is present in locations where vitamin A occurs as the free alcohol. The Kupffer cells are considered to be the site of lipase-free storage. In fact, Gomori⁶⁷¹ demonstrated the absence of lipase from these cells, by a histologic procedure. During the absorption of vitamin A, two processes are believed to be operating in the liver, namely (1) the hydrolysis of the incoming ester by serum esterase, which releases free vitamin A to form a complex capable of diffusing into the liver storage cells, and (2) phagocytic action by the Kupffer cells whereby the vitamin A ester in the chylomicrons is trapped in the liver sinusoids, and becomes incorporated into the Kupffer cells. The vitamin A (ester) is not believed to contribute to the functional vitamin A in the blood.⁶⁷⁰ However, the implication that the level of plasma vitamin A is a function of the quantity of hepatic vitamin A alcohol is not borne out experimentally (see page 468).

(4) *Factors Altering the Storage of Carotenoids and of Vitamins A*

a. **The Effect of Age on the Storage of Vitamin A.** Age has a marked effect on the proportion of vitamin A present in the liver of rats. According to Guerrant,⁶⁷² newborn rats contain only a small amount of vitamin A. The concentration of vitamin A in the livers of nursing rats doubles during the first seven days, after which it remains fairly constant over the balance of the lactation period. The amount and concentration of the vitamin A

⁶⁶⁹ N. I. Krinsky and J. Ganguly, *J. Biol. Chem.*, **202**, 227-232 (1953).

⁶⁷⁰ J. Glover and R. A. Morton, *Biochem. J.*, **43**, xii-xiii (1948).

⁶⁷¹ G. Gomori, *Arch. Pathol.*, **41**, 121-129 (1946).

⁶⁷² N. B. Guerrant, *J. Nutrition*, **37**, 37-51 (1949).

present in the liver of the normal weanling rat increases rapidly during the period of rapid growth, and reaches a maximum concentration at 170 days. After this period, additional vitamin A is stored, but the concentration in the liver remains constant. The data for these experiments are summarized in Table 1.

TABLE 1
VITAMIN A CONTENT OF THE LIVERS OF
STOCK RATS OF VARIOUS AGES^a

Group No.	No. of rats	Sex	Age of rats, days	Av. weight of rats, g.	Av. weight of livers, g.	Vitamin A in I. U.		
						Av. total	Av. per g. liver	Av. per g. body weight
1	8	?	1	6.2	0.36	7	20	1.1
2	5	?	7	11.2	0.70	25	44	3.9
3	5	Males	16	30	1.98	105	54	3.5
4	4	Males	21	42	2.6	130	50	3.1
5	4	Males	37	89	5.9	360	61	4.0
6	4	Males	51	127	7.3	700	95	5.5
7	3	Males	73	212	10.1	1,860	184	8.8
8	1	Female	117	178	8.7	5,700	655	32.0
9	6	Males	170	361	12.2	22,470	1,862	63.0
10	3	Females	330	241	10.2	18,950	1,769	82.3
11	1	Female	334	202	9.5	16,190	1,704	77.8
12	1	Female	437	279	10.4	19,840	1,908	71.3
13	4	Females	564	281	10.4	18,440	1,762	65.9
14	4	Females	741	269	10.9	18,600	1,708	69.5
15	6	Males	749	421	14.5	28,400	1,865	68.5

^a Adapted from N. B. Guarrant, *J. Nutrition*, 37, 37-51 (1949), p. 41.

The low vitamin A level reported in the livers of newborn rats (7 I.U./rat or 20 I.U./g. of liver) is in agreement with the results obtained by Williamson,³⁷³ who computed the total vitamin A in the livers of unborn rats. This figure apparently is an index of the amount of vitamin A imparted to the fetal rats through the placental circulation. Eaton and co-workers⁶⁷³ and Baker *et al.*⁶⁷⁴ observed that the vitamin A stored by the calf during intrauterine life varies with the vitamin intake of the mother, since the plasma level of vitamin A of the newborn calf is higher, and there is a greater storage of vitamin A in the liver at ten days after birth in calves

⁶⁷³ H. D. Eaton, A. A. Spielman, R. E. Johnson, and L. D. Matterson, *J. Dairy Sci.*, 32, 764-768 (1949).

⁶⁷⁴ F. H. Baker, R. MacVicar, L. S. Pope, and C. K. Whitehair, *Proc. Soc. Exptl. Biol. Med.*, 83, 571-574 (1953).

from dams fed supplementary vitamin A than in the controls. The vitamin A in the plasma and liver of the calf is closely associated with the carotene intake of the dam during the lactation period.

Vitamin A is consumed during the processes of growth. When suboptimal amounts of vitamin A are available, the quantity remaining in the liver is greater when growth is restricted than when growth proceeds normally. Thus, Johnson and Baumann⁶⁶¹ observed increased hepatic vitamin A in rats when the extent of growth was limited by diets inadequate in calories, in thiamine, or in tryptophane, or by the administration of thiouracil or of thiourea, as compared with that in the normal controls. Gillum and associates³¹³ reported that the plasma carotene and vitamin A were moderately reduced in men and women with advancing age.

b. The Effect of Dosage on the Storage of Vitamin A. No storage of vitamin A occurs in the liver until the dosage is sufficient to meet the minimum requirements for growth. Thus, Guerrant⁶⁷² reported that, when increasing doses of vitamin A, from 0.73 to 29 I.U., were fed daily to depleted rats over a period of twenty-eight days, relatively small but definite increases in hepatic vitamin A took place, although marked increases in body weight were observed. When the dosages were progressively increased from 44 I.U. to 8,800 I.U., the rise in liver vitamin A with each increment was extremely marked. Between dosage levels of 29 and 3,520 I.U. of vitamin A, the hepatic vitamin A was approximately doubled for each two-fold increase in vitamin A intake. However, when the dosage was increased from 17,600 to 75,520 I.U., no further rise in the liver vitamin A level occurred; in fact, at the highest dosage level, the hepatic vitamin A was below the maximum. Table 2 summarizes the data on the growth response of rats as related to vitamin A intake.

The average vitamin A recovered was as high as 88% at the lowest dosage; when the doses of vitamin A administered ranged between 14.7 and 3520 I.U./day, the average vitamin A recovered averaged about 14% (Range 19 to 9%). When moderately high amounts of vitamin A were given (4,400, 6,600, and 8,800 I.U.), an average of 43% (Range 51 to 38%) of that fed was recovered. When higher amounts of vitamin A were given, the proportion retained was further decreased until, at the two highest dose levels, only 6% and 5%, respectively, were recovered.⁶⁷²

The effect of dosage upon the proportion of vitamin A retained accounts for the variability in the results reported by different workers. Thus, Baumann and co-workers⁶⁵⁹ reported that only 10 to 20% of the vitamin A which they administered to rats was stored, while Golding *et al.*⁶⁷⁵ observed

⁶⁷⁵ J. Golding, S. K. Kon, and J. E. Campion, *Chem. Ind.*, 55, 400-401 (1936).

TABLE 2

THE RELATION OF THE VITAMIN A INTAKE OVER A 28-DAY PERIOD TO THE AMOUNT STORED IN THE LIVER IN RATS PREVIOUSLY DEPLETED OF VITAMIN A^a

Group no. ^b	Vitamin A fed daily, I.U.	Av. change in body weight, g. ^c	Vitamin A in I.U. (Av.)			Av. vitamin A recovered, %
			Total found	Per g. liver	Per g. body weight	
1	None	—	0	0.0	0.00	—
2	0.73	+12	18	3.9	0.12	88
3	2.2	+67	34	4.1	0.17	55
3A ^d	2.2	+64	37	4.2	0.19	60
4	4.4	+69	52	5.1	0.26	42
5	7.4	+70	59	5.7	0.31	28
6	14.7	+74	61	5.7	0.31	15
7	22	+79	68	6.1	0.33	11
8	29	+80	71	6.5	0.34	9
9	44	+98	160	12.8	0.70	13
10	88	+85	340	28.8	1.62	14
11	176	+87	550	44.8	2.57	11
12	290	+76	1,070	87.7	4.8	13
13	590	+82	2,930	210	12.9	18
14	880	+76	3,900	290	17.6	16
15	1,760	+90	8,950	640	40.3	19
16	2,640	+77	9,360	730	45.2	13
17	3,520	+85	13,690	1,140	62.5	14
18	4,400	+72	62,800	5,510	310	51
19	6,600	+65	74,100	6,330	380	40
20	8,800	+71	92,580	8,050	450	38
21	17,600	+20	105,360	12,630	700	21
22	18,380	+20	92,210	9,510	630	18
23	26,400	+8	117,260	11,380	860	16
24	35,200	-22	116,800	13,710	1,050	12
25	37,760	-27	87,330	11,640	960	6
26	75,520	-21	104,990	12,780	970	5

^a Adapted from N. B. Guarrant, *J. Nutrition*, 37, 37-51 (1949), p. 44.

^b Consisting of 5 to 12 rats per group.

^c Average weight of rats at end of depletion was 128 g.

^d This group received 3 mg. of α -tocopherol daily during the 28-day test period as additional supplement.

that 47% of the vitamin A administered to pigs was retained. Other work has confirmed the fact that the extent of utilization and storage of vitamin A is a function of the dosage employed. Thus, Lemley *et al.*⁶⁷⁶ reported

⁶⁷⁶ J. M. Lemley, R. A. Brown, O. D. Bird, and A. D. Emmett, *J. Nutrition*, 33, 53-64 (1947).

that the percentage of storage increased with the magnitude of the dose up to the optimum point, but declined when very heavy doses were given. Davies and Moore⁶⁷⁷ obtained essentially similar results when varying dosages of halibut liver oil were given to rats. They indicated that the following three principles govern storage under these conditions:

(1) At low dosages, about 100 I.U. is "lost," or applied to other purposes, before storage commences in the liver. The proportion "lost" decreases as the dose is raised, until a toxic level is reached.

(2) Following toxic overdoses a high proportion of the dose may disappear, and less storage occurs than would have been the case had the dosage been just below the toxic level.

(3) With prolonged massive dosing, without obvious toxic symptoms, the percentage of the vitamin stored may be low, even when a high concentration is attained in the liver. This is considered to be due to the "saturation" of the liver with vitamin A.

Frey *et al.*⁶⁷⁸ reported that the hepatic stores of vitamin A in cattle increased in practically a linear relationship with intake throughout the range of vitamin A supplement fed. An intake of 100 I.U./lb. of body weight was found to maintain nearly maximum serum vitamin A levels.

c. The Effect of Sex on the Storage of Vitamin A. The sex of the animal plays such an important role in the metabolism of lipids in general that it is not surprising that it should likewise be concerned with the metabolism of carotene and of vitamin A. It was reported earlier that sex differences exist in the plasma levels of carotene and of vitamin A in man. Thus, the figures for β -carotene in the blood are usually higher in the female than in the male, and those for plasma vitamin A show exactly the reverse relationship between the sexes.

Sex also constitutes an important factor in relation to the storage of vitamin A in the liver and kidneys. A number of workers have reported higher vitamin A values for hepatic vitamin A in females, as compared with males, under normal feeding conditions, not only in rats^{679,680} but also in cattle.⁶⁸¹ The same phenomenon has been shown to obtain in rats after the feeding of shark and halibut liver oils,^{354,682,683} as well as after the ad-

⁶⁷⁷ A. W. Davies and T. Moore, *Biochem. J.*, **42**, lxiii (1948).

⁶⁷⁸ P. R. Frey, R. Jensen, and W. E. Connell, *J. Nutrition*, **34**, 421-430 (1947).

⁶⁷⁹ E. C. Callison and V. H. Knowles, *Am. J. Physiol.*, **143**, 444-452 (1945).

⁶⁸⁰ T. Moore and I. M. Sharman, *Biochem. J.*, **47**, xliii-xliv (1950).

⁶⁸¹ F. Ender, *Z. Vitaminforsch.*, **3**, 247-253 (1934).

⁶⁸² A. R. Bult and C. J. Sorgdrager, *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.*, **8**, 114-116 (1938).

⁶⁸³ H. Popper and S. Brenner, *J. Nutrition*, **23**, 431-443 (1942).

ministration of carotene^{120,684} or of vitamin A itself,^{120,684-686} except following very high dosages. The difference in storage cannot be ascribed to differences in weight or growth rates,⁶⁸⁴ and it is believed to be an example of true sexual dimorphism. When moderate dosages (40 to 320 I.U./day) of vitamin A were given to rats, Moore *et al.*⁶⁸⁵ noted a greater efficiency of storage of vitamin A in females than in males. Booth⁶⁸⁴ likewise observed that the depletion of vitamin A from the liver of rats on a vitamin A-free diet was more rapid in the case of male rats than in females. Hence, the ratio of storage of the female *vs.* the male increased with time. The threshold dose below which no storage takes place was likewise found to be higher in male rats than in female rats.⁶⁸⁴ When the rats were placed on a vitamin A-free diet, deficiency symptoms appeared in the males slightly sooner than in the females;^{684,687} moreover, the females on the deficient diet outlived their brothers, although the differences in survival time were not great.⁶⁸⁴ Goodwin³⁸⁸ reported that carotenoids were more abundant in the organs of females than in those of males, in species in which they were absorbed. However, Booth⁶⁸⁴ stated that the absorption efficiency of carotene and the conversion to vitamin A are not functions of sex. Lemley and associates⁶⁷⁶ failed to note sex differences in the storage of vitamin A in rats, but it may be that some of the conditions were different from those in the experiments of other investigators. There is no doubt that the preponderance of evidence supports the concept that females store greater amounts of vitamin A in their livers than do males under similar conditions.

A sex difference in vitamin A storage in the kidney has also been recorded,⁶⁷⁹ but this appears to be in the opposite direction than is the case with liver vitamin A. When the amount of vitamin A available to rats is low, the kidney exerts a priority for storage over the livers.^{661,663,688} After moderate doses a higher level of vitamin A occurs in the kidneys of male rats as compared with female rats.^{663,680,684,685} Moore and co-workers⁶⁸⁵ reported that the sex difference in vitamin A storage in the kidney is exhibited at moderate dosages (40 to 320 I.U./day), and that it decreases at high intakes of the vitamin.

(a) *The Effect of Sex Hormones.* The removal of the sex hormones from the area of activity by castration has been shown to affect the vitamin A storage of rats. Bult and Sorgdrager⁶⁸⁹ reported that castrated rats lost

⁶⁸⁴ V. H. Booth, *Biochem. J.*, **47**, xliii (1950); *J. Nutrition*, **48**, 13-30 (1952).

⁶⁸⁵ T. Moore, I. M. Sharman, and R. J. Ward, *Biochem. J.*, **49**, xxxix-xl (1951).

⁶⁸⁶ G. C. Esh and S. Bhattacharya, *Indian J. Physiol. Allied Sci.*, **5**, 15-20 (1951).

⁶⁸⁷ J. Mayer and W. A. Krehl, *Yale J. Biol. Med.*, **20**, 403-405 (1948).

⁶⁸⁸ E. Eden and T. Moore, *Biochem. J.*, **49**, 77-79 (1951).

⁶⁸⁹ A. R. Bult and C. J. Sorgdrager, *Ned. Tijdschr. Geneesk.*, **84**, 1963-1970 (1940).

their stores of vitamin A at rates intermediate between those for males and for females. Booth⁶⁸⁴ found that castrated young male rats lost their vitamin A at a slightly slower rate than that of their unoperated controls; this is interpreted to mean that castration in the males tends to induce femininity insofar as concerns vitamin A storage. Ovariectomized females stored more vitamin A than did normal females, they lost their vitamin A more slowly, and grew faster. The kidney vitamin A values for both castrated males and ovariectomized females were found to be intermediate between the values for normal males and for normal females.

Moore and co-workers⁶⁹⁰ observed variations in the vitamin A content of kidneys, as influenced by the sex hormones. No appreciable variations were noted in the vitamin A content of kidneys of male rats after castration, without or with testosterone; the vitamin A levels of the kidneys under these conditions were 37.4 I.U./g. for normal males, 42.7 I.U./g. for castrated males given injections of testosterone, with unrestricted food intake. On the other hand, when the operated males were injected with estradiol, the kidneys contained 4.6 I.U./g., a value expected in female rats. These variations were not observed in the livers, where the concentrations were highest and almost equal in the slowly growing groups treated with testosterone and with estradiol. It is also suggested that the sex effect on vitamin A distribution can be only partially explained as a secondary effect of its influence on growth.⁶⁹⁰

d. The Effect of Thyroid Function on the Storage of Vitamin A. Although there seems to be considerable evidence that the thyroid gland influences the change of carotene to vitamin A, and hence might be indirectly concerned with the extent of storage of vitamin A after the administration of carotene, it is of interest to inquire whether or not this gland directly controls vitamin A deposition in the liver. Logaras and Drummond⁶⁹¹ and Baumann and Moore⁶⁹² reported that, when groups of normal and hyperthyroid rats containing uniform stores of vitamin A were fed a diet low in vitamin A, the resulting hepatic stores were at least as high in the thyroxine-treated animals as in the normal controls. Johnson and Baumann³⁵ also noted that hyperthyroid rats which had been fed halibut liver oil stored as much vitamin A as did the normal controls. However, in later work of Johnson and Baumann,⁶⁶¹ it was reported that, in rats of similar size, the administration of desiccated thyroid hastened the depletion of vitamin A reserves, while in hypothyroid conditions produced by

⁶⁹⁰ T. Moore, I. M. Sharman, and R. J. Ward, *Biochem. J.*, **49**, xiii-xiv (1951).

⁶⁹¹ G. Logaras and J. C. Drummond, *Biochem. J.*, **32**, 964-968 (1938).

⁶⁹² C. A. Baumann and T. Moore, *Biochem. J.*, **33**, 1639-1644 (1939).

the administration of thiouracil or thiourea, the depletion of hepatic stores was delayed. A three-fold increase in metabolic rate (produced by thyroid powder) was found to be quantitatively less important for the retention of vitamin A than a decrease in growth rate of 50%. Thiourea resulted in a particularly low content of vitamin A in the kidneys.

e. The Effect of the Method of Administration on the Storage of Vitamin

A. Teulon *et al.* observed that the storage of vitamin A in the liver was greater, and occurred sooner, when oil solutions of vitamin A were given orally than when the intramuscular route⁶⁹³ or subcutaneous injection⁶⁹⁴ was employed. On the other hand, Esh and Bhattacharya⁶⁸⁶ reported that vitamin A alcohol or acetate gave rise to higher hepatic stores of vitamin A when administered orally in a 20% Tween 20 solution than when fed in an ethyl oleate or peanut oil solution. No significant difference obtained in liver storage when natural or when synthetic vitamin A was used.⁶⁹⁴

f. The Effect of Tocopherols on Vitamin A Storage. Moore⁶⁹⁵ was the first to suggest that the vitamin E (tocopherol) intake has an important bearing on the ability of the animal to lay down administered vitamin A. Davies and Moore,⁶⁹⁶ and more recently Dubouloz and associates,⁶⁹⁷ also came to similar conclusions. According to the latter, about 22% of the vitamin A disappearing from the gastrointestinal tract of the rat was stored in the liver when tocopherol or natural antioxidants were used, while the figure was only 14.5% in the absence of antioxidants. Hickman⁵⁴² in a review, and with his co-workers,⁶⁹⁸⁻⁷⁰¹ called attention to the synergism exerted by the tocopherols upon the utilization of carotene and

⁶⁹³ H. Teulon, C. Marnay, and H. Gounelle, *Compt. rend. soc. biol.*, **145**, 831-834 (1951).

⁶⁹⁴ H. Teulon, C. Marnay, and H. Gounelle, *Compt. rend. soc. biol.*, **144**, 1546-1548 (1950).

⁶⁹⁵ T. Moore, *Biochem. J.*, **34**, 1321-1328 (1940); with I. M. Sharman, *Brit. J. Nutrition*, **5**, 119-129 (1951).

⁶⁹⁶ A. W. Davies and T. Moore, *Nature*, **147**, 794-796 (1941).

⁶⁹⁷ P. Dubouloz, G. Pistone, and R. Marville, *Bull. soc. chim. biol.*, **31**, 1273-1278 (1949).

⁶⁹⁸ K. Hickman, P. L. Harris, and M. R. Woodside, *Nature*, **150**, 91-92 (1942).

⁶⁹⁹ K. C. D. Hickman, P. L. Harris, and M. R. Woodside, *Vitamin A and E Interrelationships*, presented before Am. Chem. Soc., 104th meeting, Buffalo, Sept. 7-11, 1942; also presented before Gibson Island Conf., *Am. Assoc. Advancement Sci.*, Sect. on *Vitamins*, July, 1942; cited by K. Hickman, *Ann. Rev. Biochem.*, **12**, 353-396 (1943), p. 382.

⁷⁰⁰ K. C. D. Hickman, M. W. Kaley, and P. L. Harris, *J. Biol. Chem.*, **152**, 303-311 (1944).

⁷⁰¹ P. L. Harris, M. W. Kaley, and K. C. D. Hickman, *J. Biol. Chem.*, **152**, 313-320 (1944).

vitamin A. Thus, it was found that the apparent potency of 0.1 μ g. of carotene was multiplied by 2 to 2.5, while the effect on a 4 μ g. dose was increased by a factor of 1.25 to 1.5, and the response of rats to 40 μ g. was not improved by tocopherol. In the case of vitamin A, the response to 1 μ g. of vitamin A was increased 1.5 to 2.0 times by the optimum dose of tocopherol, but the apparent potency produced by 30 μ g. was not improved by the administration of tocopherol.⁵⁴²

When rats were fed 0.8 μ g./day of carotene, the following increases in body weight occurred over a period of thirty-six days as a result of the addition of several supplements:^{542,699} without vitamin E, 2 g.; with 0.15 mg. mixed tocopherols, 31 g.; with an equal amount of pure α -tocopherol, 27 g.; of pure β -tocopherol, 30 g.; and of pure γ -tocopherol, 30 g.; with 0.1 mg. pure ascorbic acid (vitamin C), 7.5 g.; with 1 mg. ascorbic acid, 11 g.; with 10 mg. ascorbic acid, 20 g.; with 0.13 mg. hydroquinone, 3.3 g.; with 35 mg. lauryl hydroquinone, 27.9 g.; and with 0.5 mg. palmityl-ascorbic acid, 30 g.

There are several explanations for the beneficial effect of the tocopherols on the utilization of vitamin A. Dubouloz *et al.*⁶⁹⁷ and Quackenbush and co-workers⁷⁰² were of the opinion that the tocopherols act as antioxidants in the gastrointestinal tract, thus protecting the vitamin A from destruction. On the other hand, Hickman and co-workers^{698,700} and Harris *et al.*⁷⁰¹ noted that the tocopherols enhanced the growth produced by vitamin A alcohol, by vitamin A acetate, and by standard U.S.P. reference oil. Depletion of vitamin A was found to be more difficult if tocopherols were given, while animals lived longer after the cessation of vitamin A supplementation if tocopherols were administered.⁷⁰⁰ These workers suggested that tocopherols act synergistically with carotene and vitamin A. However, it was likewise indicated that the intestine is probably the site of action of the tocopherols, inasmuch as the effect was greatly diminished when the supplements of vitamins A and E were given on alternate days, as compared with the results when they were given together.⁵⁴²

Lemley *et al.*⁷⁰³ described the beneficial effect exerted by tocopherol, in daily doses of 0.3 mg., on the liver storage of vitamin A when 100 I.U. of vitamin A were given; the effect was much less pronounced when the dose of vitamin A was doubled. On the other hand, Guerrant,⁶⁷² in a single test, was unable to demonstrate any increase in storage of vitamin A in the liver when an additional supplement of 3 mg. of α -tocopherol was added to

⁷⁰² F. W. Quackenbush, P. R. Cox, and H. Steenbock, *J. Biol. Chem.*, **145**, 169-177 (1942).

⁷⁰³ J. M. Lemley, R. A. Brown, O. D. Bird, and A. D. Emmett, *J. Nutrition*, **34**, 205-218 (1947).

the basal diet. Also, Herbert and Morgan²¹⁶ failed to observe any significant difference in the hepatic storage of vitamin A in the rat after feeding vitamin A or carotene, irrespective of whether or not tocopherols were given to the rats in daily doses of 0.5 to 1.0 mg. Actually, Johnson and Baumann⁷⁰⁴ obtained evidence of a depressing effect exerted by high levels of tocopherol upon the liver stores of vitamin A, and upon the growth of carotene-fed rats.

Sherman⁷⁰⁵ suggested that the improved utilization of vitamin A in the presence of the tocopherols is to be ascribed to the prevention of a physiologic antagonism between carotene and the unsaturated fatty acids.

g. The Effect of Carcinogenic Drugs upon Vitamin A Storage. Goerner⁶⁶⁶ was the first to show that the injection of the carcinogenic agent, 1,2,5,6-dibenzanthracene, markedly reduced the vitamin A and increased the total lipid content of hepatic mitochondria. On the other hand, it was observed that *p*-aminoazobenzene, a non-carcinogenic ring compound, had a much less pronounced effect upon liver vitamin A. Subsequently, Goerner and Goerner⁷⁰⁶ found that intraperitoneal injections of 1,2,5,6-dibenzanthracene caused a decrease in the total vitamin A stored in the liver, but that the drug was without effect upon the hepatic storage of vitamins C and D. The inhibition of growth caused by the drug paralleled a decrease in the vitamin A in the liver. Moreover, the level of glutathione in the liver was unaffected by the administration of 1,2,5,6-dibenzanthracene.⁷⁰⁷

Baumann and co-workers,⁷⁰⁸ in a further study of this problem, demonstrated that 1,2,5,6-dibenzanthracene caused a reduction in the vitamin A stores in rat liver, not only by increasing the rate of depletion fourfold, but also by preventing the entrance of vitamin A into the liver. The drug was found to be effective, not only when injected in colloidal solution intraperitoneally, but also when administered in oil by the subcutaneous route. No correlation was found to exist between the carcinogenicity of this type of compound and its effect upon vitamin A. Thus, methyleholanthrene and benzpyrene, two potent carcinogens, and 1,2-benzanthracene, a non-carcinogenic hydrocarbon, all caused a decrease in the quantity of hepatic vitamin A, but they were quantitatively much less effective than was 1,2,5,6-dibenzanthracene. Butter yellow, which is carcinogenic, and carbon black, which is non-carcinogenic, were without effect upon the vitamin A stores in the liver.⁷⁰⁸ It was likewise noted that the livers of

⁷⁰⁴ R. M. Johnson and C. A. Baumann, *J. Biol. Chem.*, **175**, 811-816 (1948).

⁷⁰⁵ W. C. Sherman, *Proc. Soc. Exptl. Biol. Med.*, **47**, 199-200 (1941).

⁷⁰⁶ A. Goerner and M. M. Goerner, *J. Nutrition*, **18**, 441-446 (1939).

⁷⁰⁷ A. Goerner and M. M. Goerner, *Am. J. Cancer*, **37**, 518-520 (1939).

⁷⁰⁸ C. A. Baumann, E. G. Foster, and P. S. Lavik, *J. Nutrition*, **21**, 431-444 (1941).

rats with spontaneous tumors, as well as of those with tumors due to methylcholanthrene, contained more vitamin A than did livers in which no tumor formation had taken place.

A problem exists as to the fate of the vitamin A which disappears from the liver after the injection of 1,2,5,6-dibenzanthracene. The hydrocarbon might promote the excretion of vitamin A, it might increase the rate of destruction of vitamin A, or it might alter the distribution of vitamin A between the liver and other organs. The first of these possibilities cannot be accepted as the explanation, since Goerner and Goerner⁷⁰⁶ found that no vitamin A was excreted in the urine after the injection of the carcinogen. Moreover, Baumann *et al.*⁷⁰⁹ were unable to detect vitamin A in the combined excreta of rats receiving 1,2,5,6-dibenzanthracene. There are no data indicating that the second possibility, *i.e.*, an increased rate of destruction of liver vitamin A, may partly account for its effect. However, Baumann and co-workers⁷⁰⁹ reported that the third possibility cited above can be readily demonstrated experimentally. Thus, it was shown that, coincident with the reduction in liver vitamin A following the injection of dibenzanthracene, there was a concomitant rise in the vitamin A content of the kidneys. Methylcholanthrene was found to promote a similar shift of vitamin A from the liver to the kidneys.⁷⁰⁹ Although as much as 50% of the total vitamin A of rats injected with dibenzanthracene was found in the kidneys (in contrast with only 5% in normal rats), the increased quantity of vitamin A stored in the kidney accounted for only 10% of that which had left the liver. The best explanation for the decreased capacity of the liver to store vitamin A under these conditions is that the liver component to which vitamin A is attached is altered or missing; presumably this component is a protein (see page 520).

h. Miscellaneous Factors Affecting Vitamin A Storage. In addition to dibenzanthracene, a number of other drugs have been found to cause a depletion in hepatic vitamin A. Thus, drugs which cause a blocking of the reticuloendothelial system, such as thorotrast,⁷¹⁰ phenylhydrazine,⁷¹¹ or bismuth,⁷¹¹ reduce the storage of vitamin A in the liver. The antimalarial drug atabrine, in addition to reducing the rate of absorption of carotene and of vitamin A, was shown by Guggenheim⁵⁶ to decrease the capacity of the liver to store vitamin A.

Alcohol also inhibits the vitamin A storage in the liver,⁷¹² although the

⁷⁰⁹ C. A. Baumann, E. G. Foster, and P. R. Moore, *J. Biol. Chem.*, **142**, 597-608 (1942).

⁷¹⁰ F. Lasch and D. Roller, *Klin. Wochschr.*, **15**, 1636-1637 (1936).

⁷¹¹ H. Wendt and D. König, *Klin. Wochschr.*, **16**, 1253-1254 (1937).

⁷¹² I. Ikegaki, *Z. Vitaminforsch.*, **7**, 113-118 (1938).

effect is less pronounced than that obtained with dibenzanthracene.⁷⁰⁹ Baumann *et al.*⁷⁰⁹ extended this finding by showing that, in contradistinction to the effect of dibenzanthracene and methylcholanthrene, the action of alcohol in lowering hepatic vitamin A does not involve a simultaneous increase in the vitamin A content of the kidney.

The vitamin A content of the liver has been found to be distinctly subnormal in patients suffering from chronic nephritis.^{713,714} This low degree of liver storage is accompanied by relatively high levels of vitamin A in the blood.^{29,84}

There are a number of abnormal conditions in which no disturbances in vitamin A storage have been noted. Thus, it is normal in a variety of clinically acute disorders,^{713,715} for example in measles, bronchopneumonia, and otitis media, as well as in the experimental loss of fat due to thiamine deficiency,⁷¹⁶ and also in the loss of liver fat due to choline.⁷¹⁷ Likewise, Bentley and Morgan⁷¹⁸ reported that the deposition of vitamin A was not affected by fatty livers or by choline deficiency. Moreover, fatty livers induced by phosphorus apparently did not alter the hepatic storage of vitamin A.^{711,719} The livers of choledochocolostomized rats did not contain less vitamin A than did those of unoperated animals maintained under the same conditions.⁷²⁰ Following the administration of phenylhydrazine, the vitamin A storage in the liver was decreased only when the Kupffer star-cells were damaged. Bismuth, which has a close affinity for the reticuloendothelial system, completely inhibited the storage of vitamin A in the liver. This is interpreted by Wendt and König⁷¹¹ to indicate the role of the reticuloendothelial system in vitamin A metabolism. In addition, Coniglio *et al.*⁷²¹ reported that doses of x-ray of 500 *r* and 650 *r* did not influence fat absorption in rats, or the concentration of vitamin A in the liver.

Food intake itself has been shown to have little effect upon the level of hepatic vitamin A. Thus, Truscott⁷²² reported that it required relatively enormous differences in food intake to change the levels of hepatic vitamin A in castrated mice. On the other hand, Molteno and Rapson⁷²³ reported

⁷¹³ L. K. Wolff, *Lancet* [110], 1932, II, 617-620 (1932).

⁷¹⁴ T. Moore, *Biochem. J.*, 31, 155-164 (1937).

⁷¹⁵ J. B. Ellison and T. Moore, *Biochem. J.*, 31, 165-171 (1937).

⁷¹⁶ W. J. Dann and T. Moore, *Biochem. J.*, 25, 914-917 (1931).

⁷¹⁷ E. J. Lease and H. Steenbock, *J. Nutrition*, 17, 85-90 (1939).

⁷¹⁸ L. S. Bentley and A. F. Morgan, *J. Nutrition*, 31, 333-346 (1946).

⁷¹⁹ F. Lasch, *Klin. Wochschr.*, 14, 1070-1073 (1935).

⁷²⁰ J. D. Greaves and C. L. A. Schmidt, *Am. J. Physiol.*, 116, 456-467 (1936).

⁷²¹ J. G. Coniglio, W. J. Darby, M. C. Wilkerson, R. Stewart, A. Stockwell, and G. W. Hudson, *Am. J. Physiol.*, 172, 86-92 (1953).

⁷²² B. L. Truscott, *Science*, 117, 63-64 (1953).

⁷²³ C. J. Molteno and W. S. Rapson, *Biochem. J.*, 33, 1390-1393 (1939).

TABLE 3
CAROTENOID DERIVATIVES HAVING PROVITAMIN A ACTIVITY

Derivative	Empirical formula	Reference All- <i>trans</i> - β -carotene = 100
β -Carotene set		
5,6-Dihydro- β -carotene	$C_{40}H_{58}$	<i>a</i>
β -Carotene monoepoxide	$C_{40}H_{56}O$	<i>b, c, d, e, f</i>
β -Carotene monofuranoxide (Mutatochrome)	$C_{40}H_{56}O$	<i>b, c, e, f, g</i>
Vitamin A-epoxide (Hepaxanthin, "574-chromogen")	$C_{20}H_{30}O_2$	<i>h</i>
β -Carotene diepoxide	$C_{40}H_{56}O_2$	<i>b, c, e, f</i>
β -Carotene difuranoxide (Aurochrome)	$C_{40}H_{56}O_2$	<i>b, c</i>
β -Carotene monoepoxide monofuranoxide	$C_{40}H_{56}O_2$	<i>b, d</i>
Rubixanthin epoxide monofuranoxide (Rubi-chrome)	$C_{40}H_{56}O_2$	<i>f, i, j</i>
β -Carotene monoxide	$C_{40}H_{56}O$	<i>k</i>
5,6-Dihydroxy- β -carotene	$C_{40}H_{56}O_2$	<i>l, m</i>
Semi- β -carotenone	$C_{40}H_{56}O_2$	<i>l, m, n</i>
β -Oxycarotene	$C_{40}H_{58}O_3$	<i>o</i>
Anhydrosemi- β -carotenone	$C_{40}H_{54}O$	<i>m</i>
β -Carotenal	$C_{30}H_{40}O$	<i>p</i>
β -Apo-2-carotenal	$C_{30}H_{40}O$	<i>j, q, r</i>
β -Apo-2-carotenol	$C_{30}H_{42}O$	<i>p, r</i>
β -Apo-4-carotenal	$C_{25}H_{34}O$	<i>j, r</i>
β -Apo-4-carotenol	$C_{25}H_{36}O$	<i>p, r</i>
β -Carotene diiodide	$C_{40}H_{56}I_2$	<i>s</i>
16,16'-Homo- β -carotene	$C_{42}H_{58}$	<i>t; 20%, u</i>
α -Carotene set		
All- <i>trans</i> - α -carotene	$C_{40}H_{56}$	<i>v; w; 53%, x</i>
5,6-Dihydro- α -carotene	$C_{40}H_{58}$	<i>a</i>
α -Carotene monoepoxide	$C_{40}H_{56}O$	<i>d, e</i>
α -Carotene diiodide	$C_{40}H_{56}I_2$	<i>s</i>
γ -Carotene set		
All- <i>trans</i> - γ -carotene	$C_{40}H_{56}$	<i>b</i>
In rat		<i>v; ca. 50%, y</i>
<i>ex Mimulus longiflorus</i> (monkey-flower)		<i>z, 28%</i>
<i>ex Pyracantha angustifolia</i> Schneid. (narrow-leaf firethorn)		<i>aa, 26%</i>
<i>ex pro-γ-carotene</i> by iodine catalysis		<i>ab, 42%</i>
In chicks		
<i>ex pro-γ-carotene</i> by stereoisomerization		<i>ac, 42%</i>
Cryptoxanthin set		
All- <i>trans</i> -cryptoxanthin	$C_{40}H_{56}O$	
In rats		<i>ad, 54-59%; ae, 50%; af, 57%; ag, 60%</i>
In chicks		<i>ah, 55%</i>
Isocryptoxanthin		<i>ai, 48%</i>
Isocryptoxanthin methyl ether		<i>ai, 52%</i>

TABLE 3 (Continued)

Derivative	Empirical formula	Reference All- <i>trans</i> - β - carotene = 100
<i>Echinenone Strongylocentrotus purpuratus</i> (purple sea-urchin), and <i>S. franciscanus</i> (red California sea-urchin)	$C_{40}H_{58}(\pm H_2)$	<i>aj</i> ; <i>ak</i> ; <i>al</i> , 54%
^a A. Polgár and L. Zechmeister, <i>J. Am. Chem. Soc.</i> , 65 , 1528-1534 (1943).		
^b W. H. Sebrell, Jr., and R. S. Harris, <i>The Vitamins</i> , vol. I, Academic Press, New York, 1954, pp. 19, 20.		
^c P. Karrer and E. Jucker, <i>Helv. Chim. Acta</i> , 28 , 427-436 (1945).		
^d P. Karrer and E. Jucker, <i>Helv. Chim. Acta</i> , 28 , 471-473 (1945).		
^e P. Karrer, <i>Helv. Chim. Acta</i> , 28 , 474-475 (1945); P. Karrer, E. Jucker, J. Rutschmann, and K. Steinlin, <i>Ibid.</i> , 1146-1156 (1945).		
^f P. Karrer, <i>Fortschr. Chem. org. Naturstoffe</i> , 5 , 1-19 (1948).		
^g P. Karrer and E. Jucker, <i>Helv. Chim. Acta</i> , 30 , 536-537 (1947).		
^h P. Karrer and E. Jucker, <i>Helv. Chim. Acta</i> , 28 , 717-723 (1945).		
ⁱ P. Karrer, E. Jucker, and K. Steinlin, <i>Helv. Chim. Acta</i> , 30 , 531-535 (1947).		
^j P. Karrer and E. Jucker, <i>Carotinoide</i> , Birkhäuser, Basel, 1948.		
^k H. v. Euler, P. Karrer, and O. Walker, <i>Helv. Chim. Acta</i> , 15 , 1507-1510 (1932).		
^l R. Kuhn and H. Brockmann, <i>Ber.</i> , 67 , 1408-1409 (1934).		
^m R. Kuhn and H. Brockmann, <i>Ann.</i> , 516 , 95-143 (1935).		
ⁿ R. Kuhn and H. Brockmann, <i>Ber.</i> , 66 , 1319-1326 (1933).		
^o R. Kuhn and H. Brockmann, <i>Ber.</i> , 65 , 894-898 (1932).		
^p P. Karrer and U. Solmssen, <i>Helv. Chim. Acta</i> , 20 , 682-690 (1937).		
^q P. Karrer, A. Rüegg, and A. Geiger, <i>Helv. Chim. Acta</i> , 21 , 1171-1174 (1938).		
^r H. v. Euler, P. Karrer, and U. Solmssen, <i>Helv. Chim. Acta</i> , 21 , 211-222 (1938).		
^s P. Karrer, U. Solmssen, and O. Walker, <i>Helv. Chim. Acta</i> , 17 , 417-419 (1934); P. Karrer, H. v. Euler, and U. Solmssen, <i>Ibid.</i> , 1169-1172 (1934); H. v. Euler, P. Karrer, and M. Rydbom, <i>Ber.</i> , 62 , 2445-2451 (1929).		
^t H. H. Inhoffen and H. Siemer, <i>Fortschr. Chem. org. Naturstoffe</i> , 9 , 1-40 (1952).		
^u H. J. Deuel, Jr., H. H. Inhoffen, J. Ganguly, L. Wallcave, and L. Zechmeister, <i>Arch. Biochem. Biophys.</i> , 40 , 352-357 (1952).		
^v R. Kuhn, H. Brockmann, A. Scheunert, and M. Schieblich, <i>Z. physiol. Chem.</i> , 221 , 129-136 (1933).		
^w H. von Euler, P. Karrer, and P. Zubrys, <i>Helv. Chim. Acta</i> , 17 , 24-29 (1934).		
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^y R. Kuhn and H. Brockmann, <i>Ber.</i> , 66 , 407-410 (1933).		
^z H. J. Deuel, Jr., C. Johnston, E. Sumner, A. Polgár, W. A. Schroeder, and L. Zechmeister, <i>Arch. Biochem.</i> , 5 , 365-371 (1944); L. Zechmeister and W. A. Schroeder, <i>Arch. Biochem.</i> , 1 , 231-238 (1942).		
^{aa} H. J. Deuel, Jr., C. H. Hendrick, E. Straub, A. Sandoval, J. H. Pinckard, and L. Zechmeister, <i>Arch. Biochem.</i> , 14 , 97-103 (1947).		
^{ab} L. Zechmeister, J. H. Pinckard, S. M. Greenberg, E. Straub, T. Fukui, and H. J. Deuel, Jr., <i>Arch. Biochem.</i> , 23 , 242-245 (1949).		
^{ac} S. M. Greenberg, C. E. Calbert, J. H. Pinckard, H. J. Deuel, Jr., and L. Zechmeister, <i>Arch. Biochem.</i> , 24 , 31-39 (1949).		
^{ad} R. Kuhn and C. Grundmann, <i>Ber.</i> , 67 , 593-595 (1934).		
^{ae} G. S. Fraps and A. R. Kemmerer, <i>Ind. Eng. Chem., Anal. Ed.</i> , 13 , 806-809 (1941).		
^{af} H. J. Deuel, Jr., E. R. Meserve, C. H. Johnston, A. Polgár, and L. Zechmeister, <i>Arch. Biochem.</i> , 7 , 447-450 (1945).		
^{ag} H. J. Deuel, Jr., S. M. Greenberg, E. Straub, T. Fukui, A. Chatterjee, and L. Zechmeister, <i>Arch. Biochem.</i> , 23 , 239-241 (1949).		
^{ah} S. M. Greenberg, A. Chatterjee, C. E. Calbert, H. J. Deuel, Jr., and L. Zechmeister, <i>Arch. Biochem.</i> , 25 , 61-65 (1950).		
^{ai} H. J. Deuel, Jr., J. Ganguly, L. Wallcave, and L. Zechmeister, <i>Arch. Biochem. Biophys.</i> , 47 , 237-240 (1953).		
^{aj} E. Lederer and T. Moore, <i>Nature</i> , 137 , 996 (1936).		
^{ak} S. M. Greenberg and B. T. Scheer. Unpublished experiments from J. Ganguly, N. I. Krinsky, J. H. Pinckard, and H. J. Deuel, Jr., <i>Z. physiol. Chem.</i> , 295 , 61-66 (1953), p. 62.		
^{al} J. Ganguly, N. I. Krinsky, J. H. Pinckard, and H. J. Deuel, Jr., <i>Z. physiol. Chem.</i> , 295 , 61-66 (1953).		

that in the South African geelbek or yellow-backed cape salmon (*Atractoscion (Otolithus) acquidens* C. and V.), which appears to correspond to the Atlantic squeteague, the total vitamin A in the liver decreased with the onset of intensive feeding, and later gradually increased. The vitamin A content of the visceral oil paralleled the total oil content of the viscera.

7. The Metabolism of Carotenoids and of Vitamins A

(1) *The Transformation to Vitamins A*

a. All-trans Carotenoids as Provitamins A. The most important reaction of the carotenoids concerning which information is available is their transformation to vitamin A. The qualitative conversion of a compound to vitamin A can be demonstrated by ascertaining whether or not the substance in question cures symptoms of hypovitaminosis A. A quantitative evaluation of the extent of this conversion can be obtained by bioassay, using vitamin A or pure all-trans- β -carotene as a standard. The biopotency of the unknown can be calculated in terms of the standard from the log dose gain in body weight curve, by the method of Coward.⁶ Only those substances which contain one intact β -ionone nucleus having a side-chain patterned after vitamin A, and which is as long or longer than that in vitamin A, can serve as precursors of this vitamin. There is some evidence that dehydro- β -carotene (isocarotene) can also act as a provitamin A₂, in spite of the fact that the β -ionone rings have two double bonds rather than one. For further information on this subject, the reader is referred to the reviews of Karrer and co-workers,⁷²⁴ of Karrer and Jucker,²⁴ to those of Zechmeister,^{79,725,726} and to that of Milas.⁷²⁷ Karrer⁷²⁸ also summarized the biologic significance of the carotenoid epoxides. Table 3 lists the various carotenoids which have been found to exert vitamin A activity.

In addition to the compounds which have been listed in Table 3 as provitamins A, a number of carotenoids exist which are devoid of provitamin A activity. The widely distributed carotenoid lycopene,¹⁷ in which both of the β -ionone rings are open, has been found to be inactive, as would be expected; the same situation obtains with the natural polycis derivative of

⁷²⁴ P. Karrer, K. Schöpp, and R. Morf, *Helv. Chim. Acta*, **15**, 1158-1165 (1932).

⁷²⁵ L. Zechmeister, *Bull. soc. chim. biol.*, **31**, 956-964 (1949).

⁷²⁶ L. Zechmeister, *Harvey Lectures*, **47**, 221-241 (1951-1952).

⁷²⁷ N. A. Milas, *Chemistry and Industrial Preparation*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins, Chemistry, Physiology, Pathology*, Vol. I, Academic Press, New York, 1954, pp. 4-58.

⁷²⁸ P. Karrer, *Fortschr. Chem. org. Naturstoffe*, **5**, 1-19 (1948).

this hydrocarbon, polycopene.⁷²⁹ Although Lund⁷³⁰ reported that zeaxanthin, which has a hydroxyl group on each of the two β -ionone rings, is a vitamin A precursor, the weight of evidence is contrary to this finding.^{731,732} Braude *et al.*⁷³¹ observed that zeaxanthin is biologically inactive in the case of both rats and pigs. Lutein is also inactive as a provitamin A.⁷³² The results of Kemmerer *et al.*,⁵¹ Kelley and Day,⁵² and of Callison and co-workers,⁵³ which involved the question of whether or not lutein in large doses decreases the provitamin A activity of β -carotene, are uniform in supporting the fact that lutein is biologically inactive as a provitamin A. Euler *et al.*⁷³² did report that both zeaxanthin and lutein are converted to products with provitamin A activity when they are treated with phosphorus tribromide (PBr₃). ϵ_1 -Carotene, C₄₀H₅₆, which contains two α -ionone rings, was synthesized by Karrer and Eugster.⁷³³ As was to be expected, it is completely inactive as a precursor of vitamin A.²

Kitol (C₄₀H₅₈(OH)₂) which is a dihydric alcohol of approximately twice the size of vitamin A, does not possess any vitamin A activity in the animal,^{540,734-736} although it may be converted to vitamin A by heat^{540,541} and by the use of radon alpha particles.⁷³⁷ Embree and Shantz⁵⁴⁰ calculated that one molecule of vitamin A was formed for each molecule of kitol destroyed, but Clough *et al.*⁵⁴¹ recorded a figure of only 0.67 to 0.75 molecule of vitamin A as arising for each molecule of kitol which disappears. Two types of kitol have been reported, one of which yields ordinary vitamin A (A₁) while the second type, found in fresh-water fishes such as the northern or Great Lakes pike (*Esox lucius*) gives vitamin A₂,⁵⁴⁰ it is therefore referred to as kitol₂. Hickman⁵⁴² is of the opinion that kitol is a detoxication product of vitamin A. Mammals, such as the whale, ordinarily are unable to tolerate as large quantities of vitamin A as can the fishes; therefore, by converting excess vitamin A to kitol, it is detoxified. Hickman⁵⁴² suggests that kitol is a *post*-vitamin A rather than a provitamin A.

⁷²⁹ H. J. Deuel, Jr., C. Johnston, E. Sumner, A. Polgár, W. A. Schroeder, and L. Zechmeister, *Arch. Biochem.*, **5**, 365-371 (1944).

⁷³⁰ A. Lund, *Beretn. Forsøgslab Kobenh.*, No. 180, 1-295 (1938); *Chem. Abst.*, **34**, 3327 (1940); cited by R. Braude, A. D. Foot, K. M. Henry, S. K. Kon, S. Y. Thompson, and T. H. Mead, *Biochem. J.*, **35**, 693-707 (1941), p. 693; *Chimie & industrie*, **42**, 569 (1939).

⁷³¹ R. Braude, A. S. Foot, K. M. Henry, S. K. Kon, S. Y. Thompson, and T. H. Mead, *Biochem. J.*, **35**, 693-707 (1941).

⁷³² H. v. Euler, P. Karrer, and A. Zubrys, *Helv. Chim. Acta*, **17**, 24-29 (1934).

⁷³³ P. Karrer and C. H. Eugster, *Helv. Chim. Acta*, **33**, 1433-1437 (1950).

⁷³⁴ H. Pritchard, H. Wilkinson, J. R. Edisbury, and R. A. Morton, *Biochem. J.*, **31**, 258-265 (1937).

⁷³⁵ H. Willstaedt and H. B. Jensen, *Nature*, **143**, 474 (1939).

⁷³⁶ H. Kringstad and J. Lee, *Tidsskr. Kjem. Bergvesen Met.*, **1**, 82-83 (1941).

⁷³⁷ D. Libermann and I. Grundland, *Compt. rend.*, **224**, 1033-1035 (1947).

Although kitol has been reported in the liver oils of some terrestrial animals such as sheep,⁵⁴⁰ it is not formed by the rat, even after excessive doses of vitamin A.⁷³⁸ For a further description of the occurrence and properties of kitol, the reader is referred to *The Lipids*, Vol. I, pages 710–713.

Only the carotenoids which have at least one intact β -ionone ring can serve as provitamins A. β -Carotene has repeatedly been found to possess the highest provitamin A activity of any of the carotenoids; its biopotency is approximately twice or more that of the other provitamins A. Euler, Karrer, and co-workers^{17,256,739} made a study of the biopotencies of β -carotene and of other carotenoids.

The biologic activity of α -carotene was found to be 53% of that of β -carotene when the evaluation was made on the basis of gain-in-weight over twenty-eight days.⁵⁷ Kuhn and co-workers⁷⁴⁰ had likewise arrived at approximately the same value in bioassays, also based upon weight gains. However, Johnson and Baumann⁶⁶² reported, on the basis of vitamin A storage in the liver, that all-*trans*- α -carotene had a biopotency of only 25% of that of all-*trans*- β -carotene. In later studies of Johnson, Swick, and Baumann,⁷⁴¹ α -carotene was found to have about one-half the effectiveness of β -carotene in the chick when the comparative biopotencies were based upon growth and prevention of vitamin A-deficiency symptoms; on the other hand, α -carotene was considerably less than one-half as potent as β -carotene, when the accumulation of vitamin A in the liver after the administration of fixed dosages of the test substances was the basis of comparison. In rats, α -carotene yielded lower stores of vitamin A than its growth-promoting power would lead one to expect.⁶⁶² The levels of bioactivity of the stereoisomeric forms of all-*trans*- α -carotene (see Table 4) would also indicate that the higher figure (53%) represents the biologic value of all-*trans*- α -carotene in rats.⁵⁷ The biopotency of echinenone⁷⁴² was found to be similar to the higher value for α -carotene, *i.e.*, 54%.

On the other hand, the all-*trans*- γ -carotenes obtained from several sources showed wide variations in calculated biologic⁷⁴³ activity. Although the biopotency of γ -carotene prepared from the Moluccan plant (*Gonocaryum pyrifforme*), described by Winterstein,⁷⁴⁴ was reported as approximately

⁷³⁸ E. M. Shantz, personal communication, Sept. 29, 1948.

⁷³⁹ B. v. Euler, H. v. Euler, and P. Karrer, *Helv. Chim. Acta*, **12**, 278–285 (1929).

⁷⁴⁰ R. Kuhn, H. Brockmann, A. Scheunert, and M. Schieblisch, *Z. physiol. Chem.*, **221**, 129–136 (1933).

⁷⁴¹ R. M. Johnson, R. W. Swick, and C. A. Baumann, *Arch. Biochem.*, **22**, 122–131 (1949).

⁷⁴² J. Ganguly, N. I. Krinsky, J. H. Pinecard, and H. J. Deuel, Jr., *Z. physiol. Chem.*, **295**, 61–66 (1953).

⁷⁴³ H. Brockmann and M. L. Tecklenburg, *Z. physiol. Chem.*, **221**, 117–128 (1933).

⁷⁴⁴ A. Winterstein, *Z. physiol. Chem.*, **219**, 249–252 (1933).

one-half of that of β -carotene,⁷⁴⁵ the value as given by the more exact methods of bioassay⁷³⁴ were recorded as 28% of that of β -carotene.^{729,746} This figure is almost identical with that (26%) obtained for the γ -carotene obtained from the berries of the narrow-leaf firethorn (*Pyracantha angustifolia* Schneid.).⁷⁴⁶ However, when all-*trans*- γ -carotene was prepared by stereoisomerization^{58,747} of pro- γ -carotene, its biopotency was found to be 42% of that of β -carotene, both in the rat⁵⁸ and in the chicken.⁷⁴⁷ A similar high biopotency (47%) was recorded for all-*trans*- γ -carotene prepared from tomato paste (see Table 4).⁵⁸ The cause of the variations in the provitamin A activity of the all-*trans*- γ -carotene obtained from the several sources is not certain.

Cryptoxanthin appears to have a biologic activity slightly higher than 50% in the rat tests (55%, 57%, 60%),⁷⁴⁸⁻⁷⁵⁰ although it is possible that these figures may be within experimental error of the 50% value. With⁷⁵¹ suggested that a qualitative difference exists between the chick and the rat in the utilization of cryptoxanthin as a provitamin A; he actually concluded that this carotenol was more potent as a source of vitamin A in the chick than was β -carotene. A variation of this nature in the metabolism of a carotenol such as cryptoxanthin might be expected in the chick since, unlike the rat, the fowls are able to store relatively large amounts of the dihydroxycarotenes such as lutein and zeaxanthin in their fat depots and elsewhere. However, Johnson, Swick and Baumann⁷⁴¹ failed to confirm the observation of With. Actually these latter investigators showed that the carotenol was inferior to β -carotene as a source of vitamin A in the chick, although the vitamin A storage in the liver following the administration of carotenol was only slightly less than that derived from a like quantity of β -carotene. Greenberg *et al.*⁷⁵⁰ reported that all-*trans*-cryptoxanthin exhibited a provitamin A biopotency of only 55% of that of all-*trans*- β -carotene in the chick, using growth as the criterion for the evaluation. It would thus appear that cryptoxanthin exhibits a similar biopotency in the

⁷⁴⁵ R. Kuhn and H. Brockmann, *Klin. Wochschr.*, 12, 972-973 (1933).

⁷⁴⁶ H. J. Deuel, Jr., C. Hendrick, E. Straub, A. Sandoval, J. H. Pinckard, and L. Zechmeister, *Arch. Biochem.*, 14, 97-103 (1947).

⁷⁴⁷ S. M. Greenberg, C. E. Calbert, J. H. Pinckard, H. J. Deuel, Jr., and L. Zechmeister, *Arch. Biochem.*, 24, 31-39 (1949).

⁷⁴⁸ H. J. Deuel, Jr., E. R. Meserve, C. H. Johnston, A. Polgár, and L. Zechmeister, *Arch. Biochem.*, 7, 447-450 (1945).

⁷⁴⁹ H. J. Deuel, Jr., S. M. Greenberg, E. Straub, T. Fukui, A. Chatterjee, and L. Zechmeister, *Arch. Biochem.*, 23, 239-241 (1949).

⁷⁵⁰ S. M. Greenberg, A. Chatterjee, C. E. Calbert, H. J. Deuel, Jr., and L. Zechmeister, *Arch. Biochem.*, 25, 61-65 (1950).

⁷⁵¹ T. K. With, *Z. Vitaminforsch.*, 17, 88-97 (1946).

rat and in the chick, and that this potency is of a lower order than that of β -carotene. Pro- γ -carotene, which is a naturally occurring polycyclic compound, is considered in the following main section (see page 542).

Other naturally-occurring provitamins A about which less is known include citroxanthin (or mutatochrome),²⁴ myxoxanthin,²⁴ aphanin, isolated by Tischer⁴³¹ from the freshwater blue-green alga, or "water-bloom" (*Aphanizomenon flosaquae*) (potency about 50% of that of β -carotene),⁷⁵² aphanacene (potency 25% that of β -carotene),⁷⁵² torularhoden ($C_{37}H_{48}O_2$),²⁴ and α -carotene epoxide.⁷⁵³ Mention should also be made of luteochrome, which Karrer and co-workers⁷⁵³ and Karrer and Jucker⁷⁵⁴ isolated and which, according to Karrer *et al.*,⁷⁵³ possesses full vitamin A activity when fed in daily doses of 18 μ g.

Synthetic 16,16'-homo- β -carotene, $C_{42}H_{58}$, which has two additional carbons centrally located on the aliphatic chain, has been reported to have 20% of the biologic activity of β -carotene.⁷⁵⁵ Two additional members of this series, decapreno- β -carotene, $C_{50}H_{68}$,^{756,757} and dodecapreno- β -carotene, $C_{60}H_{80}$,⁷⁵⁷ have been synthesized, but their biologic activity has not been reported. One would expect them to be active provitamins at a level somewhat below that of 16,16'-homo- β -carotene. The synthesis and properties of the homologues of β -carotene have been reviewed by Inhoffen and Siemer.⁷⁵⁸

(a) *The Extent of Transformation of β -Carotene to Vitamin A.* β -Carotene contains two intact β -ionone rings, while other carotenoids possessing provitamin A activity (α -carotene, γ -carotene, cryptoxanthin, echinone) have only a single intact β -ionone structure. It is known that the β -ionone structure must be unaltered if the substance is to be active as a source of vitamin A. Since β -carotene is at least twice as potent as a source of vitamin A as are the other carotenoids which possess provitamin A activity, it is only natural to ascribe this to the fact that it contains two β -ionone rings while the other active provitamins A contain only one such structure. This correlation is obviously a perfectly sound one.

The mechanism by which carotene is transformed into vitamin A is not yet completely elucidated. One theory which has been proposed, but

⁷⁵² A. Scheunert and K. H. Wagner, *Z. physiol. Chem.*, **260**, 272-275 (1939).

⁷⁵³ P. Karrer, J. Rutschmann, and K. Steinlin, *Helv. Chim. Acta*, **28**, 1146-1156 (1945).

⁷⁵⁴ P. Karrer and E. Jucker, *Helv. Chim. Acta*, **28**, 427-436 (1945).

⁷⁵⁵ H. J. Deuel, Jr., H. H. Inhoffen, J. Ganguly, L. Wallcave, and L. Zechmeister, *Arch. Biochem. Biophys.*, **40**, 352-357 (1952).

⁷⁵⁶ P. Karrer and C. H. Eugster, *Helv. Chim. Acta*, **34**, 28-33 (1951).

⁷⁵⁷ P. Karrer and C. H. Eugster, *Helv. Chim. Acta*, **34**, 1805-1814 (1951).

⁷⁵⁸ H. H. Inhoffen and H. Siemer, *Fortschr. Chem. org. Naturstoffe*, **9**, 1-40 (1952).

which is not supported by any convincing experimental data, is that the conversion of β -carotene to vitamin A involves a central fission of the molecule (between C 15 and C 15'), with the resultant formation of two molecules of vitamin A. In fact, one might also postulate that, from spatial considerations, the position 15:15' should be the favored spot for the action of the enzyme, carotenase, since it is the only position in the carotene molecule where a double bond occurs in which the two carbon atoms on each side of it are free from methyl groups. If the enzyme molecule must be adjacent to the substrate, there would be a better opportunity for close approximation here than at any other position on the molecule.

A second theory is that β -carotene is oxidized in a random manner. Since this type of oxidation should occur only relatively seldom at the central position (15:15'), but would generally take place on one side or the other, one portion of the ruptured molecule would have a shorter aliphatic chain than occurs in vitamin A, and hence would be excluded as a potential source of this vitamin. The second fragment obtained under such conditions would have a longer side-chain than occurs on the vitamin A molecule. The longer chain could be shortened by oxidation to yield vitamin A. In the case of the provitamins A other than β -carotene, the single intact β -ionone fragment would be left with an aliphatic chain too short for its conversion into vitamin A, 50% of the time. If this reasoning is correct, then only one vitamin A molecule would arise from the metabolism of each β -carotene molecule, while an average of two provitamin A molecules would be required in order to obtain one molecule of vitamin A. Glover and Redfearn⁷⁵⁹ postulated that the transformation of β -carotene into vitamin A *in vivo* involves terminal oxidation rather than central fission. Painter and Glover⁷⁶⁰ oxidized lycopene by the osmium tetroxide method of Wendler *et al.*,²⁴² and demonstrated the formation of apo-lycopenals having 9, 7, and 5 ethylenic bonds with a terminal aldehyde group. The compounds having 9 and 7 ethylenic bonds could have arisen only by terminal oxidation or by oxidation at a site other than the central double bond.

The experimental data which indicate that β -carotene is twice as potent as a provitamin A as are the carotenoids in which one of the β -ionone groups is modified would support either of the above theories. Considerable data are available in the literature indicating the superiority of vitamin A over an equal quantity of β -carotene in satisfying the vitamin A requirement. A relatively close correspondence in biologic activity can be obtained from

⁷⁵⁹ J. Glover and E. R. Redfearn, *Biochem. J.*, **58**, xv-xvi (1954).

⁷⁶⁰ R. H. Painter and J. Glover, *Biochem. J.*, **60**, xvi (1955).

one I.U. of β -carotene (which by definition weighs 0.6 $\mu\text{g.}$), and from one I.U. (or U.S.P. unit) of vitamin A which weighs 0.3 $\mu\text{g.}$ ^{761,762} Mattson,⁷⁶³ in his review of the vitamin A requirements of various species of animals, stated that the minimum daily requirement, to support growth and to prevent symptoms of deficiency in mammals, is 40 I.U. (24 $\mu\text{g.}$) per kg. body weight when given as β -carotene, or 20 I.U. when administered as vitamin A. Another typical result showing a corresponding relationship between β -carotene and vitamin A is that reported by Braude *et al.*,⁷³¹ which indicated that pigs require 100 I.U. of vitamin A per lb. of body weight, while the corresponding requirement in terms of β -carotene is 300 I.U. According to Gurcay *et al.*,²⁰⁴ the vitamin A requirement of turkey poults, to assure normal growth, was one-half as great when black-cod liver oil was used as a source of vitamin A, and one-fourth as much when crystalline vitamin A acetate was employed, as when crystalline β -carotene was used.

On the other hand, Koehn⁷⁶⁴ reported that β -carotene is quantitatively converted to vitamin A in the rat provided that adequate quantities of α -tocopherol are available. Moreover, Burns, Hauge, and Quackenbush³³ reported that there was no difference in the biologic response of rats to 1.0 $\mu\text{g.}$ of vitamin A and to a similar amount of β -carotene, respectively, when 1.0 mg. of tocopherol was given per day. However, the possibility exists that the levels of β -carotene and of vitamin A used in the tests of Koehn,⁷⁶⁴ and possibly also in the experiments of Burns *et al.*,³³ were high enough to induce a nearly maximal growth response, and hence were in the non-critical area of the growth curve. According to Harris,⁷⁶² this would render it difficult to distinguish between real differences in potency. The author is also of the opinion that the dosages are too high for a critical assay, and believes that the second theory best explains the quantitative relationships in the transformation of β -carotene, and of other carotenoids with provitamin A activity, to vitamin A. It is possible that an *in vitro* reaction might result in a greater efficiency in the transformation of β -carotene to vitamin A, although Goss and McFarlane⁷⁶⁵ obtained only 30 to 40% of the theoretical yield by oxidation of β -carotene with osmium tetroxide.

b. *Cis-Trans* Isomerization of Carotenoids, and Provitamin A Activity. Although the phenomenon of geometric isomerism has been recognized for

⁷⁶¹ J. R. Foy and K. Morgareidge, *Anal. Chem.*, **20**, 304-307 (1948).

⁷⁶² P. L. Harris, *Ann. Rev. Biochem.*, **18**, 391-434 (1949).

⁷⁶³ F. H. Mattson, *Requirements of Animals*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Acad. Press, New York, 1954, pp. 163-170.

⁷⁶⁴ C. J. Koehn, *Arch. Biochem.*, **17**, 337-344 (1948).

⁷⁶⁵ G. C. L. Goss and W. D. McFarlane, *Science*, **106**, 375 (1947).

many years as a property of organic compounds which possess a double bond, the realization that this behavior also applies to carotenoids dates back only to 1935, when Gillam and El Ridi⁷⁶⁶ reported geometric isomerism in the C₄₀ carotenoids. These authors were able to separate the several geometric isomers on an alumina column. "Pseudo- α -carotene," one of the isomers of α -carotene, was shown to have distinct properties differing from those of the all-*trans* isomer. Gillam and co-workers^{767,768} later demonstrated the reversibility of the *cis-trans* conversion.

Although the British workers attributed this isomeric change to the action of the adsorbent in the chromatographic column on the carotenoid, the subsequent investigations of Zechmeister and his colleagues proved that the reactions were spontaneous, and independent of the process of adsorption. It was proved that capsanthin solutions undergo these spontaneous and reversible changes.⁷⁶⁹ Zechmeister and Tuzson^{770,771} later described similar stereoisomeric changes in the case of β -carotene, lycopene, and cryptoxanthin which were not related to the adsorption process.

With few exceptions, the natural forms of the carotenoids are the all-*trans* compounds in which every double bond capable of *cis* $\rightarrow$ *trans* rearrangement is in the *trans* position. Pro- γ -carotene and prolycopene represent two naturally occurring carotenoids which are polycis compounds. Not all double bonds are capable of undergoing geometric isomerism, but only those in which no methyl group is attached to either of the carbon atoms adjacent to the atoms composing the double bond system. The nomenclature employed by Zechmeister⁷⁷² for the several isomers is based upon relative ease of adsorbability on the chromatographic column, as compared with the all-*trans* form. Isomers with a low degree of adsorbability, and which consequently appear below the all-*trans* form on the chromatographic column, are called neo-A, neo-B, neo-C, etc., while those with a higher adsorbability than the all-*trans* isomer, and which hence appear above the all-*trans* isomer, are called neo-T, U, V, etc. For a further discussion of *cis-trans* isomerism and stereochemistry of the carotenoids the reader is referred to *The Lipids*, Vol. I, pages 621 to 666, to the review of Zechmeister,⁷⁷² and to that of Zechmeister and Polgár.⁷⁷³

⁷⁶⁶ A. E. Gillam and M. S. El Ridi, *Nature*, **136**, 914-915 (1935).

⁷⁶⁷ A. E. Gillam and M. S. El Ridi, *Biochem. J.*, **30**, 1735-1742 (1936).

⁷⁶⁸ A. E. Gillam, M. S. El Ridi, and S. K. Kon, *Biochem. J.*, **31**, 1605-1610 (1937).

⁷⁶⁹ L. Zechmeister and L. v. Chohnoky, *Ann.*, **530**, 291-300 (1937).

⁷⁷⁰ L. Zechmeister and P. Tuzson, *Nature*, **141**, 249-250 (1938).

⁷⁷¹ L. Zechmeister and P. Tuzson, *Biochem. J.*, **32**, 1305-1311 (1938).

⁷⁷² L. Zechmeister, *Chem. Revs.*, **34**, 267-344 (1944).

⁷⁷³ L. Zechmeister and A. Polgár, *J. Am. Chem. Soc.*, **66**, 137-144 (1944).

The most complete study of the provitamin A activity of the stereoisomers of the carotenoids was made by Zechmeister, in collaboration with the author. Table 4 summarizes the results of these tests.

With the exception of pro- γ -carotene, all *cis*-isomers of the provitamins A have a lower biopotency than do the naturally occurring all-*trans* forms. Confirmatory results in support of this statement have been reported by Bahl, Sadana, and Ahmad.⁷⁷⁴ In the case of the γ -carotene set, the biopotency of the poly-*cis* pro- γ -carotene is 50% higher than that of

TABLE 4
THE RELATIVE BIOPOTENCIES OF THE VARIOUS STEREOISOMERIC
FORMS OF PROVITAMINS A, AS DETERMINED BY
BIOASSAY IN THE RAT AND CHICK

Current name	Configuration	Biopotency	
		In % of that of the all- <i>trans</i> form of the set	In % of the potency of all- <i>trans</i> - β -carotene
α -Carotene set			
α -Carotene	All- <i>trans</i>	100	53 ^a
Neo- α -carotene U	3- or 9-Mono- <i>cis</i>	25	13 ^a
Neo- α -carotene B	3,6- or 3,7-Di- <i>cis</i>	30	16 ^b
β -Carotene set			
β -Carotene	All- <i>trans</i>	100	100 ^a
Neo- β -carotene U	3-Mono- <i>cis</i>	38	38 ^{a,c}
Neo- β -carotene B	3,6- or 3,7-Di- <i>cis</i>	53	53 ^b
	15,15'-Mono- <i>cis</i>	50	50 ^d
γ -Carotene set			
In rats			
γ -Carotene (<i>Ex Mimulus longiflorus</i> , monkey-flower)	All- <i>trans</i>	100	28 ^{a,e}
γ -Carotene (<i>Ex Pyracantha angustifolia</i> Schneid., narrow-leaf firethorn)	All- <i>trans</i>	100	26 ^f
γ -Carotene (<i>Ex</i> pro- γ -carotene by iodine catalysis)	All- <i>trans</i>	—	42 ^g
γ -Carotene (<i>Ex</i> tomato paste)	All- <i>trans</i>	—	47 ^g
Neo- γ -carotene P	3-Mono- <i>cis</i>	70	19 ^f
Mixed neo- γ -carotenes	—	60	16 ^f
Pro- γ -carotene	Poly- <i>cis</i> (penta- <i>cis</i>)	157	44 ^{a,e,f}
Pro- γ -carotene	Poly- <i>cis</i> (penta- <i>cis</i>)	161	42 ^g

Table continued

⁷⁷⁴ A. N. Bahl, J. C. Sadana, and B. Ahmad, *J. Sci. Ind. Research (India)*, 7 B, No. 11, 169-171 (1950).

TABLE 4 (Continued)

Current name	Configuration	Biopotency	
		In % of that of the all- <i>trans</i> form of the set	In % of the potency of all- <i>trans</i> - β -carotene
In chicks			
γ -Carotene (<i>Ex pro</i> - γ -carotene)	All- <i>trans</i>	100	42 ^h
Pro- γ -carotene	Poly- <i>cis</i> (penta- <i>cis</i>)	189	51 ^h
Cryptoxanthin set			
In rats			
Cryptoxanthin	All- <i>trans</i>	100	57 ⁱ
Neocryptoxanthin U	3 Mono- <i>cis</i>	45	27 ^j
Neocryptoxanthin A	Central mono- <i>cis</i>	71	42 ^k
Homo- β -carotene set			
Homo- β -carotene	All- <i>trans</i> (16,16')	100	20 ^l
Di- <i>cis</i> -16,16'-homo- β -carotene	Central di- <i>cis</i>	100	20 ^l

^a H. J. Deuel, Jr., E. Sumner, C. Johnston, A. Polgár, and L. Zechmeister, *Arch. Biochem.*, **6**, 157-161 (1945).

^b H. J. Deuel, Jr., C. Johnston, E. R. Meserve, A. Polgár, and L. Zechmeister, *Arch. Biochem.*, **7**, 247-255 (1945).

^c H. J. Deuel, Jr., C. Johnston, E. Sumner, A. Polgár, and L. Zechmeister, *Arch. Biochem.*, **5**, 107-114 (1944).

^d L. Zechmeister, H. J. Deuel, Jr., H. H. Inhoffen, J. Leemann, S. M. Greenberg, and J. Ganguly, *Arch. Biochem. Biophys.*, **36**, 80-88 (1952).

^e H. J. Deuel, Jr., C. Johnston, E. Sumner, A. Polgár, W. A. Schroeder, and L. Zechmeister, *Arch. Biochem.*, **5**, 365-371 (1944).

^f H. J. Deuel, Jr., C. Hendrick, E. Straub, A. Sandoval, J. H. Pinckard, and L. Zechmeister, *Arch. Biochem.*, **14**, 97-103 (1947).

^g L. Zechmeister, J. H. Pinckard, S. M. Greenberg, E. Straub, T. Fukui, and H. J. Deuel, Jr., *Arch. Biochem.*, **23**, 242-245 (1949).

^h S. M. Greenberg, C. E. Calbert, J. H. Pinckard, H. J. Deuel, Jr., and L. Zechmeister, *Arch. Biochem.*, **24**, 31-39 (1949).

ⁱ H. J. Deuel, Jr., E. R. Meserve, C. H. Johnston, A. Polgár, and L. Zechmeister, *Arch. Biochem.*, **7**, 447-450 (1945).

^j H. J. Deuel, Jr., S. M. Greenberg, E. Straub, T. Fukui, A. Chatterjee, and L. Zechmeister, *Arch. Biochem.*, **23**, 239-241 (1949).

^k H. J. Deuel, Jr., E. R. Meserve, A. Sandoval, and L. Zechmeister, *Arch. Biochem.*, **10**, 491-496 (1946).

^l H. J. Deuel, Jr., H. H. Inhoffen, J. Ganguly, L. Wallcave, and L. Zechmeister, *Arch. Biochem. Biophys.*, **40**, 352-357 (1952).

the all-*trans* compound prepared from the monkey-flower (*Mimulus longiflorus* Grant) or from the berries of the narrow-leaf firethorn (*Pyracantha angustifolia* Schneid.), but is identical with that of the all-*trans* isomer prepared from pro- γ -carotene by iodine catalysis, as well as with that of the sample prepared from tomato paste. It is just possible that the first two samples of the all-*trans* compound described above, which have the low biologic activity, differ chemically from the all-*trans* form prepared synthetically. On the basis of the storage of vitamin A in the liver of vitamin A-

depleted rats after the test substance was fed in doses of 10 to 80 μ g. per day, Johnson and Baumann⁶⁶¹ reported the following biopotencies of several stereoisomeric forms (all-*trans*- β -carotene = 100%): neo- β -carotene U, 33; neo- β -carotene B, 48; all-*trans*- α -carotene, 25. In the case of vitamin A-depleted chicks, Johnson, Swick, and Baumann⁷⁴¹ reported that neo- β -carotene B is somewhat more than one-half as active as all-*trans*- β -carotene when the test is based upon the level of vitamin A in the liver after the feeding of test substances. It is of interest that a synthetic *cis* isomer, namely 15,15'-monocis- β -carotene (central monocis- β -carotene), likewise has about 50% of the biopotency of its all-*trans* isomer, as determined by growth tests, when it is fed to depleted rats and to chickens at a low daily level.⁷⁷⁵ This isomer cannot be efficiently utilized when fed in large amounts, as the expected higher growth responses are not observed under these conditions.

It has not been decided whether the stereoisomeric forms of the provitamins A are themselves directly active as a source of vitamin A, or whether their biopotency is in proportion to the extent to which they are reconverted to the all-*trans* form of the isomer. There is some evidence that a biologic transformation from one stereoisomeric form of a carotenoid to another is possible. Thus, Kemmerer and Fraps⁷⁷⁶ observed that neo- β -carotene U was isomerized to all-*trans*- β -carotene in rats. Deuel *et al.*¹⁴⁷ observed the presence of considerable amounts of all-*trans*- γ -carotene, as well as of neo- γ -carotenes and of a poly-*cis*- γ -carotene, not only in the feces but also in the carotenoids obtained from the tissues and gut washings of chicks after pro- γ -carotene had been fed. Moreover, a similar isomerization occurred in the case of mature hens when 15 mg. of polycopene was administered over a period of three days. In the latter tests, the following pigments (in mg.) were recovered from the feces: unchanged polycopene, 0.445; all-*trans*-lycopene, 0.712; neolycopenes, 0.562; polycis-lycopenes, 0.611; unidentified, 0.050; total recovered, 2.380. A similar distribution of isomerization products was noted in the material recovered from the tissues and gut washings. When 15 mg. of lycopenes were fed during three days to carotenoid-depleted hens, neolycopenes but no polycis-lycopenes were isolated from the feces. In the case of the synthetically-prepared 15,15'-monocis- β -carotene, Zechmeister and co-workers⁷⁷⁵ demonstrated that a considerable transformation of this monocis isomer to the all-*trans* form had taken place in the gastrointestinal tract, as determined by the composition of the fecal carotenoids.

⁷⁷⁵ L. Zechmeister, H. J. Deuel, Jr., H. H. Inhoffen, J. Leeman, S. M. Greenberg, and J. Ganguly, *Arch. Biochem.*, **36**, 80-88 (1952).

⁷⁷⁶ A. R. Kemmerer and G. S. Fraps, *J. Biol. Chem.*, **161**, 305-309 (1945).

(2) *The Transformation to Products
Other Than Vitamin A*

Little is known about transformations of carotenoids other than their conversion to vitamin A. Goodwin⁵¹² indicates that fishes are able to oxidize alimentary carotenoids to highly oxygenated pigments. When these pigments have the required structural characteristics, they may be converted into vitamin A.

It would seem probable that changes similar to those involved in the formation of vitamin A from the carotenoids classed as provitamins A would also occur in the non-provitamin A series of carotenoids, except that the product derived from the latter group would be devoid of vitamin A activity. An indication that such a change does occur in the case of this latter group is given by the experiments of Painter and Glover,⁷⁶⁰ who detected traces of apo-15-lycopenal in the intestine but not in the livers of rats which had previously received small doses of lycopene or of apo-12'-lycopenal. Although lycopene could be demonstrated in the livers of rats to the extent of 15% of the amount fed, no apo-15-lycopenal could be detected in the livers of rats previously fed lycopene or apo-15-lycopenal. It is also possible that the ionone derivatives, isolated by Prelog *et al.*⁷⁷⁷ from the urine of the pregnant mare, may be produced by the degradation of alimentary xanthophylls.

One interesting change, occurring in insects, is the transformation of β -carotene to a non-provitamin A. Thus, Goodwin⁵⁰² reported that, during the development of the eggs of the African migratory locust (*Locusta migratoria migratorioides*) and of the North African desert locust (*Schistocerca gregaria*), β -carotene begins to disappear on the sixth to seventh day, simultaneously with the appearance of astaxanthin. While β -carotene is the only pigment present in the newly fertilized eggs, astaxanthin comprises 70% of the total carotenoids present after their incubation.

(3) *Vitamin A Analogues and Isomers*

a. The Relative Biopotencies of Vitamin A₁ and Vitamin A₂. The first correct characterization of vitamin A₂ was made by Wald,⁷⁷⁸ who proved that it was a component of the visual system of freshwater fishes. Wald was the first to demonstrate that it was a vitamin. He recognized it as part of the porphyropsin system.

⁷⁷⁷ V. Prelog, J. Führer, R. Hagenbach, and R. Schneider, *Helv. Chim. Acta*, **31**, 1799-1814 (1948).

⁷⁷⁸ G. Wald, *Nature*, **139**, 1017-1018 (1937).

Lederer *et al.*⁷⁷⁹ recognized a "second vitamin A," and Edisbury and co-workers⁵³³ introduced the term vitamin A₂, based upon the earlier observations of Wald.⁷⁷⁸ Vitamin A₂ was found to have a wide distribution in the livers of fresh-water fishes (see page 498). There has been considerable difference of opinion as to its biologic activity. Although Gillam and associates⁷⁸⁰ first reported that vitamin A₂ possessed growth-promoting powers, this was denied by other investigators,⁷⁸¹ and was then later reaffirmed.^{782,783}

Vitamin A₂ has been synthesized as 3'-dehydrovitamin A²²⁷ (the Milas numbering system,⁷⁸⁴ see *The Lipids*, Vol. I, p. 708). An indication of the fact that the synthetic compound is vitamin A₂ consists in the absorption spectrum and antimony trichloride reaction, which correspond closely with those reported by Shantz⁷⁸⁵ for natural vitamin A₂. Moreover, the biopotencies of the natural and synthetic vitamin A₂ are in the same range. Shantz and Brinkman⁷⁸⁶ reported that the biologic activity of pure vitamin A₂ obtained from natural sources is equivalent to 1,300,000 I.U./g. as compared with a value of 3,300,000 I.U./g. for crystalline vitamin A₁, based upon the new U.S.P. standard (No. 3).^{787,788} Thus, natural vitamin A₂ has a biopotency of 40% of that of vitamin A₁,⁷⁸⁶ while synthetic vitamin A₂ has been found to have a bioactivity equivalent to only 30% of vitamin A₁.²²⁷ These facts would indicate that vitamin A₂ is 3'-dehydrovitamin A₁.⁷⁸⁹ This opinion is shared by Cama *et al.*,⁷⁹⁰ although it is contrary to the earlier views of Karrer, Geiger, and Bretscher⁷⁸¹ and of Shantz.⁷⁸⁵

When vitamin A₂ is given to mammals which ordinarily store vitamin A₁, both forms appear in the tissues. Thus, Shantz and co-workers⁷⁹¹ found

⁷⁷⁹ E. Lederer, V. A. Rosanova, A. E. Gillam, and I. M. Heilbron, *Nature*, **140**, 233 (1937).

⁷⁸⁰ A. E. Gillam, I. M. Heilbron, W. E. Jones, E. Lederer, and V. Rosanova, *Biochem. J.*, **32**, 405-416 (1938).

⁷⁸¹ P. Karrer, A. Geiger, and E. Bretscher, *Helv. Chim. Acta*, **24**, 161 E-172 E (1941).

⁷⁸² J. L. Jensen, E. M. Shantz, N. D. Embree, J. D. Cawley, and P. L. Harris, *J. Biol. Chem.*, **149**, 473-477 (1943).

⁷⁸³ P. Karrer and E. Bretscher, *Helv. Chim. Acta*, **26**, 1758-1778 (1943).

⁷⁸⁴ N. A. Milas, *Vitamins and Hormones*, **5**, 1-38 (1947).

⁷⁸⁵ E. M. Shantz, *Science*, **108**, 417-419 (1948).

⁷⁸⁶ E. M. Shantz and J. H. Brinkman, *J. Biol. Chem.*, **183**, 467-471 (1950).

⁷⁸⁷ J. G. Baxter and C. D. Robeson, *J. Am. Chem. Soc.*, **64**, 2411-2416 (1942).

⁷⁸⁸ J. D. Cawley, C. D. Robeson, L. Weisler, E. M. Shantz, N. D. Embree, and J. G. Baxter, *Science*, **107**, 346 (1948).

⁷⁸⁹ K. R. Farrar, J. C. Hamlet, H. B. Henbest, and E. R. H. Jones, *Chemistry & Industry*, **1951**, 49-50.

⁷⁹⁰ H. R. Cama, P. D. Dalvi, R. A. Morton, and M. K. Salah, *Biochem. J.*, **52**, 542-547 (1952).

⁷⁹¹ E. M. Shantz, N. D. Embree, H. C. Hodge, and J. H. Wills, Jr., *J. Biol. Chem.*, **163**, 455-464 (1946).

that vitamin A₂ administered to rats appeared unchanged in the livers, blood, and retinae of the animals, although the vitamin A₁ which had been present in the blood was still tenaciously held. Vitamin A₂ was found to have the capacity to assume all the functions in the rat usually mediated by vitamin A₁. Thus, rats receiving only vitamin A₂ grew normally, lived longer, and their xerophthalmia was cured. Their coats appeared glossy, and the rats became lively, ate well, and were in excellent condition. Females receiving their vitamin A only in the form of vitamin A₂ were mated, and one-half became pregnant and produced litters. However, only one female was able to raise her pup. In general, male rats seemed to respond to vitamin A₂ better than did female rats, as far as growth was concerned.

Morcos and Salah⁷⁹² observed that when vitamin A₁ was given to the Nile fishes, the air-breathing edible African catfish (*Clarias lazera*), and the boliti, a Nile food-fish (*Tilapia nilotica*), in which vitamin A₂ is the only form ordinarily stored, administered vitamin A₁ was present in the liver in unchanged form. It is apparent that the two forms of vitamin A are not readily interconverted in the animal.

The precursors of vitamin A₂ are still unknown. Collins and associates⁵⁵¹ conclude that vitamin A₂ may result from preformed vitamin A₂ in the diet, from the conversion of β -carotene or of other provitamins A to vitamin A₂, or from a specific provitamin A₂. This might be an animal carotenoid such as astaxanthin. However, there is not direct evidence supporting the last suggestion. Presumably vitamin A₂ may exist in stereoisomeric forms comparable to those of vitamin A₁. These have not as yet been prepared.

b. *Cis-Trans* Forms of Vitamin A and Their Biopotencies. Vitamin A₁ can exist in four isomeric forms. Of the five double bonds in the vitamin A molecule, only the four present in the side-chain can contribute to stereoisomerism. According to the theoretical considerations of Pauling,⁷⁹³ and of Zechmeister,^{726,772} steric hindrance should prevent double bonds 2 and 4 from assuming a *cis* position; this would leave only bonds 3 and 5 free to change from the *trans* to the *cis* configuration. On this basis, the following four vitamin A stereoisomers would be possible:⁷⁹ (1) all-*trans*-Vitamin A, (2) 3-*cis*-vitamin A, (3) 5-*cis*-vitamin A, (4) 3,5-di-*cis*-vitamin A. For the formulas of these compounds the reader is referred to *The Lipids*, Vol. I, p. 696.

In addition to all-*trans*-vitamin A, neovitamin A (2,3,4-tri-*trans*-5-*cis*

⁷⁹² S. R. Morcos and M. K. Salah, *Nature*, 167, 117-118 (1951).

⁷⁹³ L. Pauling, *Fortschr. Chem. org. Naturstoffe*, 3, 203-235 (1939).

vitamin A) has been shown to occur naturally. Robeson and Baxter⁷⁹⁴ isolated it from the "non-crystallizable portion" of natural vitamin A samples. According to these investigators, approximately 35% of the vitamin A in the liver oil of cod (*Gadus morrhua*), dogfish (*Scyllium canicula*), halibut (*Hippoglossus hippoglossus*), and California jewfish or black bass (*Stereolepis gigas*) is neovitamin A. This agrees with the results of Hayes and Petitpierre,⁷⁹⁵ who reported that neovitamin A constitutes 11 to 39% of the total vitamin A in fish liver oils. Neovitamin A is also present in the synthetic vitamin A.⁷⁹⁴

Harris and co-workers⁷⁹⁶ recorded the biopotency of neovitamin A alcohol as $87.1 \pm 3.25\%$ of that of all-*trans* vitamin A alcohol, while the neovitamin A acetate was found to possess $83.2 \pm 4.28\%$ of the bioactivity of ordinary vitamin A acetate. Both of the above assays are based upon gain-in-weight by the usual U.S.P. method. On the other hand, when the comparison of vitamin A potency was made by the liver storage procedure, the biopotency of neovitamin A was estimated to be only 75.6% of that of the all-*trans* vitamin A.⁷⁹⁶ Harris and co-workers⁷⁹⁶ assigned a potency of 2,690,000 U.S.P. units/g. to neovitamin A.

Rats are able to transform neovitamin A to the all-*trans* form, and *vice versa*. According to Harris *et al.*,⁷⁹⁶ when either isomer is fed in the pure state to rats, a mixture of 88% all-*trans* vitamin A and 12% neovitamin A is stored in the livers. This proportion varies with different species, since it has been found to be 65:35 in fish livers. Robeson and Baxter⁷⁹⁴ demonstrated that the interconversion of all-*trans* vitamin A and neovitamin A could be effected in either direction by treating their anthraquinone- β -carboxylate esters with traces of iodine in benzene solution. Neovitamin A ester was converted to vitamin A₁ ester to the extent of 70% in two hours at 25°C., while the reverse change was accomplished under similar conditions to the extent of only 30%.

It is possible that other stereoisomeric forms of vitamin A are physiologically important. While *natural* vitamin A concentrates can be converted *in vitro* to rhodopsin when opsin and two enzymes, liver alcohol dehydrogenase and cozymase, are present, the reaction fails to take place when the natural vitamin A is replaced by *synthetic* vitamin A and neovitamin A.⁷⁹⁷ On the other hand, when synthetic vitamin A is exposed to light in the presence of traces of iodine, rhodopsin is formed. This is be-

⁷⁹⁴ C. D. Robeson and J. G. Baxter, *J. Am. Chem. Soc.*, **69**, 136-141 (1947).

⁷⁹⁵ E. Hayes and M. Petitpierre, *J. Pharm. and Pharmacol.*, **4**, 879-885 (1952).

⁷⁹⁶ P. L. Harris, S. R. Ames, and J. H. Brinkman, *J. Am. Chem. Soc.*, **73**, 1252-1254 (1951).

⁷⁹⁷ R. Hubbard and G. Wald, *Science*, **115**, 60-63 (1952).

lieved to be presumptive evidence that stereoisomeric forms of vitamin A other than all-*trans* vitamin A and neovitamin A function in the animal organism.

c. Other Compounds Active as Sources of Vitamin A. (a) *Vitamin A Acid*. The synthesis of this compound, in which the primary alcohol group of vitamin A has been oxidized to a carboxyl group, was claimed by Heilbron and co-workers⁷⁹⁸ in 1936, although these findings have been questioned.⁷⁸⁴ Its biologic potency is in the same range as that of vitamin A. It required 4 μ g. to exert the same potency as 1 I.U. of vitamin A when the acid was given in peanut oil. However Milas⁷⁸⁴ reported that its biopotency was greatly increased when it was injected subcutaneously in aqueous solution as the sodium salt; 0.6 μ g. of the vitamin A acid was equivalent to one I.U. under these conditions. Bharucha and Weedon⁷⁹⁹ prepared *o*-tolyl and mesityl analogues of vitamin A acid; the latter compound had a slight vitamin A-like action, while the former derivative was found to be inactive. A 4-carboxy derivative of vitamin A acid has been found to have a slight biopotency.⁸⁰⁰ Other data on vitamin A acid are contained in the papers of Arens and van Dorp,⁸⁰¹ Karrer *et al.*,⁸⁰² and Heilbron and associates.^{803,804}

(b) *Vitamin A Aldehydes*. Several of the vitamin A aldehydes are natural products usually readily convertible to vitamin A. The all-*trans* form was first isolated from retinas by Wald⁸⁰⁵ in 1935; Morton,⁸⁰⁶ nine years later, suggested that retinene is vitamin A aldehyde, which assumption he later confirmed.⁸⁰⁷ It was soon recognized that retinene₁ was similar to vitamin A₁ aldehyde, while a second type of retinene (retinene₂) was vitamin A₂ aldehyde.^{223,523,808-810} Glover and co-workers¹⁸² were able to demonstrate that vitamin A aldehyde is rapidly converted to vitamin A

⁷⁹⁸ I. M. Heilbron, W. E. Jones, A. Lowe, and H. R. Wright, *J. Chem. Soc.*, 1936, 561-563.

⁷⁹⁹ K. R. Bharucha and B. C. L. Weedon, *J. Chem. Soc.*, 1953, 1571-1577.

⁸⁰⁰ B. M. Manly, V. Petrow, O. Stephenson, and S. W. F. Underhill, *J. Pharm. and Pharmacol.*, 4, 43-45 (1952).

⁸⁰¹ J. F. Arens and D. A. van Dorp, *Nature*, 157, 190-191 (1946).

⁸⁰² P. Karrer, E. Jucker, and E. Schick, *Helv. Chim. Acta*, 29, 704-711 (1946).

⁸⁰³ I. M. Heilbron, E. R. H. Jones, and D. G. O'Sullivan, *Nature*, 157, 485-486 (1946).

⁸⁰⁴ I. M. Heilbron, E. R. H. Jones, and D. G. O'Sullivan, *J. Chem. Soc.*, 1946, 866-869.

⁸⁰⁵ G. Wald, *J. Gen. Physiol.*, 19, 351-371, 781-795 (1935-1936).

⁸⁰⁶ R. A. Morton, *Nature*, 153, 69-71 (1944).

⁸⁰⁷ R. A. Morton, M. K. Salah, and A. L. Stubbs, *Biochem. J.*, 41, xxiv (1947).

⁸⁰⁸ R. A. Morton, M. K. Salah, and A. L. Stubbs, *Nature*, 159, 744 (1947).

⁸⁰⁹ H. R. Cama, P. D. Dalvi, R. A. Morton, M. K. Salah, G. R. Steinberg, and A. L. Stubbs, *Biochem. J.*, 52, 535-540 (1952).

⁸¹⁰ R. A. Morton, M. K. Salah, and A. L. Stubbs, *Biochem. J.*, 40, lix-lx (1946).

when administered orally, intraperitoneally, or subcutaneously into rats. This reduction can be mediated in the gut wall and in other tissues. Gounelle and collaborators,⁸¹¹ and Katsampes *et al.*⁸¹² demonstrated that vitamin A₁ aldehyde (retinene₁) is readily absorbed and converted to vitamin A₁ alcohol by man. Cama and associates⁷⁹⁰ noted that retinene₂ (vitamin A₂ aldehyde) is also readily converted to the corresponding alcohol, namely vitamin A₂, in the rat. Although the liver was first thought to be the site for the conversion of vitamin A aldehyde to vitamin A alcohol,⁸¹³ it was later noted that there is no substantial evidence to support such a theory.¹⁸²

There is considerable proof of the reverse transformation, namely that of vitamin A to vitamin A aldehyde. Thus, the aldehyde is readily prepared from vitamin A alcohol by shaking concentrates of the latter in petroleum ether with potassium permanganate, acidified with sulfuric acid.⁸¹⁴ Hawkins and Hunter⁸¹⁵ obtained the aldehyde from vitamin A alcohol by oxidation with aluminum *isopropoxide* in the presence of acet-aldehyde. Ball *et al.*²²³ obtained better yields when oxidation was produced by a specially prepared manganese dioxide kept for six to ten days in the dark; yields of aldehyde as high as 80% of the theoretical were reported. Vitamin A₁ aldehyde is obtained by the oxidation of β -carotene.^{241-243,765} The biopotency of the ordinary vitamin A₁ aldehyde (all-*trans*) was reported by Arens and van Dorp⁸¹⁶ to be essentially that of natural crystalline vitamin A, although more recent determinations^{242,727} give the value as 2,800,000 I.U./g. Retinene₂ can readily be prepared from vitamin A₂ by oxidation with manganese dioxide.^{235,808} According to Milas, only about one-half the amount of manganese dioxide (0.3 g.) is required to oxidize 10 mg. of vitamin A₂ as is needed for a like amount of vitamin A₁. Although Meunier²⁴⁴ reported that an oxidation product of lycopene was vitamin A-active and appeared to have properties similar to those of retinene₂, Painter and Glover⁷⁶⁰ were unable to demonstrate any vitamin A biopotency in apo-15-lycopenal, as determined by growth response in vitamin A-deficient rats, whereas 2 μ g. of vitamin A/day resulted in prompt recovery.

Vitamin A aldehyde, like vitamin A, exists in four stereoisomeric forms.⁸¹⁷

⁸¹¹ H. Gounelle, C. Marnay, R. Chérourx, and Y. Raoul, *Compt. rend. soc. biol.*, 146, 523-525 (1952).

⁸¹² C. P. Katsampes, A. B. McCoord, R. N. Hamburger, and S. W. Clausen, *Pediatrics*, 12, 191-197 (1953).

⁸¹³ S. Ball, J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, 41, xxiv (1947).

⁸¹⁴ R. A. Morton and T. W. Goodwin, *Nature*, 153, 405-406 (1944).

⁸¹⁵ E. G. E. Hawkins and R. F. Hunter, *J. Chem. Soc.*, 1944, 411.

⁸¹⁶ J. F. Arens and D. A. van Dorp, *Rec. trav. chim.*, 68, 604-608 (1949).

⁸¹⁷ P. Meunier and J. Jouanneteau, *Bull. soc. chim. biol.*, 30, 260-264 (1948).

The usual type is the all-*trans* form. Graham *et al.*⁸¹⁸ synthesized the 3-*cis* isomer by the same sequence of reactions as those used for the synthesis of the all-*trans* form. 5-*cis*-Vitamin A aldehyde (also called neoretinene) can be prepared by the oxidation of neovitamin A alcohol with manganese dioxide.⁸¹⁹ Graham and co-workers⁸¹⁸ reported that the biopotency of 3-*cis* vitamin A aldehyde is the same as that of vitamin A₁.

Hubbard and co-workers⁸²⁰ characterized five different retinenes which are *cis-trans* to one another. This number of isomers is possible only if a "forbidden" *cis* linkage occurs, *i.e.*, one which, because of steric hindrance, was believed to be impossible. There are indications that, under some conditions, stable compounds can be made with these "hindered" *cis* bonds.^{821,822} Collins and co-workers⁸²³ demonstrated the *in vitro* regeneration of rhodopsin when synthetic vitamin A and a phosphate buffer were used with a preparation from rat retinae and choroids.

(c) *Vitamin A Ethers*. Vitamin A methyl ether was first prepared from vitamin A alcohol by Hanze and collaborators,⁸²⁴ in 1946. It is a light yellow solid which has a biologic potency of 3,000,000 U.S.P. units per gram. Other data on vitamin A methyl ester are included in the papers of Oroshnik,⁸²⁵ and of Isler *et al.*⁸²⁶

Homovitamin A ethyl ether (C₂₁H₃₁OC₂H₅) and 5-dehydrohomovitamin A ethyl ether (C₂₁H₂₉OC₂H₅) are two biologically active synthetic compounds, each of which has one more carbon atom on the aliphatic side-chain than the number present in vitamin A. These compounds have been synthesized by Milas and associates.⁸²⁷ 5-Dehydrohomovitamin A ethyl ether is readily changed to homovitamin A ethyl ether by reduction with palladium in the presence of calcium carbonate. Both compounds possess slight biologic activity, since they are able to cure xerophthalmia and to produce normal growth of vitamin A-depleted rats. However, the biopotency is small in view of the high doses required,⁸²⁸ namely 96 mg. and

⁸¹⁸ W. Graham, D. A. van Dorp, and J. F. Arens, *Rec. trav. chim.*, **68**, 609-612 (1949).

⁸¹⁹ P. D. Dalvi and R. A. Morton, *Biochem. J.*, **50**, 43-48 (1951).

⁸²⁰ R. Hubbard, R. I. Gregerman, and G. Wald, *J. Gen. Physiol.*, **36**, 415-429 (1953).

⁸²¹ C. H. Eugster, C. F. Garbers, and P. Karrer, *Helv. Chim. Acta*, **36**, 1378-1383 (1953).

⁸²² C. F. Garbers and P. Karrer, *Helv. Chim. Acta*, **36**, 828-834 (1953).

⁸²³ F. D. Collins, J. N. Green, and R. A. Morton, *Biochem. J.*, **53**, 152-157 (1953).

⁸²⁴ A. R. Hanze, T. W. Conger, E. C. Wise, and D. I. Weisblat, *J. Am. Chem. Soc.*, **68**, 1389 (1946).

⁸²⁵ W. Oroshnik, *J. Am. Chem. Soc.*, **67**, 1627-1628 (1945).

⁸²⁶ O. Isler, M. Kofler, W. Huber, and A. Ronco, *Experientia*, **2**, 31 (1946).

⁸²⁷ N. A. Milas, S. W. Lee, C. Schuerch, Jr., R. O. Edgerton, J. T. Plati, F. X. Grossi, Z. Weiss, and M. A. Campbell, *J. Am. Chem. Soc.*, **70**, 1591-1596 (1948).

⁸²⁸ R. S. Harris; cited by N. A. Milas, *Vitamins and Hormones*, **5**, 1-38 (1947), p. 5.

60 mg., respectively.⁷⁸⁴ For a further discussion of the synthesis and properties of the above-mentioned synthetic compounds, the reader is referred to *The Lipids*, Vol. I, pages 704 to 709.

(d) *Vitamin A Hydrocarbon*. This compound was prepared by Karrer and Benz;⁸²⁹ it was called "axerophthene." The compound was reported by Euler and Karrer⁸³⁰ to be biologically active to the extent of 10% of the biopotency of vitamin A, but its lower homologues are entirely devoid of vitamin A potency. It was concluded that, in order that a substance possess vitamin A activity, the entire C₂₀-chain must be arranged in a specific manner; the substituents on the terminal carbon of the aliphatic chain are of little significance. Meunier *et al.*^{831,832} objected to the term "axerophthene," and suggested "dihydroaxerophthene" as preferable.

(e) *Anhydrovitamin A (A₁) and Rehydrovitamin A*. Anhydrovitamin A is chiefly a laboratory product which is formed when vitamin A remains in contact with small concentrations of hydrogen chloride in ethanol^{833,834} or is produced by refluxing an alcoholic solution of the natural vitamin A esters.^{834,835} Anhydrovitamin A is believed to originate from vitamin A by the loss of a molecule of water; this results in a compound having six rather than five conjugate double bonds and a CH=CH₂ end group. In 1943, Meunier *et al.*⁸³⁶ proposed that anhydrovitamin A possessed a *retro* structure, *i.e.*, an α -ionone ring connected by a double bond with the side chain (*cf.* also Shantz⁸³⁷ and Robeson⁸³⁸). Karrer and Kehrle⁸³⁹ and Oroshnik *et al.*⁸⁴⁰ furnished conclusive proof that the *retro*ionylidene structure exists. The biopotency of anhydrovitamin A was reported to be 17,500 I.U./g., by Shantz *et al.*⁸⁴¹ This may not be significant, since it might arise from rehydrovitamin A formed *in vivo* from the anhydro compounds. However, Shantz⁸³⁷ is convinced that anhydrovitamin A *per se* has biologic activity.

⁸²⁹ P. Karrer and J. Benz, *Helv. Chim. Acta*, **31**, 1048-1054 (1948).

⁸³⁰ H. v. Euler and P. Karrer, *Helv. Chim. Acta*, **32**, 461-463 (1949).

⁸³¹ P. Meunier, *Compt. rend.*, **227**, 206-207 (1948).

⁸³² A. Guérillot-Vinet, P. Meunier, J. Jouanneteau, and M. Gourevitch, *Compt. rend.*, **226**, 128-130 (1948).

⁸³³ J. R. Edisbury, A. E. Gillam, I. M. Heilbron, and R. A. Morton, *Biochem. J.*, **26**, 1164-1173 (1932).

⁸³⁴ N. D. Embree, *J. Biol. Chem.*, **128**, 187-198 (1939).

⁸³⁵ E. Le B. Gray and J. D. Cawley, *J. Nutrition*, **23**, 301-307 (1942).

⁸³⁶ P. Meunier, R. Dulou, and A. Vinet, *Bull. soc. chim. biol.*, **25**, 371-378 (1943).

⁸³⁷ E. M. Shantz, *J. Biol. Chem.*, **182**, 515-524 (1950).

⁸³⁸ C. D. Robeson, *U. S. Patent*, No. 2,583,594 (Jan. 29, 1952).

⁸³⁹ P. Karrer and J. Kehrle, *Helv. Chim. Acta*, **35**, 2570-2573 (1952).

⁸⁴⁰ W. O. Oroshnik, G. Karmas, and A. D. Mebane, *J. Am. Chem. Soc.*, **74**, 295-304 (1952).

⁸⁴¹ E. M. Shantz, J. D. Cawley, and H. D. Embree, *J. Am. Chem. Soc.*, **65**, 901-906 (1943).

Rehydrovitamin A, rather than vitamin A itself, is deposited in the livers of rats after anhydrovitamin A is fed.⁸³⁷ It was given this name for the reason that, although the new molecule is formed by the addition of a molecule of water, the double bond system differs from that of vitamin A. The biopotency of rehydrovitamin A was found by Shantz⁸³⁷ to be about one-fifteenth of that of vitamin A. For the structures and properties of anhydrovitamin A and rehydrovitamin A the reader is referred to *The Lipids*, Vol. I, pages 733-738.

d. The Vitamin A-Like Effect of Lard. Although lard is generally believed to be devoid of vitamin A, Kaunitz and Slanetz^{842,843} demonstrated the presence of a factor in a molecular distillate of lard which protected rats against the signs of vitamin A deficiency. It was first considered that this factor was distinct from vitamin A; in fact, a number of workers⁸⁴⁴⁻⁸⁴⁹ suggested the presence of substances in foods which possessed biologic activity but which were assumed to be chemically different from the known vitamins A. These substances included lard, oils from the decapod shrimp (*Penaeus foliaceus*), from the calanoid whorled Channel copepod plankton (*Temora turbinata*), and the Atlantic copepod plankton crustacean (*Centropages typicus*), and from other animal sources. Lowe and Morton⁸⁵⁰ confirmed the results of Kaunitz and Slanetz⁸⁴³ by proving that a molecular distillate of lard, which was apparently free from vitamin A or provitamin A, restored growth and cured xerophthalmia when given to vitamin A-depleted rats at levels of 0.6 g./day; it was believed that these data indicated the presence of a factor in lard capable of replacing vitamin A. However, Herb, Riemenschneider, Kaunitz, and Slanetz⁸⁵¹ isolated fractions from the molecular distillate which gave a positive Carr-Price reaction and which exhibited a typical vitamin A-absorption curve. The conclusion of these authors that the biologic value of lard is actually attributable to its vitamin A content seems entirely justified on the basis of their data. The vitamin A-content is calculated to be 0.4 to 2.0 I.U./g. of the distillate.⁸⁵¹

⁸⁴² H. Kaunitz and C. A. Slanetz, *Federation Proc.*, **9**, 335 (1950).

⁸⁴³ H. Kaunitz and C. A. Slanetz, *J. Nutrition*, **42**, 375-389 (1950).

⁸⁴⁴ L. Randoin and R. Netter, *Compt. rend.*, **198**, 2120-2122 (1934).

⁸⁴⁵ P. Dubouloz, R. Marville, and C. Chevalier, *Bull. soc. chim. biol.*, **30**, 112-115 (1948).

⁸⁴⁶ P. Le Gallic, *Compt. rend. soc. biol.*, **141**, 1214-1218 (1947); **142**, 65-67 (1948).

⁸⁴⁷ J. Mayer and W. A. Krehl, *J. Nutrition*, **35**, 523-537 (1948).

⁸⁴⁸ R. Grangaud and R. Massonet, *Bull. soc. chim. biol.*, **31**, 231-234 (1949).

⁸⁴⁹ C. E. Lane, *Science*, **111**, 471-472 (1950).

⁸⁵⁰ J. S. Lowe and R. A. Morton, *Biochem. J.*, **55**, 681-686 (1953).

⁸⁵¹ S. F. Herb, R. W. Riemenschneider, H. Kaunitz, and C. A. Slanetz, *J. Nutrition*, **51**, 393-402 (1953).

(4) *The Excretion of Carotenoids and
of Vitamin A in the Urine*

The kidney plays an important role in the metabolism of vitamin A. It has been shown to be an important organ in the storage of vitamin A, and exhibits a higher priority for this component than does the liver when the supplies of vitamin A are limited.

Vitamin A has been found in the urine of human patients suffering from cancer, renal tuberculosis, and severe general infections, and especially in conditions involving renal and hepatic dysfunction, for example, in gonotoxic icterus, obstructive jaundice, chronic nephritis and other nephroses, cirrhosis of the liver, cholelithiasis,^{777,852} and also in lobar pneumonia.⁸⁵³ The storage of vitamin A in the kidney is reduced in the case of patients with chronic nephritis;^{713,714} the level of vitamin A in the blood is concomitantly increased.^{29,84} According to Baumann *et al.*,⁷⁰⁹ following the injection of dibenzanthracene, the kidney vitamin A is maintained at higher levels than is that in the liver.

The absence of vitamin A in the diet causes an injury to the kidney. Thus, Herrin and Nicholes⁸⁵⁴ reported that the urea clearance of vitamin A-deficient dogs was well below normal, due to changes in the glomerular filtration. However, Kaiser, Francis, and Kagan⁸⁵⁵ were unable to demonstrate any differences in serum or liver vitamin A levels in male white rats which were subjected to bilateral nephrectomy, as compared with control animals. This would indicate that the normal mature kidney plays no significant role in vitamin A metabolism in such short-term experiments in the rat.

Hymans, van den Bergh, and Snapper⁸⁵⁶ believed that lutein was excreted in instances in which the blood lipochrome level was high. However, in more recent studies by Lawrie, Moore, and Rajagopal,⁸⁵⁷ no evidence of carotenuria was found. Moreover, no urinary vitamin A was recorded in physiologic conditions such as pregnancy, or in any pathologic conditions with the exception of chronic nephritis or pneumonia. In the latter condition, vitamin A was excreted in quite large amounts, apparently combined with a protein. As much as 3000 I.U. of vitamin A per twenty-

⁸⁵² R. Boller, O. Brunner, and E. Brodaty, *Wien. Arch. inn. Med.*, **31**, 1-22 (1937); *Chem. Abst.*, **31**, 7988 (1937).

⁸⁵³ R. Boller and O. Brunner, *Klin. Wochschr.*, **16**, 861-862 (1937).

⁸⁵⁴ R. C. Herrin and H. J. Nicholes, *Am. J. Physiol.*, **125**, 786-801 (1939).

⁸⁵⁵ E. Kaiser, Y. F. Francis, and B. M. Kagan, *Proc. Soc. Exptl. Biol. Med.*, **85**, 457-458 (1954).

⁸⁵⁶ A. A. Hymans, H. van den Bergh, and J. Snapper, *Deut. Arch. klin. Med.*, **110**, 540-561 (1913).

⁸⁵⁷ N. R. Lawrie, T. Moore, and K. R. Rajagopal, *Biochem. J.*, **35**, 825-836 (1941).

four hours was excreted in the urine in nephritis or in pneumonia in man; large amounts were likewise found in dog urine, but none was noted in the urine of normal or diseased cats, rabbits, or rats.⁸⁵⁷ It was observed that while normal urine would not take up vitamin A when shaken with halibut liver oil, urines containing vitamin A retained still more vitamin A under these conditions. Presumably there was a sufficient excess of urinary protein so that more vitamin A could be bound.⁸⁵⁷ Catel⁸⁵⁸ also found vitamin A in the urine of healthy dogs but never in rabbits, which are herbivorous. Vitaminuria was occasionally noted in the case of healthy premature and full-term newborn infants, but not in older children, except in nephritis and in other diseases. In these cases the vitamin A concentration in the blood was not decreased.

(5) *The Secretion of Carotenoids
and of Vitamin A into Milk*

In the case of lactating animals, large amounts of carotenoids and of vitamin A are secreted by the mammary gland. The quantity of these components, as well as the ratio in which they appear in milk, vary with species, season, and diet. The composition of milk (excluding the fat-soluble vitamin content), and the factors which determine the lipid components, are discussed in *The Lipids*, Vol. II, pages 791 to 810. A brief description of the factors affecting the carotene and vitamin A content of milks is included in *The Lipids*, Vol. I, pages 685–687.

β -Carotene is the chief carotenoid in milk fat. In some cases, small amounts of α -carotene may also occur when it is present in the rations fed the cows.⁶¹² According to Chanda,⁸⁵⁹ β -carotene makes up 65 to 85% of the total carotenoids of cow's milk. Lutein is regularly present in cow's milk and in butter.⁷³ Other xanthophylls may also be present. Gillam and Heilbron⁶³ observed the occurrence of lycopene in butter and milk.

Most of the fat-soluble yellow pigment in colostrum and in milk is carotene.⁸⁶⁰ Chanda⁸⁵⁹ found no carotenoids in goat's milk. In cow's milk the carotenoid content did not change as lactation advanced. The average amount was 5.1 $\mu\text{g/g}$. of fat.

Vitamin A is present in milk from all species, but the quantity varies under different conditions. The vitamin A is largely in the form of the ester, although Chanda⁸⁵⁹ reported that 5 to 8% of the vitamin A in cow's

⁸⁵⁸ W. Catel, *Klin. Wochschr.*, 17, 574–575 (1938).

⁸⁵⁹ R. Chanda, *Biochem. J.*, 52, ii (1952).

⁸⁶⁰ D. B. Parrish, G. H. Wise, and J. S. Hughes, *J. Biol. Chem.*, 167, 673–678 (1947).

milk occurs as the free alcohol. Parrish *et al.*⁸⁶⁰ state that practically all of the vitamin A in both the colostrum and the milk of the cow is in the ester form. In goat's milk the proportion which is esterified amounts to 98%, according to Chanda.⁸⁵⁹ Goat's milk contained 60 I.U. of vitamin A per gram of fat during the second week of lactation. The corresponding figure for cow's milk was 49 I.U. This ratio persisted throughout lactation. The higher content in goat's milk may perhaps be related to the greater activity of the thyroid gland in goats, which facilitates the absorption of carotene and its conversion to vitamin A, thus rendering more of the vitamin available for secretion by the mammary gland.⁸⁵⁹

a. The Carotenoids and Vitamin A in the Milk of Different Species. Although carotenoids are generally considered to be typical constituents of milk, there are a number of species in which only minute traces of these chromogens occur. This is true of the goat,^{859,861} and of the Egyptian buffalo.⁸⁶²

Elephant's milk contains only small amounts of vitamin A.⁸⁶³ The content is low in Indian buffalo milk, as well,⁸⁶⁴ although the butterfat contains as much vitamin A as does that of the cow. The vitamin A content of the butterfat from the Egyptian buffalo⁸⁶² is twice that of cow butterfat. Hvidsten and co-workers⁸⁶¹ reported that carotenes are absent from the colostrum of ewes. Therefore one would not expect the pigment to occur in the milk of these species. No carotenoids are present in the milk of aquatic mammals such as blue and fin-backed whales.⁴⁶⁷ Marked variations occur in the carotenoid content of the colostrum of different breeds of cows. The highest figure has been reported for the colostrum of the Jersey cow (347 $\mu\text{g.}/100\text{ ml.}$)⁸⁶⁵ followed in turn by that of the Ayrshire breed (244 $\mu\text{g.}/100\text{ ml.}$),³¹⁸ of "beef" cattle (129 $\mu\text{g.}/100\text{ ml.}$),⁸⁶⁵ of Holstein cows (59 to 236 $\mu\text{g.}/100\text{ ml.}$)⁸⁶⁶ and 100 $\mu\text{g.}/100\text{ ml.}$),⁸⁶⁵ and of the Hariana and Sahiwal breed (33.6 to 153.9 $\mu\text{g.}/100\text{ ml.}$).⁸⁶⁷ According to Gillam *et al.*,⁸⁶⁸ colostrum from several breeds of cows contained the following concentrations of carotene (expressed in mg. % fat): Friesan, 3.40; Ayrshire, 4.60; Guern-

⁸⁶¹ H. Hvidsten, L. G. Hansteen, and G. Broch, *Meieriposten*, 37, 148-153, 67-171 (1948).

⁸⁶² M. A. M. Abul-Fadl, A. M. Ismail, and M. S. El Ridi, *J. Roy. Egypt. Med. Assoc.*, 33, 521-526 (1950); *Chem. Abst.*, 45, 278 (1951).

⁸⁶³ Z. Markuze, *Biochem. J.*, 33, 198-200 (1939).

⁸⁶⁴ L. C. Dharmani and J. D. Chopra, *Indian. J. Vet. Sci.*, 16, 158-166 (1946).

⁸⁶⁵ R. W. Luecke, C. W. Duncan, and R. E. Ely, *Arch. Biochem.*, 13, 277-282 (1947).

⁸⁶⁶ R. G. Hansen, P. H. Phillips, and V. R. Smith, *J. Dairy Sci.*, 29, 809-814 (1946).

⁸⁶⁷ B. C. R. Sarkar, *J. Dairy Sci.*, 31, 479-487 (1948).

⁸⁶⁸ A. E. Gillam, I. M. Heilbron, W. S. Ferguson, and S. J. Watson, *Biochem. J.*, 30, 1728-1734 (1936).

sey, 3.61; and Shorthorn, 2.90. On the basis of a fat content of 4%, these milks would have the following carotenoid concentrations expressed in $\mu\text{g.}/100\text{ ml.}$, respectively: 136, 184, 144, 116. Dann⁸⁶⁹ and Semb *et al.*⁸⁷⁰ recorded similar variations in the carotenoid content of the colostrum of different breeds of cattle.

A number of workers, including Leshner and co-workers⁸⁷¹ in the United States, Kon and Mawson⁸⁷² in Great Britain, Chanda and associates⁸⁷³ in Scotland, and others,⁸⁷⁴⁻⁸⁷⁸ observed that the carotenoids decreased rapidly as the colostrum gave way to mature milk. Thus, the carotenoid values for cow's milk, obtained one month after the colostrum samples, by Gillam *et al.*,⁸⁶⁸ were, in some cases, only 25% of those noted in the colostrum; the milk values (expressed in $\text{mg. \% of fat}$) were: Friesan, 0.38; Ayrshire, 0.18; Guernsey, 0.91; and Shorthorn, 0.28. On the basis of a fat content of 4%, these figures expressed in $\mu\text{g.}/100\text{ ml.}$ milk are respectively as follows: 15.2, 7.2, 36.4, and 11.2. Wise and collaborators⁸⁷⁹ observed similar differences in carotenoid content between the milks of different breeds of cows.

Similar variations may be noted in the preformed vitamin A, as well as in the carotenoid, content in the milk of different breeds of cattle. Thus, the results of Gillam *et al.*⁸⁶⁸ (expressed as $\text{mg. \% fat}$), for the colostrum of several species were as follows: Friesan, 1.24; Ayrshire, 2.96; Guernsey, 1.13; and Shorthorn, 3.51. In contrast to these values, the vitamin A content in the milks was markedly reduced; the average figures observed were as follows: Friesan, 0.52; Ayrshire, 0.37; Guernsey, 0.67; and Shorthorn, 0.48. Luecke *et al.*⁸⁶⁵ report that the colostrum of cows had the following vitamin A content ($\mu\text{g.}/100\text{ ml.}$); Jersey, 401; Holstein, 52; and beef cattle, 145.

The carotenoid and vitamin A content of human milk exhibits similar

⁸⁶⁹ W. J. Dann, *Biochem. J.*, **27**, 1998-2005 (1933).

⁸⁷⁰ J. Semb, C. A. Baumann, and H. Steenbock, *J. Biol. Chem.*, **107**, 697-703 (1934).

⁸⁷¹ J. Leshner, J. K. Brody, H. H. Williams, and I. G. Macy, *Am. J. Diseases Children*, **70**, 182-192 (1945).

⁸⁷² S. K. Kon and E. H. Mawson, *Human Milk, Wartime Studies of Certain Vitamins and Other Constituents*, Med. Research Council (Brit.), Spec. Rept. Ser., No. 269, 1-188 (1950).

⁸⁷³ R. Chanda, E. C. Owen, and B. Cramond, *Brit. J. Nutrition*, **5**, 228-242 (1951).

⁸⁷⁴ W. Neuweiler, *Z. Vitaminforsch.*, **4**, 259-271 (1935).

⁸⁷⁵ N. van Eekelen and J. H. de Haas, *Geneesk. Tijdschr. Ned.-Indië*, **74**, 1201-1208 (1934).

⁸⁷⁶ O. Meulemans and J. H. de Haas, *Indian J. Pediat.*, **3**, 133-145 (1936).

⁸⁷⁷ M. Repetti, *Fol. Gynaec. (Genova)*, **31**, 337-364 (1934); *Ber. ges. Physiol. u. expll. Pharmacol.*, **86**, 242 (1935); *Chem. Abst.*, **31**, 6702 (1937).

⁸⁷⁸ E. Svensson, *Skand. Arch. Physiol.*, **73**, 237-254 (1936).

⁸⁷⁹ G. H. Wise, F. W. Atkeson, M. J. Caldwell, D. B. Parrish, and J. S. Hughes, *J. Dairy Sci.*, **30**, 279-291 (1947).

differences between the level in the colostrum and in milk. Thus, average carotenoid values recorded for human colostrum by different investigators are 51 $\mu\text{g.}/100\text{ ml.}$,⁶⁰⁵ and 25 to 34 $\mu\text{g.}/100\text{ ml.}$ ⁸⁸⁰

In general, the values (in $\mu\text{g.}/100\text{ ml.}$) for human milk are considerably lower,⁸⁸¹ for example, 15;⁸⁷² 2 to 17;¹⁹² 24 to 25;⁸⁷¹ and 17 to 60.⁸⁸² Hrubetz *et al.*²⁰⁶ reported values for vitamin A in human milk ranging between 331 and 170 I.U. per 100 ml. (100 to 51 $\mu\text{g.}/100\text{ ml.}$) at varying times during the progression of the lactation cycle. Macy,⁸⁸³ likewise, carried out extensive investigations on the proportion of vitamin A and of carotenoids in human milk during various periods of lactation, and found higher values for both carotenoids and vitamin A in colostrum than in milk.

The colostrum of swine has been reported to have a carotenoid content of 24 $\mu\text{g.}/100\text{ ml.}$,⁸⁶⁵ while that of the goat is given as 12 to 46 $\mu\text{g.}/100\text{ ml.}$ ⁸⁸⁴ Only traces of carotenoids appear in the mature milk of the latter two species. Swine colostrum was found to contain 60 $\mu\text{g.}$ of vitamin A/100 ml.⁸⁶⁵

b. The Effect of the Lactation Cycle on the Carotenoids and Vitamin A in Milk. It has been shown in the previous section that a marked variation obtains between the composition of the colostrum and that of the milk. Colostrum is more concentrated and contains a larger proportion of practically every solid component, including carotene and vitamin A, than do the milks produced subsequently.

Chanda⁸⁵⁹ reported that, in cow's milk, vitamin A decreased gradually up to the thirtieth week of lactation. Thereafter, when the yield of milk was decreasing rapidly, both the per cent of fat in the milk and the per cent of vitamin A in the fat showed small but definite increases. No change occurred in the content of carotenoids as the lactation cycle progressed. The vitamin A of milks during the second week of lactation amounted to 60 I.U./g. fat in goat's milk, while the content in cow's milk was 49 I.U./g. fat. In the case of the goat, minimum vitamin A levels were reached during the twentieth week of lactation.

In the case of swine, Heidebrecht and co-workers⁸⁸⁵ reported the following cyclic changes in the vitamin A in the milk (expressed in $\mu\text{g.}/100\text{ ml.}$): colostrum, 132; fifth day, 33; fifteenth day, 22; fifty-fifth day, 19. Swine

⁸⁸⁰ L. Skurnik and M. Hellen, *Z. Vitaminforsch.*, **15**, 52-68 (1944).

⁸⁸¹ W. J. Dann, *Biochem. J.*, **30**, 1644-1651 (1936).

⁸⁸² C. Friderichsen and T. K. With, *Ann. Paediat.*, **153**, 113-143 (1939).

⁸⁸³ I. G. Macy, *Am. J. Diseases Children*, **78**, 589-603 (1949).

⁸⁸⁴ R. Chanda and E. C. Owen, *Biochem. J.*, **51**, iv (1952).

⁸⁸⁵ A. A. Heidebrecht, R. MacVicar, O. B. Ross, and C. K. Whitehair, *J. Nutrition*, **44**, 43-50 (1951).

TABLE 5
THE CAROTENE CONTENT OF COW'S MILK
AS INFLUENCED BY SEASON^a

Breed	Carotenes in $\mu\text{g./100 ml.}$		Reference
	Winter milk	Summer milk	
Mixed	3.8-14	17-32	<i>b</i>
	5 (min.)	25 (max.)	<i>c</i>
	5	35	<i>d</i>
	5	30	<i>e</i>
	3	24	<i>f</i>
	14.07	20.4	<i>g</i>
Jersey	5.9	105	<i>h</i>
	20.5	121	<i>i</i>
	27	121	<i>j</i>
Guernsey	34.5	205	<i>i</i>
	35		<i>j</i>
Hariana and Sahiwal	10.8	43.7	<i>k</i>

^a Adapted from T. W. Goodwin, *The Comparative Biochemistry of the Carotenoids*, Chemical Publ. Co., Inc. New York, 1954, p. 242.

^b J. C. H. van Wijngaarden, *Nederland Tijdschr. Geneesk.*, 75, 2668-2671 (1934).

^c M. Deco, *Compt. rend. soc. biol.*, 130, 817-818 (1939).

^d A. Francois, *Lait*, 26, 97-116 (1946).

^e A. Lund, *Beretr. Forsøgslab. Kobenh.*, No. 218, p. 115 (1945); cited by T. W. Goodwin, *Carotenoids*, p. 242.

^f K. Skurnik and M. Hellen, *Z. Vitaminforsch.*, 15, 52-68 (1944).

^g F. Amaral Rogick and L. Amaral Rogick, *Bol. indust. animal (Sao Paulo)*, 8, No. 3-17 (1946); *Chem. Abst.*, 42, 3873 (1948).

^h P. F. Sharp and D. B. Hand, *J. Dairy Sci.*, 22, 737-741 (1939).

ⁱ T. S. Sutton and W. E. Krauss, *Bull. Ohio Agric. Expt. Sta.*, No. 548 (53rd Ann. Rept.), 59 (1935); *Chem. Abst.*, 30, 1449 (1936).

^j P. D. Boyer, K. Spitzer, C. Jensen, and P. H. Phillips, *Ind. Eng. Chem., Anal. Ed.*, 16, 101-102 (1944).

^k B. C. R. Sarkar, *J. Dairy Sci.*, 31, 479-487 (1948).

colostrum and milk were reported to contain essentially no carotene. Braude *et al.*^{886,887} and Bowland and co-workers^{888,889} reported similar values for the vitamin A content of sow's colostrum (247 I.U./100 ml.) and milk.

c. Seasonal Variations in the Carotenoid and Vitamin A Content of Milk. Season has a pronounced effect upon the carotenoid and vitamin A

⁸⁸⁶ R. Braude, S. K. Kon, and S. Y. Thompson, *J. Dairy Research*, 14, 414-418 (1946).

⁸⁸⁷ R. Braude, M. E. Coates, K. M. Henry, S. K. Kon, S. J. Rowland, S. Y. Thompson, and D. M. Walker, *Brit. J. Nutrition*, 1, 64-77 (1947).

⁸⁸⁸ J. P. Bowland, R. H. Grummer, P. H. Phillips, and G. Bohstedt, *J. Dairy Sci.*, 32, 22-28 (1949).

⁸⁸⁹ J. P. Bowland, R. H. Grummer, P. H. Phillips, and G. Bohstedt, *J. Animal Sci.*, 8, 98-106 (1949).

content of milks, which depends upon the proportion of green pasturage available to the cattle at various times of the year.^{890,891} However, Hibbs *et al.*⁸⁹² reported that a closer relation exists between the pasture carotenoids and the milk carotenoids than between pasture carotenoids and milk vitamin A. After the initial rise at the start of the pasture season, milk carotenoids follow the change in pasture carotenoids more closely when their level is below 250 $\mu\text{g./g. fat}$ than when it is higher; this indicates a maximum response in the milk at the above level. The comparative values for the carotenoid content in the milks of cows during the winter (stall-fed) and summer (pasture-fed) are given in Table 5.

Similar but less pronounced variations in the vitamin A content in milk, as related to season, may be observed. This is reflected in the vitamin A content of butter at different seasons. Thus, in a nationwide survey⁸⁹³ on the role of butter as a source of vitamin A in the diet in the United States (1947), it was found that the vitamin A content of the total output of butter averaged 15,000 I.U./lb.; that of summer butter approximated 17,000 to 18,000 I.U./lb., while the potency of winter butter was, on an average, 10,550 to 11,200 I.U./lb.

Chanda *et al.*⁸⁷³ and Nylund⁸⁹⁴ reported that seasonal variations occur in the carotenoid and vitamin A content of human milk just as in the case of cattle, according to the availability of green foods.

d. The Effect of Diet on the Carotenoids and Vitamin A in Milk. The seasonal variation in the carotenoid and vitamin A content of the milk, discussed in the previous section, is a reflection of the effect of seasonal variations in provitamin A intake.

When the vitamin A intake is increased, there is a parallel increase in the vitamin A excreted in the milk. When supplementary doses of vitamin A varying from 0 to 4,200,000 I.U./day were given to Holstein and Guernsey cows receiving a diet containing a large amount of alfalfa, a progressive rise in the vitamin A level was noted in the milk fat, as shown in Table 6.

When vitamin A was given in doses of 4,200,000 I.U. per day, it was found that the butterfat produced had a vitamin A content as high as 331 I.U./g.,¹⁹⁵ which compared with a value of 29.6 I.U./g. in the same group before the supplementation with vitamin A was started. Coincident with the increase in total vitamin A, there was a marked and steady decrease of milk carotene, as well as of blood carotene. For a further discussion of the

⁸⁹⁰ J. L. Brence and J. A. Nelson, *J. Dairy Sci.*, **34**, 960-965 (1951).

⁸⁹¹ C. A. Baumann and H. Steenbock, *J. Biol. Chem.*, **101**, 547-560 (1933).

⁸⁹² J. W. Hibbs, W. E. Krauss, and C. F. Monroe, *J. Dairy Sci.*, **32**, 955-960 (1949).

⁸⁹³ *U. S. Dept. Agr., Miscell. Bull.*, No. 636, 1-47 (1937).

⁸⁹⁴ G. Nylund, *Finska Läkare Sällsk. Handl. (Helsingfors)*, **80**, 733-766 (1937).

TABLE 6
THE EFFECT OF SUPPLEMENTARY VITAMIN A
ON THE COMPOSITION OF THE MILK FAT OF COWS^a

Daily vitamin A Supplement, I.U.	Length of supple- mentation, weeks	Carotene and vitamin A/g. fat		
		β -Carotene, μg .	Vitamin A, I.U.	Total vitamin A ^b , I.U.
<i>Holstein</i>				
0	0	4.23	24.4	31.4
700,000	8	2.88	66.0	70.8
2,100,000	3	2.77	128.2	132.8
2,800,000	6	2.23	167.9	171.6
4,200,000	8	2.15	280.4	284.0
<i>Guernsey</i>				
0	0	9.79	25.5	41.8
2,650,000	3	4.99	223.2	231.5
5,300,000	11	2.28	178.4	182.2

^a Data from H. J. Deuel, Jr., L. F. Hallman, C. Johnston, and F. Mattson, *J. Nutrition*, 23, 567-579 (1942).

^b 0.6 μ g. of β -carotene calculated as equal to one I.U.

suppressive effect of massive doses of vitamin A on carotenoid metabolism, the reader is referred to page 447.

When large doses of vitamin A were taken by women during the last trimester of pregnancy, and during lactation, the vitamin A content of the milk produced was increased.²⁰⁶ Thus, the total vitamin A in the colostrum of women who had received doses of vitamin A by mouth at several levels for the three months preceding parturition amounted to the following (I.U./100 ml.): 0 I.U. group, 424; 50,000 I.U. group, 747; 100,000 I.U. group, 1037; and 200,000 I.U. group, 1,177. The maximum level of vitamin A was noted in milk from a woman in the fourth group; the total vitamin A amounted to 2,160 I.U./100 ml. milk. In the samples of milk, which were collected at intervals over a period of sixty-one days after the start of the lactation period, the concentrations of vitamin A in the same four groups (as I.U./100 ml.) were respectively as follows: 281, 344, 885, 718. Although there was a marked decrease in milk carotene as the period of lactation progressed, there was no evidence of a suppression of the carotenoid content in the milk, such as had previously been demonstrated in the cow, following a massive vitamin A intake. Sobel and associates⁸⁹⁵

⁸⁹⁵ A. E. Sobel, A. Rosenberg, and B. Kramer, *Am. J. Diseases Children*, 80, 932-943 (1950).

failed to demonstrate increased vitamin A in human milk when fixed levels of the vitamin were given in an oily medium; however, a marked increase in the vitamin A content of milk occurred within twelve hours when similar doses of vitamin A were administered in an aqueous medium. However, when larger doses of vitamin A were given in the oil medium, an increase in milk vitamin A was observed similar to that obtained when doses of the vitamin were administered in aqueous dispersions. The same increase in the blood level of vitamin A occurred.

Parrish and co-workers⁸⁹⁶ reported that the effect of vitamin A supplements on the vitamin A content of colostrum and of early milk of cows is independent of the form or combination in which they are given. Thus, vitamin A ester, vitamin A alcohol, and vitamin A alcohol plus tocopherols appeared to have similar values in increasing the vitamin A content of colostrum and of early milk.

e. The Effect of the Thyroid Gland upon the Carotenoids and Vitamin A in Milk. In general, an increase in thyroid secretion increases the vitamin A content of milk, while a decrease in thyroid function, brought about by the administration of thiouracil, results in a suppression of the vitamin A in the milk. Chanda and co-workers⁸⁹⁷ were able to demonstrate these changes in the goat after thyroxine or thiouracil, respectively, had been administered. It was found that, on carotene-containing diets, the content of both carotene and vitamin A in the milk was increased by the administration of thyroxine. On the other hand, when cows were placed on a carotenoid-free diet, the proportion of vitamin A in unesterified form increased, and this condition was further exaggerated when thyroxine was given.⁴⁰ Thyrotrophin, the thyroid-stimulating hormone, was also shown to increase the proportion of vitamin A alcohol in cows on a carotenoid-free diet.⁸⁹⁸ The carotenoid content of milk fat was found to be increased by mastitis, while the vitamin A content was not changed; the proportion of vitamin A in the alcohol form was increased in this infection.³⁴⁰

(6) *The Secretion of Carotenoids and of Vitamin A into Eggs*

The largest proportion of the carotenoids in eggs consists of the carotenols (xanthophylls). Whereas the ratio of carotenes to xanthophylls is 1:3 in normal green food, the proportion in the eggs of hens is 1:5 to 1:7⁸⁹⁹

⁸⁹⁶ D. B. Parrish, G. H. Wise, and J. S. Hughes, *J. Dairy Sci.*, **32**, 458-464 (1949).

⁸⁹⁷ R. Chanda, H. M. Clapham, and E. C. Owen, *Biochem. J.*, **50**, iii (1951).

⁸⁹⁸ R. Chanda, H. M. Clapham, and E. C. Owen, *Biochem. J.*, **52**, xvii-xviii (1952).

⁸⁹⁹ F. E. Jacob, *Studies on the Carotenoid Pigments of Egg Yolks from Hens on Various Diets*, Thesis (Ph.D.), Univ. Missouri, 1939; Univ. Microfilms, Ann Arbor, *Publ. No. 225*, 1941; *Microfilm Abst.*, Vols. 1-3 (1938-1941), *Biol. Chem.*, pp. 1-3.

or occasionally 1:30, according to Goodwin.¹⁹² This is not due to a poor absorption of carotenes but rather to the fact that they are efficiently converted to vitamin A,^{201,399,900} presumably in the gut wall.^{219,747} However, orally-administered carotene is not utilized by chicks less than twenty-two days of age when low protein diets are employed, and utilization is delayed to thirty-five to forty-two days after hatching when high protein regimens are fed.⁹⁰¹

A number of investigators⁹⁰²⁻⁹⁰⁶ have reported that appreciable quantities of β -carotene do occur in the hen egg. Although the amount of β -carotene is small, Ganguly and co-workers⁵⁹ proved its identity by determining the spectrophotometric pattern in several solvents, and by the use of mixed chromatograms with the pure carotenoids. Nash and Zscheile⁹⁰⁷ reported that, in addition to β -carotene, ζ -carotene, which occurs in special strains of tomatoes and in carrots, is also present in eggs. ζ -Carotene is not believed to be a precursor of vitamin A.⁹⁰⁸

However, as indicated above, the carotenols are the principal carotenoids occurring in egg yolk. The amount of the carotenols deposited varies with their source, as well as with the amount ingested. Hughes and Payne⁵⁶⁹ reported that the content of carotene and cryptoxanthin in the egg varied in different hens. The percentage of xanthophyll deposited increased when the amount of yellow corn in the ration was augmented. Only 2.5% of the carotene in alfalfa was found to be deposited in the yolk. Moreover, according to the findings of Sjollem and Donath,⁹⁰⁹ as recalculated by Deuel *et al.*,²⁰⁰ carotene comprised only 7% of the total yolk carotenoids when the hens had received a diet of alfalfa, or of yellow corn and alfalfa, while it made up 11% of the total when the pigments in the diet were derived from yellow corn alone.

Lutein is the principal carotenol in egg yolk. It was first isolated in 1912 from egg yolk (from which it derives its name) by Willstätter and

⁹⁰⁰ H. J. Almquist, G. MacKinney, and E. Mecchi, *J. Biol. Chem.*, **150**, 99-105 (1943).

⁹⁰¹ T. B. Mann, *J. Agr. Sci.*, **36**, 289-300 (1946).

⁹⁰² H. v. Euler and E. Klusmann, *Z. physiol. Chem.*, **208**, 50-54 (1932).

⁹⁰³ S. M. Hauge, F. P. Zscheile, C. W. Carrick, and B. B. Bohren, *Ind. Eng. Chem.*, **36**, 1065-1068 (1944).

⁹⁰⁴ T. B. Mann, *Analyst*, **68**, 233-238 (1943).

⁹⁰⁵ C. R. Thompson, M. A. Ervan, S. M. Hauge, B. B. Bohren, and F. W. Quackenbush, *Ind. Eng. Chem., Anal. Ed.*, **18**, 113-115 (1946).

⁹⁰⁶ F. Harms, *Chem. Zentr.*, **1941**, *II*, 3007.

⁹⁰⁷ H. A. Nash and F. P. Zscheile, *Arch. Biochem.*, **7**, 305-311 (1945).

⁹⁰⁸ J. W. Porter, H. A. Nash, F. P. Zscheile, and F. W. Quackenbush, *Arch. Biochem.*, **10**, 261-265 (1946).

⁹⁰⁹ B. Sjollem and W. F. Donath, *Biochem. J.*, **34**, 736-748 (1940).

Escher.⁹¹⁰ However, it was later demonstrated by Kuhn *et al.*,⁷⁶ by the application of chromatographic techniques, that the lutein in egg yolk could actually be separated into two fractions. "Lutein" has continued to serve as the designation for the first of these, while the second one was shown to be zeaxanthin; the latter was prepared in pure form from eggs by the application of chromatographic methods, by Strain⁷⁷ and by Euler and Gard.⁷⁸ Cryptoxanthin, which is a monohydroxycarotenol and a provitamin A, has also been reported in egg yolk by Gillam and Heilbron.⁶⁷

Vitamin A is regularly present in egg yolk. The amount in the egg varies with diet. According to Sherman,⁹¹¹ vitamin A is present to the extent of 2500 to 5000 I.U./100 g. egg yolk; this amounts to 400 to 800 I.U./16 g., which is the weight of the average yolk. Russell and Taylor⁹¹² cite values of 500 to 800 I.U. per yolk, while Deuel *et al.*²⁰⁰ report figures of 803 to 872 I.U. of total vitamin A per 18 g. yolk for all birds receiving supplemental vitamin A up to 15,000 I.U./lb. of food. Vitamin A alcohol has been reported to comprise 71 to 93% of the total vitamin A in the egg.⁹¹³

Diet influences the carotenoid and vitamin A content of eggs, to about the same extent as it does that of milk. Palmer and Kempster⁹¹⁴ were the first to recognize the relationship between dietary carotenoids and the color of the egg yolk. On carotenoid-free diets, the carotenoid content in egg yolk slowly decreases.^{915, 916}

Ganguly and co-workers⁵⁹ reported that the administration of a carotenoid-free diet to hens previously on a "farm" diet resulted in a complete disappearance of β -carotene and a practically complete absence of lutein; the proportion of vitamin A was also decreased. These data are summarized in Table 7.

When hens are placed on a carotenoid-free diet after having received a carotenoid-rich one, the eggs continue to be pigmented for some time, since the xanthophylls are transferred from the skin and shanks to the eggs.⁹¹⁵ On the other hand, when hens which have been receiving a carotenoid-free diet are transferred to a high-carotenoid diet, xanthophylls appear in the yolk,⁵⁶² although from two to six eggs are laid before the maximum degree of color is noted in the egg yolk.⁵⁶⁶

⁹¹⁰ R. Willstätter and H. H. Escher, *Z. physiol. Chem.*, **76**, 214-225 (1912).

⁹¹¹ H. C. Sherman, *Chemistry of Food and Nutrition*, 7th ed., Macmillan, New York, 1947, p. 634.

⁹¹² W. C. Russell and M. W. Taylor, *J. Nutrition*, **10**, 613-623 (1935).

⁹¹³ A. W. Neff, D. B. Parrish, J. S. Hughes, and L. F. Payne, *Arch. Biochem.*, **21**, 315-320 (1949).

⁹¹⁴ L. S. Palmer and H. L. Kempster, *J. Biol. Chem.*, **39**, 331-337 (1919).

⁹¹⁵ B. B. Bohren, C. R. Thompson, and C. W. Carrick, *Poultry Sci.*, **24**, 356-362 (1945).

⁹¹⁶ F. H. Grimbleby and D. J. G. Black, *Brit. J. Nutrition*, **4**, 323-331 (1950).

TABLE 7
THE CONCENTRATION OF CAROTENOIDS AND OF VITAMIN A
IN THE YOLKS OF EGGS, AS INFLUENCED BY DIET^a

Diet	Description of hens	Average composition of egg yolk ^b		
		β -Carotene, $\mu\text{g./g.}$	Lutein, $\mu\text{g./g.}$	Vitamin A, I.U./g.
Farm	From hens immediately after purchase from farmer	1.2 ^c (0.6-1.8)	26.5 (21.9-33.8)	9.1 (5.5-14.0)
Carotenoid-free	From hens after 77 days on carotenoid-free diet	0	1.0 (0.9-1.1)	4.2 (3.5-5.0)
Carotenoid-free	From hens raised from one-day-old chicks on carotenoid-free diet	0	1.2 (0.4-1.9)	3.8 (2.8-5.0)

^a J. Ganguly, J. W. Mehl, and H. J. Deuel, Jr., *J. Nutrition*, 50, 59-72 (1953), p. 66.

^b Including range of values in parentheses.

^c Proved, by mixed chromatograms and extinction curves, in *n*-hexane and carbon disulfide, to be β -carotene.

Cruickshank and Moore⁹¹⁷ reported that the vitamin A content of eggs was doubled by the incorporation of 10% of cod-liver oil into the diet, and that it was increased five times by the administration of a vitamin A concentrate. The augmentatory effect of supplementary vitamin A in the diet on the amount of vitamin A in the egg was also recorded by Sherwood and Fraps,⁹¹⁸ as well as by Bethke *et al.*⁹¹⁹ The hens also laid 15% more eggs.⁹¹⁸ Neff and co-workers⁹¹³ reported that dietary vitamin A is transferred to the eggs more efficiently when given as vitamin A ester than when administered as either vitamin A alcohol or carotene.

When massive doses of vitamin A are given to chicks, a depression in carotenoid deposition occurs.²⁰¹ This is reminiscent of the effect of similar high doses of vitamin A on the milk and blood carotenoids of cows. In the case of chicks, the carotenols rather than the carotenes are the carotenoid pigments chiefly affected. When large dosages of vitamin A are incorporated in the diet of hens, a marked increase in the vitamin A content in the yolks occurs concomitantly with a decrease in carotenoids.²⁰⁰ The results of these experiments are summarized in Table 8.

The vitamin A content of the eggs was doubled when the hens received food containing 100,000 I.U. of vitamin A per pound, and was somewhat further increased when the level of supplementary vitamin A in the food was 200,000 I.U. per pound. The maximum level of vitamin A noted in

⁹¹⁷ E. M. Cruickshank and T. Moore, *Biochem. J.*, 31, 179-187 (1937).

⁹¹⁸ R. M. Sherwood and G. C. Fraps, *Texas Agr. Expt. Sta. Bull.*, No. 493, 5-22 (1934).

⁹¹⁹ R. M. Bethke, D. C. Kennard, and H. L. Sassaman, *J. Biol. Chem.*, 72, 695-706 (1927).

TABLE 8

THE CAROTENOID AND VITAMIN A CONTENT IN THE YOLKS OF EGGS OBTAINED FROM HENS ON A NORMAL DIET (PERIOD I) FOLLOWED BY A VITAMIN A-SUPPLEMENTED DIET (PERIOD II) OVER A PERIOD OF APPROXIMATELY SIX MONTHS^a

Group No.	Vitamin A added per lb. food, I.U.	Carotenoids in yolk			Vitamin A in yolk			Vitamin A per egg (18 g. yolk)	
		Period I (control), $\mu\text{g./g.}$	Period II		Period I (Control), I.U./g.	Amount, ^b I.U./g.	M.D.; S.E.M.D. ^c	Period I, I.U.	Period II, I.U.
			Amount, ^b $\mu\text{g./g.}$	M.D.; S.E.M.D. ^c					
1	0	38.3	32.3 $\pm$ 2.0	—	46.6	46.8 $\pm$ 0.7	—	840	844
2	1,000	36.1	29.4 $\pm$ 1.5	1.16	44.6	46.7 $\pm$ 0.8	—	803	842
3	2,000	40.2	33.0 $\pm$ 1.7	0.27	46.8	46.3 $\pm$ 1.3	—	844	835
4	15,000	36.8	25.3 $\pm$ 0.7	3.30	47.9	48.4 $\pm$ 0.7	1.62	862	872
5	30,000	39.0	21.7 $\pm$ 1.1	4.65	48.6	54.6 $\pm$ 0.8	7.35	875	982
6	60,000 ^d	(29.2)	12.9 $\pm$ 0.8	9.12	(47.4)	62.3 $\pm$ 3.3	5.56	(854)	1122
7	100,000	38.0	9.7 $\pm$ 0.6	10.82	44.3	99.3 $\pm$ 3.3	15.53	798	1785
8	200,000 ^d	(29.2)	8.4 $\pm$ 0.5	11.62	(47.4)	120.7 $\pm$ 7.1	10.38	(854)	2170

^a H. J. Deuel, Jr., M. C. Hrubetz, F. H. Mattison, M. G. Morehouse, and A. Richardson, *J. Nutrition*, 26, 673-685 (1943), p. 679.

^b Including the Standard Error of the Mean.

^c When this value exceeds 3.0, the results are considered to be highly significant.

^d Groups 6 and 8 were made up of hens previously in Groups 3 and 4. The values in parentheses represent the average values of Groups 3 and 4 for the period of eight weeks immediately preceding the transfer.

any experiment was 226 I.U./g. yolk; this value was found in one egg from a hen in Group 8. The carotenoids were reduced in the egg yolk from a normal value of about 38 $\mu\text{g./g.}$ to a minimum value of 8.4 $\mu\text{g./g.}$ No effects which could not be attributed to seasonal changes were observable until as much as 15,000 I.U. of vitamin A was added as a supplement for each pound of food.

a. The Effect of Incubation on the Carotenoids and Vitamin A in Eggs.

It was found by Parrish *et al.*⁹²⁰ that the percentage of vitamin A in ester form in the yolk increased during incubation, and that the vitamin A in the embryo liver was predominantly in the ester form, the proportion in the liver being higher than that in the yolk.

Parrish and co-workers^{920a} reported also that the embryo in eggs from hens receiving 12,000 I.U. vitamin A/lb. food assimilated vitamin A exponentially throughout the period of incubation, while this occurred only until the eighteenth to twenty-first day, or the twelfth to eighteenth day, when the eggs were from hens receiving 3000 or 1800 I.U. vitamin A/lb., respectively. In the intermediate group (3000 I.U./lb.) at the time of hatching, 28% of the original vitamin A in the incubated egg was present in the yolk-free embryo, 45% was in the unabsorbed yolk, and the balance was lost. A similar loss of vitamin A occurred when infertile eggs were incubated. Neither the carotenes⁹²¹ nor the xanthophylls⁹⁰¹ in the yolk were metabolized during incubation. When the chicks were hatched, the livers contained only about 8% of the total body carotenoids.

(γ) *Antivitamin A*

Meunier and associates^{922, 922a} prepared an oxidation derivative of vitamin A (Substance Z) which acts as an antivitamin, and hence is called "antivitamin A." In this compound, one of the double bonds on the side chain is believed to be hydroxylated. It is produced when β -carotene or vitamin A is oxidized with vanadium tetroxide (V_2O_4).^{922-922b} Retinene₁ is formed as an intermediate product. Meunier^{922a} proposed two possible formulae for antivitamin A, as given below:

⁹²⁰ D. B. Parrish, R. N. Williams, and P. E. Sanford, *Arch. Biochem.*, **34**, 64-66 (1951).

^{920a} D. B. Parrish, R. N. Williams, and L. F. Payne, *Arch. Biochem.*, **29**, 1-6 (1950).

⁹²¹ P. Suomalainen, *Suomen Kemistilehti*, **12 B**, 30 (1939); *Chem. Abst.*, **34**, 145 (1940).

⁹²² P. Meunier, J. Jouanneteau, and R. Ferrando, *Compt. rend.*, **230**, 140-142 (1950).

^{922a} P. Meunier, *Fortschr. Chem. org. Naturstoffe*, **9**, 88-113 (1952).

^{922b} P. Meunier, G. Zwingelstein, J. Jouanneteau, and R. Mallein *Compt. rend.*, **230**, 1323-1324 (1950).

Suggested Formulae for Antivitamin A^{922a}

8. The Importance of Carotenoids and of Vitamins A in Vision

It has been recognized for many years that acuity of vision is related to a dietary component. Thus, it has been known since early Egyptian times that night blindness could be alleviated by the ingestion of animal livers; this was one of the first nutritional disorders for which a specific therapy was indicated. Night blindness has made its appearance sporadically in populations subjected to restricted diets, especially as a result of wars or famine. *Nyctalopia* denotes impaired vision in dim light. This "scotopic" vision is related to the activity of the rods in the retina. In contrast, *hemeralopia* is impaired vision in bright light; this is called "photopic" or daylight vision, and it is dependent upon cone receptors. However, the two terms have ordinarily been used interchangeably; hemeralopia has been employed as synonymous with night-blindness although, strictly speaking, it is an incorrect term. However, there is increasing evidence that both rod vision and cone vision are dependent upon the presence of vitamin A. Granit⁹²³ proposed a theory to explain scotopic and photopic vision which postulates the presence of *dominators* (a sensory mechanism characterized by a broad spectral sensitivity curve which makes a wide range of wave lengths available for vision), and *modulators*, which are characterized by a narrower response curve than that of the dominators; they are relatively light-resistant, and are suited to mediate wave length dis-

⁹²³ R. Granit, *Sensory Mechanism of the Retina*, Oxford Univ. Press, London, 1947, pp. 298 ff.

crimination. The modulator action spectra fall into groups located in three spectral areas. This type of mechanism is particularly efficient for color vision. It is believed that, under suitable conditions, vitamin A or retinene might give rise to "halochromic" molecules which resemble the modulators. Ball and Morton⁹²⁴ reviewed Granit's theory. Wald²³³ gave an excellent discussion of the biochemical systems involved in vision. In an earlier review, Wald²²⁴ discussed the biochemistry of visual systems and their distribution among animals.

(1) *Biochemical Systems Involved in Vision
in Higher Animals*

a. Retinal Pigments. Two kinds of organs occur in the retinae of most vertebrates, namely the rods (dim-light vision) and the cones (color and bright-light vision). In the case of the rods, the light-sensitive pigments *rhodopsin* and *porphyropsin* are present, or at least one of them, while in the cones the single active pigment is called *iodopsin*. All these substances belong to the category of carotenoid proteins, *i.e.*, proteins to which is attached a prosthetic group related to vitamin A.

(a) *Rhodopsin*. Rhodopsin is a rose-colored carotenoid protein which, in aqueous solution, has a single broad absorption band with a maximum at about 500 m μ . When exposed to light, it bleaches to an orange intermediate ("transient orange"), then to a yellow product ("visual yellow" or "indicator yellow") and finally to a colorless product known as "visual white." Rhodopsin is found chiefly in the rods of marine fishes and of land vertebrates. Wald^{905, 925} postulated that rhodopsin undergoes a series of reactions in light and dark.

Under ordinary conditions, in aqueous solutions, retinene and opsin are the final products when light acts upon rhodopsin. However, a reductase is present in the retina which can convert retinene into vitamin A alcohol.

(b) *Porphyropsin*. Porphyropsin is a purple carotenoid protein, which has been reported in the retinae of freshwater fishes and of some anadromous fishes, of lamprey eels, and of some amphibians. Wald⁷⁷⁸ identified it in white perch (*Morone americana*), yellow perch (*Perca flavescens*), and eastern pickerel (*Esox reticulatus*). According to Wald,⁹²⁶ porphyropsin undergoes reactions which are identical with those in which rhodopsin participates; in the case of porphyropsin, retinene₂ is the first product, and this is converted into vitamin A₂. The protein portion of the carotenoid

⁹²⁴ S. Ball and R. A. Morton, *Biochem. J.*, **45**, 298-304 (1949).

⁹²⁵ G. Wald, *J. Gen. Physiol.*, **21**, 795-832 (1937-1938).

⁹²⁶ G. Wald, *J. Gen. Physiol.*, **22**, 775-794 (1938-1939).

protein is opsin, and it is identical with the protein moiety of rhodopsin. Moreover, the enzymes which activate the porphyropsin system are identical with those which function in the rhodopsin system.

(c) *Iodopsin*. In 1937, Wald⁹²⁷ was the first to report a light-sensitive pigment (iodopsin) in the cones; this was identified in the retina of the chicken. Iodopsin was found to undergo the same series of reactions as did rhodopsin and porphyropsin. The carotenoid prosthetic group was the same as that in rhodopsin, namely retinene₁, while the protein differed.²²⁶ The family of proteins associated with rod vision are called *scotopsins*, while those concerned with cone vision are referred to as *photopsins*.

b. Vitamin A Aldehyde. (a) *Vitamin A₁ Aldehyde (Retinene₁)*. This constitutes the only type of retinene originally recognized. It is the only type of retinene formed when light acts upon rhodopsin. Wald and co-workers⁹²⁸ listed lumirhodopsin (a highly unstable, orange-red product), metarhodopsin, and then, in the presence of water, retinene, and protein, as the intermediates in the rhodopsin → retinene + protein reaction. Lumirhodopsin is converted to metarhodopsin in the dark.

Retinene (or retinene₁) was found, by Ball, Goodwin, and Morton,²²³ to be the aldehyde (C₁₉H₂₇CHO) of ordinary vitamin A alcohol (C₁₉H₂₇CH₂OH). For a description of vitamin A₁ aldehyde, including the chemical and biochemical procedures for synthesizing it, the reader is referred to an earlier section (see page 451).

(b) *Vitamin A₂ Aldehyde (Retinene₂)*. Retinene₂ was first characterized by Wald^{778, 926} as a component of the visual systems of fresh-water fishes; Wald⁹²⁶ also recognized its relationship to a vitamin A other than vitamin A₁. In addition to Wald,^{523, 805} a number of other investigators^{809, 810} recognized the fact that retinene₂ is vitamin A₂ aldehyde. Cama *et al.*⁷⁹⁰ demonstrated that vitamin A₂ aldehyde (retinene₂) was readily converted to vitamin A₂ in the rat. Retinene₂ can be prepared from vitamin A₂ by oxidation with manganese dioxide.²³⁵

c. The Alcohol Dehydrogenase System. In order that the retinal pigments may function in vision, it is necessary that provisions be made for their continual regeneration. If the sensation of sight requires the formation of retinene and protein, with the consequent bleaching of the retina, a new sensation cannot be recorded until the rhodopsin supply in the retina has been restored. Although some regeneration can occur by a direct combination of retinene and the appropriate protein, this is quite ineffective

⁹²⁷ G. Wald, *Nature*, 140, 545-546 (1937).

⁹²⁸ G. Wald, J. Durrell, and R. C. C. St. George, *Science*, 111, 179-181 (1950).

in the retina. Much of the retinene may be reduced to vitamin A alcohol immediately after it is set free.

Retinene reductase is a dehydrogenating enzyme which catalyzes the reversible change of both retinene₁ and retinene₂ to vitamins A₁ and A₂, respectively. The enzyme which occurs in the retina is similar to that present in the liver, intestine, and kidney. Cozymase is a required co-factor. For a further discussion of this enzyme system, the reader is referred to an earlier section of this chapter (see page 452).

(2) *The Synthesis of Rhodopsin in Higher Animals*

Rhodopsin can be synthesized in the retina by two types of reaction, namely from retinene plus opsin, or from vitamin A plus opsin. At an early date Kühne⁹²⁹ recognized that a fairly rapid synthesis of visual purple (rhodopsin) takes place by "anagenesis" from the yellow precursors; this reaction can proceed to some degree in the isolated retina, and even in solution. The second pathway for the synthesis of rhodopsin, which Kühne⁹²⁹ recognized as a slower one, is called "neogenesis"; this takes place from colorless precursors. It was believed that this reaction could occur in the living retina only when the coöperation of the pigment epithelium was available.

a. The Synthesis of Rhodopsin from Retinene. Hecht and co-workers⁹³⁰ and also Chase and Smith⁹³¹ have confirmed the observations of Kühne⁹²⁹ that rhodopsin can be regenerated in the dark in solutions which have been completely bleached. However, the extent of this regeneration is usually only 5 to 10%, with a maximum of 15%. On the other hand, Wald and Brown⁹³² were able to effect a 70% regeneration of bleached rhodopsin solutions to rhodopsin, when replaced in the dark, by supplementing them with high concentrations of retinene. The reaction is a spontaneous one which does not require an external source of energy. The bleaching of rhodopsin, with the consequent cleavage to opsin and retinene, is the reaction which requires energy, usually in the form of light.

b. The Synthesis of Rhodopsin from Vitamin A. The synthesis of rhodopsin from vitamin A and opsin has been observed only in living tissues. The difficulty appears to be in oxidizing vitamin A to retinene under *in vitro* conditions, since the equilibrium between vitamin A and retinene

⁹²⁹ W. Kühne, *Chemische Vorgänge in der Netzhaut*, in L. Hermann, *Handbuch der Physiologie*, Vol. 3, Part 1, F. C. W. Vogel, Leipzig, 1879, 235-342.

⁹³⁰ S. Hecht, A. M. Chase, S. Schlaer, and C. Haig, *Science*, 84, 331-333 (1936).

⁹³¹ A. M. Chase and E. L. Smith, *J. Gen. Physiol.*, 23, 21-39 (1939-1940).

⁹³² G. Wald and P. K. Brown, *Proc. Nat. Acad. Sci., U.S.*, 36, 84-92 (1950).

favors reduction rather than oxidation. However, it has been found that the dehydrogenase system can bring about an oxidation if the aldehyde so formed is trapped, so that it is immediately removed from the sphere of action. Cyanide, bisulfite, and semicarbazide have been employed for this function, but Wald and Hubbard⁹³³ used hydroxylamine successfully; this behaves as shown below:

The Method of "Trapping" Retinene by Condensation with Hydroxylamine to Form Retinene Oxime²³³

Wald and Hubbard⁹³³ were able to effect a considerable oxidation of vitamin A to retinene by the alcohol dehydrogenase system, in the presence of hydroxylamine. Since opsin is a good aldehyde de-trapping agent, it should be able to bring about the formation of rhodopsin.

Fig. 1. Diagram of the rhodopsin system. (The bulk of the system lies within the outer segments of the retinal rods, but it must be supplemented with vitamin A, respiratory factors, and oxygen itself, from the pigment epithelium and from the circulatory blood.)⁹³⁴

⁹³³ G. Wald and R. Hubbard, *Proc. Nat. Acad. Sci., U.S.*, 36, 92-102 (1950).

⁹³⁴ G. Wald, *Science*, 113, 287-291 (1951).

However, it was impossible at first to bring about any appreciable synthesis of rhodopsin when ordinary vitamin A and opsin were mixed, even when the conditions outlined above were observed. Hubbard and Wald²³¹ found that the addition of succinoxidase in conjunction with retinal homogenate increased the yield of rhodopsin by 33 to 50%. The addition of vitamin A and succinoxidase from beef heart²³¹ increased the synthesis of rhodopsin by 65%.

Fig. 2. Geometrical isomers of retinene and of vitamin A in the rhodopsin cycle.⁹³⁵

The succinoxidase functions by keeping the cozymase oxidized. Hubbard and Wald²³¹ consider that the entire system consists of the following components: vitamin A (fish liver oils), alcohol dehydrogenase (horse liver), cozymase (yeast), and opsin (retina). It has been found that, with this mixture, rhodopsin is synthesized in the dark. When returned to the light, the system is again bleached. According to this concept, vitamin A functions as a source of rhodopsin only after it is oxidized to retinene under certain special conditions; the rest of the synthesis follows the same pathway as that of retinene and of opsin. Figure 1 illustrates this modified concept of the rhodopsin system. Presumably, the synthesis of porphyropsin follows the same pattern as does that of rhodopsin.

(a) *Neo-b Retinene as a Required Precursor.* Although Wald²³³ was able to demonstrate a functioning system when the vitamin A was a natural product, almost no rhodopsin was formed when a crystalline vitamin A was employed in place of the natural product. It was concluded that only certain stereoisomers of vitamin A are able to form the types of retinene which are effective in combining with opsin to form rhodopsin. In substantiation of this theory, Hubbard and Wald⁹³⁵ observed that, although no rhodopsin resulted when all-trans-vitamin A or neo-a vitamin A

⁹³⁵ R. Hubbard and G. Wald, *J. Gen. Physiol.*, **36**, 269-315 (1952-1953).

was added to the four-component system in the dark, a synthesis was immediately effected in all cases, when the inactive vitamins A were exposed to light in the presence of iodine. This latter procedure has been widely employed by Zechmeister⁷⁷² to produce isomerization of carotenoids; all possible isomers result in the mixture when any single carotenoid is exposed to the above treatment. For a further discussion of this effect, the reader is referred to *The Lipids*, Vol. I, pages 635, 636.

Although the theory as proposed by Pauling⁷⁹³ limits the number of isomers of vitamin A to four, Wald and associates^{797,820,935} examined five crystalline isomers of retinene, namely all-*trans*-retinene, neo-*a* retinene, neo-*b* retinene, iso-*a* retinene, and iso-*b* retinene. On incubation with opsin, all-*trans* retinene and neo-*a* retinene gave negative results. On the other hand, neo-*b* retinene produced a rapid synthesis of rhodopsin, which was indistinguishable from the reaction when the retinene extracted from the retina was the substrate. Iso-*a* retinene produced about the same amount of pigment as did neo-*b* vitamin A, but the η max. of this new pigment was displaced about 13 $m\mu$ below that of neoretinene. Presumably it differs from rhodopsin. This new pigment is now referred to as isorhodopsin. Although iso-*b* retinene was also found to be inactive, it was rapidly isomerized to iso-*a* retinene by a very short exposure to light; the latter product readily formed isorhodopsin. In spite of the Pauling theory, which would limit the isomers of vitamin A to four, all five products studied by Wald and co-workers^{797,820,935} were readily interconvertible, and are believed to be *cis-trans* isomers.

In view of the newer information concerning the role of the stereoisomeric retinenes in rhodopsin formation, Hubbard and Wald⁹³⁵ proposed a modification of the rhodopsin cycle, which is pictured in Figure 2.

According to the above scheme (Figure 2), retinene enters into the rhodopsin synthesis as neo-*b* retinene, but emerges from it on bleaching as all-*trans* retinene. The latter must be reisolomerized to the neo-*b* retinene before it can be used to regenerate rhodopsin.⁹³⁵ As an alternate pathway, all-*trans* retinene can be reduced to all-*trans* vitamin A, which in turn is isomerized to neo-*b* vitamin A; the latter compound is oxidized to neo-*b* retinene by the alcohol dehydrogenase system, after which it is resynthesized to rhodopsin. All-*trans* vitamin A may be discharged into the blood stream,⁸⁰⁵ and new supplies of neo-*b* vitamin A may then be selected from it.⁹³⁵ According to Wald *et al.*,²²⁶ the iodopsin in the chicken retina has precisely the same carotenoid pattern as does the rhodopsin cycle.

There is considerable evidence that stereoisomeric changes can be effected within the animal. Thus, it has already been pointed out that, when

cis-carotenoids are fed to rats and to chickens, some all-*trans* forms can be isolated from their tissues. Neo-*a* vitamin A, studied by Robeson and Baxter,⁷⁹⁴ and by Hubbard and Wald,⁹³⁵ and 3-*cis*-retinene, prepared by Graham and co-workers,⁸¹⁸ yield bioassays comparable with those obtained with the corresponding all-*trans* compounds. This fact has been cited as evidence of the biologic transformation of the *cis* and *trans* forms.²³³ Moreover, when rats were fed neovitamin A, characteristic mixtures of all-*trans* and 3-*cis* retinene were deposited in the liver.^{794,935} Although the speed of isomerization of vitamin A in the body is not known, it does occur at a rate sufficiently rapid to allow effective growth. Wald²³³ points out that, inasmuch as a temperature as low as 60°C. is known to cause stereoisomerization *in vitro* within one to two hours, the temperature of the animal (37°C.) might catalyze stereoisomeric changes at a slower rate, possibly over a period of a day. To explain the role of the retinal tissues in effecting stereoisomerism *in vivo*, Wald²³³ proposed the hypothesis that enzymes may cause stereoisomerization.

(3) *Species Variations in Biochemical Requirements for Vision*

Wald²²⁴ presented evidence that plants as well as animals employ carotenoids in the primary processes of photoreception. Light, which stimulates photokinetic responses in many different plant structures, is absorbed by the carotenes and xanthophylls in their tissues. Astaxanthin, which is ordinarily found only in animal tissues, also appears in the intermediate forms of the unicellular zoophytes (*Protista*), for example in the green euglena (*Euglena viridis*), in other species of *Euglena*, and in the red, freshwater protozoan (*Haematococcus* spp.), where it is concentrated in the eye-spots. Although the chemical mechanism of photoreception in invertebrates is not well understood, vitamin A₁ and retinene₁ occur in the image-forming eyes of the molluscs and arthropods. Vitamin A₁ and retinene₁ continue to play the dominant role among the retinal pigments of the higher species, except that, in the case of freshwater species of fishes, rhodopsin is replaced by porphyropsin, and vitamin A₁ and retinene₁ are replaced by vitamin A₂ and retinene₂. The salmon and eels which inhabit both fresh water and salt water have both the porphyropsin and the rhodopsin systems concomitantly. Both systems likewise occur in some amphibia. In marine fishes, as well as in land vertebrates, vitamin A₁ and retinene₁ appear alone in the retinal pigments. Astaxanthin is present not only in the eye-spots of the *Protista*, but also in the eyes (and integument) of most of the crustaceans, and in the cones of certain birds and turtles, where it is

believed to play a role in color differentiation. It is also restricted to the retina in the case of the chicken. It would therefore seem probable that the carotenoids and vitamins A have a molecular structure which renders them particularly adaptable in photoreception. For a further discussion of the distribution and photoreceptor function of carotenoids and of vitamins A, the reader is referred to the classical review of Wald,²²⁴ and to the discussion in *The Lipids*, Vol. I, pages 680 to 683.

9. The Requirements of Animals for Carotenoids and for Vitamins A

(1) Introduction

In assessing the requirement of an animal for vitamin A, one is faced with the problem as to what criteria should be employed for such an evaluation. In the past, it has usually been considered that the minimum requirement necessary to provide satisfactory growth and to prevent lesions and dysfunctions due to vitamin A deficiency represents an approximation of the level in question. However, the above procedure for determining the vitamin A requirement is arbitrary.

Various physiologic and pathologic conditions cause a variation in the vitamin A requirement. Moreover, factors which affect the absorption of vitamin A will influence the amount needed in the diet. Any situation which alters the conversion of β -carotene to vitamin A will influence the quantity of vitamin A required insofar as the source of the vitamin is provitamin A. The vitamin requirements of a number of species have been reviewed recently, including that of the rat by Brown and Sturtevant,⁹³⁶ that of the mouse by Morris,⁹³⁷ that of chickens, pigeons, turkeys, and ducks by Bird,⁹³⁸ that of the cotton rat and the hamster by Schweigert,⁹³⁹ and that of the guinea pig by Mannering.⁹⁴⁰ The most up-to-date review of vitamin A requirements is that of Rubin and De Ritter,⁹⁴¹ published in 1954. Other recent articles include the summary of the requirements of animals by Mattson,⁷⁶³ and of human beings reported by Mason.⁹⁴²

The criteria which have been employed for determining the vitamin A requirement include the amount necessary for good or optimum growth,⁹⁵⁰

⁹³⁶ R. A. Brown and M. Sturtevant, *Vitamins and Hormones*, 7, 171-199 (1949).

⁹³⁷ H. P. Morris, *Vitamins and Hormones*, 5, 175-195 (1947).

⁹³⁸ H. R. Bird, *Vitamins and Hormones*, 5, 163-173 (1947).

⁹³⁹ B. S. Schweigert, *Vitamins and Hormones*, 6, 55-67 (1948).

⁹⁴⁰ G. J. Mannering, *Vitamins and Hormones*, 7, 201-221 (1949).

⁹⁴¹ S. H. Rubin and E. De Ritter, *Vitamins and Hormones*, 12, 101-135 (1954).

⁹⁴² K. E. Mason, *Requirements of Human Beings*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 1954, pp. 171-175.

for maximum life span,⁹⁴³⁻⁹⁴⁶ for satisfactory tooth structure,⁹⁴⁶ for the maintenance of an optimum plasma vitamin A level,³⁵⁰ for the provision of adequate liver stores,⁹⁴⁷ and the amount needed to produce the optimum vitamin A content in the retina.³⁵⁰ The vaginal smear method has been employed in rats^{948,949} since the discovery by Evans and Bishop⁹⁵⁰ of the relationship of the vaginal epithelium to vitamin A intake. Mason and Ellison⁹⁵¹ described the method in detail. Tooth color is a useful criterion,^{946,952} and the curative action of the product on xerophthalmia offers another approach to the subject.^{353,659,953} Lewis *et al.*³⁵⁰ used the amount of vitamin A required to prevent deficiency symptoms (xerophthalmia, loss of weight) as a criterion.

The growth method as described by Coward⁶ has been widely employed as a bioassay method for the determination of vitamin A. With certain modifications, it has been accepted as the official method for bioassay by the U. S. Pharmacopeia Convention.⁹⁵⁴ Guerrant⁹⁵⁵ and Bliss and György⁹⁵⁶ described (1951) the growth method and other assay procedures in more detail. While the growth method is extremely accurate when the sensitive and linear portion of the log-dose-growth response curve is studied, the determination of the requirement for optimum growth makes use of the extreme portions of the curve, where there is great likelihood of error, and the results may be quite inaccurate. According to Hegsted,⁹⁵⁷ the errors in this measurement (statistical dose-response) are such as to lead to low estimates for requirements.

In the case of chickens, hatchability of eggs and survival of the chicks

⁹⁴³ H. C. Sherman, H. L. Campbell, M. Udiljak, and H. Yarmolinsky, *Proc. Nat. Acad. Sci., U.S.*, **31**, 107-109 (1945).

⁹⁴⁴ H. C. Sherman and H. Y. Trupp, *Proc. Nat. Acad. Sci., U.S.*, **35**, 90-92 (1949).

⁹⁴⁵ H. C. Sherman and H. Y. Trupp, *J. Nutrition*, **37**, 467-474 (1949).

⁹⁴⁶ H. E. Paul and M. F. Paul, *J. Nutrition*, **31**, 67-78 (1946).

⁹⁴⁷ K. Guggenheim and W. Koch, *Biochem. J.*, **38**, 256-260 (1944).

⁹⁴⁸ H. Goss and H. R. Guilbert, *J. Nutrition*, **18**, 169-179 (1939).

⁹⁴⁹ H. R. Guilbert, C. E. Howell, and G. H. Hart, *J. Nutrition*, **19**, 91-103 (1940).

⁹⁵⁰ H. M. Evans and K. S. Bishop, *Anat. Record*, **23**, 17-18 (1922).

⁹⁵¹ K. E. Mason and E. T. Ellison, *J. Nutrition*, **10**, 1-11 (1935).

⁹⁵² J. T. Irving and M. B. Richards, *Nature*, **144**, 908-909 (1939).

⁹⁵³ C. A. Baumann, B. M. Riising, and H. Steenbock, *Z. Vitaminforsch.*, **3**, 81-89 (1934).

⁹⁵⁴ Anonymous, *U. S. Pharmacopeia*, XIV, 1950, pp. 787-792.

⁹⁵⁵ N. B. Guerrant, *General Aspects of Small Animal Experimentation*, in P. György, *Vitamin Methods*, Vol. 2, Academic Press, New York, 1951, pp. 1-40.

⁹⁵⁶ C. I. Bliss and P. György, *The Animal Vitamin Assays*, in P. György, *Vitamin Methods*, Vol. 2, Academic Press, New York, 1951, pp. 41-275.

⁹⁵⁷ D. M. Hegsted, *J. Nutrition*, **35**, 399-409 (1948).

have been among the criteria used;⁹⁵⁸ Sherwood and Fraps⁹⁵⁹ included fertility and egg production as well. The quantity of vitamin A necessary to prevent or cure night blindness was employed by Guilbert *et al.*⁹⁴⁹ to ascertain the vitamin A requirement of swine, cattle, sheep, and horses. The dark adaptation test is an application of a similar physiologic condition in man⁹⁶⁰⁻⁹⁶⁴ which is widely used for a clinical evaluation of the vitamin A requirement. Lactation is a criterion employed for the evaluation of the vitamin A requirement of sheep^{965,966} and of horses.⁹⁶⁶

(2) *Factors Altering the Requirement for
β-Carotene and for Vitamin A*

a. Species. If the vitamin A requirement is based upon the amount necessary for the whole animal to satisfy its daily vitamin A needs, it is obvious that marked variations will obtain, according to the size of the animal. Guilbert and Hart⁹⁶⁷ first called attention to this point in 1935, and subsequent studies have confirmed and strengthened the hypothesis. As in other biometric phenomena, it seems that vitamin A requirements might be more uniform in different animals with wide ranges of body weight if expressed on the basis of surface area rather than on that of body weight.

According to the summary of Mattson,⁷⁶³ the average minimum daily requirement to support growth and to prevent gross symptoms of deficiency in mammals is 24 μg. of β-carotene (40 I.U.) or 20 I.U. of vitamin A per kg. of body weight. When the figure is based upon storage, reproduction, and lactation, considerably higher values are observed for the vitamin A requirement. The proportion of carotene or of vitamin A required by chickens and turkeys is considerably higher than that needed by mammals.

⁹⁵⁸ H. J. Almquist and E. Mecchi, *Poultry Sci.*, **18**, 129-137 (1939).

⁹⁵⁹ R. M. Sherwood and G. S. Fraps, *Texas Agr. Expt. Sta., Bull. No. 573*, 5-21 (1940).

⁹⁶⁰ S. Hecht and S. Shlaer, *J. Opt. Soc. Amer.*, **28**, 269-275 (1938).

⁹⁶¹ J. M. Lewis and C. Haig, *J. Pediat.*, **15**, 812-823 (1939).

⁹⁶² L. E. Booher and E. C. Callison, *J. Nutrition*, **18**, 459-471 (1939).

⁹⁶³ E. L. Batchelder and J. C. Ebbs, *J. Nutrition*, **27**, 295-302 (1944).

⁹⁶⁴ E. M. Hume and H. A. Krebs, *Vitamin A Requirement of Human Adults. An Experimental Study of Vitamin A Deprivation in Man* (A Report of the Vitamin A Subcommittee of the Accessory Food Factors Committee), *Med. Research Council (Brit.)*, *Spec. Rept. Ser. No. 264* (1949).

⁹⁶⁵ P. B. Pearson, H. M. Briggs, W. G. Kammlade, J. I. Miller, and R. F. Miller, *Recommended Nutrient Allowances for Domestic Animals. V. Recommended Nutrient Allowances for Sheep*, Committee on Animal Nutrition, Nat. Research Council, Washington, D. C. (1945), pp. 1-20.

⁹⁶⁶ H. R. Guilbert and J. K. Loosli, *J. Animal Sci.*, **10**, 22-41 (1951).

⁹⁶⁷ H. R. Guilbert and G. H. Hart, *J. Nutrition*, **10**, 409-427 (1935).

Whereas β -carotene is only about one-half as effective as a source of vitamin A as is the preformed vitamin A itself at a moderate intake, the relative potency of the β -carotene decreases with increased intakes, both in mammals⁹⁴⁹ and in birds.⁹⁶⁸

(a) *Rat*. Since the rat has been widely used for nutrition experiments, considerable data have been amassed on the vitamin A requirements of this

Fig. 3. The relation of the vitamin A intake per day to the growth rate of rats.³⁵² (From the original of Fig. 1, Lewis, Bodansky, Falk, and McGuire, *J. Nutrition*, 23, 354 (1942).)

species.⁹⁶⁹ Most of the information is based upon the amount of vitamin A required to prevent deficiencies in growth, and anomalies such as cornification of the epithelial cells, xerophthalmia, reduction of the erythrocyte count and of the hemoglobin content, severe anemia, infertility, and fetal resorption, as well as gross dental and ocular abnormalities.

On the basis of daily requirements, the following averages (in I.U.) for the vitamin A needs of rats have been recorded. Several different criteria for evaluation were employed. When the requirement was calculated as

⁹⁶⁸ R. B. Nestler, J. V. Derby, and J. B. DeWitt, *J. Nutrition*, 36, 323-329 (1948).

⁹⁶⁹ R. W. Little, A. W. Thomas, and H. C. Sherman, *J. Biol. Chem.*, 148, 441-443 (1943).

I.U./day/kg. body weight, the minimum level required to prevent vaginal cornification (as determined by vaginal smears)^{679,948,949} was found to be 18 to 22; to avoid gross and histologic evidence of deficiency, 20;³⁵⁰ to ensure growth, 80;⁶⁷⁹ for adequate growth, longevity and tooth color, 100;⁹⁴⁶ for optimum growth, 25;³⁵⁰ for minimum liver storage, 50 to 80;⁶⁷⁹ for adequate liver storage, 100;³⁵⁰ for maintenance of normal blood levels, 50.³⁵⁰

When the daily requirement was based upon I.U./100 g. of food, growth was found to require 20⁶⁷⁹ and 40;⁹³⁶ and optimum longevity, 1,200.^{943,945,970}

When the basis of the vitamin A requirement of the rat was the amount (in I.U.) needed per whole rat per day, the results also presented wide variations, according to the procedures employed, *viz.*, liver storage, 12⁹⁶⁹ and 100;³⁵⁰ blood level, 20³⁵³ and 50;³⁵⁰ growth;³⁵⁰ cure of xerophthalmia, 4;³⁵³ and tooth color, 3.⁹⁵²

As an illustration of the data upon which the above estimates of the vitamin A requirements of the rats are based, one may observe the effect upon growth of different amounts of vitamin A, as shown in Figure 3.

TABLE 9
THE RELATION OF THE VITAMIN A INTAKE OF RATS
TO THE VITAMIN A CONTENT OF PLASMA,
LIVER, AND RETINA^a

Daily vitamin A intake for 6 weeks, I.U.	Animals in group No.	Vitamin A		
		In blood plasma, I.U./100 ml.	In liver, I.U./g.	In retina, I.U./g.
0	13	0	0	14
1	11	7	0	—
2	31	14	0	20
10	31	35	0	25
25	16	69	3	20
50	15	100	34	—
100	21	112	113	26
1000	26	110	1270	25

^a Adapted from J. M. Lewis, O. Bodansky, K. G. Falk, and G. McGuire, *J. Nutrition*, 23, 351-363 (1952).

Other experimental data which have been employed to assess the vitamin A requirement of rats, based upon blood level, liver storage, and the concentration of vitamin A in the retina, are given in Table 9, while Table 10 summarizes the relationship of life span to vitamin A intake.

⁹⁷⁰ H. C. Sherman and H. L. Campbell, *Proc. Nat. Acad. Sci., U. S.*, 31, 164-166 (1945).

TABLE 10
THE EFFECT OF THE VITAMIN A INTAKE ON THE
AVERAGE SURVIVAL TIME OF MALE AND
FEMALE RATS^a

Daily vitamin A intake, I.U./100 g. body weight	Number of rats		Average age at death		
	Males	Females	Males, days	Females, days	All rats, days
1	9	14	81	79	80 ± 2
2	9	10	204	260	234 ± 18
4	10	10	478	564	521 ± 28
20	10	10	622	675	649 ± 30

^a H. E. Paul and M. F. Paul, *J. Nutrition*, 31, 67-78 (1946).

It is thus obvious that the optimum requirement of the rat for vitamin A has been reported at values varying between 10 and 100 I.U. per kg. per day, depending upon what criterion is used for the assessment. The usual criteria, such as good growth and prevention of deficiency symptoms, can be satisfied when daily intakes between 20 and 100 I.U. per 100 kg. body weight are used as the basis.

(b) *Mouse*. According to McCarthy and Cerecedo,^{971,972} the vitamin A requirement of the mouse is somewhat less than one I.U. per animal per day. The weanling mouse is able to withstand long periods of vitamin A depletion because of the large, initial embryonic stores present at weaning.⁹⁷³ It was recorded that more stringent conditions are required to produce deficiency in the mouse than in the rat.⁹⁷³ When the young from mothers on a vitamin A-deficient diet were continued on the same diet, death occurred in some cases before the development of any extensive changes in the epithelium, or of structural changes in the eye; however, some injury did occur to the eyes, as evidenced by the production of a thick, clear, colorless exudate. The reproductive function in the male mouse is especially sensitive to a deficiency in vitamin A; however, one I.U. is quite adequate for the normal sex function in the male.⁹⁷²

(c) *Guinea Pig*. Little is known about the requirements of the guinea pig for vitamin A, although Mannering⁹⁴⁰ recorded the fact that this species requires a frequent intake of the vitamin in the diet. According to Bentley and Morgan,⁵⁹³ at least 2 mg. of carotene (3330 I.U.) per kg. body weight are required before an appreciable amount of vitamin A is stored in the

⁹⁷¹ P. McCarthy and L. R. Cerecedo, *Federation Proc.*, 9, 364 (1950).

⁹⁷² P. T. McCarthy and L. R. Cerecedo, *J. Nutrition*, 49, 357-367 (1953).

⁹⁷³ P. T. McCarthy and L. R. Cerecedo, *J. Nutrition*, 46, 361-376 (1952).

liver. Preformed vitamin A was found to be six times as effective as carotene in promoting storage of vitamin A in the liver.

(d) *Rabbit*. Although Pirie and Wood⁹⁷⁴ demonstrated that the rabbit requires vitamin A, no quantitative data are available. In the case of animals on vitamin A-free diets, impairment of growth, corneal lesions, and reduction of the ascorbic acid content of the aqueous humor were noted. Rabbits fed on a vitamin A-free diet were maintained in a satisfactory state of health when they were supplemented with carotene in an amount of 50 $\mu\text{g.}/\text{kg.}$ of body weight/day.⁹⁷⁵ The remedial dose was considerably more than 70 $\mu\text{g.}$

(e) *Dog*. On the basis of the rate of depletion of the vitamin A supply in the liver, Crimm and Short⁹⁷⁶ cited the daily requirement of the dog as 22 to 47 I.U. of vitamin A/kg. of body weight. Frohring⁹⁷⁷ cited a figure of 100 I.U./kg./day, based upon liver storage of vitamin A. On the other hand, Morgan⁹⁷⁸ recorded a value of somewhat over 800 I.U./kg./day when "optimum health" was the criterion, while Michaud and Elvehjem⁹⁷⁹ estimated the requirement as 20 $\mu\text{g.}$ (66 I.U.) per kg. body weight as vitamin A, or 40 to 60 $\mu\text{g.}$ (66 to 100 I.U.) per kg. weight using β -carotene as the source of the vitamin A. The deficiency symptoms in the dog included loss of appetite, eye infections, a peculiar, divergent strabismus, atoxia, nervous running around the cage in circles, as well as the usual skin symptoms and corneal lesions.

(f) *Chickens, Turkeys, and Other Fowl*. The estimates of the requirement of fowl for vitamin A have been made under two categories, *i.e.*, that of the young growing bird and that of the adult birds, including the laying hen. With and Wanscher⁹⁸⁰ found that chicks required 100 to 200 I.U./kg. body weight daily for growth, and 210 I.U./kg. weight for adequate liver storage. When growth is used as the main criterion, and the estimates are based upon I.U./100 g. feed, there is a wide range in the estimates for chicks, varying from a low figure of 87 I.U./100 g. food⁹⁸¹ to a high value of 355.⁹⁸² Other estimates based upon growth fall between these extreme

⁹⁷⁴ A. Pirie and C. Wood, *Biochem. J.*, **40**, 557-560 (1946).

⁹⁷⁵ P. H. Phillips and G. J. Bohstedt, *J. Nutrition*, **15**, 309-319 (1938).

⁹⁷⁶ P. D. Crimm and D. M. Short, *Am. J. Physiol.*, **118**, 477-482 (1937).

⁹⁷⁷ W. O. Frohring, *Vet. Med.*, **32**, 76-82 (1937).

⁹⁷⁸ A. F. Morgan, *North. Am. Veterinarian*, **21**, 476-486 (1940).

⁹⁷⁹ L. Michaud and C. A. Elvehjem, *Nutrition Abst. & Revs.*, **13**, 323-331 (1944).

⁹⁸⁰ T. K. With and O. Wanscher, *Vitamine u. Hormone*, **4**, 341-366 (1943).

⁹⁸¹ G. E. Bearse and M. W. Miller, *Poultry Sci.*, **16**, 34-38 (1937).

⁹⁸² E. L. Johnson, C. W. Carrick, and S. M. Hauge, *Poultry Sci.*, **27**, 308-314 (1948).

limits.^{956,959,983-986} The vitamin A requirement for laying hens is estimated as 483 I.U./100 g. food.⁹⁵⁷ On the basis of liver storage, a figure as high as 375 I.U./100 g. food (2,250 μ g. carotene/100 g. ration) was proposed by Bolin *et al.*⁹⁸⁸ The recommendation of the National Research Council Committee on Nutrient Allowances for Poultry⁹⁸⁹ is that the allowance of vitamin A for growing chicks be set at 266 I.U./100 g. food.

The vitamin A requirements of adult chickens, and especially of laying hens, are considerably higher than those for the growing chicks. This is to be expected, since the egg contains a fairly large amount of vitamin A. The suggested levels for vitamin A in the diet for satisfactory nutritional results vary from a low value of 440 to 460 I.U./100 g. food^{958,990} to a high figure of 920 I.U./100 g. food.⁹⁵⁹ The requirement for pullets is given as 333 I.U./100 g. food. The level needed for efficient egg production is 833 I.U./100 g. food, and 1000 I.U. if the eggs are to be used for hatching.⁹⁵⁹ Rubin and Bird⁹⁹¹ give this requirement as 1250.4 I.U. of vitamin A. The recommendation of the National Research Council (Bird *et al.*⁹⁸⁹) is 440 I.U./100 g. food. Intermediate levels of vitamin A have been recommended by several investigators.^{983,987} In arriving at the recommended daily allowance of vitamin A for adult birds, not only egg-production has been considered, but also hatchability of eggs, survival of chicks, mortality of hens, maintenance of laying hens, maintenance of breeding hens, and liver storage. Heywang⁹⁹² found that 551 I.U./100 g. feed was sufficient for egg production and hatchability in cool weather, but that the requirement was higher in hot weather.

Several groups of investigators^{983,993,994} reported that carotene is as ac-

⁹⁸³ F. F. Castano, R. V. Boucher, and E. W. Callenbach, *J. Nutrition*, **45**, 131-141 (1951).

⁹⁸⁴ H. J. Almquist, T. H. Jukes, and W. E. Newlon, *Calif. Agr. Extension Circ.*, **108**, 1-38 (Aug. 1938).

⁹⁸⁵ M. W. Taylor and W. C. Russell, *Poultry Sci.*, **26**, 234-242 (1947).

⁹⁸⁶ R. E. Gray and H. E. Robinson, *Poultry Sci.*, **20**, 36-41 (1941).

⁹⁸⁷ G. F. McClary, V. L. Miller, and G. E. Bearse, *Western Wash. Expt. Sta. Bull.*, Dept. Poultry Husbandry (Dec. 1942), pp. 56-57.

⁹⁸⁸ D. W. Bolin, C. E. Lampman, and L. R. Berg, *Poultry Sci.*, **22**, 348-353 (1943).

⁹⁸⁹ H. R. Bird, H. J. Almquist, W. W. Cravens, F. W. Hill, and J. McGinnis, *Nutrient Requirements for Domestic Animals. I. Nutrient Requirements for Poultry*, Nat. Research Council (U.S.), Agr. Bd., Div. Biol. Agr., Washington, 1954, pp. 1-27.

⁹⁹⁰ M. W. Taylor, J. R. Stern, W. C. Russell, and E. Jungherr, *Poultry Sci.*, **26**, 243-254 (1947).

⁹⁹¹ M. Rubin and H. R. Bird, *Univ. Maryland Agr. Expt. Sta.*, **55**, *Bull. A-12*, 339-350 (1942).

⁹⁹² B. W. Heywang, *Poultry Sci.*, **31**, 294-301 (1952).

⁹⁹³ R. M. Bethke, P. R. Record, and O. H. M. Wilder, *Poultry Sci.*, **18**, 179-187 (1939).

⁹⁹⁴ J. B. Reynolds, D. B. Parrish, H. L. Mitchell, and L. F. Payne, *Poultry Sci.*, **27**, 182-185 (1948).

tive as vitamin A in the chick when the criterion of activity is growth. On the other hand, Castano and co-workers⁹⁸³ observed that vitamin A exhibits a greater efficiency in the liver storage test than does β -carotene. Johnson *et al.*⁹⁸² reported the opposite result, and Rubin and Bird⁹⁹⁵ found that β -carotene and vitamin A were equally effective in this respect. There appears to be little doubt that vitamin A is more readily absorbed in the chicken than is carotene, especially when fat is absent from the diet.¹⁰³ Hogan⁹⁹⁶ contributed a critical review on the subject of vitamin A requirements of chicks and of laying hens.

The figures for the vitamin A requirement of turkey poults are consistently higher than those for growing chicks. The following values (expressed as I.U./100 g. food) have been recorded: growth, 440;²⁰⁴ growth and signs of deficiency, 300 to 500;⁹⁹⁷ and 500;^{998,999} growth and mortality, 600;¹⁰⁰⁰ optimum growth for broad-breasted turkeys, and liver storage, 1700;¹⁰⁰¹ liver storage, deficiency symptoms, and mortality, 1733, according to Hinshaw and Lloyd,¹⁰⁰² as cited by Hogan.⁹⁹⁶ Asmundson and Jukes¹⁰⁰³ recommended 1000 I.U./100 g. food as the proper level of vitamin A in the ration of turkey poults, but the basis for reaching their decision was not stated. Bird *et al.*⁹⁹⁹ estimated the satisfactory level for growing turkeys as 533 I.U./100 g. food. According to Gurcay and co-workers,²⁰⁴ vitamin A acetate is four times as effective as β -carotene, and twice as active as black-cod liver oil, in promoting growth in turkey poults. On the other hand, vitamin A acetate was found to be twenty to thirty times as effective as β -carotene, and about twice as active as black-cod liver oil in producing liver storage of vitamin A. However, Kramke *et al.*⁹⁹⁹ stated that 500 I.U./100 g. food was effective for optimum growth, whether the vitamin A was given as the alcohol, as natural or synthetic esters, or in the form of carotene in alfalfa or in wheat-germ oil.

The vitamin A requirement of the North American quail or bobwhite (*Colinus (Ortyx) virginianus*) was reported as 640 I.U./100 g. feed, by Nest-

⁹⁹⁵ M. Rubin and H. R. Bird, *Poultry Sci.*, 20, 155-160, 291-297 (1941).

⁹⁹⁶ A. G. Hogan, *Nutrition Abst. & Revs.*, 19, 751-791 (1950).

⁹⁹⁷ R. Van Reen, M. W. Taylor, and W. C. Russell, *J. Nutrition*, 43, 235-254 (1951).

⁹⁹⁸ W. C. Russell, M. W. Taylor, and R. Van Reen, *Federation Proc.*, 8, 394 (1949).

⁹⁹⁹ E. H. Kramke, M. D. Lloyd, and J. C. Fritz, *Poultry Sci.*, 31, 49-53 (1952).

¹⁰⁰⁰ H. S. Wilgus, Jr., *Colorado Farm Bull.*, 2, 3-4 (1940).

¹⁰⁰¹ F. D. Wharton, L. D. Matterson, H. M. Scott, and C. I. Bliss, *J. Nutrition*, 39, 543-554 (1949).

¹⁰⁰² W. R. Hinshaw and W. E. Lloyd, *Hilgardia*, 8, 281-304 (1934).

¹⁰⁰³ V. S. Asmundson and T. H. Jukes, *California Agr. Expt. Sta., Ext. Serv. Circ.*, No. 110, 1-78 (March, 1939).

ler and associates.⁹⁶⁸ Harper and co-workers¹⁰⁰⁴ cited somewhat higher values for the quail, namely, 1222 I.U./100 g. food for liver storage, and 777 I.U./100 g. food for growth and survival. Although Wolbach and Hegsted¹⁰⁰⁵ demonstrated that the duck requires vitamin A, the exact quantity of vitamin A needed was not determined.

(g) *Swine*. The requirement of swine for vitamin A is in the same range as that of other animals, but varies according to the criterion under investigation. The smallest amount is required to prevent night blindness (18 to 24 I.U./kg. body weight),⁹⁴⁹ and somewhat more for growth (22 I.U./kg. body weight).⁷³¹ Guilbert and Loosli⁹⁶⁶ indicated that 66 I.U./kg. body weight is sufficient for growth and reproduction, as well as for providing a safety factor. Carotene is much less efficiently utilized by swine as a source of vitamin A than is the preformed vitamin A.⁹⁴⁹ Thus, 25 to 39 μ g./kg. body weight are necessary to prevent night blindness, 40 μ g. are needed for growth, and 125 to 195 μ g. are required to assure satisfactory lactation and growth. The deficiency symptoms in the swine include night blindness, diarrhea, infertility of sows, muscular incoördination, and an unthrifty appearance.

(h) *Cattle*. Cattle exhibit the vitamin A deficiency symptoms described by Guilbert *et al.*¹⁰⁰⁶ and by Lewis and Wilson.¹⁰⁰⁷ These include night blindness, severe xerophthalmia, keratinization of the epithelium and, in the case of pregnant cows, the birth of weak or dead calves. To prevent night blindness, 21 to 27 I.U. of vitamin A, or 26 to 33 μ g. of carotene/kg. body weight are necessary.⁹⁴⁹ The following figures for vitamin A have been reported for older calves: growth and deficiency signs, 32 I.U./kg. body weight;¹⁰⁰⁷ optimum growth, 64 I.U./kg. body weight;¹⁰⁰⁷ growth and liver storage, 250 I.U./kg. body weight;¹⁰⁰⁷ and maximum blood concentration of vitamin A, 512 I.U./kg. body weight.¹⁰⁰⁷ In the case of dairy cattle, Guilbert and Loosli⁹⁶⁶ report the vitamin A figure as 66 I.U./kg. body weight on the basis of growth, reproduction, plus a safety factor, or 132 μ g. as β -carotene/kg. body weight. Thus, the requirement for carotene is 6.6 times as much for carotene as for vitamin A. Baker *et al.*⁶⁷⁴ reported that a carotene intake of 130 μ g./kg. body weight was insufficient to maintain liver stores of vitamin A or constant vitamin A values in the plasma. On the other hand, an intake of 730 μ g./kg. body weight of β -

¹⁰⁰⁴ T. A. Harper, R. V. Boucher, and E. W. Callenbach, *Poultry Sci.*, **31**, 273-283 (1952).

¹⁰⁰⁵ S. B. Wolbach and D. M. Hegsted, *Arch. Pathol.*, **54**, 548-563 (1952).

¹⁰⁰⁶ H. R. Guilbert, R. F. Miller, and E. H. Hughes, *J. Nutrition*, **13**, 543-564 (1937).

¹⁰⁰⁷ J. M. Lewis and L. T. Wilson, *J. Nutrition*, **30**, 467-475 (1945).

carotene for dairy cows during lactation was adequate to increase liver stores and plasma levels of vitamin A.

TABLE 11
ESTIMATES OF THE VITAMIN A REQUIREMENT OF CATTLE^a

I.U. fed/kg. body weight/day	Criterion used	References
32	Growth, nyctalopia	<i>b</i>
44	Growth	<i>c</i>
43-55	Nyctalopia	<i>d</i>
60	Growth	<i>e</i>
64	Growth	<i>b</i>
83	Fertility in bulls	<i>f</i>
100	Protection under winter conditions	<i>c</i>
110	Spinal fluid pressure in Holstein calves	<i>g</i>
115	Spinal fluid pressure in Jersey calves	<i>g</i>
125	Spinal fluid pressure in Guernsey calves	<i>g</i>
125	Blood level in Holsteins	<i>h</i>
165	Reproduction	<i>i</i>
210	Blood level in Guernseys	<i>h</i>
220	Exigencies of normal conditions	<i>g</i>
250	Growth, liver storage	<i>b</i>
220	National Research Council recommendation	<i>j,k</i>

^a F. H. Mattson, *Requirements of Animals*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 1954, p. 168.

^b J. M. Lewis and L. T. Wilson, *J. Nutrition*, **30**, 467-475 (1945).

^c H. A. Keener, S. I. Bechdel, N. B. Guerrant, and W. T. S. Thorp, *J. Dairy Sci.*, **25**, 571-583 (1942).

^d H. R. Guilbert, C. E. Howell, and G. H. Hart, *J. Nutrition*, **19**, 91-103 (1940).

^e J. H. Jones, J. K. Riggs, G. S. Fraps, J. M. Jones, H. Schmidt, R. E. Dickson, P. E. Howe, and W. H. Black, *Proc. Am. Soc. Animal Production*, **31**, 94-102 (1938).

^f L. L. Madsen, O. N. Eaton, L. Heemstra, R. E. Davis, C. A. Cabell, and B. Knapp, *J. Animal Sci.*, **7**, 60-69 (1948).

^g L. A. Moore, J. F. Sykes, W. C. Jacobson, and H. G. Wiseman, *J. Dairy Sci.*, **31**, 533-538 (1948).

^h P. D. Boyer, P. H. Phillips, N. S. Lundquist, C. W. Jensen, and I. W. Rupel, *J. Dairy Sci.*, **25**, 433-440 (1942).

ⁱ A. H. Kuhlman and W. D. Gallup, *J. Dairy Sci.*, **25**, 688-689 (1942).

^j J. K. Loosli, C. F. Huffman, W. E. Petersen, and P. H. Phillips, *Recommended Nutrient Allowances for Domestic Animals, III, Recommended Nutrient Allowances for Dairy Cattle*, Comm. Animal Nutrition, Nat. Research Council (U.S.), Washington, 1950, pp. 1-30.

^k H. H. Guilbert, P. Gerlaugh, and L. L. Madsen, *Recommended Nutrient Allowances for Domestic Animals, IV. Recommended Nutrient Allowances for Beef Cattle*, Comm. Animal Nutrition, Nat. Research Council (U.S.), Washington, 1945, pp. 1-32.

In the case of non-lactating female cattle and males, different levels of vitamin A intake, from 32 to 220 I.U./kg. body weight per day have been

recommended.^{763,1005,1009} The vitamin A requirements of cattle, as recalculated by Mattson⁷⁶³ from various sources, are given in Table 11.

(i) *Sheep*. The vitamin A requirement of sheep was reported by Guilbert *et al.*⁹⁴⁹ to be 17 to 26 I.U. of vitamin A/kg. body weight when night blindness was used as the criterion. When the requirements for growth, reproduction, and storage were considered as the criteria, values of 66 I.U.⁹⁶⁶ and 66 to 92 I.U./kg. body weight⁹⁶⁵ were observed. Carotene is believed to have an activity of one-sixth that of vitamin A, on the weight basis.

(j) *Horse*. The vitamin A requirements of the horse were recorded by Guilbert *et al.*⁹⁴⁹ as 17 to 22 I.U. of vitamin A/kg. body weight, or 20 to 30 μ g. of β -carotene, when the amount needed to prevent or cure night blindness was the basis of the test. When growth, reproduction, and lactation were the physiologic functions tested, 66 I.U. of vitamin A/kg. body weight were required, or 132 μ g. of β -carotene (220 I.U.)/kg. body weight.⁹⁶⁶

(k) *Other Animals*. Undoubtedly the hamster and the cotton rat require vitamin A, but no data are available on the quantitative aspects of this requirement. In the case of the macaque or rhesus monkey (*Macaca mulatta*), Truscott and van Wagenen¹⁰¹⁰ demonstrated a requirement for vitamin A, but no quantitative figures were reported. In various species, the deficiency symptoms include diarrhea as the most consistent sign, while xerophthalmia, night blindness, edema, cessation of the menstrual cycle, mild gingivitis, reduction of bile secretion, and a mild increase in leucocytes have been recorded.

Foxes develop certain characteristic symptoms when deficient in vitamin A. Using this criterion as a method of evaluation, Smith¹⁰¹¹ placed the daily vitamin A requirement of this species in the range of 15 to 25 I.U. of vitamin A/kg. body weight. According to Bassett *et al.*,¹⁰¹² an intake of 25 I.U./kg./day is the minimal level which allows normal growth and a detectable content of vitamin A in the blood stream. Storage of vitamin A in the liver did not occur until the vitamin A intake was 50 to 100 I.U./kg./day.

¹⁰⁰⁸ J. K. Loosli, C. F. Huffman, W. E. Petersen, and P. H. Phillips, *Recommended Nutrient Allowances for Domestic Animals. III. Recommended Nutrient Allowances for Dairy Cattle*, Comm. Animal Nutrition, Nat. Research Council (U.S.), Washington, D.C., 1950, pp. 1-30.

¹⁰⁰⁹ H. R. Guilbert, P. Gerlaugh, and L. L. Madsen, *Recommended Nutrient Allowances for Domestic Animals. IV. Recommended Nutrient Allowances for Beef Cattle*, Comm. Animal Nutrition, Nat. Research Council (U.S.), Washington, D.C., 1950, pp. 1-32.

¹⁰¹⁰ B. L. Truscott and G. van Wagenen, *Yale J. Biol. and Med.*, 25, 139-146 (1952).

¹⁰¹¹ S. E. Smith, *J. Nutrition*, 24, 97-107 (1942).

¹⁰¹² C. F. Bassett, J. K. Loosli, and F. Wilke, *J. Nutrition*, 35, 629-638 (1948).

In the case of the mink, it is believed that the vitamin A requirement is somewhat less than that of the fox.¹⁰¹²

(l) *Man*. In the case of human subjects, the dark adaptation test has been the most widely accepted method for the evaluation of the vitamin A requirement. Lewis and Haig⁹⁶¹ gave a figure of 25 I.U. of vitamin A/kg.

TABLE 12
THE RECOMMENDED DAILY ALLOWANCES OF VITAMIN A BY THE
FOOD AND NUTRITION BOARD OF THE NATIONAL RESEARCH COUNCIL^a

Category	Age, years	Approximate body weight, kg.	Daily vitamin A allowances ^b	
			I.U.	I.U./kg.
Infants	<1	7.5	1500	200
Children	1-3	12	2000	167
	4-6	18	2500	140
	7-9	27	3500	130
	10-12	35	4500	130
Boys	13-15	49	5000	100
	16-20	63	6000	95
Girls	16-20	54	5000	95
Men	>20	65	5200	75
Women	>20	55	5000	90
Pregnancy, latter half		65	6000	90
Lactation		55	8000	145

^a Adapted from *Recommended Daily Allowances*, Food and Nutrition Board, National Research Council, *Publ. No. 302*, 1-36, revised, 1953; cited by K. E. Mason, *Requirements of Human Beings*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 1954, p. 173.

^b Based upon the assumption that two-thirds of the intake is provided as carotene, and that carotene has one-half or less than one-half of the biologic value of vitamin A.

body weight for infants, while Lewis and Bodansky¹⁰¹³ cited figures of 100 to 200 I.U. of vitamin A/kg. body weight when blood levels were the criteria. In the case of adults, the vitamin A requirements per kg. body weight observed by the use of growth and dark adaptation tests were given as 31 I.U.,¹⁰¹⁴ 47 to 57 I.U.,⁹⁶² and 81 I.U.⁹⁶³ (dark adaptation only). Hume and Krebs⁹⁶⁴ cited as the twenty-four-hour requirement for adults a value of 2500 I.U. based upon dark adaptation, plasma levels, and liver storage. The vitamin A requirements of human subjects were reviewed by Mason.⁹⁴²

The daily allowance recommended by the National Research Council¹⁰¹⁵

¹⁰¹³ J. M. Lewis and O. Bodansky, *Proc. Soc. Exptl. Biol. Med.*, 52, 265-266 (1943).

¹⁰¹⁴ K. H. Wagner, *Z. physiol. Chem.*, 264, 153-188 (1940).

¹⁰¹⁵ Anonymous, *Recommended Dietary Allowances*, Nat. Research Council, Food and Nutrition Board, *Publ. No. 302*, 1-36 (1953).

for normal adults is 5000 I.U., as provided by 4000 I.U. of carotene and 1000 I.U. of vitamin A. The recommended daily allowances of vitamin A established by the U. S. Food and Drug Administration¹⁰¹⁶ are given as 1500 U.S.P. units for infants, 3000 U.S.P. units for children one to eleven years of age, 4000 U.S.P. units for children over twelve years of age and for adults. The recommended daily allowances of vitamin A as proposed by the Food and Nutrition Board of the National Research Council are given in Table 12.

b. Sex. It has long been recognized that the sex of the animal is an important factor in the metabolism of the carotenoids and vitamins A. Thus, in general, higher levels of β -carotene obtain in the plasma of women than in that of men, while the situation is reversed insofar as the level of blood vitamin A is concerned. Moreover, the sex of the animal is important in relation to the storage of vitamin A in the tissues, including the liver and kidney. Females usually store more vitamin A in their livers than do the males. However, in the experiments of Paul and Paul,⁹⁴⁶ in which the level of vitamin A intake and longevity were correlated, it was evident that given doses of vitamin A prolonged the life span to a greater extent in females than in males. One may interpret this to indicate a lower requirement in the female (see Table 10, page 581). In general, the vitamin A requirements of the males and females are considered to be practically identical. Only during pregnancy and lactation can it be demonstrated that the requirement of the female is increased appreciably over that of the male. However, Week and Sevigne¹¹⁸ observed essentially different responses to test doses of vitamin A in women as compared with men.

c. The Administration in Aqueous Dispersions. The absorption of vitamin A is considerably improved when it is administered in aqueous dispersions,^{123-126,128} as compared with its utilization when given in oil solutions; hence smaller amounts of the vitamin A are required when it is administered in water dispersion. Aqueous dispersions prove particularly helpful in diseases associated with impaired absorption as, for example, celiac disease, pancreatic fibrosis, and occlusion of the bile duct. It has also been observed that chicks utilize vitamin A better, as indicated by greater growth, when the vitamin is given in water emulsion rather than in vegetable oil.¹⁰¹⁷

d. The Form of Vitamin A. Experiments designed to demonstrate pos-

¹⁰¹⁶ Anonymous, *Federal Register*, 6, Sect. 403 (j), Nov. 22, 1941, p. 5925; 1949 ed., *Code Federal Regulations*, Title 21, Sect. 125.3 (4) (b) (1), p. 108.

¹⁰¹⁷ G. R. Halpern and J. Biely, *J Biol. Chem.*, 174, 817-826 (1948).

sible differences in the effectiveness of different forms of vitamin A have produced conflicting results. Kagan and co-workers¹²⁶ found no difference in the effectiveness of vitamin A alcohol or vitamin A palmitate when given in aqueous dispersion to normal children, irrespective of whether the free alcohol or the palmitate was employed. On the other hand, Week and Sevigne¹¹⁷ found that the liver storage of vitamin A in chickens after the feeding of vitamin A alcohol and vitamin A acetate was greater than when the palmitate ester of the vitamin was employed, especially when the solvent employed was jojoba oil. In a later study by these workers,¹¹⁸ similar results were obtained on human subjects when the test dose of vitamin A was given in a vegetable margarine. Thus, vitamin A alcohol was found to be superior in its ability to increase plasma vitamin A as compared with vitamin A acetate, which, in turn, gave better results than did one natural vitamin A ester tested. However, this was not true in the case of a second ester. On the other hand, Popper *et al.*¹⁰¹⁸ obtained opposite results after the two forms of vitamin A were fed in aqueous dispersion or in oily solution. The plasma vitamin A level was slightly higher after the intake of vitamin A esters than after the alcohol had been ingested. Vitamin A in an aqueous medium resulted in a higher plasma level than did an equal amount given in oil. The increase was mainly due to an increase in vitamin A esters, regardless of whether the alcohol or the ester form had been administered. Carotene has been reported, on the basis of growth tests, to be better utilized when administered in margarine fat than when given in other oils.³⁰

e. Stress Factors. Ershoff¹⁰¹⁹ demonstrated that the vitamin A requirement in immature rats was augmented when they were maintained in a cold environment (2°C.), as compared with a normal temperature (23°C.). Thus, when rats were kept in the cold on a vitamin A-deficient diet, they exhibited a more rapid depletion of vitamin A, and they survived for a shorter period than did animals kept under similar conditions but at a higher environmental temperature.

The vitamin A requirements are likewise related to the activity of the thyroid gland. Thus, Greaves and Schmidt⁷²⁰ observed that thyroxine accelerated the release of vitamin A from the livers of rats on a vitamin A-free regimen; thyroxine was also shown to increase the vitamin A storage of carotene in the liver, but not that of preformed vitamin A.³⁵ In the case of cows and goats, Chanda and Owen⁴⁰ noted that the administration

¹⁰¹⁸ H. Popper, F. Steigmann, and H. A. Dyniewicz, *Proc. Soc. Exptl. Biol. Med.*, 73, 188-190 (1950).

¹⁰¹⁹ B. H. Ershoff, *Proc. Soc. Exptl. Biol. Med.*, 74, 586-587 (1950).

of thyroxine increased the vitamin A content of the milk when carotene-containing diets were given. On the other hand, when no carotene was present in the feed, the administration of thyroxine increased the proportion of vitamin A alcohol in the milk, due to the mobilization of hepatic reserves. One must interpret these data as indicating that thyroxine increases the vitamin A requirement when the source of the vitamin is vitamin A itself; when the source of vitamin A is the provitamin A, carotene, thyroxine may not adversely affect vitamin A requirements, since the increased excretion of the vitamin is partially compensated by a more efficient transformation of carotene to vitamin A.

f. Dietary Components Fed Simultaneously. (a) *Tocopherols.* In 1940, Moore⁶⁹⁵ called attention to the fact that the utilization of vitamin A as denoted by liver storage was dependent upon vitamin E intake. More recently, Moore and Sharman⁶⁹⁵ demonstrated that the vitamin A storage in the livers of female rats receiving 1,000 I.U. weekly did not reach the optimum level until 1 to 3 mg. of DL- α -tocopherol was given weekly. On the other hand, Herbert and Morgan²¹⁶ were unable to demonstrate any augmentatory effect of 0.5 mg. per day of α -tocopherol on the liver storage of vitamin A in partially vitamin A-depleted rats receiving 35 to 129 μ g. of vitamin A daily, although an increased storage was noted in rats receiving comparable doses of β -carotene (24 to 174 μ g. daily) when 0.5 mg. of α -tocopherol per day was given. The sparing effect of the tocopherols on vitamin A was also shown by Hickman *et al.*,⁷⁰⁰ by means of growth rate and survival studies. Furthermore, it was found that the tocopherols exert a synergistic effect upon growth after the administration of small and moderate doses of the provitamin A.⁷⁰¹ Hickman and co-workers¹⁰²⁰ observed that α -, β -, and γ -tocopherols are equally effective in sparing vitamin A.

Tocopherols exhibit a similar sparing action upon vitamin A in the case of chickens. Dam *et al.*¹⁰²¹ reported increased growth and a greater storage of vitamin A in the liver when synthetic tocopherol was added to a diet containing 10% of cod liver oil, free tocopherol was found to be more effective than esterified tocopherol. It was recorded that methylene blue, thiodiphenylamine, and Antabuse exhibited a similar behavior toward vitamin A. However, the effect of the tocopherol was not noted in chickens when this vitamin was added to a fat-free diet or to one containing lard.

¹⁰²⁰ K. C. D. Hickman, M. W. Kaley, and P. L. Harris, *J. Biol. Chem.*, **152**, 321-328 (1944).

¹⁰²¹ H. Dam, I. Prange, and E. Søndergaard, *Acta Pharmacol. et Toxicol.*, **8**, 1-22 (1952).

(b) *Vitamin B₁₂*. When rats were fed a diet low in vitamin B₁₂, the effect of supplementary vitamin A upon growth was found by High and Wilson²¹⁴ to be less than when larger amounts of vitamin B₁₂ were given; however, an increased vitamin B₁₂ intake did not raise the level of storage of vitamin A in the liver. In contradistinction to these results, the administration of vitamin B₁₂ to rats on a diet containing β -carotene augmented both growth and vitamin A storage in the liver.

(c) *Proteins*. According to Mayer and Krehl,⁸⁴⁷ high levels of dietary protein increased the severity of vitamin A deficiency symptoms in rats; at least part of these effects are believed to be mediated through the concomitant decrease in vitamin C reserve. The type of protein in the food appears to influence the fate of ingested β -carotene. Thus, James and El Gindi¹⁰²² reported that rats receiving lactalbumin or gluten in their diets excreted more carotene in the feces than they did when casein or zein was the dietary protein. These workers believe that vitamin A is consumed during an hepatic process concerned with the change of moieties from ingested proteins to other proteins or to amino acids. The vitamin A levels in the plasma of rats receiving casein or lactalbumin were higher than was the case in rats on regimens containing zein or gluten.

(d) *Choline*. Bentley and Morgan⁷¹⁸ reported that hepatic storage of vitamin A by depleted rats was not affected by choline when a high-fat, low-protein diet was employed. On the other hand, when the diet was a low-fat, low-protein regimen, the addition of choline increased the vitamin A content of the livers. Vitamin A deposition after the ingestion of carotene was best in the group of rats receiving the low-fat regimen, but choline had little influence upon the extent of deposition of vitamin A.

(e) *Antibiotics*. Although Burgess *et al.*²¹¹ did note an increase in the vitamin A storage in chicks when penicillin was added to the diet, Coates and co-workers¹⁰²³ stated that the effect was non-specific, since it occurred only when the growth of the chicks was also stimulated by the penicillin. Aureomycin had no effect upon liver storage of vitamin A.¹⁰²⁴

(f) *Mineral Oil*. It is well known that the consumption of mineral oil reduces the absorption of β -carotene to an appreciable extent.^{43,44} Mineral oil exerts less effect upon the absorption of vitamin A than on that of carotene,¹⁰⁹⁻¹¹¹ although some workers^{5,42,45-47,112-115} recorded a decrease in the absorption of the vitamin when mineral oil was consumed. Steigmann and

¹⁰²² W. H. James and I. M. El Gindi, *J. Nutrition*, **51**, 97-108 (1953).

¹⁰²³ M. E. Coates, G. F. Harrison, S. K. Kon, J. W. G. Porter, and S. Y. Thompson, *Chemistry & Industry*, **1952**, 149.

¹⁰²⁴ E. W. Hartsook, E. Batchelor, and B. C. Johnson, *Proc. Soc. Exptl. Biol. Med.*, **83**, 43-45 (1953).

co-workers¹⁰²⁵ are of the opinion that, in human subjects, this is a practical problem only when appreciable quantities of mineral oil are taken at meals. Doses of 2.5 ml. of mineral oil three times daily at mealtime did not affect the absorption of vitamin A, nor was there any interference when 30 ml. of the oil were taken at bedtime.

10. Hypovitaminosis A (Avitaminosis A)

(1) Introduction

The first indication that a nutritional adjunct is necessary for growth in addition to protein, carbohydrate, fat, minerals, and water came as a result of the deficiency symptoms of animals receiving diets which we now know were devoid of vitamin A. Although the first evidence of deficiency involves such generalized symptoms as poor growth, unthrifty appearance of the skin, and especially of the fur, and infertility, a series of specific deficiency effects can now be attributed to the lack of vitamin A. The morphologic effects of vitamin A deficiency can be demonstrated in many epithelial structures, as well as in the epiphyseal cartilage of growing bones. In the latter structures, changes may occur before any decrease in growth rate is evident. When skeletal growth is retarded, beyond the normal growth period, by lack of vitamin A, it may be resumed after the administration of vitamin A, long after growth would normally cease.

The deficiency in the epithelial tissues brought about by a vitamin A deficiency is characterized, in growing and adult animals, by keratinization of the epithelia which are renewed by multiplication of the basal cells. The fetus exhibits many types of malformation. All vertebrates examined (including the mouse, rat, guinea pig, fowl, rabbit, dog, fox, pig, cow, monkey, and man)^{1026, 1027} require vitamin A or a carotenoid precursor for vision, for the maintenance of many types of epithelia, and for the development of teeth and skeletal structure. The most recent (1954) comprehensive discussion on the pathology of avitaminosis A in animals is that of Wolbach.¹⁰²⁸ Mason made a similar contribution on the effects of vitamin A deficiency in human subjects.¹⁰²⁹

¹⁰²⁵ F. Steigmann, H. Popper, H. Dyniewicz, and I. Maxwell, *Gastroenterology*, 20, 587-594 (1952).

¹⁰²⁶ S. B. Wolbach, *J. Am. Med. Assoc.*, 108, 7-13 (1937).

¹⁰²⁷ R. H. Follis, Jr., *The Pathology of Nutritional Disease*, C C Thomas, Springfield, Ill., 1948.

¹⁰²⁸ S. B. Wolbach, *Effects of Vitamin A Deficiency and Hypervitaminosis A, in Animals*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 1954, pp. 106-137.

¹⁰²⁹ K. E. Mason, *Effects of Vitamin A Deficiency in Human Beings*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, vol. I, Academic Press, New York, 1954, pp. 137-163.

(2) *Deficiency Symptoms in Animals Other Than Man*

a. Prenatal Effects of Vitamin A Deficiency. When the intake of vitamin A is severely restricted during pregnancy, death and resorption of the fetus have been shown to occur in rats and pigs. The maternal and placental epithelia are also damaged.¹⁰³⁰ When the vitamin A deficiency is less acute, the pregnancy may continue to term, but various abnormalities associated with arrested development of the skeleton and of various organs may be evident in the fetus.¹⁰³⁰ Warkany and associates¹⁰³¹⁻¹⁰³⁴ described many congenital malformations occurring in the offspring of vitamin A-deficient rats. Approximately 75% of the young exhibited anomalies in one or more organs. These abnormalities involved the eyes predominantly, and with decreasing frequency the genitourinary system, the diaphragm, the major arteries derived from the embryonic aortic arch system, and the lungs. The anomalies appear to arise during the process of organogenesis, and may be prevented by the administration of vitamin A to the mother on the tenth to twelfth day of pregnancy.

Other workers also reported anomalies of the heart and circulatory system in rats born to vitamin A-deficient mothers.^{1035,1036} Andersen¹⁰³⁷ described a congenital diaphragmatic hernia in an inbred stock of albino rats, which was aggravated (18.9% incidence) on a vitamin A-free diet, or was inhibited (0.9% incidence) when vitamin A was added to the maternal diet. This is the first instance in which an antenatal precipitating factor has been demonstrated as a result of the diet of the mother.

In the experiments of Hale¹⁰³⁸ on pigs, reported by Warkany,¹⁰³⁹ gilts were kept before breeding on a vitamin A-deficient diet until deficiency symptoms were apparent, and were then given cod liver oil after thirty days of pregnancy. The young born to these mothers exhibited malformations such as arrested formation of eyes at various stages, or even complete absence of eye-balls, harelip, cleft palate, misplaced kidneys, and extra ear-like growths.

b. Postnatal Effects of Vitamin A Deficiency. (a) *Effect on Epithelial*

¹⁰³⁰ K. E. Mason, *Am. J. Anat.*, **57**, 303-344 (1935).

¹⁰³¹ J. Warkany, C. B. Roth, and J. G. Wilson, *Pediatrics*, **1**, 462-471 (1948).

¹⁰³² J. Warkany and E. Schraffenberger, *Arch. Ophthalmol.*, **35**, 150-169 (1946).

¹⁰³³ J. G. Wilson, C. B. Roth, and J. Warkany, *Am. J. Anat.*, **92**, 189-217 (1953).

¹⁰³⁴ J. Warkany and C. B. Roth, *J. Nutrition*, **35**, 1-11 (1948).

¹⁰³⁵ J. G. Wilson and S. Barch, *Proc. Soc. Exptl. Biol. Med.*, **72**, 687-693 (1949).

¹⁰³⁶ J. G. Wilson and J. Warkany, *Pediatrics*, **5**, 708-725 (1950).

¹⁰³⁷ D. H. Andersen, *Am. J. Pathol.*, **25**, 163-184 (1949).

¹⁰³⁸ F. Hale, *J. Heredity*, **24**, 105-106 (1933); *Am. J. Ophthalmol.*, **18**, 1087-1093 (1935); *Texas State J. Med.*, **33**, 228-232 (1937).

¹⁰³⁹ J. Warkany, *Vitamins and Hormones*, **3**, 73-103 (1945).

Tissues and on Organs. The effects of vitamin A deficiency are similar, irrespective of whether the epithelium is of ectodermal, mesodermal, or entodermal origin. In all cases, atrophy occurs, a reparative proliferation by the basal cells follows, and growth and differentiation of new cells into a stratified keratinizing epithelium take place. The replacement of epithelium, regardless of the site of damage and of its original function, is identical everywhere, and is comparable to the layers of the epidermis.^{1026, 1027, 1040} When vitamin A is given, the morphologic structure of the tissue returns to normal in each area, and normal function is resumed.¹⁰⁴¹

The sequence in which the organic deficiency symptoms appear varies somewhat with species, but the same organs are usually involved. The epithelia which atrophy and which become replaced by stratified keratinizing epithelia are those which possess the function of secretion, in addition to acting as a covering layer. These functioning cells are not capable of division. The order of appearance of keratinizing metaplasia, according to Wolbach,¹⁰²⁸ is as follows in man as well as in animals: salivary glands, including submaxillary, parotid, and all the accessory buccal glands of the mouth, tongue, and pharynx; the respiratory tract, together with the nares and accessory sinuses; Jacobson's organ, the trachea and bronchi; the genitourinary tract, including the bladder, ureter, renal pelvis, epididymis, prostate, seminal vesicles, coagulating gland, uterus, oviduct, and accessory sex glands of the vulva; the eyes and paraocular glands, including the corneal and palpebral conjunctivae, the Harderian, intraorbital and extraorbital lacrimal glands, and the Meibomian glands; and the skin.

a'. Effect on the Eyes: The effects of vitamin A deficiency on both the internal functioning and the external structure of the eye are evident. In the first case, night blindness is a result of vitamin A deprivation, but there are no demonstrable morphologic changes in the retina.¹⁰²⁸ The condition is reversible in the adult. On the other hand, when irreversible loss of vision does occur in young animals deprived of vitamin A, it may be traced to the effect of pressure on the blood vessels and optic nerve due to the retardation in growth of the skull.¹⁰²⁸ According to Johnson,¹⁰⁴² when twenty-three-day-old rats were placed on a vitamin A-free diet, edema of the retina was the first symptom. This was followed by slight degenerative changes of the outer segments of the rods. When vitamin A was given, the normal condition was rapidly restored. Following prolonged deprivation of vitamin A, more profound degeneration of the outer seg-

¹⁰⁴⁰ S. B. Wolbach and O. A. Bessey, *Physiol. Revs.*, **22**, 233-289 (1942).

¹⁰⁴¹ S. B. Wolbach and P. R. Howe, *J. Exptl. Med.*, **57**, 511-526 (1933).

¹⁰⁴² M. L. Johnson, *Arch. Ophthalmol. (Chicago)*, **29**, 793-810 (1943).

ments of the rod occurred, the correction of which required longer periods of vitamin A therapy. When the lesions involved the greater part of the outer nuclear layer, the damage became irreparable, even when vitamin A was given.

In addition to the changes in the retina, the epithelia of the cornea and the bulbar and palpebral conjunctivae become keratinized.¹⁰²³ Concurrently with the epithelial changes in the cornea, blood vessels grow into the tunica propria from the limbic plexus. The ducts of the lacrimal glands undergo keratinization, and the glands themselves become atrophied. Xerophthalmia results when these keratinized cells accumulate in the conjunctival sacs and excite inflammatory reactions. Keratomalacia, which entails edema and necrosis of the cornea, may occur. Bacterial invasion is favored by the accumulation of keratinized cells, and this may result in ulceration of the cornea and hypopyon.¹⁰²⁸ The gross ocular symptoms occur rather late in vitamin A deficiency, in the case of both animals and man.^{1043,1044}

b'. Effect on the Respiratory Tract: Extensive keratinization occurs early in the sequence of changes associated with avitaminosis A. This involves the entire respiratory tract, from the nares to the bronchi.¹⁰²⁸ Bacterial invasion occurs, resulting in atrophy of the epithelium and the loss of ciliary action, even before a replacement by keratinizing epithelium occurs. According to Blackfan and Wolbach,¹⁰⁴³ these conditions are responsible for lobar and bronchial pneumonia, which frequently is the immediate cause of death.¹⁰⁴⁴

c'. Effect on the Gastrointestinal Tract: Although the esophagus may become hyperkeratotic, changes in the epithelia of the stomach and intestine are slight, and the cells do not undergo keratinizing replacement. A keratinizing metaplasia may be observed in the pancreatic ducts, but not in the biliary ducts.¹⁰²⁸

d'. Effect on the Genitourinary Tract: In all species, a keratinizing metaplasia occurs throughout the urinary tract, as well as in the epididymis, uterus, oviduct, and sex glands.¹⁰²⁸ The accumulation of keratinized cells may result in obstruction of the urinary system. This may be the immediate cause of death in rats suffering from vitamin A deficiency. Grossly visible lesions of this type have been reported in human infants and adults as well.^{1043,1044} One of the first symptoms of deficiency of "the fat-soluble vitamin," now known to be specifically a vitamin A deficiency, re-

¹⁰⁴³ K. D. Blackfan and S. B. Wolbach, *J. Pediat.*, 3, 679-706 (1933).

¹⁰⁴⁴ L. K. Sweet and H. J. K'ang, *Am. J. Diseases Children*, 50, 699-734 (1935).

ported in the early days by Osborne and Mendel,¹⁰⁴⁵ was urolithiasis, which consists in the tendency to form calcium phosphate concretions in the urinary tract. Van Leersum¹⁰⁴⁶ confirmed the results of Osborne and Mendel. It was suggested that a morbid change of the epithelial cells in the tubules takes place, which gives rise to the formation of calcium casts. This suggestion was supported by the later work of van Leersum.¹⁰⁴⁷ Lamming and co-workers¹⁰⁴⁸ reported that vitamin A deficiency did not reduce the formation of decidua in the rabbit, although it resulted in the production of premature degeneration of ova, and reduced the number of fertilized ova present forty and ninety-six hours after mating. It was also shown¹⁰⁴⁹ that incipient vitamin A deficiency reduced, by 14%, the number of animals which mated normally, while the number of animals which conceived was reduced to the extent of 18% by the hypovitaminosis A. Kahn¹⁰⁵⁰ noted an interrelation between vitamin A, estrogen, and the keratinization of the vaginal epithelium. A dose of vitamin A as low as 0.6 I.U. significantly inhibits the cornification resulting from a total dose of 0.6 I.U. of α -estradiol. Thus, the effect of the lack of vitamin A on the estrous cycle of animals can be correlated with its failure to react with the estrogenic hormones.

e'. Effect on the Skin: One of the most prominent symptoms of vitamin A deficiency is the unthrifty appearance of the skin, and epithelial appendages such as hair, fur, and feathers. Hyperkeratosis of the hair follicles has been reported in adult humans deficient in vitamin A,^{1041, 1051, 1052} and large amounts of vitamin A will undoubtedly restore the normal condition. However, it apparently does not occur in rats and guinea pigs deprived of vitamin A. Wolbach¹⁰⁵³ reported that atrophy of the hair bulb and of the external sheath cells occurs in newly-formed hair follicles, following the application of keratinizing agents such as benzpyrene and methylcholanthrene. Some hyperkeratinization of the epidermis and hair follicles has been pro-

¹⁰⁴⁵ T. B. Osborne and L. B. Mendel, *J. Am. Med. Assoc.*, 69, 32-33 (1917).

¹⁰⁴⁶ E. C. van Leersum, *J. Biol. Chem.*, 76, 137-142 (1928).

¹⁰⁴⁷ E. C. van Leersum, *J. Biol. Chem.*, 79, 461-463 (1928).

¹⁰⁴⁸ G. E. Lamming, G. W. Salisbury, R. L. Hays, and K. A. Kendall, *J. Nutrition*, 52, 217-225 (1954).

¹⁰⁴⁹ G. E. Lamming, G. W. Salisbury, R. L. Hays, and K. A. Kendall, *J. Nutrition*, 52, 227-239 (1954).

¹⁰⁵⁰ R. H. Kahn, *Am. J. Anat.*, 95, 309-335 (1954).

¹⁰⁵¹ C. N. Frazier and C.-K. Hu, *Arch. Dermatol. and Syphilol.*, 33, 825-852 (1936).

¹⁰⁵² O. A. Bessey and S. B. Wolbach, *Vitamin A, Physiology and Pathology*, in *The Vitamins*, Sympos. Council on Pharm. and Chem.; Council on Foods, Am. Med. Assoc., Chap. II, Chicago, 1939, pp. 27-54.

¹⁰⁵³ S. B. Wolbach, *Ann. N. Y. Acad. Sci.*, 53, 517-536 (1951).

duced by withdrawing vitamin A from rats previously subjected to a diet lacking in some of the heat-stable members of the vitamin B group.¹⁰⁵⁴

f'. *Effect on the Teeth*: A deficiency in vitamin A has an especially severe effect upon the incisors of rats and of guinea pigs.¹⁰²⁸ These teeth are growing continuously, and thus constantly require vitamin A for growth processes. The main site of action of vitamin A is on the odontogenic epithelium. In the absence of vitamin A, growth continues at the basal formative end, but differentiation is incomplete. There is also a loss of the organizing influence, and this results in an inadequate and defective dentine formation, and atrophic odontoblasts. The thinness of the dentine layer causes it to assume an abnormal shape, and plication and buckling of the dentine frequently occur near the formative end of the tooth.¹⁰²⁸ The teeth of vitamin A-deficient animals present a thick dentine layer on the labial side, and excessively thin layers elsewhere. Skaloud¹⁰⁵⁵ reported that the dental deterioration and malformation due to vitamin A deficiency may be compensated for by the administration of vitamins C and D.

(b) *Effect on the Bones*. Although a deficiency of vitamin A does not affect the structure of mature bones, it does have a profound effect upon growing bones.¹⁰²⁸ Vitamin A deficiency presents a different type of retardation of skeletal growth and interference with growth pattern than does inanition or deficiency in other vitamins. For a description of the histologic skeletal changes, the reader is referred to Wolbach.¹⁰⁵⁶ When a vitamin A deficiency exists, a retardation of skeletal growth may occur without affecting that of the soft tissues of the nervous system.¹⁰⁵⁷ However, according to Wolbach,¹⁰⁵⁶ in vitamin A deficiency endochondral bone growth ceases before the growth rate of the animal as a whole is seriously affected. This results in disastrous imbalance between the development of the skeleton and of the soft tissues in young animals. The cranial cavity and spinal canal become too small for the central nervous system, resulting in pressure upon the brain, spinal cord and nerve roots, with consequent paralysis and other symptoms. Although the skeletal growth of the human infant has not been studied in relation to vitamin A intake, the results of studies on the rat, guinea pig, dog, duck, and chick are uniform in indicating a prompt cessation of epiphyseal growth in the absence of vitamin A. Furthermore, all growth of the skeleton dependent upon re-

¹⁰⁵⁴ M. Sullivan and V. J. Evans, *Arch. Dermatol. and Syphilol.*, 51, 17-25 (1945).

¹⁰⁵⁵ F. Skaloud, *Z. Vitaminforsch.*, 19, 2-34 (1947).

¹⁰⁵⁶ S. B. Wolbach, *J. Bone and Joint Surg.*, 29, 171-192 (1947).

¹⁰⁵⁷ S. B. Wolbach and O. A. Bessey, *Arch. Pathol.*, 32, 689-722 (1941).

placement or upon endochondral bone formation ceases during the deficiency.¹⁰²⁸

Secondary symptoms may occur as a result of the abnormal bone growth. In the petrous bone of the dog, a continuation of the appositional bone growth causes a compression of the eighth nerve,^{1058, 1059} resulting in deafness.¹⁰⁵⁸ However, this does not occur in the rat.¹⁰²⁸ Moore¹⁰⁶⁰ reported blindness in calves as a result of compression of the optic nerve, but this, also, has not been observed in rats.¹⁰²⁸ In the case of the rabbit, certain malformations occur in the bones of the ear following long-continued vitamin A deficiency.¹⁰⁶¹ According to Mellanby,¹⁰⁶² vitamin A deficiency causes skeletal changes in dogs, due to its effect upon the position and activity of osteoclasts and osteoblasts. When vitamin A therapy is resumed, the sequences in bone growth return to normal, although the order in which the resumption of normal activity takes place has not been demonstrated. According to Wolbach and Bessey,¹⁰⁵⁷ the epiphyseal sequences promptly return to normal in the vitamin A-deficient rat after vitamin therapy is started.

Vitamin A plays an important role in the case of all animals. It is necessary for growth and differentiation of epiphyseal cartilage; it is likewise required for differentiation of many epithelial structures, but is not concerned with their growth. In addition to the above important functions, vitamin A is primarily concerned with black and white vision, and also with color vision. When deprivation of vitamin A occurs, all of these functions operate with impaired efficiency, or they may entirely cease.

(3) *Deficiency Symptoms in Man*

a. Introduction. Two categories of vitamin A deficiency are recognized in human nutrition, namely "primary" and "secondary."¹⁰²⁹ The first condition obtains when the deficiency results from an inadequate intake of vitamin A or of carotene. The "secondary"avitaminosis A results when there are: (1) disorders which interfere with the absorption of vitamin A and carotene, as for example celiac disease, cystic fibrosis of the pancreas, sprue, giardiasis, congenital absence or obstruction of the bile duct, cirrhosis of the liver, severe diarrhea, and ulcerative colitis, (2) disorders

¹⁰⁵⁸ E. Mellanby, *J. Physiol.*, *94*, 380-398 (1938).

¹⁰⁵⁹ E. Mellanby, *J. Physiol.*, *99*, 467-486 (1941).

¹⁰⁶⁰ L. A. Moore, *J. Nutrition*, *17*, 443-459 (1939).

¹⁰⁶¹ H. B. Perlman, *Arch. Otolaryngol.*, *50*, 20-35 (1947).

¹⁰⁶² E. Mellanby, *A Story of Nutritional Research: The Effect of Some Dietary Factors on Bones and the Nervous System*, Williams & Wilkins, Baltimore, 1950.

which decrease the ability of the body to transform carotene to vitamin A (diabetes mellitus and hypothyroidism), and (3) factors which cause an unusually rapid utilization of the vitamin or an increased rate of loss of vitamin A (acute or chronic infections which are associated with a high and sustained fever). These conditions exert their effect by reducing the vitamin A content of the plasma and of several tissues, rather than by depleting the liver stores of vitamin A.¹⁰²⁹

The vitamin A stores ordinarily present are so extensive that two or three years on a vitamin A-deficient diet would be required to deplete the reserve supplies of vitamin A in the case of the adult. The deficiency can be brought about more rapidly in infants and young children, in whose case the prior storage of vitamin A is less extensive, and a rapid rate of utilization due to growth normally takes place. Although outright vitamin A deficiency is more common in the young, it does not often occur in these cases as a simple deficiency state. The most recent authoritative discussion of avitaminosis A in man is that of Mason.¹⁰²⁹

b. Experimental Avitaminosis A in Man. Mason¹⁰²⁹ points out that eight series of experiments were performed between 1937 and 1943 in which an attempt was made to demonstrate the symptoms which supervene in man following a vitamin A-free regimen, and the effects of vitamin A on this condition. Most of the assessment of vitamin A deficiency has been made by the use of dark adaptation tests. As an example of this type of study, Hume and Krebs⁹⁶⁴ studied the dark adaptation time in the case of twenty-three subjects (twenty men and three women) who lived on a diet essentially devoid of vitamin A and of carotene. One subject maintained this dietary regimen for as long as two years, and several followed it for one and one-half years without any appreciable signs of vitamin A deficiency. There was slight evidence of deficiency symptoms in three of the twenty-three subjects, as indicated by reduced plasma vitamin A levels and a slightly increased dark adaptation time. It is thus evident that there is a marked variation between different subjects with regard to their ability to resist vitamin A deficiency when they are continued on a vitamin A-free diet over a prolonged period. For example, Blanchard and Harper¹⁰⁶³ reported that, although only moderate changes in dark adaptation time were observed in the majority of their ten young male subjects, as a result of a stringent vitamin A-low diet over a period of forty-two to forty-five days, in at least one case the dark adaptation time was increased from a normal value of two or three minutes to a period of over nineteen minutes on the twenty-ninth day of deprivation of vitamin A. Hume and Krebs⁹⁶⁴ reported that eight months of depletion were required before most of the

human subjects exhibited an appreciable diminution of plasma vitamin A. Within three months values of 40 I.U./100 ml. were noted, but only in the case of three subjects. One man, however, showed no appreciable decrease, even after twenty-two months of deprivation. Some additional data have been made available in nutrition surveys and clinicopathologic studies.

c. Effects of Vitamin A Deficiency in Man. (a) *Night Blindness.* The inability to see in dim light represents one of the oldest recognized diseases known to man; it was early shown to respond to treatment with liver. It is now recognized that vitamin A in the form of vitamin A aldehyde (retinene) comprises the most important part of the visual purple, which is made up of the retinal pigments. Rhodopsin and porphyropsin are present in the rods, while iodopsin occurs in the cones. These retinal pigments are readily broken down when exposed to light, and they can then be regenerated in the dark. New retinene can be formed from vitamin A by the retinene reductase system, in which DPN is a coenzyme.

When the amount of vitamin A available in the plasma is decreased, it would be expected that the regeneration of the visual purple would be delayed. In order to estimate the speed of the regeneration, which can be employed as an index of vitamin A requirements, various types of biophotometers and adaptometers have been employed to determine the so-called "dark adaptation time." These instruments afford a reliable measurement of dark adaptation except where the visual defect is minimal.¹⁰⁶³⁻¹⁰⁶⁵

(b) *Xerophthalmia and Keratomalacia.* Xerophthalmia is manifested by a roughened state (or xerosis) of the conjunctiva and cornea. It is often associated with the presence of irregular whitish plaques (the so-called Bitot's spots) on the scleral conjunctiva. When the condition progresses further, and is associated with edema, infiltration, and necrotic softening of the cornea, which may be followed by perforation, hypopyon and panophthalmitis, the syndrome is referred to as keratomalacia.

These disorders of the eye have been recognized for about one hundred years, during which time their frequent association with night blindness was recognized. It was also known that liver and cod liver oil were effective curative agents. In 1863, Bitot¹⁰⁶⁶ first described the whitish plaques on the corneoconjunctival mucosa, occurring in association with night blindness. He described them as "hemeralopic plaques." They were success-

¹⁰⁶³ E. L. Blanchard and H. A. Harper, *Arch. Internal Med.*, **66**, 661-669 (1940).

¹⁰⁶⁴ P. C. Jeans, E. L. Blanchard, and F. E. Satterthwaite, *J. Pediat.*, **18**, 170-194 (1941).

¹⁰⁶⁵ R. E. Eckardt and L. V. Johnson, *J. Pediat.*, **18**, 195-199 (1941).

¹⁰⁶⁶ P. Bitot, *Gaz. méd. Paris*, **18**, 435-438 (1863).

fully treated with cod liver oil by Snell,¹⁰⁶⁷ in 1876. A number of cases of these conditions have been recorded in the literature, including the report of de Gouvea¹⁰⁶⁸ on a series of cases of plantation slaves in Rio di Janeiro who were treated effectively with cod liver oil, and the classic studies of Mori,¹⁰⁶⁹ who described 1511 cases of xerophthalmia and 116 cases of keratomalacia observed in Japan between 1899 and 1902. There are also the reports of Bloch¹⁰⁷⁰⁻¹⁰⁷² and of Blegvad¹⁰⁷³ which, combined, furnish data on 211 cases of xerophthalmia and 453 cases of keratomalacia observed in Denmark during the years 1919 and 1920. Mori¹⁰⁶⁹ observed the highest incidence of the diseases in summer, when they were associated with the so-called summer diarrhea. Although Mori considered a lack of fat to be the cause of the disorder, it was recognized that cod liver oil played a unique role in the cure of these conditions.

Xerophthalmia and keratomalacia have been reported in insufficiently breast-fed children,^{1074,1075} as well as in newborn babies.^{1076,1077} Bouman and van Creveld¹⁰⁷⁷ noted that keratomalacia occurred in an infant whose mother, during pregnancy, had had a diet deficient in vitamin A, in the provitamins A and in fat, consisting mainly of bread and tea with unfortified margarine. Although the mother exhibited no symptoms of vitamin A deficiency, the infant was born with keratomalacia, which resulted in complete and permanent blindness. For a further description of the external manifestations and the histopathology of this condition, the reader is referred to the review of Mason.¹⁰²⁹

(c) *Metaplasia of Epithelia.* The association of symptoms which can be attributed to a metaplasia of the epithelia of the respiratory tract, with keratomalacia, was early recognized. Thus, symptoms such as dryness of oral, nasal, and pharyngeal membranes were observed in patients with keratomalacia, together with hoarseness, husky voice, and a dry cough, frequently associated with pneumonia. Keratinization of the mucosa of

¹⁰⁶⁷ S. Snell, *Lancet*, 1876, I, 8-9.

¹⁰⁶⁸ H. de Gouvea, *Albrecht von Graefe's Arch. Ophthalmol.*, 29, Part I, 167-200 (1883).

¹⁰⁶⁹ M. Mori, *Jahrb. Kinderheilk.*, 59, 175-195 (1904).

¹⁰⁷⁰ C. E. Bloch, *J. Hyg.*, 19, 283-304 (1921).

¹⁰⁷¹ C. E. Bloch, *Am. J. Diseases Children*, 27, 139-148 (1924).

¹⁰⁷² C. E. Bloch, *Am. J. Diseases Children*, 28, 659-667 (1924).

¹⁰⁷³ O. Blegvad, *Am. J. Ophthalmol.* [2], 7, 89-117 (1924).

¹⁰⁷⁴ T. Thalberg, *Arch. Augenheilk.*, 12, 315-332 (1883).

¹⁰⁷⁵ J. H. de Haas, J. H. Posthuma, and O. Meulemans, *Indian J. Pediat.*, 8, 139-157 (1941).

¹⁰⁷⁶ J. P. Maxwell, *J. Obstet. Gynecol. Brit. Empire*, 39, 764-776 (1932).

¹⁰⁷⁷ H. D. Bouman and S. van Creveld, *Z. Vitaminforsch.*, 10, 192-197 (1940).

the renal pelvis was recorded by Leber.¹⁰⁷⁸ Wilson and DuBois¹⁰⁷⁹ called attention to the widespread keratinizing metaplasia of the epithelia of the respiratory tract and of various ducts in the case of a five-months-old infant who had succumbed to keratomalacia. More recent reports of metaplasia in cases of vitamin A deficiency include those of Thatcher and Sure,¹⁰⁸⁰ (one case), Blackfan and Wolbach¹⁰⁴³ (twelve cases) and Sweet and K'ang¹⁰⁴⁴ (seventeen cases). The keratinization of epithelia on the internal surfaces of the body is a relatively late manifestation of avitaminosis A. In addition to the epithelia of the respiratory tract, those of the gastrointestinal tract, and of the genitourinary tract, are involved.

(d) *Cutaneous Lesions.* The skin and its appendages are the first to exhibit the effect of avitaminosis A by an unthrifty appearance. The first definite association of the skin condition with keratomalacia was that of Pillat,¹⁰⁸¹ who studied it in six patients. Their hair was described as dull, dry, coarse, or brittle, while the skin over the entire body (except the face) was generally desiccated, finely wrinkled, covered with fine scales, and earthy brown to slate gray in color. The presence of a follicular type of skin lesion in soldiers with ocular difficulties was recorded by Frazier and Hu;¹⁰⁵¹ they referred to this type of lesion as "follicular hyperkeratosis." Cutaneous lesions of this nature were observed in the case of 180 patients with xerophthalmia and twenty-seven without this condition. Follicular hyperkeratosis has also been found in association with night blindness.¹⁰⁸² Confirmation that avitaminosis A is the cause of follicular hyperkeratosis was presented by Nicholls,¹⁰⁸³ who studied laborers and convicts in Ceylon, and by Loewenthal,^{1084, 1085} who observed prisoners in Uganda, East Africa. It was given the name "phrynoderma" by Nicholls, but is more commonly known as follicular hyperkeratosis. A number of investigators¹⁰⁸⁶⁻¹⁰⁹¹ confirmed the fact that avitaminosis A causes cutaneous lesions which re-

¹⁰⁷⁸ T. Leber, *Albrecht von Graefe's Arch. Ophthalmol.*, 29, Part III, 225-290 (1883).

¹⁰⁷⁹ J. R. Wilson and R. O. DuBois, *Am. J. Diseases Children*, 26, 431-446 (1923).

¹⁰⁸⁰ H. S. Thatcher and B. Sure, *Arch. Pathol.*, 13, 756-765 (1932).

¹⁰⁸¹ A. Pillat, *Arch. Ophthalmol. (Chicago)*, 2, 256-287, 399-415 (1929).

¹⁰⁸² F. Reiss, *Chinese Med. J.*, 50, 945-948 (1936).

¹⁰⁸³ L. Nicholls, *Indian Med. Gaz.*, 68, 681-687 (1933).

¹⁰⁸⁴ L. J. A. Loewenthal, *E. African Med. J.*, 10, 58-59 (1933).

¹⁰⁸⁵ L. J. A. Loewenthal, *Arch. Dermatol. and Syphilol.*, 28, 700-708 (1933).

¹⁰⁸⁶ W. E. Giblin, *Med. J. Australia*, 1936, I, 202-203.

¹⁰⁸⁷ M. V. Radhakrishna Rao, *Indian J. Med. Research*, 24, 727-736; 25, 39-44 (1937).

¹⁰⁸⁸ P. Fasal, *Arch. Dermatol. and Syphilol.*, 50, 160-166 (1944).

¹⁰⁸⁹ L. F. Steffens, H. L. Bari, and C. Sheard, *Am. J. Ophthalmol.*, 23, 1325-1340 (1940).

¹⁰⁹⁰ J. B. Youmans and M. B. Corlette, *Am. J. Med. Sci.*, 195, 644-650 (1938).

¹⁰⁹¹ B. M. Nicol, *Brit. J. Nutrition*, 3, 25-43 (1949).

spond to vitamin A therapy. Papular lesions resembling those found in follicular hyperkeratosis were also demonstrated in the case of twenty-four out of thirty-one subjects who voluntarily partook of a partial starvation diet containing 1810 I.U. of carotene.¹⁰⁹²

There has sometimes been a lack of definite correlation between the incidence of skin lesions and of other evidence of vitamin A deficiency in children and adults.¹⁰⁹³⁻¹⁰⁹⁶ However, Nicholls^{1093,1093} attributed blindness in children in Ceylon to corneal xerophthalmia and keratomalacia due to vitamin A deficiency, and Loewenthal¹⁰⁹⁴ suggested a causal connection between phrynoderma and this deficiency. Pal¹⁰⁹⁷ and Ramalingaswami¹⁰⁹⁸ reported a high incidence of Bitot's spots, keratomalacia, and severe diarrhea, associated with a dry, inelastic skin, in children in Coonoor, South India. Marmelzat¹⁰⁹⁹ also recorded the case of a ten-year-old white girl in Galveston, Texas, in whose case Bitot's spots, typical follicular hyperkeratotic lesions, and metabolic calcinosis in both kidneys were observed simultaneously. Medication with 25,000 I.U. of vitamin A daily cleared up the Bitot's spots in twenty-four days, while most of the hyperkeratosis had disappeared within two months. For a further discussion of this subject, the reader is referred to the review of Mason.¹⁰²⁹

11. Hypervitaminosis A

(1) Introduction

Within the last few years it has been recognized that the ingestion of excessive amounts of vitamin A results in the appearance of toxic symptoms. If the abnormal doses of vitamin A are continued, the animals subjected to them may die. It has long been known that the ingestion of polar bear liver by Eskimos and by Arctic travelers will bring on severe illness in man as well as in dogs. It is also claimed that the liver of the bearded seal (*Phoca barbata*) is poisonous, although opinions on this point are not unanimous. According to Richardson,¹¹⁰⁰ as early as 1596 members of an

¹⁰⁹² A. Keys, J. Brožek, A. Henschel, O. Mickelsen, and H. L. Taylor, *The Biology of Human Starvation*, Vols. I and II, Univ. Minnesota Press, Minneapolis, 1950.

¹⁰⁹³ L. Nicholls, *Indian Med. Gaz.*, 69, 241-251 (1934); 70, 14-16, 550-552 (1935).

¹⁰⁹⁴ L. J. A. Loewenthal, *Ann. Trop. Med. Parasitol.*, 29, 349-360, 407-413 (1935).

¹⁰⁹⁵ W. R. Aykroyd and K. Rajagopal, *Indian J. Med. Research*, 24, 419-437 (1936).

¹⁰⁹⁶ W. R. Aykroyd and B. G. Krishnan, *Indian J. Med. Research*, 24, 707-725 (1937).

¹⁰⁹⁷ D. Pal, *Indian J. Pediat.*, 16, 1-5 (1949).

¹⁰⁹⁸ V. Ramalingaswami, *Indian J. Med. Sci.*, 2, 665-674 (1948).

¹⁰⁹⁹ W. L. Marmelzat, *Arch. Dermatol. and Syphilol.*, 63, 759-763 (1951).

¹¹⁰⁰ J. Richardson, *Polar Regions*, A. and B. Black, Edinburgh, 1861, p. 71; cited by K. Rodahl and T. Moore, *Biochem. J.*, 37, 166-168 (1943), p. 166.

expedition led by Barentzoon to Nova Zembla became ill after having partaken of polar bear liver (*Ursus (Thalassarcos) maritima*). In three cases, the illness was so severe that there was loss of skin from head to foot. On the other hand, Kane¹¹⁰¹ reported inconsistent results of poisoning due to bear liver. On two occasions, the whole company became ill after eating it, but in other instances there were no untoward effects. Another report is that of Koettlitz,¹¹⁰² who wrote of an English expedition to Franz Josef Land between 1894 and 1897 in which all the members suffered as a consequence of having eaten polar bear liver. Lindhard¹¹⁰³ likewise reported the poisoning of the members of another expedition after they had prepared and eaten a stew made out of the liver, heart, and kidney of a freshly-shot polar bear. All nineteen men who partook of the stew became sick. The distress started in some cases within three hours after the meal, and all the rest of the group became ill during the night. The symptoms of the poisoning included drowsiness, sluggishness, irritability or irresistible desire to sleep, visual disturbances, tonic and clonic attacks of cramp in the extremities, severe headache, and vomiting. During the second twenty-four hours, the skin of ten of the nineteen men began to peel around the mouth, and gradually over larger areas. In some cases the peeling was confined to the face, but, in the case of three men, it involved the entire surface from head to foot. Nansen¹¹⁰⁴ observed that on two occasions small amounts of bear liver were eaten without a deleterious effect. This fact would indicate that the poisonous effects occur only when large amounts of the liver are consumed. Doutt¹¹⁰⁵ found that the ingestion of fresh bear liver resulted in violent headaches, nausea, giddiness and torpor. In 1943, Rodahl and Moore⁵⁹⁴ reported that three specimens of bear liver contained 15,000 to 18,000 I.U. of vitamin A/g. wet weight, while a sample of seal liver was found to contain 13,000 I.U. of vitamin A/g. wet weight. When the liver was fed to rats, a hypervitaminosis A occurred whenever the consumption was high; however, when only small amounts of liver were eaten, no obvious symptoms of injury occurred. These data led Rodahl and Moore⁵⁹⁴ to ascribe the poisonous action of bear liver to its high content of vitamin A. In addition to the paper listed above, the

¹¹⁰¹ E. K. Kane, *Arctic Explorations in the Years 1854, 1855*, Childs & Peterson, Philadelphia, 1856, pp. 392, 393.

¹¹⁰² R. Koettlitz; cited by W. S. Bruce and W. E. Clarke, *Proc. Roy. Phys. Soc. Edinburgh*, 14, 78-86 (1897-1898), p. 80.

¹¹⁰³ J. Lindhard, *Medd. Grønland*, 41, 459-468 (1913).

¹¹⁰⁴ F. Nansen, *Blant sel og bjørn, Kristiania*, 1924; cited by K. Rodahl and T. Moore, *Biochem. J.*, 37, 166-168 (1943), p. 167.

¹¹⁰⁵ J. K. Doutt, *J. Mammalogy*, 21, 356-357 (1940).

reader is referred to the excellent review on hypervitaminosis A by Nieman and Klein Obbink¹¹⁰⁶ which appeared in 1954, and to the chapter of Wolbach¹⁰²⁸ and that of Mason¹⁰²⁹ in the recent monograph on *The Vitamins*.²

(2) Symptoms of Hypervitaminosis A in Animals

Two types of experimental hypervitaminosis A occur, namely acute and chronic intoxication. Acute intoxication supervenes within a few hours after the administration of excessive doses of vitamin A, which are usually given by injection. As an example, the toxic symptoms following the subcutaneous injection of 0.5 c.c. of a 25% solution of "biosterol" (or "fat-soluble A") into rats and mice, as reported by Takahashi *et al.*¹¹⁰⁷ and by Matsuoka,¹¹⁰⁸ which were followed by death, are an example of the acute type of toxicity. From fifteen to twenty minutes after the injection, the hind legs became paralyzed, and generalized cramps developed, associated with anemia, and a mucous discharge from the mouth. The animals became unconscious and died.¹¹⁰⁷ On the other hand, chronic intoxication with vitamin A appears when smaller doses of vitamin A are continued over a period of time. In general, hypervitaminosis A results in arrested growth, leading to a loss in weight, and marked emaciation, accompanied by anemia and cachexia. Rodahl¹¹⁰⁹ is of the opinion that the reduction in the growth rate results from a decreased food intake attributable to the lessening of appetite caused by the overdoses of vitamin A.

a. Skin Symptoms. Trophic changes occur in the epithelium which lead to a localized loss of hair; the bare patches are frequently colored red, indicating a hyperemia, and the hair becomes matted. That this effect of hypervitaminosis A is generalized is indicated by the fact that it is observed in the hedgehog (complete loss of quills thirty-four days after the injection of 100,000 I.U. of vitamin A¹¹¹⁰), and in the rat. In the latter animal, baldness ensued within a month after the injection of high dosages of the vitamin.¹¹¹⁰

Local application of vitamin A to the skin produces a strong contact

¹¹⁰⁶ C. Nieman and H. J. Klein Obbink, *Vitamins and Hormones*, 12, 69-99 (1954).

¹¹⁰⁷ L. Takahashi, Z. Nakamiya, K. Kawakami, and T. Kitasato, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, 3, 81-145 (1925).

¹¹⁰⁸ M. Matsuoka, *Bull. Inst. Phys. Chem. Research (Tokyo)*, 13, No. 10, 1267-1283 (1934); *Chem. Abst.*, 29, 5509 (1935); *Sci. Papers Inst. Phys. Chem. Research*, 25, *Abst.*, 71-73 (Oct., 1934).

¹¹⁰⁹ K. Rodahl, *The Toxic Effect of Polar Bear Liver*, *Norsk Polarinstitutt Skr.*, No. 92, 1-90 (1949).

¹¹¹⁰ W. Weslaw, B. Wroński, A. Wróblewski, and B. Wróblewski, *Klin. Wochschr.*, 17, 777-781, 879-884 (1938).

effect, with peeling. Escarras and Paillas¹¹¹¹ observed hyperkeratinization and hyperplasia of the blood vessels as a result of a localized action of the vitamin when applied to the skin of the guinea pig. In hypervitaminosis, the most characteristic change is the great increase in lipids in the horny layer of the squamous epithelium,^{1112,1113} as well as in the number of young non-keratinized cells.¹¹¹⁴ Keratinization was suppressed in rats,¹¹¹⁵ and in chick embryo ectoderm explants,¹¹¹⁶ by excess vitamin A in the cultures.

b. Skeletal Lesions. The most characteristic disorders in hypervitaminosis A are bone fractures. Von Euler and Widell¹¹¹⁷ called attention to the possibility of disturbed bone growth in young rats treated with cod liver oil, although they gave no definite data. The typical bone fractures were described almost simultaneously by Bomskov and Seemann,¹¹¹⁸ by Collazo and Rodriguez,¹¹¹⁹ and by Davies and Moore.¹¹²⁰ Multiple spontaneous fractures occur in the tibia and femur, producing a condition resembling rickets.¹¹²¹ Whereas the spontaneous fractures occur almost exclusively in the case of young rats,^{594,1119,1122} hypochromic anemia and hemorrhage are the more important abnormalities in older animals exposed to hypervitaminosis A. The most characteristic pathologic change, according to Strauss,¹¹²¹ is either increased activity of the osteoclasts, or decreased activity of the osteoblasts.¹¹²³ Longitudinal growth of the bone is augmented more than lateral growth in hypervitaminosis A. Wolbach and Bessey¹⁰⁴⁰ and later Wolbach alone^{1056,1124} postulated that, in hypervitaminosis A, periosteal bone growth, *i.e.*, the sequence of the growth phases in the cartilage which precedes the endochondral bone formation and the modeling of the bone, is increased, and the cartilage becomes cal-

¹¹¹¹ A. Escarras and J. Paillas, *Compt. rend. soc. biol.*, 129, 312-314 (1938).

¹¹¹² T. Moll, O. Dalmer, P. v. Dobeneck, G. Domagk, and F. Laquer, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, 170, 176-207 (1933).

¹¹¹³ T. Moll, G. Domagk, and F. Laquer, *Klin. Wochschr.*, 12, 465-467 (1933).

¹¹¹⁴ G. Domagk and P. v. Dobeneck, *Virchow's Arch. pathol. Anat. u. Physiol.*, 290, 385-395 (1933).

¹¹¹⁵ J. D. Sabella, H. A. Bern, and R. H. Kahn, *Proc. Soc. Exptl. Biol. Med.*, 76, 499-503 (1951).

¹¹¹⁶ H. B. Fell and E. Mellanby, *J. Physiol.*, 119, 470-488 (1953).

¹¹¹⁷ H. v. Euler and H. Widell, *Z. physiol. Chem.*, 144, 132-136 (1925).

¹¹¹⁸ C. Bomskov and G. Seemann, *Z. ges. exptl. Med.*, 89, 771-779 (1933).

¹¹¹⁹ J. A. Collazo and J. S. Rodriguez, *Klin. Wochschr.*, 12, 1768-1771 (1933).

¹¹²⁰ A. W. Davies and T. Moore, *Biochem. J.*, 28, 288-295 (1934).

¹¹²¹ K. S. Strauss, *Beitr. pathol. Anat. u. allgem. Pathol.*, 94, 345-352 (1934).

¹¹²² W. Papke, *Z. ges. exptl. Med.*, 101, 648-658 (1937).

¹¹²³ J. T. Irving, *J. Physiol.*, 108, 92-101 (1949).

¹¹²⁴ S. B. Wolbach, *Proc. Inst. Med. Chicago*, 16, 118-145 (1946).

cified, and is disposed of by osteoclasia. Studer¹¹²⁵ states that hyperplasia begins between the diaphysis and the epiphysis.

Hypervitaminosis A also causes changes in the teeth. According to Nieman and Klein Obbink,¹¹⁰⁶ the incisor teeth present pathologic changes, but Pohto¹¹²⁶ confirmed this finding only in part. A reduction in the formation of dentine occurs, together with thinning or disappearance of the lingual alveolar bone.¹¹²³ Rodahl¹¹²⁷ is of the opinion that hypervitaminosis A causes a deposit of calcium in the pulp and dentine, and degeneration of the odontoblasts. Fell and Mellanby¹¹²⁸⁻¹¹³⁰ developed *in vitro* methods for studying the effect of an excess of vitamin A on bone tissue. They noted resorption and disappearance of the fetal bone tissue *in vitro*, and dwindling of the cartilage matrix, which sometimes disappeared completely.¹¹²⁸⁻¹¹³⁰

c. Hemorrhage and Inflammation of Mucous Membranes. Internal hemorrhage is a characteristic reaction following hypervitaminosis A in experimental animals, and especially in rats. Hemorrhage occurs chiefly in the older animals, in contradistinction to bone fractures, which are observed principally in the younger ones. The hemorrhages may be subcutaneous and intramuscular, although they occur chiefly in the lungs and viscera. Walker *et al.*¹¹³¹ reported that excessive doses of vitamin A increase the prothrombin time in the rat. Although this animal is able to synthesize vitamin K in the intestinal tract, about 90% of the rats suffering from hypervitaminosis A die as a consequence of hemorrhage, when they are on a vitamin K-free diet.¹¹³² Rodahl¹¹³³ confirmed the hypothesis that hemorrhage is a symptom of hypervitaminosis A.

d. Endocrine Symptoms. Definite changes in some of the endocrine organs result from hypervitaminosis. The adrenal glands present the most conspicuous change when an excess of vitamin A is present; the effect is mild in the rat and more noticeable in the guinea pig. The changes which occur in the adrenals of the guinea pig in hypervitaminosis A are

¹¹²⁵ A. Studer, *Verhandl. schweiz. naturforsch. Ges.*, 128 Jahresversamml. (St. Gallen), 1948, p. 182.

¹¹²⁶ M. Pohto, *Mikroskopische Untersuchungen über die Schneidezähne der Ratten bei der A-Avitaminose, der Heilung derselben, und der A-Hypervitaminose*, Diss. Med. Chem. Lab. Odont. Inst., Univ. Helsinki, 1938; cited by S. B. Wolbach and O. A. Bessey, *Physiol. Revs.*, 22, 233-289 (1942), p. 236.

¹¹²⁷ K. Rodahl, *Nature*, 164, 531 (1949).

¹¹²⁸ H. B. Fell and E. Mellanby, *Brit. Med. J.*, 1950, II, 535-539.

¹¹²⁹ H. B. Fell and E. Mellanby, *J. Physiol.*, 115, 4P-6P (1951).

¹¹³⁰ H. B. Fell and E. Mellanby, *J. Physiol.*, 116, 320-349 (1952).

¹¹³¹ S. E. Walker, E. Eyllenburg, and T. Moore, *Biochem. J.*, 41, 575-580 (1947).

¹¹³² C. L. Maddock, S. B. Wolbach, and D. Jensen, *Federation Proc.*, 7, 275 (1948).

¹¹³³ K. Rodahl, *Hypervitaminosis A*, *Norsk Polarinstitut Skr.*, No. 95, 1-206 (1950).

identical with those of scurvy,^{1106,1127} and resemble symptoms resulting from underfeeding in the rat, rabbit, and guinea pig.¹¹³⁴⁻¹¹³⁶ The administration of vitamin C in doses as high as 250 mg. daily did not affect the response of this gland to excessive doses of vitamin A.¹⁰³³

The thyroid gland is of especial interest because of its reputed relationship to the metabolism of vitamin A. Although Takahashi *et al.*¹¹⁰⁷ reported that excessive doses of vitamin A caused hypertrophy of the thyroid gland, no histologic changes occurred in the gland under these conditions.¹¹³⁷ In fact, Wolbach¹⁰²³ stated that the thyroid glands of rats and of guinea pigs which had suffered severe skeletal changes from overdosing with vitamin A presented essentially the same picture as did glands from normal animals, insofar as colloid content, vacuolization, staining and height of alveolar epithelium were concerned. No variations in the thyroid glands were evident, even when comparisons were made with sections of the thyroid gland from A-avitaminotic rats. In fact, Wolbach stated that "the conclusion was unavoidable that this gland is not primarily influenced by vitamin A."

Congestion of the spleen,¹¹³⁸ hypertrophy of the islets of Langerhans in the pancreas,¹¹³⁸ and basophil hyperplasia of the anterior lobe of the pituitary¹¹³⁹ occur as a result of a vitamin A excess. Masin^{1140,1141} reported that estrus was prolonged in rats by the injection of 20,000 to 37,000 I.U. of vitamin A, due to the "luteinizing" effect of the vitamin. However, when pregnant rats were subjected to a continued excess of vitamin A, the litter rate was diminished (to 10%), and congenital malformations occurred in more than 54% of the surviving young.¹¹⁴² Gineste¹¹⁴³ found that an overdosage of vitamin A reduced the growth rate of the thymus gland by 21%. This was, however, in proportion to the decrease in the growth of the animal as a whole. No histologic changes were noted in the thymus of the rats with hypervitaminosis A, as compared with the findings

¹¹³⁴ H. Popper, *Physiol. Revs.*, **24**, 205-224 (1944).

¹¹³⁵ H. T. Blumenthal and L. Loeb, *Am. J. Pathol.*, **18**, 615-631 (1942).

¹¹³⁶ R. Whitehead, *J. Pathol. Bacteriol.*, **54**, 169-176 (1942).

¹¹³⁷ H. Noetzel, *Z. ges. exper. Med.*, **105**, 83-88 (1939).

¹¹³⁸ L. Cornil, A. Chevallier, and J. E. Paillas, *Ann. anat. pathol. et anat. normale méd.-chir.*, **16**, 74-83 (1939).

¹¹³⁹ L. Cornil, A. Chevallier, J. E. Paillas, and J. Chouquet, *Ann. anat. pathol. et anat. normale méd.-chir.*, **16**, 83-86 (1939).

¹¹⁴⁰ F. Masin, *Acta cient. venezolana*, **1**, 116-120 (1950).

¹¹⁴¹ F. Masin, *Acta cient. venezolana*, **1**, 26-29 (1950).

¹¹⁴² S. Q. Cohlman, *Science*, **117**, 535-536 (1953).

¹¹⁴³ P. J. Gineste, *Compt. rend. soc. biol.*, **135**, 974-975 (1941).

in the controls.¹¹⁴¹ Hypophysectomized rats were found to respond to excessive dosage of vitamin A in the same manner as did normal rats.¹¹⁴⁴

e. Other Effects of Hypervitaminosis A. Another outstanding change caused by an excessive intake of vitamin A is the degenerative atrophy of a number of the organs.¹¹⁴⁵ Prominent among these changes is the pathologic deposition of fat in the R.E. (reticuloendothelial) system of the liver, *i.e.*, in the Kupffer star cells.^{1114,1119,1137,1145-1147} Degenerative changes in the testicles were noted after long-continued administration of toxic doses of vitamin A to weanling rats. Adult rats were not affected.¹¹⁴⁸

Anemia likewise develops in hypervitaminosis A, as might be expected from the fact that a certain amount of hemorrhage occurs in this condition.¹¹⁴⁹ However, Lewis and Reti¹¹⁴⁹ do not consider the symptom specific. The erythrocyte count is reduced, and the blood contains erythroblasts.^{1106,1121} The abnormality in the blood picture has been ascribed to hypochromic anemia with hyperplastic bone marrow,¹¹²² as well as to an erythroblastic anemia.¹¹⁵⁰ According to Rodahl,¹¹²⁷ a variable hypochromic anemia obtains, associated with subperiosteal hematomata. Hemoglobin is reduced.¹¹²² It has also been reported that no storage of liver lipids takes place,¹¹⁵¹ and that a temporary increase in the blood lipids occurs.^{173,1151,1152} This is in line with the suggestion that hypervitaminosis A is related to fat metabolism, a hypothesis which is also supported by the fatty infiltration of the R.E. system.

(3) *Symptoms of Hypervitaminosis A in Man*

Hypervitaminosis A occurs only rarely in man and, in most cases, results from overdosages of vitamin A concentrates in the case of children. As discussed in the Introduction, the first report of this condition was that of Arctic explorers, who consumed large amounts of the vitamin A-rich liver of the polar bear or of other Arctic mammals. In addition to the masterly review of Nieman and Klein Obbink,¹¹⁰⁶ other reviews on the effect of A-hypervitaminosis in man include those of Klein Obbink and Nieman,¹¹⁵³

¹¹⁴⁴ S. B. Wolbach and C. L. Maddock, *Arch. Pathol.*, 53, 273-278 (1952).

¹¹⁴⁵ J. A. Collazo and J. S. Rodriguez, *Klin. Wochschr.*, 12, 1732-1734 (1933).

¹¹⁴⁶ W. v. Drigalski and W. Laubmann, *Klin. Wochschr.*, 12, 1171-1174 (1933).

¹¹⁴⁷ U. Uotila and P. E. Simola, *Arch. pathol. Anat. u. Physiol.*, 301, 523-534 (1938).

¹¹⁴⁸ C. L. Maddock, J. Cohen, and S. B. Wolbach, *Arch. Pathol.*, 56, 333-340 (1953).

¹¹⁴⁹ J. T. Lewis and L. Reti, *Compt. rend. soc. biol.*, 118, 577-580 (1935).

¹¹⁵⁰ G. Poumeau-Delille, *Compt. rend. soc. biol.*, 137, 373, 604-605 (1943).

¹¹⁵¹ H. Fasold and H. Peters, *Z. ges. expil. Med.*, 92, 57-62 (1934).

¹¹⁵² H. Wendt, *Münch. med. Wochschr.*, 82, 1160-1162 (1935); *Deut. med. Wochschr.*, 62, 1213-1215 (1936).

¹¹⁵³ H. J. Klein Obbink and C. Nieman, *Ned. Tijdschr. Geneesk.*, 97, 872-877 (1953).

Cubertier,¹¹⁵⁴ Palacios Mateos,¹¹⁵⁵ Knudson and Rothman,¹¹⁵⁶ and a short discussion in *Nutrition Reviews*.¹¹⁵⁷

In some cases the symptoms of hypervitaminosis A appear after the ingestion of a single excessive dose of vitamin A. Doutt¹¹⁰⁵ listed the following symptoms as typical of hypervitaminosis A in man: nausea, violent headache, dizziness, and torpor. As noted earlier, the peeling of the skin from a large part of the body may also be considered as a typical symptom.^{1158, 1159} In the case of children and infants with acute hypervitaminosis A, vomiting and drowsiness were observed, as well as the bulging of the fontanelle (acute benign hydrocephalus).^{1156, 1160, 1161}

Hypervitaminosis A symptoms were most frequently produced in children by long-continued excessive doses of vitamin A preparations. Symptoms included anorexia, growth failure, irritability, skeletal lesions, and skin symptoms such as itching fissures at the corners of the mouth and nostrils, coarsening of the hair, and a gradual development of alopecia.¹¹⁰⁶ Plasma vitamin A was largely increased, and usually the phosphatase level also rose. An excess of vitamin A in the blood of children who had received large doses of vitamin A was noted by Caffey.¹¹⁶² Hyperostoses in various bony structures were also a prominent symptom. In the twenty-five cases of known hypervitaminosis A recorded in humans by Nieman and Klein Obbink,¹¹⁰⁶ only two concerned this condition in the adult.^{1163, 1164}

(4) *The Level of Vitamin A Intake Which Causes Toxicity*

As is the case with other drugs, the level at which toxicity may be produced depends upon whether single or multiple injections are given, and upon the route of administration.

a. Acute Toxicity in the Rat. It was indicated by the experiments of

¹¹⁵⁴ S. Cubertier, *Sur les accidents toxiques causés par des fortes doses de vitamine A. Étude chez l'animal et chez l'enfant*. Thèse, Paris, 1949; cited by C. Nieman and H. J. Klein Obbink, *Vitamins and Hormones*, 12, 69-99 (1954), p. 77.

¹¹⁵⁵ J. M. Palacios Mateos, *Rev. clín. españ.*, 42, 269-271 (1951).

¹¹⁵⁶ A. G. Knudson, Jr., and P. E. Rothman, *Am. J. Diseases Children*, 85, 316-334 (1953).

¹¹⁵⁷ Anonymous, *Nutrition Revs.*, 6, 261-263 (1948).

¹¹⁵⁸ E. V. Poslavskii and A. G. Bogatkina, *Klin. Med. (U.S.S.R.)*, 11, 49-55 (1948); cited by C. Nieman and H. J. Klein Obbink, *Vitamins and Hormones*, 12, 69-99 (1954), pp. 77, 82.

¹¹⁵⁹ T. C. Lonie, *New Zealand Med. J.*, 49, 680-685 (1950).

¹¹⁶⁰ J. Marie and G. Sée, *Semaine hôp. Paris*, 27, 1744-1746 (1951).

¹¹⁶¹ J. M. Chuet García, *Acta Pediat. Españ. (Madrid)*, 9, No. 108, 1448-1449 (1951).

¹¹⁶² J. Caffey, *Pediatrics*, 5, 672-688 (1950).

¹¹⁶³ M. B. Sulzberger and M. P. Lazar, *J. Am. Med. Assoc.*, 146, 788-793 (1951).

¹¹⁶⁴ J. R. Frey and M. A. Schoch, *Dermatologica*, 104, 80-86 (1952).

Moore and Wang¹¹⁶⁵ that the lower limits of acute toxicity in young rats are within the range of 40,000 to 90,000 I.U., 50,000 I.U. being the average figure when the vitamin is administered parenterally. On the other hand, Rodahl¹¹³³ believes that if no puncture of the blood vessels occurs during the injection of the vitamin A, and hence no fat embolism develops, a single injection of a high-potency concentrate of 1,000,000 I.U. will produce no effect.

In the case of orally-administered vitamin A, the lethal dose given in oily solution to rats is reported by v. Drigalski¹¹⁶⁶ to be *ca.* 120,000 I.U. Rodahl¹¹³³ obtained only a temporary effect in adult rats when the dose was 1,600,000 I.U.

b. Chronic Toxicity in the Rat. (*a*) *The Level of Toxicity in Young Rats.* Various investigators have demonstrated that the onset of toxicity in young rats, as evaluated by different symptoms, occurs at *ca.* 10,000 to 12,500 I.U. per day, and at 20,000 to 25,000 I.U. per day for the lethal dose.¹¹⁰⁶ Thus, although Bell *et al.*¹¹⁶⁷ were unable to demonstrate toxicity when 13,500 I.U. of vitamin A were given daily, Studer¹¹²⁵ did report that a change in the blood pattern occurred in the rat after two days when a dosage of 10,000 I.U./day was given. Confirmatory evidence of this latter figure was obtained by Rodahl.¹¹³³ Van Metre¹¹⁶⁸ noted fractures and disturbances of bone growth in rats which had received 1250 I.U. per g. body weight per day, while Guerrant⁶⁷² noted some delay in the growth of rats given 8,800 I.U. of this vitamin per day. When rats received 13,500 I.U. of vitamin A/day, they survived, but growth was arrested and their pelts became coarse. This was defined as a "subtoxic dose."¹¹⁰⁶ On the other hand, more severe symptoms such as loss of weight and skin lesions were shown by Weslaw *et al.*¹¹¹⁰ to occur in young rats, when the vitamin A intake, as Vogan or Cresavit, was at a level of 20,000 I.U./day. (Vogan and Cresavit Merck are sesame oil solutions of vitamin A obtained from A-rich fish liver oils). Harris and Moore¹¹⁶⁹ observed loss of weight, marasmus, lack of appetite, difficulty in walking and sometimes paralysis in rats given 25,000 times the minimum dose of vitamin A. According to Wolbach and Bessey,¹⁰⁴⁰ and Moore and Wang,¹¹⁷⁰ bone fractures occurred within seven to twenty days when young rats were fed 25,000 to 50,000 I.U./day.

¹¹⁶⁵ T. Moore and Y. L. Wang, *Biochem. J.*, **39**, 222-228 (1945).

¹¹⁶⁶ W. v. Drigalski, *Klin. Wochschr.*, **12**, 308-309 (1933).

¹¹⁶⁷ M. E. Bell, E. Gregory, and J. C. Drummond, *Z. Vitaminforsch.*, **2**, 161-182 (1933).

¹¹⁶⁸ T. E. Van Metre, *Johns Hopkins Hosp. Bull.*, **81**, 305-311 (1947).

¹¹⁶⁹ L. J. Harris and T. Moore, *Biochem. J.*, **22**, 1461-1477 (1928); **23**, 1114-1121 (1929).

¹¹⁷⁰ T. Moore and Y. L. Wang, *Biochem. J.*, **37**, viii-ix (1943).

(b) *Age as a Factor in Toxicity.* Hypervitaminosis A is produced with a much lower dosage in young rats than in older animals. Thus, amounts as high as 26,500 I.U./day¹¹⁰⁶ or 30,000 I.U./day¹²⁹ were not toxic in adult rats, but reduced the metabolic rate.¹²⁹ This is nearly twice the level which is considered to be toxic in young rats. Inasmuch as the feeding of 50,000 I.U./day does bring on definite hypervitaminotic symptoms (fatal uterine hemorrhage) in the older animals,¹¹⁷⁰ Nieman and Klein Obbink¹¹⁰⁶ consider that chronic oral toxicity begins at 25,000 I.U./day, and that the lethal dose is *ca.* 50,000 I.U./day in adult rats.

(c) *Relative Toxicity When Vitamin A Is Administered Orally or Parenterally to Rats.* The toxic dosage of vitamin A is apparently approximately the same when given subcutaneously as when administered orally. Although Chauchard¹¹⁷¹ observed a reduction in nerve and muscle chronaxia and an excitation of the nervous system when only 3,500 I.U. of vitamin A daily (two drops of a solution containing 120,000 I.U. per c.c.) were injected subcutaneously, Weslaw *et al.*¹¹¹⁰ stated that the lethal dosage of vitamin A is lower for subcutaneous than for oral administration. Bone fractures appeared after rats had been injected daily for two weeks with 25,000 I.U. of vitamin A; moreover, Poumeau-Delille¹¹⁵⁰ observed a lethal effect in rats following the daily administration of 100,000 I.U. to 400,000 I.U. subcutaneously for two weeks. Nieman and Klein Obbink¹¹⁰⁶ are of the opinion that there is no great difference in toxicity between oral and subcutaneous administration. However, Rodahl¹¹³³ reported that larger doses of vitamin A are required to produce equal intoxication when the subcutaneous route is employed than when the vitamin is administered orally.

(d) *Toxicity in Animals Other Than the Rat.* The amount of vitamin A required to produce toxic effects in animals other than the rat is to a great extent in proportion to body weight. Thus, in the case of the mouse, Moll and co-workers^{1112,1113} observed a toxic effect when 4500 I.U. of vitamin A (as Vogan) were given per day, while Ypsilanti¹¹⁷² reported that adult mice succumbed after 1.5 weeks when *ca.* 11,000 I.U. of vitamin A were injected daily. This establishes the effective quantity of vitamin A to produce toxicity in mice as about one-fourth that of rats.

In the case of cats and rabbits, naturally, much larger amounts of vitamin A can be tolerated. Moll and associates^{1112,1113} stated that as much as 2,000,000 to 6,000,000 I.U. of vitamin A could be administered to animals of these species without ill effects. However, Wendt and Schroeder¹¹⁷³

¹¹⁷¹ P. Chauchard, *Compt. rend. soc. biol.*, 137, 429-430 (1943).

¹¹⁷² H. Ypsilanti, *Klin. Wochschr.*, 14, 90-91 (1935).

¹¹⁷³ H. Wendt and H. Schroeder, *Z. Vitaminforsch.*, 4, 206-212 (1935).

noted that guinea pigs began to lose weight within five days when 1.5 c.c. of Vogan (180,000 I.U. of vitamin A) were given daily. Rodahl¹⁰³³ reported similar toxic effects in rats, guinea pigs, rabbits, and young cockerels after the administration of vitamin A in doses comparable on the weight basis; symptoms of toxicity began with dosages of 50 to 130 I.U./g. body weight. Rigdon *et al.*²⁰⁵ observed failure to gain weight in ducks following doses of 200,000 I.U. of vitamin A/day, although no bone fractures occurred.

c. Acute and Chronic Toxicity in Man. The acute toxic dose of vitamin A, when given orally to adult humans, is in excess of 1,000,000 I.U. Values recorded by various investigators are as follows: Getz and co-workers, 2,000,000 I.U. (dull headache);¹¹⁷⁴ Poslavskii and Bogatkina, 1,000,000 to 5,000,000 I.U. (as whale liver oil);¹¹⁵⁸ Rodahl and Moore, 7,500,000 I.U. (irritability, sluggishness, drowsiness, headache, vomiting);⁵⁹⁴ and Oyenhart and Wassermann, 300,000 to 1,200,000 I.U. (no untoward effects).¹¹⁷⁵

When vitamin A is given repeatedly, the level for chronic toxicity appears to be about 600,000 I.U./day. According to Frey and Schoch,¹¹⁶⁴ daily dosages of 400,000 I.U. can be readily tolerated by adults, over an extended period. Moreover, Van Bruggen and Straumfjord³⁶⁰ reported no untoward effects resulting from the administration of 100,000 I.U. of vitamin A per day over a period of eighteen months, and Argonz and Abinzano^{1176, 1177} consider the treatment of premenstrual tension with 200,000 I.U. of vitamin A daily as harmless, and, in most cases, beneficial. On the other hand, several investigators^{1163, 1164} found that a daily intake of 600,000 to 1,000,000 I.U. of vitamin A precipitated the symptoms of hypervitaminosis A. Rodahl and Moore⁵⁹⁴ reported that several forms of indisposition, including dizziness, appeared within five days, in men, when they were given 6,000,000 I.U. of vitamin A daily.

Although the daily administration of 300,000 I.U. of vitamin A over several weeks did not precipitate a hypervitaminotic condition in children,¹¹⁷⁸ toxicity was evident in the case of a two-year-old tuberculous child who received 400,000 I.U. (in four divided doses) weekly; the effect was indicated by a reduced erythrocyte count and an increased sedimentation

¹¹⁷⁴ H. R. Getz, G. B. Hildebrand, and M. Finn, *J. Am. Med. Assoc.*, **112**, 1308-1311 (1939).

¹¹⁷⁵ J. C. Oyenhart and C. Wassermann, *El día méd.*, **12** (1950); cited by C. Nieman and J. Klein Obbink, *Vitamins and Hormones*, **12**, 69-99 (1954), p. 83.

¹¹⁷⁶ J. Argonz and C. Abinzano, *Semana méd. (Buenos Aires)*, **56**, 407-417 (1949).

¹¹⁷⁷ J. Argonz and C. Abinzano, *J. Clin. Endocrinol.*, **10**, 1579-1590 (1950).

¹¹⁷⁸ E. Lehman and H. G. Rapaport, *J. Am. Med. Assoc.*, **114**, 386-393 (1940).

time.¹¹⁷⁹ Nieman and Klein Obbink¹¹⁰⁶ calculate the toxic dosage for children at 100,000 I.U. per day. However, Caffey¹¹⁶² believes that 75,000 I.U. of vitamin A will bring on a hypervitaminosis A within six months.

(5) *The Interaction of Excess Vitamin A
with Other Vitamins and with Hormones*

a. Interaction with Thyroxine. The thyroid gland is intimately connected with the utilization and metabolism of vitamin A. It appears to be concerned with the transformation of carotene into vitamin A, with the storage of vitamin A, as well as with the secretion of carotenoids into milk. It has also been reported that an antagonism exists between excess vitamin A and thyroxine in hypervitaminosis A.

Takahashi *et al.*¹¹⁰⁷ were the first to report that hypertrophy and hypofunction of the thyroid gland occur in hypervitaminosis A. In fact, several investigators successfully employed large doses of vitamin A in the treatment of Basedow's disease¹¹⁵² or of general hyperthyroidism.¹¹⁸⁰ Three doses of 30 drops each of Vogan reduced the basal metabolism in Basedow's disease to practically normal values.¹¹⁵² Peters¹¹⁸¹ and Fasold and Peters¹¹⁵¹ demonstrated a definite antagonism between vitamin A and thyroxine. Hypervitaminosis A could be prevented in young rats by the injection of thyroxine; conversely, normal growth could be maintained in hyperthyroidism by the simultaneous administration of overdoses of vitamin A. According to Chevallier and Baert,¹¹⁸² the basal metabolism of young rats is reduced when excess vitamin A is given. It is suggested that the toxicity of excessive amounts of vitamin A may be related to its antagonism toward the thyroid hormone.¹¹⁸³ Confirmation of the effect of large doses of vitamin A on thyroxine has been adduced by examination of the gland itself. Thus, a reduction in the colloid content of the acini was observed by Sherwood *et al.*¹¹⁸⁴ after large doses of vitamin A. The daily administration of 30,000 I.U. of vitamin A caused a reduction in the weight of the thyroid gland of rats, together with a reduction of the basal metabolism.¹²⁹ Sadhu and Truscott¹¹⁸⁵ are of the opinion that hypervitaminosis A results successively in a reduction in the breakdown of thyroxine by the

¹¹⁷⁹ A. Masi and M. Giusti, *Riv. pediat. siciliana*, 2, 266-267 (1947); *Chem. Abst.*, 43, 3499 (1949).

¹¹⁸⁰ S. Simkins, *J. Clin. Endocrinol.*, 7, 574-585 (1947).

¹¹⁸¹ H. Peters, *Klin. Wochschr.*, 12, 1977 (1933).

¹¹⁸² A. Chevallier and H. Baert, *Compt. rend. soc. biol.*, 116, 1037-1039 (1934).

¹¹⁸³ J. Chalier and M. Jeune, *Compt. rend. soc. biol.*, 129, 604-606 (1938).

¹¹⁸⁴ T. C. Sherwood, L. A. Toth, and K. Carr, *Endocrinology*, 18, 254-255 (1934).

¹¹⁸⁵ D. P. Sadhu and B. L. Truscott, *Endocrinology*, 43, 120-123 (1948).

liver, hyperthyroxinemia, reduced secretion of thyrotropic hormone, and loss in weight of the thyroid gland, accompanied by a reduction in the content of protein-linked iodine in the gland.

b. Interaction with Calcium and Phosphorus. Since bone fracture is a prominent symptom of hypervitaminosis A, it appears possible that the excess vitamin A might have the capacity to react with phosphorus and calcium in the animal organism. An increased excretion of calcium and phosphorus in the urine was shown to occur after the subcutaneous injection of biosterol ("fat-soluble A"),¹¹⁸⁶ possibly due to the destruction of digestive organs and muscles consequent to the hypervitaminosis. The action of the vitamin D, which is also present in biosterol, cannot be entirely discounted. The decrease in these minerals in the bones following the administration of Vogan has been cited as the cause for the tendency to bone fractures in hypervitaminosis A.¹¹¹⁸ However, the calcium and phosphate levels of the blood of rats with hypervitaminosis A have been reported to be normal,^{1125, 1187} and no reduction of these substances in bone ash could be demonstrated in rats treated with large doses of vitamin A.^{1133, 1165} Hypervitaminosis A results in a marked increase in the content of alkaline phosphatase in the epiphyseal junction,¹¹⁸⁸ although the phosphatase content in other bones is decreased.

c. Interaction with the Vitamin B Complex. Höjer,¹¹⁸⁹ in 1927, and Bell *et al.*¹¹⁶⁷ several years later, reported that certain B vitamins exert a favorable effect upon symptoms of hypervitaminosis A produced by cod-liver oil. It is now believed that the beneficial effect in these cases can be attributed to the antagonism of the B complex toward the toxicity of the saponifiable fraction of the fat. Moore and Wang¹¹⁶⁵ are of the opinion that yeast does not influence the course of gestation in hypervitaminosis A in rats. Scheunert and Wagner¹¹⁹⁰ did not observe any synergism or antagonism between thiamine and toxic doses of vitamin A. This conclusion is concurred in by Rodahl.¹¹³³ Vedder and Rosenberg¹¹⁹¹ had earlier reported that 500 I.U. of thiamine increased the toxicity of high dosages of vitamin A given in jewfish liver oil.

d. Interaction with Ascorbic Acid. A number of the earlier investigations indicated that an antagonism existed between vitamin A and ascorbic

¹¹⁸⁶ T. Munehisa, *Sei-i-kwai Med. J. (Tokyo)*, 48, No. 5, 57-81, *Abst. Sect.*, 2-3; No. 10, 77-107, *English Sect.*, 4 (1929).

¹¹⁸⁷ K. Rodahl, *J. Nutrition*, 41, 399-421 (1950).

¹¹⁸⁸ K. S. Ludwig, *Int. Z. Vitaminforsch.*, 25, 98-103 (1953).

¹¹⁸⁹ A. Höjer, *Acta Paediat.*, 6, 180-190 (1927).

¹¹⁹⁰ A. Scheunert and K. H. Wagner, *Z. physiol. Chem.*, 256, 111-121 (1938).

¹¹⁹¹ E. B. Vedder and C. Rosenberg, *J. Nutrition*, 16, 57-68 (1938).

acid (vitamin C).¹¹⁹¹ Thus, it was reported that fish liver oils counteracted the antiscorbutic effect of ascorbic acid in guinea pigs;^{1192,1193} conversely, v. Euler and Widell¹¹¹⁷ showed that ascorbic acid had a beneficial effect upon the incidence of bone fractures, and upon the reduction in growth of rats, resulting from the administration of large amounts of cod liver oil. Similar favorable results of ascorbic acid on hypervitaminosis A were reported by Wendt and Schroeder,¹¹⁷³ in the case of both rats and guinea pigs; ascorbic acid had a marked delaying action on the deposition of vitamin A in the liver. In contradistinction to these positive results, a number of workers have failed to confirm the hypothesis that an interrelationship exists between vitamin A and ascorbic acid. Thus, Collett and Eriksen¹¹⁹⁴ failed to demonstrate any antagonistic action of vitamin A against vitamin C, except in very high dosages (in guinea pigs). Simola and Kauppinen¹¹⁹⁵ observed that the ascorbic acid content of the tissues of A-hypervitaminotic guinea pigs and rats was normal. Moreover, overdoses of vitamin A were not found to increase the ascorbic acid requirement of guinea pigs.¹¹⁹⁶ Jonsson and co-workers¹¹⁹⁷ state that scurvy may occur as a secondary symptom in rats deficient in vitamin A.

One suggestion is that thyroxine acts as a third factor in the interrelationship of vitamin A and ascorbic acid,^{1198,1199} and that an antithyroid factor is present in scurvy.¹¹⁹⁹ Additional evidence of the relationship of vitamins A and C is based upon the reduction of ascorbic acid in A hypervitaminotic rats, in contrast to the findings of Simola and Kauppinen.¹¹⁹⁵ Thus, when an overdosage of vitamin A-rich oil concentrate was given to rats, the urinary ascorbic acid disappeared.¹¹⁹¹ Abnormally low ascorbic acid values were found by Rodahl¹¹²⁷ in the serum and liver of A-hypervitaminotic rats. Moreover, the toxic effect of excessive doses of vitamin A was found to be greater in guinea pigs on a scorbutigenic diet than on one furnishing adequate amounts of vitamin C. Rodahl¹¹³³ reported that rats could be protected against moderate dosages of vitamin A, up to 50,000 I.U./day, by the subcutaneous injection of ascorbic acid. However, a number of workers^{1131,1165,1200} failed to observe abnormal ascorbic acid

¹¹⁹² G. Mouriquand and P. Michel, *Compt. rend. soc. biol.*, **86**, 1170-1172 (1922).

¹¹⁹³ G. Mouriquand, P. Michel, and R. Sanyas, *Lyon méd.*, **132**, 360-362 (1923).

¹¹⁹⁴ E. Collett and B. Eriksen, *Biochem. J.*, **32**, 2299-2303 (1938).

¹¹⁹⁵ P. E. Simola and T. K. Kauppinen, *Skand. Arch. Physiol.*, **71**, 31-32 (1935).

¹¹⁹⁶ J. Melka, *Compt. rend. soc. biol.*, **141**, 521-523 (1947).

¹¹⁹⁷ A. G. Jonsson, A. L. Obel, and K. Sjöberg, *Z. Vitaminforsch.*, **12**, 300-320 (1942).

¹¹⁹⁸ G. Török, *Magyar Orvosi Arch. (Budapest)*, n.s. **39**, 315 (1938); *Chem. Abst.*, **33**, 227 (1939).

¹¹⁹⁹ G. Balassa and G. Szántó, *Z. Vitaminforsch.*, **8**, 233-237 (1939).

¹²⁰⁰ N. Eeg-Larsen and A. Pihl, *Acta Pharmacol. Toxicol.*, **7**, 367-375 (1951).

metabolism in animals suffering from hypervitaminosis A. Van Bruggen and Straumfjord³⁶⁰ found a normal vitamin C content in the serum of a man who had received 100,000 I.U. of vitamin A/day for three years.

e. Interaction with Vitamin D. In view of the disturbed bone formation and the effect on blood clotting in hypervitaminosis A, it seemed that it might have some relationship to the action of vitamin D. In fact, it was early reported that vitamin A interferes with the curative effect of vitamin D in rickets.¹¹¹⁸ In addition, Javillier and Emerique-Blum¹²⁰¹ reported that the onset of experimental rickets was accelerated in rats given an overdose of vitamin A.

Conversely, Vedder and Rosenberg¹¹⁹¹ noted that the toxicity of large doses of vitamin A could be reduced by the administration of a high dosage of vitamin D. The antirachitic vitamin likewise counteracted the adverse effects of high doses of vitamin A on blood clotting.¹²⁰² According to Maddock and co-workers,¹¹³² hypervitaminosis A was accompanied by hemorrhages only when the experimental rats were receiving a rachitogenic diet.

However, hypervitaminosis A and avitaminosis D differ. Vitamin D₂ will not prevent the appearance of bone fractures in A-hypervitaminotic rats.¹¹⁶⁵ On the other hand, corneal injury produced by the external application of vitamin A was inhibited by the application of vitamin D.¹²⁰³ Rodahl¹¹³³ concluded (in 1950) that neither the prophylactic effect nor the curative action of vitamin D was affected by hypervitaminosis A. On the other hand, A-hypervitaminotic symptoms appeared to develop more rapidly in rats with avitaminosis D than in animals having a normal supply of the antirachitic vitamin. There is therefore little question that an interaction does exist between vitamins A and D, but more experimental work will be necessary before an explanation of the mechanism is forthcoming.

f. Interaction with Vitamin K. A hypoprothrombinemia in A-hypervitaminosis was reported by Light and co-workers,¹²⁰⁴ and by Walker *et al.*¹¹³¹ This is believed to be related to the characteristic hemorrhages in this condition. According to Maddock and associates,¹¹³² who also observed a hypoprothrombinemia in hypervitaminosis A, the clotting time of rats was increased from a normal value of twenty-five seconds to 70 to 183 seconds simply by the feeding of excessive doses of vitamin A/day for five

¹²⁰¹ M. Javillier and L. Emerique-Blum, *Compt. rend.*, 212, 289-292 (1941); *Chem. Zentr.*, 113, Part I, 3112 (1942).

¹²⁰² Y. Raoul and N. Ragheb-Hanna, *Compt. rend. soc. biol.*, 144, 17-19 (1950).

¹²⁰³ W. J. Kapuschinski and J. Zebrowski, *Klin. Oczna (Warsaw)*, 20, 328-331 (1950); *Chem. Abst.*, 46, 5158 (1952); *Am. J. Ophthalmol.*, 35, 283 (1952).

¹²⁰⁴ R. F. Light, R. P. Alscher, and C. N. Frey, *Science*, 100, 225-226 (1944).

days or longer. However, the clotting time of these rats could be restored to normal by the administration of an excess of vitamin K.¹¹³¹ While a reduction in the incidence of the hemorrhages also followed vitamin K supplementation, this treatment afforded no relief from the bone fractures.¹¹³¹ Quick and Stefanini¹²⁰⁵ are of the opinion that the excess vitamin A interferes with the microbiologic synthesis of vitamin K by the intestinal flora. In the case of the chick, which derives its vitamin K exclusively from its food, the coagulation time is not increased when 50,000 I.U. of vitamin A are given.

¹²⁰⁵ A. J. Quick and M. Stefanini, *J. Biol. Chem.*, 175, 945-952 (1948).

CHAPTER X

THE METABOLISM AND NUTRITIONAL VALUE OF THE VITAMINS D

Although the physiologic role of the fat-soluble vitamins other than vitamin A is not so well understood as is that of the latter vitamin, recent discoveries have indicated that each fat-soluble vitamin mediates important specific independent physiologic changes. The following three chapters will briefly review the background material presented earlier in Volumes I and II of *The Lipids* concerning each of the three fat-soluble vitamins, D, E, and K. This will be followed in each case by a discussion of the metabolic aspects and the physiologic roles played by each vitamin.

1. Introduction

(1) *Rickets*

As in the case of vitamin A, vitamin D was discovered because of the therapeutic effect of preparations in which it was present on the avitaminosis occurring when this vitamin was absent from the diet of animals and of humans. The deficiency disease arising in animals and in children lacking vitamin D is referred to as rickets. According to Nicolaysen and Eeg-Larsen,¹ the terminology for this condition originated from the verb in the local Dorsetshire dialect, "to rucket," which meant "to breathe with difficulty." Bills,² writing in Sebrell-Harris *The Vitamins*, attributed the origin of the term, *rickets*, to the word, *wrygates*, which in turn means "crooked goings." In contradistinction to the use of the terms, *rickets*, *ricketic*, and *antiricketic* in the English literature, *rachitis* is the word employed by the Germans to describe this abnormality. The latter term is presumably derived from the Greek word, *rachis*, meaning spine.¹ The term *rachitis*, and the related *rachitic* and *antirachitic*, have been widely employed in the American literature. The French have used a similar root in

¹ R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, 11, 29-60 (1953).

² C. E. Bills, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. II, Academic Press, New York, 1954, pp. 132-223.

coining the name of the deficiency in their term, *rachitisme*. The most important development in the understanding of rickets is to be attributed to the two English doctors, Glisson and Whistler, who lived in the seventeenth century.¹ Later reviews and monographs on this deficiency condition are those of Orgler³ (1912), Korenchevsky⁴ (1922), György⁵ (1929), Hess⁶ (1929), and that of the Medical Research Council⁷ (1932).

As in the case of vitamin A, the deficiency syndrome resulting from the absence of vitamin D in the diet was recognized long before the presence of the vitamin itself was understood. Trousseau,⁸ as early as 1873, recommended cod-liver oil for the treatment of rickets. Hopkins^{9,10} and Funk¹¹ were the first to postulate, many years later, that rickets was caused by the absence of an "accessory foodstuff" in the diet.

The understanding of the nature of the curative agent in cod-liver oil against rickets began to emerge soon after Mellanby¹²⁻¹⁴ produced rickets in dogs, and was able to alleviate the condition by the use of certain animal fats. This investigator was of the opinion that the curative factor was "fat-soluble A," or that its distribution was similar to that of the latter principle.^{12,13} Further investigation of the nature of the antirachitic principle was made possible by the experimental production of rickets in rats. This development was based upon the pioneer studies of Sherman and Pappenheimer,^{15,16} as well as upon the brilliant research of the McCollum

³ A. Orgler, *Ergeb. inn. Med. Kinderheilk.*, 8, 142-182 (1912).

⁴ V. Korenchevsky, *The Aetiology and Pathology of Rickets from an Experimental Point of View*, Med. Research Council (Brit.), Spec. Rept. Ser. No. 71, (1922); *Brit. Med. J.*, 1921, 2, 547-550; *New York Med. J.*, 115, 612-614 (1922).

⁵ P. György, *Die Behandlung und Verhütung der Rachitis und Tetanie*, Springer, Berlin, 1929; cited by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, 11, 29-60 (1953), p. 30; *Ergeb. inn. Med. Kinderheilk.*, 36, 752-966 (1929).

⁶ A. Hess, *Rickets, Including Osteomalacia and Tetany*, Lea & Febiger, Philadelphia (1929).

⁷ Anonymous, *Vitamins: A Survey of Present Knowledge*, Med. Research Council (Brit.), Spec. Rept. Ser. No. 167, (1932), pp. 48-49.

⁸ A. Trousseau, *Lectures on Clinical Medicine*, Blakiston, 1873 (translated by J. R. Cormack and P. V. Bazire from the 3rd ed. of *Clinique medicale de l'Hotel Dieu de Paris*, Ballière, Paris, 1898).

⁹ F. G. Hopkins, *Analyst*, 31, 385-404 (1906).

¹⁰ F. G. Hopkins, *J. Physiol.*, 44, 425-460 (1912).

¹¹ C. Funk, *Die Vitamine, Ihre Bedeutung für die Physiologie und Pathologie, mit besonderer Berücksichtigung der Avitaminosen*, J. F. Bergmann, Wiesbaden, 1914, 201 pp.

¹² E. Mellanby, *J. Physiol.*, 52, liii-liv (1919).

¹³ E. Mellanby, *Lancet*, 196, I, 407-412 (1919).

¹⁴ E. Mellanby, *Experimental Rickets*, Med. Research Council (Brit.), Spec. Rept. Ser. No. 61 (1921); cited by C. E. Bills, in H. Sebrell and R. S. Harris, *The Vitamins*, Vol. II, 132-223 (1954), p. 133; *Lancet*, 1920, I, 604.

¹⁵ H. C. Sherman and A. M. Pappenheimer, *Proc. Soc. Exptl. Biol. Med.*, 18, 193-197 (1921).

¹⁶ H. C. Sherman and A. M. Pappenheimer, *J. Exptl. Med.*, 34, 189-198 (1921).

group.¹⁷⁻²¹ Zucker, *et al.*²² observed that the antirachitic property of fish liver oils was concentrated in the non-glyceride portion, while McCollum and co-workers²³ soon followed with the proof that the antirachitic activity and the antixerophthalmic activity represent two distinct chemical principles (see *Lipids*, volume I, pp. 740-742).

(2) *The Provitamins D*

a. Structural Requirements for Activity. The clue to the identity of vitamin D was furnished by the discovery of the effect of ultraviolet radiation in curing rickets. It had been reported as early as 1822 by Sniadecki²⁴ that sunlight constitutes an effective treatment for rickets. For early reports²⁵⁻³¹ on the beneficial effect of sunlight and of quartz-lamp radiation, see *Lipids*, volume I, p. 741. Hess and Gutman³² concluded that the curative action of ultraviolet irradiation was identical with that of cod-liver oil in the diet.

The simultaneous demonstration by Steenbock *et al.*³³⁻³⁶ and by Hess

¹⁷ E. V. McCollum, N. Simmonds, H. T. Parsons, P. G. Shipley, and E. A. Park, *J. Biol. Chem.*, **45**, 333-341 (1920-1921).

¹⁸ P. G. Shipley, E. A. Park, E. V. McCollum, N. Simmonds, and H. T. Parsons, *J. Biol. Chem.*, **45**, 343-348 (1920-1921).

¹⁹ E. V. McCollum, N. Simmonds, P. G. Shipley, and E. A. Park, *J. Biol. Chem.*, **47**, 507-527 (1921).

²⁰ P. G. Shipley, E. V. McCollum, and N. Simmonds, *J. Biol. Chem.*, **49**, 399-410 (1921).

²¹ E. V. McCollum, N. Simmonds, P. G. Shipley, and E. A. Park, *J. Biol. Chem.*, **50**, 5-30, vi (1922).

²² T. F. Zucker, A. M. Pappenheimer, and M. Barnett, *Proc. Soc. Exptl. Biol. Med.*, **19**, 167-169 (1922).

²³ E. V. McCollum, N. Simmonds, J. E. Becker, and P. G. Shipley, *J. Biol. Chem.*, **53**, 252-312 (1922).

²⁴ J. Sniadecki, *On the Physical Education of Children*, Wilno, 1822; cited by W. Mozolowski, *Nature*, **143**, 121 (1939).

²⁵ T. A. Palm, *Practitioner*, **45**, 271-279, 321-342 (1890).

²⁶ E. Buchholz, *Verhandl. 21ten Versamml. Gesell. Kinderheilk.*, **76 ten Versamml. deut. Naturforsch. Aerzte**, Breslau (1904), **21**, 116-122 (1905), Wiesbaden.

²⁷ J. Raczynski, *Compt. rend. assoc. intern. pcdiat.*, **1913**, 308; cited by H. R. Rosenberg, *Chemistry and Physiology of the Vitamins*, Interscience, New York, 1945, p. 342.

²⁸ K. Huldschinsky, *Deut. med. Wochschr.*, **45**, 712-713 (1919).

²⁹ K. Huldschinsky, *Z. orthop. Chim.*, **39**, 426-451 (1919-1920).

³⁰ A. F. Hess and L. J. Unger, *Proc. Soc. Exptl. Biol. Med.*, **18**, 298 (1921).

³¹ A. F. Hess, L. J. Unger, and A. M. Pappenheimer, *Proc. Soc. Exptl. Biol. Med.*, **19**, 8-12 (1921-1922).

³² A. F. Hess and M. G. Gutman, *J. Am. Med. Assoc.*, **78**, 29-31 (1922).

³³ H. Steenbock, *Science*, **60**, 224-225 (1924).

³⁴ H. Steenbock and A. Black, *J. Biol. Chem.*, **61**, 405-421 (1924).

³⁵ H. Steenbock and M. T. Nelson, *J. Biol. Chem.*, **62**, 209-216 (1924).

³⁶ H. Steenbock, *U. S. Patent No. 1,680,818* (Aug. 14, 1928).

and Weinstock,³⁷⁻³⁹ in 1924-1925, that antirachitic activity could be imparted to certain foods by ultraviolet irradiation opened the avenue for the synthesis and isolation of vitamin D. It was realized immediately that the precursor of the active principle was in the category of a provitamin, and that this was transformed into the physiologically-active form by the photochemical effect of the ultraviolet rays. Shortly after the original findings, various workers reported that the activated substance was originally a sterol-like compound without antirachitic potency.⁴⁰⁻⁴² Several groups demonstrated almost simultaneously that provitamin D was ergosterol, or a sterol with a structure similar to that of dehydrocholesterol.⁴³⁻⁴⁷ It was discovered that there were several provitamins D, including not only ergosterol but also 22-dihydroergosterol,⁴⁸ and 7-dehydrocholesterol. The physiologic nature of the latter provitamin D was demonstrated when it was isolated from hog skin.⁴⁹ It had previously been synthesized by Boer *et al.*⁵⁰ Moreover, a number of investigators⁵¹⁻⁵⁵ showed that the vitamin D in halibut and tuna oils consisted almost entirely of an ester identical with the product obtained from activated 7-dehydrocholesterol. Crystalline vitamin D₂ (the form prepared from ergosterol) was isolated by Reerink and van Wijk^{56,57} in 1929, although Linsert⁵⁸ and Windaus, Linsert *et al.*⁵⁹ are

³⁷ A. F. Hess, *Am. J. Diseases Children*, **28**, 517-521 (1924).

³⁸ A. F. Hess and M. Weinstock, *Proc. Soc. Exptl. Biol. Med.*, **22**, 5-6, 6-7 (1924-1925).

³⁹ A. F. Hess and M. Weinstock, *J. Biol. Chem.*, **62**, 301-313 (1924).

⁴⁰ A. F. Hess, M. Weinstock, and F. D. Helman, *J. Biol. Chem.*, **63**, 305-308 (1925).

⁴¹ H. Steenbock and A. Black, *J. Biol. Chem.*, **64**, 263-298 (1925).

⁴² O. Rosenheim and T. A. Webster, *Lancet*, 1925, **I**, 1025-1026.

⁴³ R. Pohl, *Nachr. Ges. Wiss. Göttingen, Math-physik. Klasse*, **III**, 185-191 (1926).

⁴⁴ A. Windaus and A. F. Hess, *Nachr. Ges. Wiss. Göttingen, Math-physik. Klasse*, **III**, 175-184 (1926).

⁴⁵ A. Windaus and F. Holtz, *Nachr. Ges. Wiss. Göttingen, Math-physik. Klasse*, **III**, 217-225 (1927).

⁴⁶ O. Rosenheim and T. A. Webster, *Lancet*, 1927, **I**, 306-307.

⁴⁷ O. Rosenheim and T. A. Webster, *Biochem. J.*, **21**, 389-397 (1927).

⁴⁸ A. Windaus and R. Langer, *Ann.*, **508**, 105-114 (1933).

⁴⁹ A. Windaus and F. Bock, *Z. physiol. Chem.*, **245**, 168-170 (1937).

⁵⁰ A. G. Boer, E. H. Reerink, A. van Wijk, and J. van Niekerk, *Proc. Acad. Sci. Amsterdam*, **39**, 622-632 (1936).

⁵¹ H. Brockmann, *Z. physiol. Chem.*, **241**, 104-115 (1936).

⁵² H. Brockmann, *Z. physiol. Chem.*, **245**, 96-102 (1937).

⁵³ H. Brockmann and A. Busse, *Z. physiol. Chem.*, **249**, 176-180 (1937).

⁵⁴ E. J. H. Simons and T. F. Zucker, *J. Am. Chem. Soc.*, **58**, 2655 (1936).

⁵⁵ G. A. D. Haslewood and J. C. Drummond, *Chemistry & Industry*, **14** (*J. Soc. Chem. Ind.*, **55**) 598-599 (1936).

⁵⁶ E. H. Reerink and A. van Wijk, *Biochem. J.*, **23**, 1294-1307 (1929).

⁵⁷ E. H. Reerink and A. van Wijk, *Biochem. J.*, **25**, 1001-1010 (1931).

⁵⁸ O. Linsert, annotation in *Ann.*, **489**, 269 (1931).

⁵⁹ A. Windaus, O. Linsert, A. Lüttringhaus, and G. Weidlich, *Ann.*, **492**, 226-241 (1931).

credited with having made the first pure preparation. Schenck⁶⁰ prepared pure vitamin D₃ from irradiated 7-dehydrocholesterol in 1937.

The terminology still used in relation to the vitamins D is that introduced by the Askew and Angus groups⁶¹⁻⁶³ and by Windaus *et al.*^{59,64,65} The product originally given the designation of vitamin D₁ was later shown to be impure, and therefore this term is not employed. Vitamin D₂ is the name given to the active principle obtained on irradiation of ergosterol; it is likewise referred to as activated ergosterol and also as *calciferol*. Windaus and co-workers⁶⁶ assigned the name, vitamin D₃, to the product prepared by activation of 7-dehydrocholesterol, while the designation vitamin D₄ is used for the active vitamin obtained by Windaus and Langer⁴⁸ on irradiation of 22-dihydroergosterol.

In addition to ergosterol, 7-dehydrocholesterol and 22-dihydroergosterol themselves, many of their derivatives have been found to be highly potent provitamins D. Thus, the following esters of cholesterol (7-dehydrocholesterol) can be activated into potent antirachitic substances: acetate,⁶⁷⁻⁶⁹ benzoate,⁶⁹ isobutyrate,⁶⁹ palmitate,⁶⁷ and phosphate.⁷⁰ Ergosterol also is as readily activated in the form of the ester as in that of the free alcohol.² On the other hand, it is reported that the esters of calciferol are not uniformly converted to active vitamin D on irradiation. Although irradiated ergosteryl acetate,^{71,72} irradiated ergosteryl ethyl carbonate,⁷² and irradiated diergosteryl phosphate⁷³ have been found to be equally active, the provitamin activity of the benzoate ester was reported to be considerably reduced,⁷² except when doses of 10 m μ or over were given.⁷⁴ It had

⁶⁰ F. Schenck, *Naturwissenschaften*, **25**, 159 (1937).

⁶¹ F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. G. C. Jenkins, and T. A. Webster, *Proc. Roy. Soc. (London)*, **B 107**, 76-90, 91-100 (1930).

⁶² T. C. Angus, F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. K. Callow, C. Fischermann, J. S. L. Philpot, and T. A. Webster, *Proc. Roy. Soc. (London)*, **B 108**, 340-359 (1931).

⁶³ F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. K. Callow, J. S. L. Philpot, and T. A. Webster, *Proc. Roy. Soc. (London)*, **B 109**, 488-506 (1932).

⁶⁴ A. Windaus, A. Lüttringhaus, and M. Deppe, *Ann.*, **489**, 252-269 (1931).

⁶⁵ A. Windaus, F. v. Werder, and A. Lüttringhaus, *Ann.*, **499**, 188-200 (1932).

⁶⁶ A. Windaus, F. Schenck, and F. v. Werder, *Z. physiol. Chem.*, **241**, 101-103 (1936).

⁶⁷ O. Rosenheim and T. A. Webster, *Biochem. J.*, **20**, 537-544 (1926).

⁶⁸ A. F. Hess, M. Weinstock, and E. Sherman, *J. Biol. Chem.*, **67**, 413-423 (1926).

⁶⁹ C. E. Bills and F. G. McDonald, *J. Biol. Chem.*, **72**, 13-19 (1927).

⁷⁰ H. von Euler and A. Bernton, *Ber.*, **60**, 1720-1725 (1927).

⁷¹ A. Rosenheim and T. A. Webster, *Lancet*, **1927**, *II*, 622-625; *Biochem. J.*, **22**, 762-766 (1928).

⁷² R. K. Callow, *Biochem. J.*, **25**, 79-86 (1931).

⁷³ H. von Euler, A. Wolf, and H. Hellström, *Ber.*, **62**, 2451-2456 (1929).

⁷⁴ A. Windaus and O. Rygh, *Nachr. Ges. Wiss. Göttingen, Math.-physik. Klasse*, **III**, 202-216 (1928).

been reported by Rosenheim and Webster⁷¹ to be activated to the same degree as was ergosterol itself. Ergosteryl palmitate⁷⁴ and vitamin D₂ palmitate⁷⁵ were found to be less readily activated than is free ergosterol, while the allophanate, cinnamate, diphenylacetate, oxalate, α -naphthylurethane, and phenylurethane derivatives are all completely inactive.⁷⁴ This variation in effectiveness as provitamins D is not due to the fact that the sterol portion of the molecule cannot be activated by ultraviolet light, but rather to the circumstance that the esters cannot be utilized by the animal. When the inactive esters are saponified, the product exerts a biologic potency equal to that of activated free ergosterol.⁷⁴ Bailey⁷⁵ reported that the activities of vitamins D in different fish oils were increased when the vitamin D esters present were saponified; the percentage increase varied from 0 to 100%, according to the oil tested.

Considerable variations in activity obtain when substitutions other than those involving esterification are made on the C₃. Thus, when the hydroxyl group is replaced by a chlorine or bromine atom, the new compound produced still possesses the capacity to undergo activation when exposed to ultraviolet light. However, these halides, which are known to be resistant to saponification, were found by Bernstein *et al.*⁷⁶ to be devoid of activity when fed to rats or chickens. Similarly, when the hydroxyl group of 7-dehydrocholesterol was replaced with a thiol group, the compound produced on irradiation, although it had a spectral picture similar to that of activated 7-dehydrocholesterol, was not antirachitic, according to the chick bone ash test.^{77,78} It is not known whether or not activated 7-dehydro-3-homocholesterol, as prepared by Strating and Backer,⁷⁹ is physiologically active; in this case, the hydroxyl of 7-dehydrocholesterol is replaced by a HOCH₂— group.

The variation in structure due to the asymmetry of the C₃ position apparently does not affect the biopotency of the provitamins. Thus, both epiergosterol and 7-dehydroepicholesterol are readily activated.^{2,80} According to the Windaus group,⁸¹ the irradiated 7-dehydroepicholesterol is about one-tenth as active as the corresponding product prepared from 7-dehydrocholesterol. On the other hand, when the asymmetry, is on the C₉

⁷⁵ B. E. Bailey, *J. Fisheries Research Board Can.*, **6**, 103–108 (1943).

⁷⁶ S. Bernstein, J. J. Oleson, H. B. Ritter, and K. J. Sax, *J. Am. Chem. Soc.*, **71**, 2576–2577 (1949).

⁷⁷ J. Strating and H. J. Backer, *Rec. trav. chim.*, **69**, 909–920 (1950).

⁷⁸ S. Bernstein and H. J. Sax, *J. Org. Chem.*, **16**, 685–693 (1951).

⁷⁹ J. Strating and H. J. Backer, *Rec. trav. chim.*, **70**, 389–402 (1951).

⁸⁰ A. Windaus and K. Buchholz, *Ber.*, **72**, 597–599 (1939).

⁸¹ A. Windaus and J. Naggatz, *Ann.*, **542**, 204–218 (1939).

position, the normal configuration on this carbon is a necessary condition for activation. Thus, isopyrocalciferol, which differs from ergosterol only in the position of the C₉ carbon, cannot be activated to produce a potent vitamin D.² Lumisterol, however, in which an epimerization of the methyl group at C₁₀ poses the only structural difference from ergosterol, is fully activatable.² Apparently the 9 position but not the 10 position determines the potential activity.

The side-chain is an important determinant of activity. The sex hormone, 3,17-dihydroxyandrostadiene, which is structurally similar to ergosterol and 7-dehydrocholesterol except that it contains no side-chain and has a hydroxyl group on carbon-17, was shown by Dimroth and Paland⁸² to be inactive as a precursor of vitamin D. Furthermore, Haslewood⁸³ found that the cholanic acid analogues of ergosterol and of 7-dehydrocholesterol, which are similar to the provitamins D₂ and D₃ except that they have a shorter side-chain, have practically no activity. The side-chain can be modified by desaturation or saturation between C₂₂ and C₂₃ without seriously lowering the effectiveness of the sterol as a provitamin D. This has been demonstrated not only in the case of 22-dihydroergosterol, as contrasted with ergosterol, but also with the natural compound, $\Delta^{5,7,22}$ -cholestatriene-3-ol (22-dehydro-7-dehydrocholesterol). Presumably it is present in the provitamin D mixture of the edible sea mussel (*Mytilus edulis*);⁸⁴ it is questionable whether or not it contributes to the potency of the irradiated mixture.⁸⁴

It is evident that a large number of compounds may serve as potential provitamins D. As early as 1935, Bills⁸⁵ had recognized this fact; he first estimated that there were six such compounds,⁸⁵ this was later revised to eight.⁸⁶ In addition to the three most active provitamins D already mentioned, namely ergosterol, 7-dehydrocholesterol,⁸⁷ and 22-dihydroergosterol, the additional active compounds include 7-dehydrositosterol,⁸⁸ 7-hydroxycholesterol and 7-dehydrocampesterol, as prepared by Ruigh.⁸⁹ Although 7-dehydrostigmasterol differs from ergosterol only by having an ethyl group in the C₂₄ position in place of the methyl group in ergosterol, it is practi-

⁸² K. Dimroth and J. Paland, *Ber.*, **72**, 187-190 (1939).

⁸³ G. A. D. Haslewood, *Biochem. J.*, **38**, 454-456 (1939).

⁸⁴ J. van der Vliet, *Rec. trav. chim.*, **67**, 246-256, 265-281 (1948).

⁸⁵ C. E. Bills, *Cold Spring Harbor Symposia Quant. Biol.*, **3**, 328-340 (1935).

⁸⁶ C. E. Bills, Paper presented at convention Am. Med. Assoc., Kansas City, May 14, 1936; cited by C. E. Bills, O. N. Massengale, M. Imboden, and H. Hall, *J. Nutrition*, **13**, 435-452 (1937), p. 435.

⁸⁷ E. Geiger and S. Lassen, *Proc. Soc. Exptl. Biol. Med.*, **52**, 11-12 (1934).

⁸⁸ W. Wunderlich, *Z. physiol. Chem.*, **241**, 116-124 (1936).

⁸⁹ W. L. Ruigh, *J. Am. Chem. Soc.*, **64**, 1900-1902 (1942).

cally devoid of activity as a vitamin D after irradiation.⁹⁰ 7-Dehydrositosterol, which also carries an ethyl group on C₂₄, was originally found to be capable of activation.^{87,91} However, Ruigh⁸⁸ later presented evidence that the positive effects were to be ascribed to the presence of a small amount of 7-dehydrocampesterol, or other provitamins D which were present as impurities in the 7-dehydrositosterol. Although there are no data as to whether or not 7-dehydroclionasterol, which is a C₂₄-epimer of 7-dehydrositosterol, is a potential provitamin D, Bills² is of the opinion that it is not active, since the ethyl group on the C₂₄ carbon would preclude this possibility.

TABLE I
THE DISTRIBUTION OF PROVITAMINS D IN
PHANEROGAMS AND CRYPTOGAMS^a

Source	Provitamin D, parts per 1000 total sterol
Phanerogams	
Cottonseed oil	28
Rye grass	15
Scopolia root	14
Spinach	10
Wheat germ oil	10
Cocksfoot grass	8
Horse chestnut	8
Rutabaga	2.8
Carrot	1.7
Bean	1.0
Cabbage	0.5
Cryptogams	
<i>Aspergillus niger</i> , saprophytic mold	±1000
<i>Cortinarius shiitake</i> , agaricoid root-fungus	±1000
<i>Claviceps purpurea</i> , ergot	900
<i>Saccharomyces cerevisiae</i> , brewery yeast	800
<i>Penicillium puberulum</i> , velvety blue-green mold	280
<i>Fucus vesiculosus</i> , olive-green rockweed, alga	0.8

^a Adapted from W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Academic Press Inc., New York, 1954, Volume II, p. 149.

b. Occurrence of Provitamins D in General. Although the provitamins D are widely distributed in nature, they are seldom found in sufficient amounts and sufficiently free from other sterols to make it possible to

⁹⁰ O. Linsert, *Z. physiol. Chem.*, **241**, 125-128 (1936).

⁹¹ W. Grab, *Z. physiol. Chem.*, **243**, 63-89 (1936).

isolate them in pure form. Ergosterol provides an exception to this rule, as does the so-called provitamin D_m, recently isolated by Petering and Waddell⁹² from the ribbed mussel (*Arcuatula (Modiola) demissa*). According to the data of Bills,² the proportion of the total sterols which are provitamins D are usually under 6% in the vertebrates; in the case of invertebrates, proportions of provitamins D as high as 8% occur in the *Cnidaria*, 21% in the *Annelida*, 12% in the *Arthropoda*, and 37% in the case of one type of *Mollusca* (*Arcuatula demissa*). On the other hand, the maximum proportion of provitamin D was only 0.45% of the total sterol in the *Echino-dermata*. Plant sources included among the *Phanerogams* also had a very low proportion of provitamins D (maximum of 2.8% of total sterol), but in the case of the *Cryptogams*, practically 100% of the sterols were provitamins D (see Table 1). The quantitative results of Windaus⁹³ of Gillam and Heilbron,⁹⁴ and of van der Vliet,⁹⁵ as summarized by Bills, in Sebrell and Harris,² are given in Tables 2 and 3.

c. Occurrence of Specific Provitamins D. (a) *Ergosterol*. Tanret⁹⁶ was the first to describe a sterol in the parasitic fungus ergot (*Claviceps purpurea*) in 1879; because of its source, it was called ergotinine and later ergosterine.⁹⁷ This investigator recognized that it differed in composition from cholesterol and from the phytosterols.⁹⁸ Gérard^{99,100} reported the distribution of ergosterol in a wide variety of plants; he found that there was a taxonomic relation in the occurrence of this sterol, since it was characteristic of the cryptogams just as the phytosterols are characteristic of the phanerogams. According to Heidushka and Lindner,¹⁰¹ the ergosterol content of ten fungi, including yeasts, was found to vary between 0.29 and 1.17% of the dry matter. Bills and co-workers¹⁰² observed an ergosterol content varying from a trace (in *Saccharomyces logos*, which ferments melibiose) to as much as 2% of the dry material (in *Saccharomyces carlsbergensis*,

⁹² H. G. Petering and J. Waddell, *J. Biol. Chem.*, **191**, 765-773 (1951).

⁹³ A. Windaus, *Nachr. Ges. Wiss. Göttingen, Math.-physik. Klasse, Fachgruppe III*, n.s. **1**, 185-192 (1936).

⁹⁴ A. E. Gillam and I. M. Heilbron, *Biochem. J.*, **30**, 1253-1256 (1936).

⁹⁵ J. van der Vliet, *Chem. Weebkad*, **39**, 271-276 (1942); *Chem. Zentr.*, **1942**, **II**, 1353.

⁹⁶ C. Tanret, *Ann. chim. phys.* [5], **17**, 493-512 (1879).

⁹⁷ C. Tanret, *Compt. rend.*, **108**, 98-100 (1889).

⁹⁸ C. Tanret, *Ann. chim. phys.* [6], **20**, 289-297 (1890).

⁹⁹ E. Gérard, *Compt. rend.*, **114**, 1544-1546 (1892); **121**, 723-726 (1895); **126**, 909-911 (1898).

¹⁰⁰ E. Gérard, *J. pharm. chim.*, [6], **1**, 601-608 (1895).

¹⁰¹ A. Heidushka and H. Lindner, *Z. physiol. Chem.*, **181**, 15-23 (1929).

¹⁰² C. E. Bills, O. N. Massengale, and P. S. Prickett, *J. Biol. Chem.*, **87**, 259-264 (1930).

TABLE 2

THE DISTRIBUTION OF PROVITAMINS D IN VARIOUS INVERTEBRATES^a

Systematic name	Source	Common name	Provitamin D, parts per 1000 total sterol
<i>Porifera</i>			
—		Commercial sponges	20
<i>Cliona celata</i>		Boring sponge	<10
<i>Spheciospongia vesparia</i>		Loggerhead sponge, West Indies	<10
<i>Halichondria panicea</i>		Green-brown "crumb of bread" sponge	6
<i>Coelenterata (Cnidaria)</i>			
<i>Metridium dianthus</i>		Sea clove, snowy or red sea anemone	86
<i>Metridium dianthus</i>		Sea clove, snowy or red sea anemone	52
<i>Actinia equina</i>		Sea anemone, purple	50
<i>Urticina crassicornis</i>		Sea anemone, variegated	45
<i>Pennatula (Leioptilus) quadrangularis</i>		Sea pen	42
<i>Alcyonium digitatum</i>		Sea finger, pink	34
<i>Anemonia sulcata</i>		Sea anemone, tentacled	17
<i>Madreporaria</i>		Commercial corals (true corals)	10
—		Sea anemone (Australia)	9
—		Sea anemone (unidentified)	Trace
<i>Bryozoa</i>			
<i>Flustra securifrens</i>		Sea mat, straw-yellow	65
<i>Annelida</i>			
<i>Tubifex</i> , spp.		Waterworm	210
<i>Lumbricus terrestris</i>		Earthworm	170
<i>Arenicola marina</i>		Lugworm or sandworm	55
<i>Hirudo medicinalis</i>		Leech, medicinal	39
<i>Nereis virens</i>		Marine worm, creeper, clam-worm	16
<i>Arthropoda</i>			
<i>Tenebrio molitor</i>		Mealworm, darling beetle	120
<i>Melolontha vulgaris</i>		Cockchafer, May beetle	5
<i>Melolontha vulgaris</i>		Cockchafer grub	89
<i>Chironomus</i> , Fabr., spp.		Gnat, midge, Harlequin fly	61
<i>Dytiscus marginatus</i>		Diving water-beetle	40
<i>Carausius morosus</i>		Stick insect (eggs)	25
<i>Aleurobius farinae</i>		Meal mite	25
<i>Eriocheir sinensis</i>		Chinese wool hand crab (Yangtse-Kiang river)	23
<i>Blatta orientalis</i>		Oriental cockroach, black beetle	23
<i>Gastropacha pini</i>		Pine caterpillar	22

TABLE 2 (Continued)

Systematic name	Source	Common name	Provitamin D, parts per 1000 total sterol
<i>Cantharis vesicatorius</i>		Spanish fly, blister beetle	22
<i>Cancer pagurus</i>		Common edible crab	15
<i>Bombyx mori</i>		Chinese mulberry silkworm (eggs)	8.9
<i>Daphnia</i> , spp.		Waterflea	7.5
<i>Musca domestica</i>		Housefly	7.0
<i>Apis mellifica</i>		Honeybee	4.5
<i>Crangon vulgaris</i>		European shrimp, prawn	3.8
<i>Munida bamffia</i>		British crab (Bamffshire)	3.5
<i>Homarus vulgaris</i>		Spring lobster, European	2.5
<i>Mollusca</i>			
<i>Arcuatula demissa</i>		Ribbed mussel	370
<i>Arion empiricorium</i>		Slug, red road snail	220
<i>Buccinum undatum</i>		Whelk, wave-horn snail	180
<i>Littorina littorea</i>		European edible periwinkle	170
<i>Archidoris tuberculata</i>		Yellow sea slug	150
<i>Ostrea angasi</i>		Australian oyster	130
<i>Arion ater</i>		Black road slug	120
<i>Mytilus edulis</i>		Edible sea mussel	100
<i>Helix pomatia</i>		Edible Roman land snail, vineyard snail	97
<i>Anodonta cygnea</i>		Swan mussel, freshwater	80
<i>Ostrea virginica</i>		American-Canadian oyster	80
<i>Pecten australis</i>		Australian scallop	65
<i>Mytilus planulatus</i>		Australian flat mussel	62
<i>Cardium edule</i>		European cockle, sand shell	50
<i>Cardium tenuicostatum</i>		Australian cockle	41
<i>Ostrea edulis</i>		European flat oyster	34
<i>Limax agrestis</i>		Field slug	32
<i>Sepia officinalis</i>		Atlantic cuttlefish	12
<i>Echinodermata</i>			
<i>Astropecten irregularis</i>		Little sea star	4.5
<i>Asterias rubens</i>		Red starfish, "five-finger"	3.8

^a Adapted from W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Academic Press, Inc., New York, 1954, Volume II, pp. 150-152.

a brewery bottom yeast) in samples of twenty-nine strains of yeast, grown under standard conditions.

Ergosterol has been found to occur in lower plants such as brown algae, slime fungi, bacteria such as *Staphylococcus* spp., fungi (*Mucor* spp.), molds

TABLE 3
THE DISTRIBUTION OF PROVITAMINS D IN
VARIOUS TISSUES OF VERTEBRATES^a

Tissue	Animal	Provitamin D, parts per 1000 total sterol
Skin	Pig	46
	Chicken (feet)	25
	Rat	19
	Wild pig	16
	Mouse	9
	Calf	7
	Human adult	4.2
	Human infant	1.5
	Cow	1.8
	Deer	1.6
	Eel	1.2
	Chicken (trunk)	0.06
Liver	Tuna (Japanese)	11
	Cod (Atlantic)	4.4
	Shark	1.0
	Tuna	1.0
	Pig	1.0
	Cod (Japanese)	0.90
	Halibut	0.60
	Bluefin tuna	0.40
	Whale	0.12
Brain	Rabbit	0.70
	Lumpfish	0.50
	Human fetus	0.50
	Sheep	0.40
	Deer	0.33
	Human infant	0.20
	Horse	0.19
	Cow	0.13
	Human adult	0.06
Eggs	Duck (Chinese)	60
	Duck (Dutch)	13
	Cod (roe)	5.5
	Herring (roe)	2.4
	Hen	1.6
	Cormorant	1.0
	Mire crow	1.0
	Silver gull	1.0
	Lumpfish (roe)	0.3
Body	Frog	8.8
Venom	Toad	4.6

TABLE 3 (Continued)

Tissue	Animal	Provitamin D, parts per 1000 total sterol
Wool fat	Sheep	3.9
Milk	Cow	2.3
Placenta	Cow	1.8
Pancreas	Beef	1.8
Blood serum	Cow	1.5
Spinal cord	Beef	1.2
Gutted carcasses	Mice	0.80
Colostrum	Cow	0.70
Thymus	Cow	0.70
Bile	Ox	0.50
Body oil	Herring	0.50
Spleen	Cow	0.45
Milt	Herring	0.40
Heart	Calf	0.32
Lymph	Dog	0.30
Gutted carcasses	Rat	0.30
Gallstones	Man	0.25
Lung	Calf	0.25
Blood	Dog	0.10
Sclerotic aortas	Man	0

^a Adapted from W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Academic Press, Inc., New York, 1954, Volume II, pp. 149, 150.

(*Penicillium* spp.), yeasts and lichens.^{103,104} According to Savard and Grant,¹⁰⁵ ergosterol can be prepared from clay mold (*Penicillium notatum*). Although considerable amounts of ergosterol are present in *Penicillium*, this mold has not become a commercial source, since the yields are quite low. The most practical source of ergosterol is dried yeast, in which the provitamin makes up 90 to 100% of the total sterols.¹⁰⁶ Strains of brewery yeast (*Saccharomyces cerevisiae*), grown in aerated wort, serve for this purpose.

Although the main sources of ergosterol are the *Cryptogams*, there are well authenticated reports that its presence has been observed in animals. Thus, Windaus⁹³ and Bock and Wetter¹⁰⁷ reported that ergosterol comprises up to 25% of the total sterols of the red road snail (*Arion empiricorium*), and ap-

¹⁰³ C. E. Bills, *Physiol. Revs.*, 15, 1-97 (1935).

¹⁰⁴ C. Tanret, *Ann. chim. phys.* [8], 15, 313-330 (1908).

¹⁰⁵ K. Savard and G. A. Grant, *Science*, 104, 459-460 (1946).

¹⁰⁶ H. R. Rosenberg, *Chemistry and Physiology of the Vitamins*, Interscience, New York, 1945.

¹⁰⁷ F. Bock and F. Wetter, *Z. physiol. Chem.*, 256, 33-41 (1938).

proximately 22% of the sterols of the earthworm (*Lumbricus terrestris*) have also been shown to consist of ergosterol. Ergosterol has likewise been isolated from the bodies of the Roman land snail (*Helix pomatia*).¹⁰⁷ However, there is some question as to the universal occurrence of ergosterol in the earthworm. Boer and co-workers^{108,109} reported that "spectroscopically pure" provitamins of this species which they isolated had constants which differed from those of ergosterol; in fact, these workers referred to the sterol as a "chicken provitamin D." Moreover, van Vliet⁹⁵ recorded the fact that the activated earthworm provitamin D had a potency equal to that of chicken provitamin D. This would not have been the case if it had been ordinary ergosterol.

The presence of ergosterol has likewise been proved without question in the higher vertebrate forms. Thus, Windaus and Stange¹¹⁰ noted the presence of ergosterol in the eggs of chickens, although it was believed that this represented an adventitious occurrence, which was to be ascribed to the presence of ergosterol in the feed. A similar explanation has been advanced by Bethke *et al.*^{111,112} to explain the presence of vitamin D₂ (activated ergosterol) in hen's eggs, inasmuch as this is not the natural form of vitamin D in this species. In fact, these workers stated that the type of vitamin D present in the tissues and egg-yolks of fowl depended upon whether irradiated ergosterol or cod-liver oil had been the main source of vitamin D in the diet. Variations in the type of vitamin D present in the milk of the cow were also found to be dependent upon the dietary sterol fed.¹¹³ Although the presence of vitamin D₂ in the higher forms may in all probability be traced to the presence of ergosterol in their feed, it is difficult to explain the occurrence of small amounts of vitamin D₂ in tuna fish liver oils as recorded by Brockmann and Busse,¹¹⁴ although the vitamin was mainly in the form of vitamin D₃ in this case. A possible explanation for the occurrence of ergosterol and vitamin D₂ in the tissues and eggs of chickens^{115,116} is the fact that this species can absorb a slight amount of

¹⁰⁸ A. G. Boer, J. van Niekerk, E. H. Reerink, and A. van Wijk, *U. S. Patent No. 2,163,659* (June 27, 1939).

¹⁰⁹ A. G. Boer, J. van Niekerk, E. H. Reerink, and A. van Wijk, *U. S. Patent No. 2,266,674* (Dec. 16, 1941).

¹¹⁰ A. Windaus and O. Stange, *Z. physiol. Chem.*, **244**, 218-220 (1936).

¹¹¹ R. M. Bethke, P. R. Record, C. H. Kirk, and D. C. Kennard, *Poultry Sci.*, **15**, 326-335 (1936).

¹¹² R. M. Bethke, P. R. Record, O. H. M. Wilder, and C. H. Kirk, *Poultry Sci.*, **15**, 336-344 (1936).

¹¹³ R. M. Bethke, W. E. Krauss, P. R. Record, and O. H. M. Wilder, *J. Nutrition*, **11**, 21-30 (1936).

¹¹⁴ H. Brockmann and A. Busse, *Z. physiol. Chem.*, **256**, 252-270 (1938).

¹¹⁵ R. Schönheimer and H. Dam, *Z. physiol. Chem.*, **211**, 242-245 (1932).

¹¹⁶ W. Menschick and I. H. Page, *Z. physiol. Chem.*, **211**, 246-252 (1932).

ergosterol in the gastrointestinal tract, in contradistinction to the inability of mice, rats, rabbits, or dogs to do so.¹¹⁵

Other unusual sources of ergosterol in nature include cocks foot grass,¹¹⁷ wheat germ oil,¹¹⁸ and *Scopolia* root sterols.¹¹⁹ Rosenberg¹⁰⁶ recorded values in *Scopolia* up to 1.4% of the total sterols. According to Bills,² no provitamin D other than ergosterol has been isolated from vegetable sources, with the exception of 22-dihydroergosterol, which was separated chromatographically from the minor sterols of ergot by Santos Ruiz.¹²⁰

(b) *7-Dehydrocholesterol*. This is believed to be the most common provitamin D occurring in the higher animals and man. The occurrence of this provitamin D as a contaminant of cholesterol was first demonstrated by Boer *et al.*⁵⁰ Moreover, Boer *et al.*^{50,121} noted the presence of cholesterol in duck's eggs, which contained as much as 4.5% of this provitamin D.² Why this should vary from the type of provitamin D in hens' eggs was not determined, but it is presumably related to the nature of the predominant sterol fed. As already recorded, Windaus and Bock⁴⁹ observed the presence of 7-dehydrocholesterol in pig skin. It has been demonstrated in the whelk or the so-called wave-horn snail (*Buccinum undatum*)¹⁰⁷ as well, although the other two species of snails investigated were found to contain ergosterol. On the basis of the high efficacy ratios of vitamin D observed following irradiation of most animal tissues, it has generally been assumed that all the provitamin D in mammalian sources is 7-dehydrocholesterol (provitamin D₃). This is true of the commercial cholesterol obtained from dried animal tissues, identified by Bills² as the spinal cords of cattle, and used by Waddell,¹²² as well as of the activated samples of butter, lard, and brain fat studied by Bethke and co-workers.¹²³ Moreover, similar high efficacy ratios were observed by Haman and Steenbock¹²⁴ with irradiated lard and chicken fat, indicating that the provitamin D in these cases is 7-dehydrocholesterol.

(c) *Miscellaneous Provitamins D*. Several provitamins D other than

¹¹⁷ A. Pollard, *Biochem. J.*, **30**, 382-386 (1936).

¹¹⁸ A. Windaus and F. Bock, *Z. physiol. Chem.*, **256**, 47-48 (1938).

¹¹⁹ A. Windaus and F. Bock, *Z. physiol. Chem.*, **250**, 258-261 (1937).

¹²⁰ A. Santos Ruiz, *Anales real acad. farm.*, **3**, 201-231 (1941); *Chem. Abst.*, **38**, 503 (1944).

¹²¹ A. G. Boer, J. van Niekerk, E. H. Reerink, and A. van Wijk, *Dutch Patent No. 45,849* (June 15, 1939); *German Patent No. 678,533* (July 17, 1939); *Chem. Abst.*, **33**, 8852 (1939).

¹²² J. Waddell, *J. Biol. Chem.*, **105**, 711-739 (1934).

¹²³ R. M. Bethke, P. R. Record, and O. H. M. Wilder, *J. Biol. Chem.*, **112**, 231-238 (1935).

¹²⁴ R. W. Haman and H. Steenbock, *J. Biol. Chem.*, **114**, 505-514 (1936).

ergosterol, 7-dehydrocholesterol, and 22-dihydroergosterol have been reported from various sources. Boer and his group^{108, 109, 125} described the preparation of provitamins D in patents granted. They obtained the provitamins from various invertebrates: the stick-insect (*Carausius morosus*), the leech (*Hirudo medicinalis*), the mealworm (*Tenebrio molitor*), the diving water-beetle (*Dytiscus marginatus*), the earthworm (*Lumbricus*, spp.), the periwinkle (*Littorina littorea*), the swan mussel (*Anodonta cygnea*), the whelk (*Buccinum undatum*), the field slug (*Limax agrestis*), the edible crab (*Cancer pagurus*), the European flat oyster (*Ostrea edulis*), the edible sea-mussel (*Mytilus edulis*) and the waterworm (*Tubifex*, spp.).^{108, 109, 125} The provitamin D obtained from the earthworm, *Lumbricus terrestris*, is believed to be a mixture of ergosterol and a second undefined provitamin D. A second one from the waterworm, *Tubifex*, according to Bills,² is probably a mixture, although van Vliet⁹⁵ reported an efficacy ratio of 100% for the activated material. *Mytilus edulis* is believed by van Vliet⁹⁵ to consist of three components, which are 7-dehydrocholesterol, ergosterol, and a substance tentatively identified as 22-dehydro-7-dehydrocholesterol.² Rosenberg and Waddell¹²⁶ report an efficacy ratio of 71% for this provitamin D; in all probability this low ratio is ascribable to its high ergosterol content.² Kind and Harman¹²⁷ are of the opinion that the provitamin D in the European periwinkle, *Littorina littorea*, is 7-dehydroclionasterol, although the purified activated product was reported⁹⁵ to have an efficacy ratio of 100%. Bills² considers that this activity is inconsistent with the presence of an ethyl group in the side chain, which would rule out 7-dehydroclionasterol as the mother substance. The so-called provitamin D_m (in which "m" refers to *Modiolus*), was isolated from the provitamin D of the ribbed mussel (formerly known as *Modiolus demissus*, Dillwyn, but designated in the new terminology as *Arcuatula demissa*), by Petering and Waddell.⁹² It is believed to have twenty-nine carbons. It yields a vitamin D which has an efficacy ratio 10 to 20% higher than that of cod-liver oil.¹²⁸ A number of workers have prepared the provitamin D from the ribbed mussel.^{92, 126, 128}

(3) The Vitamins D

A number of different compounds have been found to possess antirachitic potency, although they are known to vary from each other both qualita-

¹²⁵ A. G. Boer, J. van Niekerk, E. H. Reerink, and A. van Wijk, *U. S. Patent No. 2,216,719* (Oct. 8, 1940).

¹²⁶ H. R. Rosenberg and J. Waddell, *J. Biol. Chem.*, **191**, 757-763 (1951).

¹²⁷ C. A. Kind and S. C. Harman, *J. Org. Chem.*, **13**, 867-869 (1948).

¹²⁸ H. R. Rosenberg, *U. S. Patent No. 2,475,917* (July 12, 1949).

tively and quantitatively as regards their action. Four of the vitamins were prepared in pure form; two of these were derived from natural sources. It is believed that the other vitamins D actually do exist, since a number of different provitamins D can be activated. The vitamins D themselves have not been demonstrated in pure form. The inability to prepare pure minor members of the vitamin D group is probably to be ascribed to their presence in extremely small amounts, and especially to the fact that they frequently occur in mixtures with other D vitamins which are present in a much larger proportion. Ten different provitamins D are known. Thus it must be surmised that there are ten different D vitamins corresponding in structure to each of the ten precursors. In addition to these compounds, the possibility exists that purely synthetic compounds may be produced which have the structural features required for an active vitamin D. Thus, Raoul and collaborators¹²⁹ reported that bicholestadiene (2,4,2',4'-bicholestadiene), on irradiation, becomes highly active as an antirachitic agent.

Vitamin D₂ (activated ergosterol) and vitamin D₃ (activated 7-dehydrocholesterol) are the two principal D vitamins on the basis of the extent of their distribution, and of the fact that the pure compounds are known to mediate the physiologic changes usually ascribed to the D vitamins. Vitamins D₂ and D₃ possess similar biologic activities in the case of the rat. Vitamin D₂ has only a feeble antirachitic potency in the case of chicks, but vitamin D₃ possesses a normal biopotency in this species. Vitamin D₄ is the terminology assigned to the activated 22-dihydroergosterol,¹³⁰ while vitamin D₅ is believed to be activated 7-dehydrositosterol. The structure of the common provitamins D and of the vitamins D is given below. Further information as to the structure of related compounds is given in *The Lipids*, Vol. I, pp. 749-755 and 782-791.

a. General Structural Requirements for the Vitamins D. All of the D vitamins have a similar structure insofar as the cyclic portion of the molecule is concerned. The differences in structure of the several vitamins D are confined to the side chain. In the activation of the provitamins D, the main structural alteration involves the destruction of the steroid ring B by its rupture between carbons 9 and 10. The methyl side chain on carbon 10 becomes a methylene group with a double bond attaching it to carbon 10; carbon 9 becomes $\text{—CH}_2\text{—}$ by the addition of one hydrogen atom.

¹²⁹ Y. Raoul, N. Le Boulch, J. Chopin, P. Meunier, and A. Guerillot-Vinet, *Compt. rend.*, **234**, 2493-2495 (1952).

¹³⁰ A. Windaus and G. Trautmann, *Z. physiol. Chem.*, **247**, 185-188 (1937).

Ergosterol (Provitamin D₂) 7-Dehydrocholesterol (Provitamin D₃) Vitamin D₂ Vitamin D₃

In addition to the open B ring, all active members of the vitamin D group have conjugated bonds at the 10, 18-; 5, 6-; and 7,8-positions. The hydroxyl group on carbon 3 is another prerequisite; vitamins D are active only when this hydroxyl group is free, or when the ester is one which can be readily hydrolyzed in the animal body. Windaus and Rygh⁷⁴ reported that non-hydrolyzable esters of vitamin D are relatively inactive biologically. When the position of the hydroxyl group on carbon 3 is altered by

epimerization, the biopotency is greatly decreased, although the epi-compounds still possess some activity.

The presence of a side chain on carbon 17 appears to be another requirement for biologic activity. When the side chain is completely absent, as in the case of $\Delta^{5,7}$ -androstadiene-3,17-diol,¹³¹ or even when the chain is shortened to a length of four carbons, as in the case of 3-hydroxy- $\Delta^{5,7}$ -choleadienic acid,⁸³ the activated compound is almost without biologic potency. Slight changes in the normal side chains, such as replacement of methyl groups by ethyl groups or the variation in the spatial arrangement of such ethyl side chains, can result in profound alterations in biologic activity. For a more complete discussion of the structural relationships of the provitamins D, of the vitamins D, and of their derivatives, the reader is referred to pages 749 to 756, and pages 782 to 791 of Volume I, *The Lipids*.

b. Production of the Vitamins D from the Provitamins D. In order to effect the change from a physiologically inert compound to one possessing the high biologic activity exhibited by the vitamins D, it is necessary to impart a definite quantity of energy to the provitamin D molecule. This can be brought about by the application of the ultraviolet light present in sunlight, or of that supplied by artificial sources of radiant energy, as well as by several types of physical treatment. It was originally believed that light having wave lengths between 230 and 305 $m\mu$ (which is the principal absorption range of ergosterol) activated ergosterol with equal efficiency.¹³²⁻¹³⁴ Rosenberg¹⁰⁶ is of the opinion that the greatest efficiency results when light having a wave length of 281 $m\mu$ is employed; this coincides with the position of the chief absorption band of ergosterol. Bunker *et al.*¹²⁵ obtained the best results with 7-dehydrocholesterol when monochromatic light having a wavelength of 296.7 $m\mu$ was used. These investigators¹³⁵ reported that the most effective treatment of rachitic rats with ultraviolet radiation was by the use of rays having this same wave length (296.7 $m\mu$). This finding is disputed by Knudson and Benford,¹³⁶ who observed that light with a wave length of 280.4 $m\mu$ was most effective in curing rickets. In addition to sunlight, the ultraviolet light obtained from magnesium and carbon arcs has been used, as well as the mercury vapor

¹³¹ A. Butenandt, E. Hausmann, J. Paland, D. v. Dresler, and U. Meinerts, *Ber.*, 71, 1316-1322 (1938).

¹³² S. K. Kon, F. Daniels, and H. Steenbock, *J. Am. Chem. Soc.*, 50, 2573-2581 (1928).

¹³³ T. A. Webster and R. B. Bourdillon, *Biochem. J.*, 22, 1223-1230 (1928).

¹³⁴ A. L. Marshall and A. Knudson, *J. Am. Chem. Soc.*, 52, 2304-2314 (1930).

¹³⁵ J. W. M. Bunker, R. S. Harris, and L. M. Mosher, *J. Am. Chem. Soc.*, 62, 508-511 (1940).

¹³⁶ A. Knudson and F. Benford, *J. Biol. Chem.*, 124, 287-299 (1938).

lamp and cored carbon electrodes impregnated with different metals.¹⁰⁶ Reerink and van Wijk¹³⁷ employed a bismuth vapor lamp with satisfactory results. For a description of the filters employed, and other details, the reader is referred to Volume I, *The Lipids*, pp. 759, 760, and to the review of Bills,² who described various specific technical methods for carrying out irradiation of special products.

In addition to the usual types of irradiation, other methods have been shown to accomplish the activation of the provitamin D molecule. These procedures include the use of cathode rays,^{138,139} alone or with catalysts such as iron or uranium salts.¹⁴⁰ Canal rays and the α -, β -, and γ -rays of radioactive elements have been used. Radium emanation provides another method for the activation of the provitamins, D,¹⁴¹ and corpuscular rays for accomplishing this activation was described in a German patent.¹⁴² In addition, electrons of high frequency have been widely employed,^{143,144} as well as alternating current of high frequency.^{145,146} Goldblatt¹⁴⁷ observed that x-rays were unable to effect the activation of provitamins D. On the other hand, later experiments¹⁴⁸ established the fact that irradiation by cold quartz mercury lamp rendered ergosterol powerfully antirachitic. Bills¹⁰³ found radio waves of high intensity and short wave length entirely ineffective.

The process of activation is not instantaneous, but requires a given amount of time. A series of compounds are formed in order, prior to vitamin D. When irradiation is continued for a prolonged period, the vitamin D is decomposed, resulting in the formation of products which may be toxic. Analogous series of compounds are formed from each of the provitamins D, but each series varies from the other in the structure of the side chain. The following is the sequence of products formed on irradiation of ergosterol:

¹³⁷ E. H. Reerink and A. van Wijk (to N. V. Philips' Gloeilampenfabrieken, Holland), *U. S. Patent No. 1,904,751* (Apr. 18, 1933).

¹³⁸ A. Knudson and C. N. Moore, *J. Biol. Chem.*, **81**, 49-64 (1929).

¹³⁹ R. M. Hoffman and F. Daniels, *J. Biol. Chem.*, **115**, 119-130 (1936).

¹⁴⁰ A. J. Pacini (to Research Products, Inc.) *U. S. Patent No. 1,983,944* (Dec. 11, 1934).

¹⁴¹ R. B. Moore and T. de Vries, *J. Am. Chem. Soc.*, **53**, 2676-2681 (1931).

¹⁴² K. Hembd and Vitam Fabrik, *German Patent No. 577,170* (Aug. 10, 1933).

¹⁴³ Brit. Thompson-Houston Co., *British Patent No. 292,926* (Aug. 22, 1928).

¹⁴⁴ A. Knudson (to Sun-A-Sured, Inc.), *U. S. Patent No. 2,007,765* (July 9, 1935).

¹⁴⁵ I. G. Farbenindustrie, *Austrian Patent No. 119,210* (Apr. 15, 1930).

¹⁴⁶ C. C. Whittier, *U. S. Patent Nos. 2,106,779; 2,106,780* (to Nutrition Research Labs., Inc., Chicago), *2,106,781; 2,106,782* (Feb. 1, 1938).

¹⁴⁷ H. Goldblatt, *Ergeb. allgem. Pathol. path. Anat., Abt. 2*, **25**, 58-491 (1931).

¹⁴⁸ H. Goldblatt, *Proc. Soc. Exptl. Biol. Med.*, **30**, 380-383 (1932).

Products Formed on the Irradiation of Ergosterol^{106, 149}

The substances formed during the activation of the provitamins D are similar, irrespective of the method of activation. None of the steps in the irradiation mechanism is reversible. The names for the several irradiation products were assigned by Windaus, Deppe, and Wunderlich.¹⁵⁰ The irradiation products are well-defined compounds whose structures are definite, and which are well known. The ease with which the provitamins D can be converted to the corresponding vitamins D varies with the wave length of the light employed in the irradiation, with the physical state of the provitamin, and with the solvent. The best results are obtained when the provitamin D is in solution, especially when the solution is agitated. The efficiency of activation may be increased when a substance is present which protects the vitamin D after it is produced. Temperature has little effect upon the activation reaction. For a further discussion of the irradiation products, and of factors influencing the irradiation reaction, the reader is referred to Volume I, *The Lipids*, pp. 758 to 770, as well as to Rosenberg¹⁰⁶ and to Bills.²

c. Occurrence of the Vitamins D. In contradistinction to the wide distribution of the provitamins D, the vitamins D themselves are present to only a limited extent.^{151, 152} They are practically absent from the plant kingdom, although significant amounts of the provitamins D do occur in vegetables. The chief sources of vitamin D among the plants are in the mushroom and related fungi. Thus, it has been demonstrated in mushrooms growing in the open woods, in the morel or May mushroom (*Morchella esculenta*), the edible boletus (*Boletus edulis*, *B. badius*), the chanterelle (*Cantharellus cibarius*), and the edible turban-top (*Helvella mitra (esculenta)*).^{153, 154} Scheunert and Reschke¹⁵³ observed that there are only insignificant amounts of vitamins D in the edible meadow (button) mushroom (*Agaricus (Psalliota) campestris*) grown in dark cellars, although the vitamin D was found to be present to a somewhat greater extent when the mushrooms were

¹⁴⁹ P. Setz, *Z. physiol. Chem.*, **215**, 183-192 (1933).

¹⁵⁰ A. Windaus, M. Deppe, and W. Wunderlich, *Ann.*, **533**, 118-127 (1937).

¹⁵¹ E. P. Daniel and H. E. Munsell, *U.S. Dept. Agr., Miscell. Pub. No. 275*, 1-176 (1937).

¹⁵² L. E. Booher, E. R. Hartzler, and E. M. Hewston, *Vitamin Values of Foods*, Chem. Pub. Co., Brooklyn, 1942.

¹⁵³ A. Scheunert and J. Reschke, *Deut. med. Wochschr.*, **57**, 349-351 (1931).

¹⁵⁴ A. Scheunert, M. Schieblich, and J. Reschke, *Z. physiol. Chem.*, **235**, 91-96 (1935).

grown in the sunlight.¹⁵⁴ The plant provitamins D may under exceptional conditions be transformed to the corresponding vitamins D during processing of the plant. Knapp and Coward¹⁵⁵ reported a transformation of the provitamin D in the cacao shells (*Theobroma cacao*) dried in sunlight, while Steenbock and co-workers¹⁵⁶ demonstrated a similar transformation in dried hay. Bills and co-workers¹⁰² and Massengale *et al.*¹⁵⁷ noted that yeast is relatively devoid of active vitamin D, although the content varies widely for different species.¹⁰² It may be activated to a concentration of 10,000 I.U./g. when exposed to sunlight for approximately thirty minutes¹⁵⁷ under controlled conditions.² This process is the basis of a patent by Bills.¹⁵⁸

Vitamin D is widely distributed in the animal kingdom, in contrast to its sparse distribution in the plants. Even in the animal world, the species which contain significant amounts of these vitamins are relatively few.¹⁵⁹ Since the highest concentrations of the D vitamins are to be found in the fish liver oils, it is of considerable interest whether or not the marine plants are rich in this vitamin. In general, most workers have failed to demonstrate any appreciable concentration of vitamin D in the marine vegetation. Thus, Leigh-Clare¹⁶⁰ reported that the pennate, free-floating marine diatom (*Nitzschia closterium*), failed to synthesize any vitamin D, even when cultured under conditions of maximum insolation. According to Drummond and Gunther,^{161,162} no vitamin D, or only negligible amounts, are to be found in mixed phytoplankton taken from the ocean. Vitamin D was found to be absent from two forms of algae, namely, the green tubular seaweed of the Monterey Peninsula (*Enteromorpha intestinalis*) and the ruffled brown blade-kelp or "tangle" (*Laminaria saccharina*). The green rockweed (*Fucus vesiculosus*) was found to have a slight antirachitic potency.¹⁶³ Oil from phytoplankton contained some provitamins D but no active vitamin D, except when it was near the surface of the water, and thus exposed to sunlight. In this case small amounts of the vitamin were

¹⁵⁵ W. A. Knapp and K. H. Coward, *Analyst*, **59**, 474-478 (1934).

¹⁵⁶ H. Steenbock, E. B. Hart, C. A. Elvehjem, and S. W. F. Kletzien, *J. Biol. Chem.*, **66**, 425-440 (1925).

¹⁵⁷ O. N. Massengale, C. E. Bills, and P. S. Prickett, *J. Biol. Chem.*, **94**, 213-219 (1931).

¹⁵⁸ C. E. Bills, *U. S. Patent No. 1,877,382* (Sept. 13, 1932).

¹⁵⁹ P. A. Coppens and G. A. Metz, *Arch. néerland. Physiol.* **18**, 407-415 (1933); *Biochem. Z.*, **266**, 169-174 (1933).

¹⁶⁰ J. L. Leigh-Clare, *Biochem. J.*, **21**, 368-372 (1927).

¹⁶¹ J. C. Drummond and E. R. Gunther, *Nature*, **126**, 398 (1930).

¹⁶² J. C. Drummond and E. R. Gunther, *J. Exptl. Biol.* **11**, 203-209 (1934).

¹⁶³ N. G. Johnson and T. Levring, *Svensk. Hydrograf.-Biol. Skrifter*, Ser. 3, **1**, No. 3, 1-7 (1947); *Chem. Abst.*, **45**, 236 (1951).

found.¹⁶³ One source of vitamin D in sea plants is the gulfweed (*Sargassum*, spp.), a floating brown alga produced in enormous quantities in the area near the Tortugas Islands.¹⁶⁴ Large masses of these plants break away, and are carried in the Gulf stream to as far north as Iceland. The alga becomes a major food for fish, especially after it becomes infested with mollusks, shrimps, and other invertebrates. According to Darby and Clarke,¹⁶⁴ the sea-weed freed of extraneous matter contained about 3% of oil on the dry basis, and thus was found to have an antirachitic potency comparable to that of some of the poorer fish liver oils. It is uncertain to what degree *Sargassum* may act as a contributor to the vitamin D in fish liver oils. Copping¹⁶⁵ reported that copepods contain some vitamin D.

(a) *Vitamins D in Fish Oils.* The highest concentrations of vitamin D found in nature are those present in fish oils; in most cases the vitamin D is almost entirely concentrated in the liver although, in some cases, relatively large amounts may be concentrated in the viscera, and moderate amounts are found in fish roe.¹⁵¹ Vitamin D occurs in most fish oils, but there are marked differences in the quantities present, not only in different species, but also in the case of different members of the same species. Bills,² in his most recent compilation of the vitamin D content of various fish liver oils, recorded a maximum average value of 45,000 I.U./g. in the liver oil of the oriental tuna (*Thunnus orientalis*) while European sturgeon liver (*Acipenser sturio*) is practically devoid of this vitamin, bioassays showing biopotencies of less than one unit/g. liver oil. Morton¹⁶⁶ stated in 1942 that the vitamin D content of fish liver oils may reach 200,000 I.U./g. in exceptional cases. The vitamin D in fish oils has been found to occur both in free and in esterified forms.^{167,168} Table 4 records the values for the vitamin D content of one-hundred species of fishes as given by Bills,² on the basis of earlier findings of Bills *et al.*¹⁶⁹

Although vitamin D is largely concentrated in the liver, Bills¹⁷¹ first

¹⁶⁴ H. H. Darby and H. T. Clarke, *Science*, **85**, 318-319 (1927).

¹⁶⁵ A. M. Copping, *Biochem. J.*, **28**, 1516-1520 (1934).

¹⁶⁶ R. A. Morton, *Ann. Rev. Biochem.*, **11**, 365-390 (1942).

¹⁶⁷ K. C. D. Hickman, *Ind. Eng. Chem.*, **29**, 1107-1111 (1937).

¹⁶⁸ K. C. D. Hickman and E. L. Gray, *Ind. Eng. Chem.*, **30**, 796-802 (1938).

¹⁶⁹ C. E. Bills, F. G. McDonald, O. N. Massengale, M. Imboden, H. Hall, W. D. Hergert, and J. C. Wallenmeyer, *J. Biol. Chem.*, **109**, vii (1935).

¹⁷⁰ D. S. Jordan, B. W. Evermann, and H. W. Clark, *Check List of Fishes and Fishlike Vertebrates of North and Middle America North of the Northern Boundary of Venezuela and Columbia*. Report of the U. S. Commissioner of Fisheries for the Fiscal Year 1928, with Appendices, Part 2, Govt. Printing Office, Washington, 1930.

¹⁷¹ C. E. Bills, *Studies on the Antirachitic Vitamin*, Dissertation, Johns Hopkins Press, Baltimore, 1924; cited by W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. II, 1954, p. 164.

TABLE 4

THE VITAMIN D CONTENT IN THE LIVER OILS OF VARIOUS SPECIES OF FISH^a

Common name of fish	Systematic name	Zoological order	Vitamin D I.U./g.
<i>Thunnus orientalis</i>	Oriental tuna	Percomorphi	45,000
<i>Auxis thazard</i>	Frigate mackerel (South Seas)	Percomorphi	44,000
<i>Thunnus saliens</i>	California bluefin tuna	Percomorphi	42,000
<i>Katsuwonus pelamis</i>	"Skipjack" oceanic bonito	Percomorphi	42,000
<i>Parathunnus sibi</i>	Meji tuna	Percomorphi	38,000
<i>Sarda lineolata</i>	California bonito	Percomorphi	35,000
<i>Seriola dorsalis</i>	California yellowtail	Percomorphi	25,000
<i>Lutianus campechanus</i>	Red snapper, Mexican snapper	Percomorphi	22,000
<i>Thunnus secundodorsalis</i>	Atlantic tuna	Percomorphi	16,000
<i>Thunnus gerono</i>	Albacore	Percomorphi	13,000
<i>Neothunnus macropterus</i>	Pacific yellowfin tuna	Percomorphi	12,000
<i>Cynoscion nobilis</i>	White sea-bass	Percomorphi	11,000
<i>Stereolepis gigas</i>	Jewfish, California black sea-bass	Percomorphi	9,000
<i>Stereolepis ishinagi</i>	Ishinagi	Percomorphi	7,000
<i>Xiphias gladius</i>	Swordfish, broadbill	Percomorphi	7,000
<i>Embiotoca jacksoni</i>	Bay black perch	Holeonoti	7,000
<i>Lepidopsetta bilineata</i>	Rock sole	Heterosomata	6,800
<i>Scomber japonicus</i>	Oriental mackerel	Percomorphi	6,300
<i>Sebastes rosaceus</i>	Rosy rockfish	Cataphracti	5,800
<i>Decapturus muroadsi</i>	Mackerel scads	Percomorphi	5,800
<i>Epinephelus morio</i>	Grouper, red	Percomorphi	4,800
<i>Sphyræna argentea</i>	Barracuda, California	Percomorphi	4,700
<i>Sebastes constellatus</i>	Starry rockfish	Cataphracti	4,500
<i>Sebastesomus flavidus</i>	Yellowtail rockfish	Cataphracti	4,000
<i>Rosicola miniatus</i>	Vermilion rockfish	Cataphracti	2,600
<i>Cynoscion macdonaldi</i>	Totuava	Percomorphi	2,500
<i>Atherinopsis californiensis</i>	Jack smelt	Percomorphi	2,400
<i>Makaira mitsukurii</i>	Striped marlin, sailfish	Percomorphi	2,300
<i>Paralichthys californicus</i>	California halibut	Heterosomata	2,300
<i>Sardinia caerulea</i>	Pacific sardine	Isospondyli	2,300
<i>Pteropodus vexillaris</i>	California rockfish	Cataphracti	2,200
<i>Sebastespyr ruberrimus</i>	Tambor	Cataphracti	2,100
<i>Thyristes atun</i>	Snock	Percomorphi	2,000
<i>Sebastes paucispinis</i>	Bocaccio, rockfish	Cataphracti	1,800
<i>Pteropodus maliger</i>	Quill-back rockfish	Cataphracti	1,800
<i>Merluccius productus</i>	Pacific hake	Anacanthini	1,500
<i>Sebastesomus mystinus</i>	Blue rockfish, priest fish	Cataphracti	1,500
<i>Pteropodus nebulosus</i>	China rockfish	Cataphracti	1,400
<i>Psittichthys melanostictus</i>	Sand sole	Heterosomata	1,400

TABLE 4 (Continued)

Common name of fish	Systematic name	Zoological order	Vitamin D, I.U./g.
<i>Hippoglossus hippoglossus</i>	Atlantic halibut	Heterosomata	1,200
<i>Alosa sapidissima</i>	American shad	Isospondyli	1,200
<i>Roccus saxatilis</i>	Striped bass	Percomorphi	1,200
<i>Rosicola pinniger</i>	Orange rockfish	Cataphracti	1,100
<i>Sebastes chlorostictus</i>	Green spotted rockfish	Cataphracti	1,100
<i>Acutomentum entomelas</i>	Widow rockfish	Cataphracti	1,100
<i>Hyperprosopon argenteum</i>	Wall-eyed surf perch	Holconoti	1,100
<i>Cyclichthys schoepfi</i>	Rabbitfish, balloonfish	Plectonathi	1,100
<i>Platichthys stellatus</i>	Starry flounder	Heterosomata	1,000
<i>Hispaniscus elongatus</i>	Green-striped rockfish, reina	Cataphracti	990
<i>Hexagrammos otakii</i>	Ainame, greenling	Cataphracti	950
<i>Ophiodon elongatus</i>	Ling cod	Cataphracti	920
<i>Taeniotoca lateralis</i>	Striped sea-perch	Holconoti	900
<i>Rhacochilus toxotes</i>	Rubberlip sea-perch	Holconoti	890
<i>Sebastesomus melanops</i>	Black rockfish	Cataphracti	830
<i>Hispaniscus rubrivinctus</i>	Flag rockfish	Cataphracti	810
<i>Scomber scombrus</i>	Atlantic mackerel	Percomorphi	750
<i>Pneumatophorus diego</i>	Pacific mackerel	Percomorphi	730
<i>Sphoeroides maculatus</i>	Atlantic pufferfish	Plectonathi	570
<i>Scorpaenichthys marmoratus</i>	Cabezon	Cataphracti	530
<i>Eopsetta jordani</i>	Petrale sole	Heterosomata	520
<i>Sciaena mitsukurii</i>	Nibe, croaker	Percomorphi	500
<i>Phanerodon furcatus</i>	Pacific white sea-perch	Holconoti	460
<i>Damalichthys argyrosomus</i>	Fork-tail sea-perch	Holconoti	410
<i>Anoplopoma fimbria</i>	Sablefish	Cataphracti	310
<i>Sebastes goodei</i>	Chilipepper	Cataphracti	270
<i>Epinephelus analogus</i>	Cabrilla	Percomorphi	260
<i>Reinhardtius hippoglossoides</i>	Greenland halibut	Heterosomata	260
<i>Gadus macrocephalus</i>	Pacific cod	Anacanthini	190
<i>Salmo salar</i>	Atlantic salmon	Isospondyli	180
<i>Sebastesomus alascanus</i>	Channel rockfish, slim thorn-head	Cataphracti	150
<i>Errex zachirus</i>	Rex sole	Heterosomata	140
<i>Parophrys vetulus</i>	English sole	Heterosomata	140
<i>Scorpaenia guttata</i>	California scorpion	Cataphracti	140
<i>Citharichthys sordidus</i>	Pacific sand dab	Heterosomata	120
<i>Urophycis</i> (spp.)	Atlantic hake	Anacanthini	120
<i>Pleuronichthys decurrens</i>	Curlfin turbot	Heterosomata	110
<i>Sebastes baramenue</i>	Menuke rockfish	Cataphracti	100
<i>Gadus morhua</i>	Atlantic cod	Anacanthini	100
<i>Pseudopleuronectes dignabilis</i>	Winter flounder	Heterosomata	87
<i>Acutomentum ovale</i>	Speckled rockfish	Cataphracti	82
<i>Pneumatophorus grex</i>	Tinker mackerel, chub mackerel	Percomorphi	77

TABLE 4 (Continued)

Common name of fish	Systematic name	Zoological order	Vitamin D, I.U./g.
<i>Pollachius virens</i>	Atlantic pollack	Anacanthini	70
<i>Theragra chalcogramma</i>	Pacific wall-eye pollack	Anacanthini	67
<i>Pimelometopon pulchrum</i>	Sheepshead, California red-fish	Pharyngognathi	62
<i>Cypselurus californicus</i>	California flying fish	Syngnathini	51
<i>Menticirrhus undulatus</i>	California corbina	Percomorphi	51
<i>Sebastes marinus</i>	Rosefish	Cataphracti	33
<i>Raja inornata</i>	California skate	Batoidei	25
<i>Hippoglossoides dubius</i>	Abura karei flounder	Heterosomata	25
<i>Raja binoculata</i>	Big skate of California	Batoidei	24
<i>Sebastes macrochir</i>	Kichiji, thornhead	Cataphracti	22
<i>Anarhichas lupus</i>	Atlantic wolffish	Jugulares	19
<i>Squalus suckleyi</i>	Pacific dogfish	Tectospondyli	13
<i>Clidoderma asperrimum</i>	Same karei flounder	Heterosomata	12
<i>Alopias vulpinus</i>	Thresher shark	Euselachii	9
<i>Cetorhinus maximus</i>	Basking shark	Euselachii	6
<i>Squalus acanthus</i>	Atlantic spiny dogfish	Tectospondyli	3
<i>Hydrolagus collieri</i>	Ratfish	Chimaeroidei	2
<i>Glyptocephalus cynoglossus</i>	Witch flounder	Heterosomata	<2
<i>Acipenser fulvescens</i>	Lake sturgeon	Glanioptomi	<1

^a Adapted from C. E. Bills in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. II, Academic Press, Inc., New York, 1954, pp. 165, 166. The nomenclature for the species is that of Jordan *et al.*¹⁷⁰ rather than that chiefly employed in this and other volumes of *The Lipids*, which is largely based upon the report entitled *A List of Common and Scientific Names of Better-Known Fishes of the United States and Canada*, which appears as Transactions of American Fisheries Society, 75, Special Publication No. 1, 355-397, Ann Arbor, Michigan, 1948, and upon the manuscript List of "*Fishes of California*" prepared by C. L. Hobbs and W. I. Follett, California Acad. Sciences (San Francisco) 1956. This may account for some discrepancies in systematic nomenclature between this table and the text.

noted its presence in the commercial menhaden oil, which is a body oil. The liver and body oils of a given species were generally found to be quite similar, but the vitamin concentration is invariably higher in the liver oil. Thus, in the case of the tuna, the liver oil may be several thousand times richer in vitamin D than the body oil. Bills¹⁰³ gives the following figures for the vitamin D content of fish mesentery oils: "black horse" or blue sucker (*Cyprinus elongatus*), 400 I.U./g.; golden perch or fresh water drum (*Aplodinotus grunniens*), 11 I.U./g.; big-mouth buffalo fish (*Ictiobus megastomatobus*) *cyprinella*, 10 I.U./g.; and spotted channel catfish (*Ictalurus punctatus*), 5 I.U./g. Values for the vitamin D in the oil from the entire bodies of the fishes include the following: herring (*Clupea harengus*), 100 I.U./g.; California sardine or pilchard (*Sardinops caerulea*), 80

I.U./g.; menhaden (*Brevoortia tyrannus*), 50 I.U./g.; flathead mud catfish (*Pilodictis olivaris*), 20 I.U./g.; and capelin (*Mallotus villosus*), 3 I.U./g. In the case of the Greenland halibut (*Reinhardtius hippoglossoides*), the average vitamin D content of the body fat (obtained from carcass without liver) was 30 I.U./g.

Many factors, such as season, sex, age, food supply, climate, and nutritional condition¹⁷² influence the amount of vitamin D in fish liver oils. Liver oil from spawning, undernourished cod contains considerably more vitamin D than does liver oil from well-nourished cod.¹⁷² For example, Hess and co-workers¹⁷³ observed that the vitamin D content of the livers of the cod varied inversely with the oil content. Oil from some cod livers studied was as much as 1,000 times as potent as that of others of the same species.¹⁷³ A similar phenomenon was noted in the case of livers from the haddock and pollack. According to Bills *et al.*,¹⁷⁴ the oil content of pooled halibut livers obtained near Seattle in January was at the lowest point, 12%, while the vitamin D content reached its highest value, namely 1400 I.U./g. oil. On the other hand, in August, the oil content of the livers showed its maximum value, namely 25%, coincidentally with the lowest concentration of vitamin D, *i.e.*, 900 I.U./g. Pugsley¹⁷⁵ observed that the vitamin D content of the liver and intestinal oils of the Pacific gray cod (*Gadus macrocephalus*) varied inversely with the amount of fat in the livers. The same inverse relationship existed between the fatness of the liver and the vitamin D concentration in the oil of the Atlantic cod (*Gadus morrhua*).¹⁷⁶ On the other hand, the vitamin D content of the liver oil of this species was found to remain constant when calculated as units per gram of body weight of fish in a given age group, although the vitamin D content of the liver increased with the age of the fish. Bailey¹⁷⁷ reported an inverse relationship in the oil yield of the whole pilchard (*Sardinops caerulea*), including stomach contents, and its vitamin D potency. The vitamin D content of the Norwegian cod, which is caught at the time when its nutritional condition is poor, is higher than that of the Newfoundland cod, which is in a much better nutritional state when caught.

Although the milt and roe of fishes such as the cod were found to con-

¹⁷² E. Poulsson and F. Ender, *Skand. Arch. Physiol.* 66, 92-96 (1933).

¹⁷³ A. F. Hess, C. E. Bills, and E. M. Honeywell, *J. Am. Med. Assoc.*, 92, 226-228 (1929).

¹⁷⁴ C. E. Bills, M. Imboden, and J. C. Wallenmeyer, *J. Biol. Chem.*, 105, x (1934).

¹⁷⁵ L. I. Pugsley, *J. Fisheries Research Board, Can.*, 4, 405-408 (1939).

¹⁷⁶ L. I. Pugsley, C. A. Morrell, and J. T. Kelly, *Can. J. Research, F* 23, 243-252 (1945).

¹⁷⁷ B. E. Bailey, *Canada, Biol. Bd., Pacific Biol. Sta.* (Nanaimo, B.C.), and *Fisheries Expt. Sta.* (Prince Rupert, B.C.), *Progress Repts.*, No. 19, 5-6 (April, 1934).

tain considerable amounts of vitamin D, the newly-hatched fish were practically devoid of this vitamin, indicating that it had been consumed in the course of development of the larvae.¹⁷⁸ This indicates that fishes start life with adequate vitamin D, then exhaust the supply, only to accumulate it again later. An especially large amount of the vitamin is lost by the adult fish at the time of spawning.

(b) *Vitamins D in Higher Animals.* In the higher animals, vitamin D does not ordinarily occur in the body fat, but is usually concentrated in the eggs and milk in amounts sufficient to supply that necessary for the growth and development of the newborn. However, in the case of marine birds, which live largely on fish, vitamin D has been found in the body fat.

The proportion of vitamin D in milk depends upon many factors, including species, season, period of lactation, and vitamin D intake. Cow's milk is relatively low in vitamin D.¹⁷⁹ Values recorded for this fluid include 5.3 to 8.3 I.U./quart for milk from cows kept indoors, as contrasted with 17 to 26 I.U./quart for that from cows kept out-of-doors in June.¹⁸⁰ This agrees well with the figures of Bechtel and Hoppert,¹⁸¹ who cited 5 I.U./quart for winter milk and 44 I.U./quart for summer milk. In a comprehensive study of the effect of season on the vitamin D content of cow's milk, Henry and Kon¹⁸² reported that, during the 1940-1941 season in Great Britain, the values for vitamin D varied from less than 0.1 I.U./g. fat in the samples collected from November to March to 0.55 I.U./g. fat in July and to a maximum figure of 0.97 I.U./g. fat in August.

Dikshit and Ranganathan¹⁸³ reported high values for the vitamin D content of South Indian butters and ghee, *i.e.*, 6 to 18 I.U./g. fat in the non-saponified residue of the fat, and 10 to 32 I.U./g. fat in the fat itself. These values are much higher than those reported by Fixsen and Roscoe¹⁸⁴ for vitamin D in English and Danish butter, or than the highest figure reported by Coward and Morgan¹⁸⁵ for English butters in summer, namely 4 I.U./g. The high values for vitamin D in the Indian butters is attributed to the strength of the sun to which the cows and fodder are exposed in

¹⁷⁸ A. F. Hess, C. E. Bills, M. Weinstock, E. Honeywell, and H. Rivkin, *Proc. Soc. Exptl. Biol. Med.*, **25**, 652-653 (1928).

¹⁷⁹ H. E. Honeywell, R. A. Dutcher, and C. D. Dahle, *J. Nutrition*, **2**, 251-256 (1930).

¹⁸⁰ J. E. Campion, K. M. Henry, S. K. Kon, and J. Mackintosh, *Biochem. J.*, **31**, 81-88 (1937).

¹⁸¹ A. E. Bechtel and C. A. Hoppert, *J. Nutrition*, **11**, 537-549 (1936).

¹⁸² K. M. Henry and S. K. Kon, *Biochem. J.*, **36**, 456-459 (1942).

¹⁸³ P. K. Dikshit and S. Ranganathan, *Indian J. Med. Research*, **38**, 37-40 (1950).

¹⁸⁴ M. A. B. Fixsen and M. H. Roscoe, *Nutrition Abst. & Revs.*, **7**, 823-867 (1937-1938).

¹⁸⁵ K. H. Coward and B. G. E. Morgan, *Brit. Med. J.*, 1935, II, 1041-1044.

south India. However, Henry and Kon¹⁸⁶ were unable to confirm the high figures for vitamin D in Indian butters. According to the latter workers, the values for vitamin D in the three samples studied were as follows (expressed in I.U./g. fat): Bangalore (sample 1), 0.56; Bangalore (sample 2), 0.41; and Coimbatore, 0.29. Patwardhan¹⁸⁷ cited a figure of 1.2 I.U. of vitamin D/g. fat in a Bangalore butter. It would therefore appear questionable that butters from different areas exposed to moderate or to excessive amounts of sunshine show any such marked variations in vitamin D. According to the Heinz charts,¹⁸⁸ the figure of 35 I.U./quart of milk is based upon values for vitamin D in butter fat of 8 to 60 I.U./100 g.

The vitamin D content of human milk has been found to be somewhat higher than that of cow milk. Drummond and co-workers¹⁸⁹ reported an average figure of 60 I.U./quart in mother's milk. Greater amounts of vitamin D occur in the colostrum than in the mature milk. This early secretion contains six to ten times as much of the vitamin as in the milk secreted later in the case of the cow,¹⁹⁰ and about three times the amount in the case of human mothers.¹⁸⁹

The low concentrations of vitamin D in cow milk can be augmented in commercial practice by any of several procedures. Thus, vitamin D may be added directly to the milk in a suitable solvent such as propylene glycol, to yield a product with a potency of 150 or 300 I.U./quart.^{191,192} A second procedure which is frequently employed involves the direct irradiation of the cow with sunlight or with artificial ultraviolet light,^{180,181} but the method is of little importance commercially.

Another procedure for increasing the vitamin D in milk which has considerable application involves the direct irradiation of the milk. This is the most economical method, but it is difficult to produce satisfactory results, inasmuch as the milk acquires an unpleasant taste and odor if over-irradiated.¹⁹³ Diemair¹⁹⁴ suggested several procedures for overcoming this difficulty and increasing the amount of vitamin D in the milk.

¹⁸⁶ K. M. Henry and S. K. Kon, *J. Dairy Research* 21, 81-82 (1954).

¹⁸⁷ V. N. Patwardhan, Personal communication, 1953; cited by K. M. Henry and S. K. Kon, *J. Dairy Research*, 21, 81-82 (1954)

¹⁸⁸ H. J. Heinz, *Nutritional Data*, Pittsburgh (1950).

¹⁸⁹ J. C. Drummond, C. H. May, and N. E. G. Richardson, *Brit. Med. J.*, 1939, II, 757-760.

¹⁹⁰ J. van Niekerk and M. S. C. Blik, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, 9, 25-26 (1939).

¹⁹¹ T. F. Zucker, *Am. J. Pub. Health*, 23, 10-18 (1923).

¹⁹² D. J. Barnes, *J. Mich. State Med. Soc.*, 22, 242-246 (1933).

¹⁹³ K. G. Weckel and H. C. Jackson, *Food Research*, 1, 419-426 (1936).

¹⁹⁴ W. Diemair, *Chem. Fabrik*, 14, 51-54 (1941).

There is a sufficient concentration of the provitamins D in milk to render possible a maximum production of 200 I.U. vitamin D/quart, but the commercial irradiated milks have been standardized to contain 135 I.U./quart.

An additional procedure for increasing the vitamin D in milk, and the most physiologic method, involves increasing the vitamin D intake on the part of the cow. This is usually done by the feeding of limited amounts of irradiated yeast to the animals. When controlled quantities of yeast are given, milk having as much as 400 I.U./quart is produced. This is the so-called "metabolized" vitamin D milk. Kraus *et al.*^{195,196} demonstrated that milk and butter containing increased amounts of vitamin D were produced after irradiated ergosterol (vitamin D₂) was administered to the cows; however, Thomas and MacLeod¹⁹⁷ and Kraus and Bethke¹⁹⁸ found that an even greater increase in the vitamin D content of milk resulted when irradiated yeast was fed in place of the irradiated ergosterol. Cod-liver oil concentrate (Vitex), also, has been satisfactorily employed for this purpose.¹⁹⁹ Kirk²⁰⁰ recorded an increased concentration of vitamin D in the milk of the ewe after feeding irradiated yeast. Irradiated ergosterol exerted less augmentatory effect in this species than it did in the cow.

In the eggs of birds, vitamin D is usually present in fairly large amounts; it occurs exclusively in the yolk. An average figure for the yolks of hen's eggs is 150 to 400 I.U./100 g.¹⁸⁸ The vitamin D content in the egg is increased when the hen is irradiated,²⁰¹ as well as when the food contains an increased quantity of vitamin D.^{202,203}

In the case of milk and eggs, the increased vitamin D produced by vitamin D feeding is of the same type as that present in the food. Thus, Bethke *et al.*¹¹³ reported that, when the vitamin D in the milk was increased by feeding irradiated yeast, it required about ten times as much of this vitamin D (presumably vitamin D₂) to satisfy the requirement for the chick as was the case when the source of the vitamin D was irradiated milk (presumably vitamin D₃ produced by activation of the provitamin D₃, 7-

¹⁹⁵ W. E. Kraus and R. M. Bethke, *J. Biol. Chem.*, **92**, x-xi (1931).

¹⁹⁶ W. E. Kraus, R. M. Bethke, and C. F. Monroe, *J. Nutrition*, **5**, 467-477 (1932); *Ohio Agr. Expt. Sta. Bimonthly Bull.*, No. 156, 117-121 (1932).

¹⁹⁷ B. H. Thomas and F. L. MacLeod, *Science*, **73**, 618-620 (1931).

¹⁹⁸ W. E. Kraus and R. M. Bethke, *Ohio Agr. Expt. Sta. Bimonthly Bull.*, **18**, No. 162, 77-80 (1933).

¹⁹⁹ W. E. Kraus, R. M. Bethke, and M. Wilder, *Ohio Agr. Exptl. Sta. Bimonthly Bull.*, **18**, No. 1, 15-19 (1933).

²⁰⁰ W. O. Kirk, *Iowa State Coll. J. Sci.*, **13**, 235-238 (1939).

²⁰¹ G. H. Maughan and E. Maughan, *Brit. J. Phys. Med.*, **7**, 137-138 (1932).

²⁰² G. H. Maughan and E. Maughan, *Science* **77**, 198 (1932).

²⁰³ G. M. DeVaney, H. E. Munsell, and H. W. Titus, *Poultry Sci.*, **12**, 215-222 (1933).

dehydrocholesterol). It is known from other work that vitamin D₃ can be used readily by the chick, but that vitamin D₂ is quite ineffective as a source of vitamin D for this species. Bethke and associates¹¹² likewise demonstrated that the increased vitamin present in the hen's egg after the feeding of higher levels of vitamin D corresponds to the type of vitamin D introduced into the diet.

2. The Digestion, Absorption, and Transport of the Vitamins D

(1) *The Absorption of the Vitamins D*

Much less information is available concerning the absorption of the vitamins D from the intestine than about that of the other fat-soluble vitamins. This is largely due to the fact that the methods hitherto employed for the estimation of the D vitamins were cumbersome, and required a rather large amount of biologic material. However, much of this difficulty has been overcome by the use of C¹⁴-tagged vitamin D, which was employed in the studies of Kodicek,²⁰⁴ as well as by the more recent chemical method of Kodicek and Ashby²⁰⁵ which employs reversed-phase paper chromatography.

There is no question that the vitamins D can be absorbed to an appreciable extent from the gastrointestinal tract. This is the general route of administration when bioassay studies are made and, in fact, when synthetic diets employing vitamin D are given. The vitamins D can also be utilized when injected into animals. This would seem to indicate that, under normal conditions, vitamin D is absorbed unchanged from the gut.

The capacity of the gut for absorption of the vitamins D is not known. Morgan and Shimotori²⁰⁶ reported the absence of any appreciable amount of vitamin D from the feces of young dogs after the first day of dosing with 20,000 I.U./kg. body weight, given in the form of cholesterol (an activated animal sterol). Cruickshank and Kodicek²⁰⁷ were able to recover only about 17% of the administered vitamin D in the feces of rats during two days after 40,000 I.U. (1 mg. calciferol) had been administered in a single dose. However, these workers were able to account for only 6% of the total dose in the body, and it was not known what occurred to the remaining 77%. It has recently (1955) been demonstrated, by the use of C¹⁴-

²⁰⁴ E. Kodicek, *Bone Structure and Metabolism*, Ciba Lecture; Unpublished data made available to the author, 1955.

²⁰⁵ E. Kodicek and D. R. Ashby, *Biochem. J.*, **57**, xii (1954).

²⁰⁶ A. F. Morgan and N. Shimotori, *J. Biol. Chem.*, **147**, 189-200 (1943).

²⁰⁷ E. M. Cruickshank and E. Kodicek, *Biochem. J.*, **51**, xlii (1952); also personal communications from the authors, October, 1955.

labeled vitamin D, that most of the missing vitamin D is excreted *via* the intestine, not as an active vitamin, however, but as an inactive derivative.²⁰⁴ It is not known whether this ingested vitamin D was inactivated in the gastrointestinal tract, or whether it was absorbed unchanged from the tract, to be inactivated in the body and reëxcreted into the intestinal tract.

There are several factors which are known to influence the absorption of vitamins D from the small intestine. In the first place, Knudson and Floody²⁰⁸ demonstrated that fat improved the utilization of the anti-rachitic vitamin. Thus, the extent of healing of rickets in rats was greater when a known amount of vitamin D was given in conjunction with a diet containing 5% of fat than when a fat-free regimen was employed. It was noted that better results were obtained when the diets contained 10 or 20% of fat than when fat-free diets were given, although the results were less satisfactory than when the rations contained 5% of fat. It was not possible to determine whether the improvement in the utilization of vitamin D in the fat series was to be ascribed to a better absorption, or to some effect on the vitamin D after absorption. Boer²⁰⁹ likewise reported that rachitogenic diets no longer produced rickets when 10% of the saponifiable fraction of margarine was added; he interpreted this as evidence that fat exerts a sparing action on vitamin D. Kon and Booth²¹⁰ also produced evidence to substantiate this conclusion. Finally, McDougall²¹¹ observed that, when 11% of lard or olive oil was added to a low-calcium diet, the rats no longer developed rickets.

A second requirement for effective absorption of the vitamins D is bile. Thus, the presence of this secretion is required in the case of the vitamins D, as in that of the other fat-soluble vitamins and actually of fat itself. Düttmann²¹² found that, when bile was absent from the gastrointestinal tract of man and of other animals, osteoporotic malacia developed, due to acidotic disturbance of the calcium metabolism and of the digestion of fat, with resultant lack of absorption of vitamin D. Seifert²¹³ suggested that vitamin D is not absorbed in the absence of bile, and that therefore the utilization of calcium in the intermediary metabolism is decreased. This abnormal condition can be alleviated by the parenteral administration of

²⁰⁸ A. Knudson and R. J. Floody, *J. Nutrition*, **20**, 317-325 (1940).

²⁰⁹ J. Boer, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **9**, 67-68 (1939).

²¹⁰ S. K. Kon and R. G. Booth, *Biochem. J.*, **28**, 111-120, 121-130 (1934).

²¹¹ E. J. McDougall, *Biochem. J.*, **32**, 194-202 (1938).

²¹² G. Düttmann, *Brun's Beitr. klin. Chir.*, **139**, 720-729 (1927).

²¹³ E. Seifert, *Brun's Beitr. klin. Chir.*, **136**, 496-498 (1926).

bile²¹² and vitamin A.²¹³ Tammann²¹⁴ found that the administration of vitamin D and of activated cholesterol had a beneficial effect upon the rachitic bone condition, and upon the disturbance of the calcium-phosphorus balance. The question was clarified by the comprehensive work of Greaves and Schmidt,²¹⁵ who studied the absorption of vitamin D in choledochocolostomized rats, using calcium and phosphorus balances as the criterion of the absorption of vitamin D. Bile fistula rats were found to have a negative calcium and phosphorus balance, and little or no irradiated ergosterol was absorbed through the intestinal wall following the operation. Heymann²¹⁶ confirmed these findings on dogs. In dogs whose bile ducts had been ligated, vitamin D was absent from the blood. A hyperphosphatemic reaction did not occur following the administration of viosterol (vitamin D₂ in oil) or drisdol (vitamin D₃ in propylene glycol). Thus, vitamin D was not absorbed unless bile was present in the chyme. It has been reported that the presence of mineral oil in the diet usually reduces the absorption of vitamin D. Although Dutcher and co-workers,²¹⁷ and Jackson²¹⁸ were unable to obtain evidence of any deleterious effect on the part of mineral oil on the absorption of vitamin D, Smith and Spector²¹⁹⁻²²¹ recorded an interference in the absorption of vitamin D resulting from mineral oil, in both rats and dogs.

Absorbed vitamin D can apparently be excreted into the intestine through the bile, at least for the first three or four weeks after its administration.^{215, 216, 222-225} According to Heymann,²²⁶ the upper third of the small intestine, also, is probably active in the excretion of this vitamin.

It is generally accepted that the esters of the vitamins D are hydrolyzed in the small intestine before being absorbed. This hypothesis is based

²¹⁴ H. Tammann, *Brun's Beitr. klin. Chir.*, 142, 83-120 (1928).

²¹⁵ J. D. Greaves and C. L. A. Schmidt, *J. Biol. Chem.*, 102, 101-112 (1933).

²¹⁶ W. Heymann, *J. Biol. Chem.*, 122, 249-256 (1937-1938).

²¹⁷ R. A. Dutcher, J. O. Ely, and H. E. Honeywell, *Proc. Soc. Exptl. Biol. Med.*, 24, 953-955 (1927).

²¹⁸ R. W. Jackson, *J. Nutrition*, 7, 617-622 (1934).

²¹⁹ M. C. Smith and H. Spector, *J. Nutrition*, 20, 197-202 (1940).

²²⁰ M. C. Smith and H. Spector, *J. Nutrition*, 20, 19-30 (1940).

²²¹ M. C. Smith and H. S. Spector, *Univ. Arizona, Coll. Agr. Ariz. Exptl. Sta., Tech. Bull. No. 84*, 373-395 (1940).

²²² N. B. Taylor, C. B. Weld, and J. F. Sykes, *Trans. Roy. Soc. Can., Sect. V*, 26, 29-31 (1942).

²²³ N. B. Taylor, C. B. Weld, and J. F. Sykes, *Brit. J. Exptl. Pathol.*, 16, 302-309 (1935).

²²⁴ J. D. Greaves and C. L. A. Schmidt, *Univ. Calif. Pub. Physiol.*, 8, 43-47, 49-60 (1934).

²²⁵ W. Heymann, *Am. J. Diseases Children*, 55, 913-923 (1938).

²²⁶ W. Heymann, *J. Biol. Chem.*, 122, 257-262 (1937).

upon the fact that esters which cannot be hydrolyzed have no antirachitic potency⁷⁵ although it has not been unequivocally proved that such non-utilization of unhydrolyzable esters is to be ascribed to their failure to be absorbed rather than to the biologic activity of the conjugated form after absorption (if, in fat, these esters are absorbed without hydrolysis).

No differences in absorption of the several vitamins D are noted in normal dogs. The absorption of vitamins D₂ and D₃ in unchanged form after they have been fed to hens is proved by the classical experiments of Bethke *et al.*^{111,112} in which it was demonstrated that the kind of vitamin D in the egg yolk corresponds to that in the diet. A similar phenomenon was reported by Bethke and co-workers¹¹³ for the cow, as proved by the relationship between the type of vitamin D in the milk and in the diet. However, according to McChesney,²²⁷ the chick does not absorb vitamin D₂ effectively. Whereas the amount of vitamin D₃ found in the carcasses and feces accounts for all the vitamin D₃ given, 35% of the vitamin D₂ administered cannot be traced.

(2) *The Transport of the Vitamins D*

There is no experimental evidence concerning the pathway for the transport of the vitamins D from the lumen of the intestine to the tissues. However, there is no reason to suppose that this route differs from that of the other fat-soluble vitamins or from that of the fats themselves. This would involve the lymphatic system as the principal if not the only route for the transfer of vitamin D from the epithelial cells to the blood stream. The portal system would probably be of no more importance in this transfer than it is in the case of vitamin A and the carotenoids, which is *nil*.

Presumably, the blood effects the further transfer of the vitamin D to the tissues after the vitamin has been emptied into the subclavian vein from the thoracic duct. The fact that vitamin D is present in blood has been established by a number of investigators. Warkany^{228,229} reported an average concentration of 100 I.U.% (50 to 135 I.U.%) in human serum, while the mean content recorded in the case of rabbits was 50 I.U.%. Hess and co-workers²³⁰ observed that the plasma vitamin D in cows was increased after irradiated ergosterol was given. The plasma of cows was found to contain four times the concentration of vitamin D which

²²⁷ E. W. McChesney, *Proc. Soc. Exptl. Biol. Med.*, **58**, 300-303 (1945).

²²⁸ J. Warkany, *Am. J. Diseases Children*, **52**, 831-847 (1936).

²²⁹ J. Warkany, *Biochem. Z.*, **293**, 415-426 (1937).

²³⁰ A. F. Hess, M. Weinstock, and J. Gross, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1357-1358 (1933).

was present in the corpuscles.²³¹ Warkany²²⁹ gives figures for vitamin D in whole blood varying between 40 and 67 I.U./100 ml., depending upon the quantity of the vitamin D administered. In the case of cattle, pigs, a dog and a rabbit, Warkany²²⁹ recorded figures for vitamin D in the blood ranging from 50 to 100 I.U./100 ml. In the case of children and adults, Warkany and Mabon²³² found the average for serum vitamin D to be 110 I.U./100 ml. Warkany and collaborators²³³ observed that, when patients were treated with high doses of vitamin D, the serum vitamin D rose steadily until values of 9,000 to 13,000 I.U./100 ml. were obtained when doses of 400,000 to 500,000 I.U. of vitamin D were given daily. After the termination of the vitamin D dosing, the levels of vitamin D in the blood returned to normal in the course of three to six months. On the other hand, when Cruickshank and Kodicek²⁰⁷ fed 40,000 I.U. of vitamin D to rats in a single dose, inconsequential amounts of the vitamin were recovered from the blood.

3. The Further Metabolism of the Vitamins D in the Tissues

Little information is available as to the fate of the vitamins D after they leave the blood. In studies on dogs Morgan and Shimotori²⁰⁶ recorded the following values for vitamin D (expressed in I.U./g. tissue): kidneys, 3 to 8; heart, *ca.* 5; wall of large intestine, *ca.* 5; and brain, lung, spleen, muscle, stomach, abdominal fat, skin and hair, 0.3 to 1.0.

Figures are also available for the vitamin D concentration in human tissues, usually after large (and unphysiologic) dosages. Thus, in the case of an infant who died of pneumonia four days after the intramuscular injection of 15 mg. of calciferol (vitamin D), Houet²³⁴ observed the following values (expressed in mg. per total tissue): brain, 1.6; kidneys, 0.64; liver, 0.04; and muscle, 3.8 mg.

It is impossible to arrive at anything in the nature of a quantitative recovery of unchanged vitamin D when large doses are given to animals. Although McChesney and Giacomino²³⁵ accounted for 1000 I.U. in the carcasses of chicks fed 2400 I.U. of vitamin D₂ (about 40%), Morgan and Shimotori²⁰⁶ were never able to recover more than 10% of the quantity ingested in the case of dogs. Moreover, Kodicek²⁰⁴ demonstrated only 6% of the ingested vitamin D in the carcasses of weanling rats fed 40,000 I.U.

²³¹ A. F. Hess, R. F. Light, C. N. Frey, and J. Gross, *J. Biol. Chem.*, **97**, 369-377 (1932).

²³² J. Warkany and H. E. Mabon, *Am. J. Diseases Children*, **60**, 606-614 (1940).

²³³ J. Warkany, G. M. Guest, and F. J. Grabill, *J. Lab. Clin. Med.*, **27**, 557-565 (1942).

²³⁴ R. Houet, *Ann. Paediat.*, **166**, 169-176 (1946).

²³⁵ E. W. McChesney and N. J. Giacomino, *J. Nutrition*, **29**, 229-235 (1945).

of vitamin D, although 17% more was excreted in the feces during the following two days. This totals a 23% recovery.²³⁶ The body vitamin D was present mainly in the liver (3.7%) and intestinal tissue (0.6%), while the remainder of the body contained 1.7%.²³⁶ In a more recent report, Cruickshank *et al.*²³⁷ noted the distribution of vitamin D₂ in the following amounts (% of dose) forty-eight hours after it had been administered: muscle, 0.9; skin, 0.6; kidneys, 0.5; lungs, 0.1; spleen, 0.05, brain, 0.03; and adrenals, 0.01. In the most recent study of this group reported by Kodicek,²⁰⁴ in which the recovery of C¹⁴-ergocalciferol was tested when given in a 1 mg. dose orally to rachitic rats, a complete recovery

TABLE 5
THE RECOVERY OF C¹⁴-ERGOCALCIFEROL WHEN GIVEN ORALLY
IN A 1 MG. DOSE TO RACHITIC RATS^a

Tissue or excretion product	Recovery in % of dose given			
	As determined chemically	As determined from C ¹⁴ content		
		As vitamin D	As breakdown products	Total
Urine	0	0	2.1	2.1
Feces	21.0	19.7	59.6	79.3
Carbon dioxide	—	—	?	?
<i>Total</i>	<i>21.0</i>	<i>19.7</i>	<i>61.7</i>	<i>81.4</i>
Liver	7.6	5.7	5.7	11.4
Bones	1.4 ^b	(1.4) ^c	2.6	4.0
Intestines	0.63	0.6	0.9	1.5
Blood	—	(1.2)	0	1.2
Kidneys	—	0.2	0	0.2
Lungs	—	(0.2)	0	0.2
Brain	—	0	—	0
Rest of organs	—	(0.5)	—	0.5
Muscle and skin	—	0.8	—	0.8
<i>Total</i>	—	<i>10.6</i>	<i>9.2</i>	<i>19.8</i>
<i>Grand total</i>	—	<i>30.3</i>	<i>70.9</i>	<i>101.2</i>

^a Data adapted from E. Kodicek, Ciba Lecture. Unpublished data made available to author.

^b Based upon biologic rat assay.

^c Figures in parenthesis represent results in which no chemical identification was made but which are considered to be vitamin D.

was observed, based upon the C¹⁴ assay; 30.3% was accounted for as vitamin D, while 70.9% was present in the form of breakdown products. These data are summarized in Table 5.

²³⁶ E. M. Cruickshank, E. Kodicek, and P. Armitage, *Biochem. J.*, **54**, 337-347 (1953).

²³⁷ E. M. Cruickshank, E. Kodicek, and P. Armitage, *Biochem. J.*, **58**, 172-175 (1954).

According to the most recent data summarized in Table 5, as much as 4% of the total vitamin D and its breakdown products are present in the bones. It is the opinion of Kodicek²³⁸ that vitamin D is concentrated in the sites where the turnover of inorganic phosphorus is taking place, such as the liver, bones, and intestinal wall.

The further fate of vitamin D is not known. It is possible that it remains in the several tissues for a period of time, and is gradually destroyed *in situ* as it carries out its physiologic function, or that it is gradually excreted *via* the bile without decomposition in the tissues. It has already been recorded that a certain amount of vitamin D can be reëxcreted into the intestine through the bile,^{215,216,222-225} as well as *via* the walls of the upper one-third of the small intestine.²²⁶ Heymann^{216,226} observed that a slow reëxcretion of vitamin D in the bile of dogs continued for months after the administration of the vitamin had been discontinued. Coppens and Metz¹⁵⁹ reported that an enzyme occurs in the lungs which destroys vitamin D. In the case of pregnant females, there is also the possibility that some vitamin D would be transferred to the fetuses. Thus, Embleton and Collings²³⁹ have reported that young rats receive supplies of vitamin D from the doe either *via* the placenta or from the milk during lactation.

4. The *in vivo* Activation of the Provitamins D

The activation of the several provitamins D in the test tube is well understood from both the physical and the chemical viewpoints, and has been discussed in an earlier section of this chapter (see pages 639 to 641). Until 1943 there had been less information concerning the *in vivo* transformation of the provitamins D to their corresponding vitamins D. In this year, Geiger and Lassen⁸⁷ found that the subcutaneous or oral administration of 7-dehydrocholesterol which had been activated by arc lamp irradiation had a pronounced curative effect upon rachitic rats. The subcutaneous injection of the provitamin D into the depleted rats was effective only if the rats were irradiated daily. However, irradiation alone was ineffective. In later studies these workers^{239a} demonstrated that 7-hydroxycholesterol and 7-ketocholesterol, injected subcutaneously into rats subsequently exposed to ultraviolet irradiation, had a greater anti-rachitic potency than did 7-dehydrocholesterol administered under similar conditions. Administration of these provitamins D *per os* was less effective than was the subcutaneous method, indicating faulty resorption of

²³⁸ E. Kodicek, Personal communication to the author, October 10, 1955.

²³⁹ J. Embleton and A. J. Collings, *Nature*, 159, 340-341 (1947).

^{239a} S. Lassen and E. Geiger, *Proc. Soc. Exptl. Biol. Med.*, 53, 181-183 (1943).

these compounds from the intestinal tract. Activated 7-dehydrocholesterol was the exception.⁸⁷ There seems to be little doubt that the provitamins D can be activated in the skin, and that this explains the action of sunlight or of ultraviolet light in preventing rickets as effectively as do the vitamins D themselves.

One of the facts which offers presumptive proof of the *in vivo* conversion of provitamins D to vitamins D is that the former are found in higher concentrations in the skin than elsewhere. This is the logical site for the transformation to take place. 7-Dehydrocholesterol (provitamin D₃) is a constant contaminant of cholesterol,²⁴⁰ but Rosenberg¹⁰⁶ points out that it usually occurs in higher proportion in the skin lipids than in those of the inner tissues. Windaus and Bock⁴⁹ separated 7-dehydrocholesterol in 3 to 6% of the total sterols of pigskin. Rosenberg¹⁰⁶ recorded the following percentages of total sterols which are present as provitamins D in the skin of several species: man, 0.15 to 0.43; cattle, 0.18; calf, 0.68; mouse, 0.87; and chicken (feet) 1.0 to 4.0. Rosenberg²⁴¹ has recently confirmed the presence of 7-dehydrocholesterol (provitamin D₃) in the feet of chickens. Moore and Baumann²⁴² noted the presence of 22 to 36% of a sterol, which reacts rapidly with the Schoenheimer-Sperry reagents, in the sterols of rat skin. Since cholesterol itself does not possess the ability to react promptly with the above reagent, while 7-dehydrocholesterol and 7-hydroxycholesterol are known to have this property, it is presumed that this represents provitamin D in the rat skin. Idler and Baumann²⁴³ demonstrated that the major component of the fast-reacting sterol fraction, as determined by chromatographic separation of its azoyl ester on silica, is Δ^7 -cholestene-3 β -ol. Glover, Glover, and Morton²⁴⁴ reported that cholesterol is dehydrogenated to 7-dehydrocholesterol in the mucous membrane of the small intestine.

5. The Effect of the Vitamins D on Various Physiologic Processes

(1) Introduction

Clues as to the nature of the several physiologic processes which are under the control of the vitamins D were first obtained by investigation of the abnormal conditions which result in avitaminosis D. Thus, in all

²⁴⁰ A. G. Boer, E. H. Reerink, A. van Wijk, and J. van Niekerk, *Proc. Acad. Sci., Amsterdam*, **39**, 622-632 (1936).

²⁴¹ H. R. Rosenberg, *Arch. Biochem. Biophys.*, **42**, 7-11 (1953).

²⁴² P. R. Moore and C. A. Baumann, *J. Biol. Chem.*, **195**, 615-621 (1952).

²⁴³ D. R. Idler and C. A. Baumann, *J. Biol. Chem.*, **195**, 623-628 (1952).

²⁴⁴ M. Glover, J. Glover, and R. A. Morton, *Biochem. J.*, **51**, 1-9 (1952).

species of animals studied, a decrease in the bone ash content occurs in rickets, and a marked disturbance in ossification can be observed. An enlargement of the growing epiphyseal cartilage takes place in all tubular bones, which can readily be demonstrated by x-ray examination. The deranged bone formation has been found to result from a reduction of a Ca x P product in the blood, due to a decrease in the utilization of calcium and of phosphate. This may be caused by a number of different factors.

(2) *The Absorption of Calcium and Phosphate*

It is logical that an investigation of the pathology of rickets should start with a study of what changes, if any, take place in the composition of bones. Thus, Korenchevsky⁴ cited Marchand,²⁴⁵ and others^{246,247} as having demonstrated that rachitic bones were richer in water and poorer in calcium than were normal bones; the main mineral constituents of the latter were known to be calcium and phosphorus. The condition of rickets was therefore ascribed to some abnormality in calcium and phosphorus metabolism which resulted in the production of bones deficient in these elements. The bone composition in osteomalacia was shown to be identical with that in rickets, as reported by Frey,²⁴⁸ Weber,²⁴⁹ and Huppert.²⁵⁰ Weber²⁴⁹ found a large proportion of lactic acid in the bone substance and a reduced amount of calcium. These findings led to studies of mineral metabolism, particularly that of calcium, in relation to the composition of bones. Classical reports of the early period include that of Chossat²⁵¹ in 1842, and later those of Edwards,²⁵² Weiske,²⁵³ and Voit.²⁵⁴

a. Balance Experiments on Calcium and Phosphate Metabolism in Rachitic Infants without and with Vitamin D. Evidence has been accumulated which indicates that an increased loss of calcium and phosphate in the

²⁴⁵ R. F. Marchand, *J. prakt. Chem.*, 27, 83-97 (1942); also cited by V. Korenchevsky, Med. Research Council (London), *Spec. Rept. Ser.*, No. 71 (1922).

²⁴⁶ E. Bibra, *Chemische Untersuchungen über die Knochen und Zähne des Menschen und der Wirbelthiere, mit Rücksichtigung auf ihre physiologischen und pathologischen Verhältnisse*, Schweinfurt, 1844; cited by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, 11, 29-60 (1953), p. 30.

²⁴⁷ A. Friedleben, *Physikalische und chemische Constitution wachsender und rachitischer Knochen*, Wien, 1860; cited by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, 11, 29-60 (1953), p. 30.

²⁴⁸ H. Frey, *Zentr. med. Wissensch.*, 1, 28-29 (1863).

²⁴⁹ O. Weber, *Virchow's Arch. pathol. Anat. u. Physiol.*, 38, 1-15 (1867).

²⁵⁰ H. Huppert, *Zentr. med. Wissensch.*, 5, 458 (1867).

²⁵¹ M. Chossat, *Compt. rend.*, 14, 451-456 (1842).

²⁵² M. A. M. Edwards, *Compt. rend.*, 52, 1327-1329 (1861).

²⁵³ H. Weiske, *Z. Biol.* 7, 179-184, 333-337 (1871).

²⁵⁴ F. Voit, *Z. Biol.*, 29, 325-397 (1892).

TABLE 6
BALANCE STUDIES OF CALCIUM AND PHOSPHORUS IN RACHITIC CHILDREN
BEFORE AND AFTER SEVERAL FORMS OF THERAPY^a
(All figures in g./24 hr.)

Treatment	Mineral studied	Intake		Urine		Feces		Balance	
		Before	After	Before	After	Before	After	Before	After
Cod liver oil ^b	Ca	0.30	0.33	0.01	0.002	0.30	0.14	-0.01	+0.188
	P	0.45	0.47	0.20	0.23	0.26	0.12	-0.01	+0.12
	Ca	0.98	1.03	0	0	0.88	0.24	+0.10	+0.79
	P	0.84	0.87	0.32	0.42	0.46	0.07	+0.06	+0.38
	Ca	0.78	0.77	0	0	0.79	0.43	-0.01	+0.34
Cod liver oil ^c	P	0.67	0.67	—	—	0.37	0.17		
	Ca	1.17	1.17	0.01	0.01	0.86	0.66	+0.30	+0.50
	P	0.94	0.98	0.25	0.41	0.66	0.27	+0.03	+0.30
	Ca	1.32	0.98	0	0.01	1.30	0.27	+0.02	+0.70
	P	0.94	0.84	0.21	0.40	0.70	0.09	+0.03	+0.35
After ultraviolet light ^d	Ca	1.43	1.47	0.01	0.01	1.15	0.83	+0.27	+0.63
	P	1.11	1.18	0.27	0.42	0.65	0.44	+0.19	+0.32
	Ca	1.4	1.3	0.01	0.01	1.45	0.45	-0.06	+0.81
	P	1.3	0.97	0.30	0.32	0.8	0.19	+0.2	+0.46
	Ca	1.5	1.34	0.01	0.04	1.36	0.90	+0.13	+0.40
	P	1.4	1.0	0.23	0.40	0.83	0.34	+0.34	+0.26
	Ca	1.06	0.96	0.01	0.01	0.92	0.38	+0.13	+0.57
	P	0.77	0.74	0.32	0.41	0.41	0.11	+0.04	+0.22

^a Adapted from data summarized by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, 11, 29-60 (1953).

^b J. A. Schabard, *Z. klin. Med.*, 69, 435-474 (1910).

^c S. V. Telfer, *Quart. J. Med.*, 20, 7-25 (1926).

^d W. J. Orr, L. E. Holt, Jr., L. Wilkins, and F. H. Boone, *Am. J. Diseases Children*, 20, 362-372 (1923).

feces occurs in rickets.¹ As a consequence, the ash content of the blood is reduced, and less calcium and phosphate are deposited in the bones. Table 6 summarizes some of the earlier data in regard to the calcium and phosphorus balances in rachitic children before and after treatment with cod liver oil or ultraviolet light.

There can be little doubt that cod liver oil and ultraviolet light therapy bring about a profound alteration in the metabolism of calcium and of phosphate in rickets. Thus, in all cases, the equilibrium of these components is changed from a slightly negative or slightly positive balance in rickets to a strongly positive one, by the therapy designed to bring increased vitamin D to the tissues. The calcium loss in the stools is invariably greatly reduced after the rachitic children have been given cod liver oil or after they have been irradiated with ultraviolet light. The excretion of calcium in the urine, which, in any case, is quite insignificant, may tend to increase slightly after the treatment of the rachitic infants. When the rise does occur, it must be a reflection of the greatly increased proportion of calcium which has been absorbed.

Phosphorus, in the form of phosphate, is greatly decreased in the feces when sources of vitamin D such as cod liver oil or ultraviolet irradiation are given to rachitic infants. However, the considerable phosphate elimination in the urine, which occurs in the case of rachitic infants, is still further increased when the rachitic condition is improved by this form of medication. The reduction of fecal phosphate is so much greater than the increase in urinary phosphate that the phosphate balance inclines strongly to the positive side when the rachitic infant improves in health.

McCance²⁵⁵ furnished considerable data of a similar nature in the case of a young girl with "resistant rickets," due to a raised resistance to vitamin D which had persisted since her childhood. She was treated for almost a year with vitamin D. As soon as the medication was begun, the pattern of the calcium and phosphate metabolism as obtained from excretion and absorption data changed from the rachitic to the normal. This normal pattern of metabolism of calcium and phosphate was maintained throughout the year during which the medication was continued and the balances were followed. Liu and co-workers²⁵⁶ reported that vitamin D exerted a similar effect on calcium and in phosphate excretion in the case of an adult woman suffering from osteomalacia. Harris²⁵⁷ points out that

²⁵⁵ R. A. McCance, *Quart. J. Med.*, 40, (n.s. 16), 33-46 (1946).

²⁵⁶ S. H. Liu, H. I. Hsu, H. C. Chao, and S. H. Cheu, *J. Clin. Invest.*, 20, 255-271 (1941).

²⁵⁷ L. J. Harris, *Vitamin D and Bone*, chap. XIX in G. H. Bourne, *Biochemistry and Physiology of Bone*, Acad. Press, New York, 1956, pp. 581-622.

adult osteomalacia is due to lack of vitamin D or to a contributory shortage of calcium and phosphate in the diet, as is rickets. The chief pathologic difference between the two conditions is that osteomalacia is manifested by a decalcification of the bone, rather than by inadequate calcification of the growing end of the bone as observed in the rachitic infant. Both conditions respond to treatment with vitamin D.

b. Vitamins D in Relation to Calcium and Phosphate Metabolism in Species Other Than Man. The effect of rickets on the utilization of calcium and phosphate in dogs and chickens appears to be the same as in avitaminosis D in man. However, the metabolism of these inorganic constituents in the rat appears to differ markedly from that in other species.

Mellanby¹⁴ demonstrated in 1921 in his classical studies on puppies that rickets is a deficiency disease in this species; it was reported that the fecal calcium progressively approached the level of the ingested calcium during the development of the disease. In a later study, Mellanby²⁵⁸ showed that a reduction of the positive calcium balance occurred as a result of the increased fecal excretion of calcium, concomitantly with the development of the rickets. According to Wanscher²⁵⁹ and to McChesney and Giacomino,²³⁵ the effect of vitamin D on the absorption of calcium and phosphate is the same in the chick as it is in children and puppies.

In contradistinction to the decreased absorption of calcium and phosphate during rickets in man, dogs, and chicks, the rat, at least during its period of rapid growth, presents no comparable picture. It has been demonstrated that, even during advanced rickets, rats can absorb considerable quantities of calcium and phosphate. Under ordinary conditions rickets could not be produced in this species merely by the omission of vitamin D from the diet, but it was necessary that the Ca:P ratio be unbalanced. Rickets would then occur in the absence of vitamin D when the Ca:P ratio was high or when it was low. However, the hypertrophic uncalcified cartilage was found to be much more pronounced on diets having a high Ca:P ratio; therefore this dietary procedure has usually been employed in the study of experimental rickets. Dikshit and Patwardhan²⁶⁰ suggested that calcification depends mainly upon the state of the cartilage cell and matrix. Vitamin D probably influences either the metabolism or the structure of the epiphyseal cartilage cell and osteoblasts, and their matrices, rendering them capable of laying down the bone salt.

²⁵⁸ E. Mellanby, *J. Physiol.* 109, 488-533 (1949).

²⁵⁹ O. Wanscher, Thesis, A. Busck, Nyt Nordisk Forlag, Copenhagen, 1939; cited by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, 11, 29-60 (1953), p. 33.

²⁶⁰ P. K. Dikshit and V. N. Patwardhan, *Indian J. Med. Sci.*, 6, 107-116 (1952).

c. **On the Mechanism of Action of the Vitamins D on Calcium and Phosphate Balances.** The data which have been reviewed in the previous sections can be interpreted as evidence not only that vitamin D increases the absorption of calcium and phosphate but also that it may decrease the reëxcretion of these minerals into the intestine. Much work has been carried out to determine whether the action of vitamin D can best be explained by the absorption or by the reëxcretion theory.

The reëxcretion theory was a development of the current teaching during the latter part of the nineteenth century that the calcium and phosphate in the body was regulated by reëxcretion into the intestine, presumably chiefly into the large intestine. Thus, the intestine would act as a sort of auxiliary kidney in the regulation of body minerals. Bergeim²⁶¹ believed that his ferric oxide method supported the reëxcretion theory. Gyorgy⁵ accepted the reëxcretion theory, as did Harris.²⁶²

However, a number of observations have been recorded which appear to give strong support to the absorption theory in explaining the action of vitamin D. These render the reëxcretion theory quite untenable. In 1923, Orr and co-workers²⁶³ interpreted their data as evidence that vitamin D increased the absorption of calcium and phosphate. However, there was no proof that the reduction of the amount of fecal calcium and phosphate resulted from increased absorption of the minerals following vitamin D therapy. Nicolaysen²⁶⁴ was the first to attempt to obtain definite evidence as to which of these theories was the more probable. His data seem to indicate fairly conclusively that vitamin D acts by increasing the absorption of calcium. Thus, when the base level of fecal calcium in dogs on a calcium-free diet was determined, it was found that the fecal calcium was not increased by overloading the blood stream with injected calcium, although an increased excretion of calcium *via* the urine occurred. Moreover, no augmentation of the amount of calcium in the feces was noted when the bone salts were mobilized by means of the parathyroid hormone. Finally, it was found that no change in fecal calcium was produced when the entire colon was separated as a Thiery-Vella fistula and the feces were formed only in the distal part of the ileum, which was anastomosed to the rectum. The basal calcium excreted in the feces undoubtedly arises from the digestive juices, which contribute approximately 1 g. of calcium to the intestinal contents per day.

²⁶¹ O. Bergeim, *J. Biol. Chem.*, 70, 47-50, 51-58 (1926).

²⁶² L. J. Harris, *Ann. Rev. Biochem.*, 3, 247-294 (1934).

²⁶³ W. J. Orr, L. E. Holt, Jr., L. Wilkins, and F. H. Boone, *Am. J. Diseases Children*, 26, 362-372 (1923).

²⁶⁴ R. Nicolaysen, *Skand. Arch. Physiol.*, 69, suppl., 1-66 (1934).

In later tests by Nicolaysen,^{265,266} it was observed that the loss of body calcium and phosphate in the feces on a calcium-free and phosphorus-free diet, in vitamin D deficiency, was greater than in parallel experiments in which liberal amounts of vitamin D were present. This is interpreted as evidence that vitamin D increases the absorption of endogenous calcium. Moreover, it was found that, on phosphorus-free diets, the fecal excretion of calcium was greater when the intake of calcium was progressively increased. In all cases rats receiving vitamin D absorbed more calcium than did those on the vitamin D-free diets. Finally, it was observed that the absorption of phosphate was complete on calcium-free diets in vitamin D-deficient rats, irrespective of the source of the phosphate.

Further evidence that vitamin D acts by improving the absorption of calcium from the gut was obtained from studies of isolated loops of intestine by Nicolaysen,²⁶⁷ who noted that calcium disappeared more slowly from these isolated loops of intestine of vitamin D-deficient rats than from corresponding loops when vitamin D was present. On the other hand, glycerophosphate was absorbed as rapidly in isolated loops from vitamin D-deficient rats as in those from normal animals.²⁶⁸ Patwardhan and Chitre²⁶⁹ were unable to demonstrate any difference in the absorption of calcium in the entire isolated small intestine of rats, over a three-hour period, irrespective of whether the animals had previously been on a vitamin D-free regimen or had received vitamin D in the ration. Nicolaysen²⁷⁰ obtained opposite results when the isolated loop experiments were extended to twenty-four hours.

Experiments using the Ca^{45} isotope have given additional support to the absorption theory as the mechanism for the action of vitamin D. Greenberg²⁷¹ observed no increased excretion as related to vitamin D content of rats, but he did note a marked acceleration in the absorption rate of this isotope when vitamin D was administered. Harrison and Harrison²⁷² noted only a slight effect exerted by vitamin D on the absorption of Ca^{45} and the effect occurred chiefly in the large intestine and not in the small intestine. On the other hand, Lindquist²⁷³ demonstrated a marked augmentation of absorption which was evident within twelve hours after the

²⁶⁵ R. Nicolaysen, *Biochem. J.*, **31**, 107-121 (1937).

²⁶⁶ R. Nicolaysen, *Biochem. J.*, **31**, 122-129 (1937).

²⁶⁷ R. Nicolaysen, *Biochem. J.*, **31**, 323-328 (1937).

²⁶⁸ R. Nicolaysen, *Biochem. J.*, **31**, 1086-1088 (1937).

²⁶⁹ V. Patwardhan and Chitre, *Indian J. Med. Research.*, **30**, 81-89 (1942).

²⁷⁰ R. Nicolaysen, *Acta Physiol. Scand.*, **22**, 260-266 (1951).

²⁷¹ D. M. Greenberg, *J. Biol. Chem.*, **157**, 99-104 (1945).

²⁷² H. E. Harrison and H. C. Harrison, *J. Biol. Chem.*, **188**, 83-90 (1951).

²⁷³ B. Lindquist, *Kgl. Fysiograf. Sällskap. Lund Förh.*, **20**, 243-249 (1950).

vitamin was administered. The specific activity of the serum calcium was higher in rachitic chicks than in non-rachitic chicks after the injection of Ca^{45} , and the rate of decline of the specific activity was the same in both groups.²⁷⁴ All of these observations tend to support the conception that vitamin D decreases the calcium loss in the feces by increasing its rate of absorption. On the other hand, Dols *et al.*²⁷⁵ was unable to show any alteration in the rate of absorption of P^{32} from the intestine of vitamin D-free rats as compared with the rate in normal animals.

(3) *The Effect on Phosphate Metabolism*

Although the effect of vitamin D on the absorption of phosphate would appear to be indirect, occurring only because the vitamin increases the calcium absorption (and thus reduces the amount of Ca^{2+} which can combine with PO_4^{3-} in the intestine to produce insoluble $\text{Ca}_3(\text{PO}_4)_2$), some investigators have attributed to vitamin D a direct action upon the absorption of the phosphate group. Thus, Heymann²⁷⁶ reported that rachitic infants excreted almost 100% of injected phosphate in the urine, whereas normal infants excreted only 50%. In further experiments with rats, dogs, and rabbits, it was shown that organic phosphate passed through the entire wall of the intestinal loops more slowly in the case of preparations from vitamin D-deficient animals than in those from normal animals.^{277, 278} This was also true *in vivo* in human subjects.²⁷⁹ Zetterström and Ljunggren²⁸⁰ suggested that vitamin D may stimulate the action of phosphatase. They demonstrated this effect *in vitro* on the phosphorylases prepared from bone, kidney, or intestines by a water-soluble phosphorylated form of vitamin D. Zetterström²⁸¹ was of the opinion that, since phosphorylated vitamin D increased the activity of purified alkaline phosphatase, phosphatase is required for phosphate absorption. However, Moore²⁸² suggested that the *in vitro* experiments of Zetterström and Ljunggren²⁸⁰ may be criticized on the grounds that no natural phosphate of calciferol

²⁷⁴ B. B. Migicovsky and A. M. Nielson, *Arch. Biochem. Biophys.*, **34**, 105-111 (1951).

²⁷⁵ M. J. L. Dols, B. C. P. Jansen, G. L. Sizoo, and J. de Vries, *Nature*, **139**, 1068 (1937).

²⁷⁶ W. Heymann, *Z. Kinderheilk.*, **45**, 232-241 (1928).

²⁷⁷ W. Heymann, *Z. Kinderheilk.*, **49**, 748-760, 761-767 (1930).

²⁷⁸ W. Heymann, *Z. Kinderheilk.*, **51**, 673-707 (1931).

²⁷⁹ W. Heymann, *Z. Kinderheilk.*, **55**, 92-100 (1933).

²⁸⁰ R. Zetterström, and M. Ljunggren, *Acta Chem. Scand.*, **5**, 283-290 (1951).

²⁸¹ R. Zetterström, *Studies on the Mode of Action of Vitamin D*, Thesis, A. Bonniers Boktryckeri, Stockholm, 1951; cited by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, **11**, 29-60 (1953), p. 39.

²⁸² T. Moore, *Proc. Nutrition Soc.*, **12**, 121-128 (1953).

is known to exist. Nicolaysen and Eeg-Larsen¹ also refuted these claims by the facts, repeatedly proved, that the absorption of phosphate is rapid and complete in vitamin D deficiency, provided no calcium salts are present. Moreover, it is pointed out¹ that no attention was paid to oxygen supply in Heymann's experiments, while the mucous membrane of the gut has been shown to be extremely sensitive to oxygen deprivation.²⁸³

Rominger,²⁸⁴ in a review based upon earlier publications,^{285,286} expressed the view that vitamin D acts primarily upon the phosphate metabolism of children. McGowan²⁸⁷ and McGowan *et al.*²⁸⁸ postulated that vitamin D acts by setting free inorganic phosphorus from the body lipids, the relative deficiency of which is regarded as the essential cause of rickets. However, there was no proof to substantiate the conjecture. Nicolaysen²⁸⁹ also observed a decrease of the organic phosphorus + phosphocreatine fraction in the muscles, and of the inorganic phosphorus in the liver and kidneys of rachitic rats on a vitamin D-free diet containing calcium and phosphorus. A reduction was noted in the phospholipids and protein-phosphorus in the livers of these rachitic rats, but not in the muscles. This condition was corrected by vitamin D therapy, and the addition of phosphate without vitamin D was able to effect the same change. This investigator^{1,289} is of the opinion that phosphorus starvation is responsible for the aforementioned condition, and that vitamin D is not the factor controlling the balance between the different forms of phosphorus in the organism.

In the well-controlled experiments of Rapoport and Guest,^{290,291} it was observed that, in the case of rats rendered rachitic by a diet high in calcium and low in phosphorus, an initial decrease in the inorganic phosphorus and adenosinetriphosphate (ATP) of the blood cells was followed by a reduction of diphosphoglycerate in the blood. When healing of the rickets was induced by a minimum dose of vitamin D, by the addition of phosphate to the diet, or by fasting, these components progressively increased in the blood cells in the reverse order, *i.e.*, first diphosphoglycerate, then ATP, and inorganic phosphate. Diglycerophosphate was believed

²⁸³ R. B. Fisher and D. B. Parsons, *J. Physiol.*, **110**, 36-46 (1949).

²⁸⁴ E. Rominger, *Ergeb. Vitamin-u. Hormonforsch.*, **2**, 104-159 (1939).

²⁸⁵ E. Rominger, H. Meyer, and C. Bomskov, *Z. ges. exptl. Med.*, **73**, 343-381 (1930); *Klin. Wochschr.*, **9**, 1391-1394 (1930).

²⁸⁶ E. Rominger, H. Meyer, and C. Bomskov, *Z. ges. exptl. Med.*, **78**, 259-271, 272-286 (1931).

²⁸⁷ J. P. McGowan, *Biochem. J.*, **27**, 943-950 (1933).

²⁸⁸ J. P. McGowan, I. J. Cunningham, and D. W. Auchinachie, *Biochem. J.*, **25**, 1295-1303 (1931).

²⁸⁹ R. Nicolaysen, *Biochem. J.*, **30**, 1329-1337 (1936).

²⁹⁰ S. Rapoport and G. M. Guest, *J. Biol. Chem.*, **126**, 749-761 (1938).

²⁹¹ G. M. Guest and S. Rapoport, *Am. J. Diseases Children*, **58**, 1072-1089 (1939).

to be the key substance, and a decrease in its concentration was always a reflection of an increasing deficiency of phosphorus in the rats. Guest and Rapoport²⁹² suggested that the "rapid increase in the concentration of diphosphoglycerate in the blood cells following any of the procedures which induced healing appeared to be a sign of rapid mobilization of phosphorus in the body and of the availability of such phosphorus for transport to the calcifying bone." This theory is a most attractive one, and while it has not been confirmed by other workers, it has not been refuted.

According to Nicolaysen and Eeg-Larsen,¹ other reports of changes in the phosphate fraction are indecisive. This is true of the results of the Finnish workers, Rih *et al.*²⁹³ and Peitsara,²⁹⁴ who recorded the presence of a difficultly hydrolyzable acid-soluble phosphate fraction in the muscles of rachitic dogs which increased further during exercise. In the case of normal dogs, this fraction decreased. The results of Cohn and Greenberg²⁹⁵ are almost unconfirmed. They concluded that vitamin D increases the conversion of organic phosphorus to the inorganic form in the bone. The data of Underwood and co-workers,²⁹⁶ who believed that the inorganic phosphorus was increased in the blood of fasted rachitic rats which had been given vitamin D, are also open to question.

One possible explanation for the action of vitamin D, already suggested for phosphatase in general, is that its effect may be limited to kidney phosphatase. Thus, Zetterstrm²⁹⁷ observed that phosphorylated vitamin D₂ exerted an appreciable effect upon the activity of purified kidney phosphatase. Later, the same worker²⁹⁸ postulated that the phosphorylated vitamin D stimulated the oxygen uptake of the kidney mitochondria. However, the data of Pardee and Potter²⁹⁹ do not agree quantitatively with those of Zetterstrm,²⁹⁸ while Eeg-Larsen and Gran (in unpublished work cited by Nicolaysen and Eeg-Larsen)¹ noted an identical O₂ uptake in kidney homogenates from normal and from rachitic rats.

a. Vitamin D and Phosphate Resorption in the Kidneys. One of the fairly recent suggestions to account for the changes in phosphate metabolism in rickets is that the diminished plasma phosphate results not only from the deficient absorption in the intestine, but also from the unusually

²⁹² G. M. Guest and S. Rapoport, *Physiol. Revs.*, **21**, 410-437 (1941).

²⁹³ C. E. Rih, E. Helske, H. Peitsara, and E. Vehniinen, *Acta Paediat.*, **19**, 335-369 (1937).

²⁹⁴ H. Peitsara, *Acta Paediat.*, **31**, Suppl. 3, 1-244 (1944).

²⁹⁵ W. E. Cohn and D. M. Greenberg, *J. Biol. Chem.*, **130**, 625-634 (1939).

²⁹⁶ E. Underwood, S. Fisch, and H. C. Hodge, *Am. J. Physiol.*, **166**, 387-393 (1951).

²⁹⁷ R. Zetterstrm, *Nature*, **167**, 409-410 (1951).

²⁹⁸ R. Zetterstrm, *Acta Chem. Scand.*, **5**, 343-352 (1951).

²⁹⁹ A. B. Pardee and V. R. Potter, *J. Biol. Chem.*, **181**, 739-753 (1949).

high excretion of this phosphate in the urine.³⁰⁰ The data presented by Harrison and Harrison³⁰⁰ appear to demonstrate that reabsorption of phosphate by the renal tubules is increased in the kidneys of rachitic puppies when vitamin D is given. However, it is possible that the increased reabsorption of phosphate under these conditions is an indirect result following the primary effect of the vitamin D in increasing the absorption of calcium from the intestine.¹ This would increase plasma calcium, which itself might decrease phosphate excretion *via* the kidney by causing an interaction of calcium and phosphate in the tubules. In fact, Wolf and Ball³⁰¹ demonstrated that, when a calcium salt was given to dogs intravenously as a slow infusion, only small changes in the blood calcium were noted, while no alteration obtained in the blood inorganic phosphate. However, a striking depression in phosphate excretion in the urine was observed. The suggestion that the effect of vitamin D in increasing phosphate retention by increasing kidney reabsorption would seem to be a valid one, but, in all probability, its occurrence is secondary to the effect of the vitamin in augmenting the intestinal absorption of calcium.

b. Phytic Acid and the Action of Vitamin D. Phytic acid, which is hydrolyzed to inositol and six orthophosphate molecules by the enzyme, phytase (see *The Lipids*, Vol. II, pp. 67-69), may likewise be related to the action of vitamin D. Mellanby^{14,302} and Bruce and Callow³⁰³ were the first to identify phytic acid as the rickets-producing substance known to be present in cereals. Even in the absence of calcium, phytic acid is not completely available to the body as a source of phosphate. When calcium is present, phytic acid is even less effective as a source of phosphate, as its digestion is depressed under these conditions.³⁰⁴ On the other hand, Harrison and Mellanby³⁰⁵ noted that the calcification of the bones of puppies was reduced when phytic acid was included in the diet. The explanation offered for the latter phenomenon was that phytic acid prevents calcium absorption; this suggestion was confirmed later experimentally by Mellanby.²⁵⁸ According to Harris,²⁵⁷ phytic acid interferes with the absorption

³⁰⁰ H. E. Harrison and H. C. Harrison, *J. Clin. Invest.*, **20**, 47-55 (1941).

³⁰¹ A. V. Wolf and S. M. Ball, *Am. J. Physiol.*, **158**, 205-217 (1949).

³⁰² E. Mellanby, *Experimental Rickets. The Effect of Cereals and Their Interaction with Other Factors of Diet and Environment in Producing Rickets*. Med. Research Council (London), *Spec. Rept. Ser. No. 93*, (1925); cited by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, **11**, 29-60 (1953), p. 42.

³⁰³ H. M. Bruce and R. K. Callow, *Biochem. J.*, **28**, 517-528 (1934).

³⁰⁴ J. T. Lowe and H. Steenbock, *Biochem. J.*, **30**, 1991-1995 (1936).

³⁰⁵ D. C. Harrison and E. Mellanby, *Biochem. J.*, **33**, 1660-1680 (1939).

of calcium by forming insoluble salts with it. He considers it to be an "antivitamin D."

c. Phytase and the Action of Vitamin D. It is of considerable interest to determine whether or not the beneficial effect of vitamin D when administered to rats on a phytin-containing diet is attributable to its stimulating effect upon the phytin-hydrolyzing enzyme, phytase. Partwardhan³⁰⁶ noted that an extract of the mucous membrane of rat intestine possessed some phytase activity. Mellanby³⁰⁷ also observed phytase activity in an extract from rat intestine, but very little from the intestines of puppies, and only in the duodenal and rectal contents.

When vitamin D is administered to animals which have been fed phytic acid, less phytic acid is excreted than occurs in vitamin D-deficient animals.²⁵⁸ In later work³⁰⁸⁻³¹¹ it was found that vitamin D exerted a constant beneficial effect upon phytin-treated rats, as indicated by bone ash studies. It was believed that vitamin D resulted in an increased utilization of phytin. However, since there was no increased activity in the phytase of the intestine of vitamin D-treated rats, Spitzer and co-workers³¹¹ concluded that the action of the vitamin was of a secondary nature. One is led to conclude that the improved utilization of phytic acid following the administration of vitamin D is probably not to be ascribed to any action of phytase.

A second possibility to explain the increased utilization of phytin following vitamin D therapy, as suggested by Mellanby,³⁰⁷ is that the vitamin D counteracts the calcium-depriving action of phytate. A number of facts support this second hypothesis. The fact that extra calcium in the diet reduced the digestibility of phytate-phosphorus in man,³¹² in rats,³⁰⁴ and in puppies^{258,307} suggests that, in rickets, the greater amount of calcium in the gut would bring about the same depression in the digestion of phytin, both by combining with it and by inhibiting phytase. The action of vitamin D in vitamin D-deficient rats would increase phytin utilization by causing the absorption of calcium, and would thus lessen its phytin-

³⁰⁶ V. N. Patwardhan, *Biochem. J.*, **31**, 560-564 (1937).

³⁰⁷ E. Mellanby, *A Story of Nutritional Research*, Williams & Wilkins, Baltimore, 1950.

³⁰⁸ R. K. Boutwell, R. P. Geyer, A. W. Halverson, and E. B. Hart, *J. Nutrition*, **31**, 193-202 (1946).

³⁰⁹ C. H. Krieger, R. Bunkfeldt, and H. Steenbock, *J. Nutrition*, **20**, 7-14 (1940).

³¹⁰ C. H. Krieger and H. Steenbock, *J. Nutrition*, **20**, 125-132 (1940).

³¹¹ R. R. Spitzer, G. Maruyama, L. Michaud, and P. H. Phillips, *J. Nutrition*, **35**, 185-193 (1948).

³¹² E. W. H. Cruickshank, J. Duckworth, H. W. Kosterlitz, and G. M. Warnock, *J. Physiol.*, **104**, 41-46 (1945).

inhibiting action in the intestine. For a further discussion of phytase, the reader is referred to *The Lipids*, Vol. II, pp. 67 to 69.

(4) *The Action on Bone Formation*

There can be no doubt that vitamin D exerts a profound effect upon calcification of bone, and that it is able to correct the abnormal condition of these structures in rickets. The question which many physiologists have raised, and which is still unsolved, is whether or not the effect of vitamin D on calcification is direct or whether it results indirectly from the effect of the vitamin upon calcium metabolism.²⁵⁷

Vitamin D causes an increased deposition of bone salts in rachitic animals which can be demonstrated histologically or by chemical analysis of the ash content of the bones. According to Wolbach and Bessey,³¹³ it is generally accepted that, in rickets (1) a failure of calcification of cartilage columns in the so-called zone of provisional calcification, and failure of calcification of the osteoid occur; (2) that a continued growth and consequent increase in thickness of the diaphyseal cartilage and osteoid result; (3) that there is a lack of vascular growth into the cartilage; (4) and that a resorption of bone formed before the deficiency in this area takes place. Wolbach and Bessey³¹³ also state the undisputed fact that experimental rickets in animals duplicates completely the spontaneous disease in man and animals.

However, one concept proposed by Wolbach and Bessey³¹³ is challenged by Nicolaysen and Eeg-Larsen.¹ This is the statement of the former authors³¹³ that rickets is due to the impaired absorption of calcium and phosphate, and "that there is no reason to believe that the cells and matrices concerned in bone growth and maintenance are defective in rickets or are directly acted upon by the vitamin D." This generalized conclusion is largely based upon the experiments of Shohl and Wolbach,³¹⁴ and is correct on the basis of the experimental results obtained. However, in an extended study by Nicolaysen and Jansen,³¹⁵ data are presented which lead to the conclusion that vitamin D does influence the structure, *viz.*, the matrices of the bones. The anatomical picture was strikingly different in the presence and absence of vitamin D. Thus, a dense network of trabeculae was always evident in the bones of the vitamin D-deficient rats, in contrast to the few trabeculae which were present in the case of vitamin

³¹³ S. B. Wolbach and O. A. Bessey, *Physiol. Revs.*, **22**, 233-289 (1942).

³¹⁴ A. T. Shohl and S. B. Wolbach, *J. Nutrition*, **11**, 275-291 (1936).

³¹⁵ R. Nicolaysen and J. Jansen, *Acta Paediat.*, **23**, 405-433 (1939).

D-treated rats. The results of Reed and Reed,^{316,317} based upon x-ray diffractograms from normal and rachitic rats, indicated different structures which were interpreted as due to a disorientation of the crystal pattern; this would suggest a peripheral catalytic action by vitamin D. Ramalingaswami *et al.*³¹⁸ made a histochemical study of the rachitic cartilage of rat tibiae during calcification under the influence of vitamin D. They suggested that failure of rachitic cartilage to calcify in vitamin D deficiency is due to a disturbance of glycogen metabolism, the progress of which may be essential to the formation of the matrix, and to the deposition of bone salts. The administration of vitamin D removes the block, permits normal glycogen metabolism, and therefore the formation of the matrix, and calcification.

a. As Related to Citrate Content. It was established by Dickens³¹⁹ in 1941 that very high concentrations of citrate occur normally in bones (which may represent 70% of that contained in the entire body), while a 50% reduction in this component was observed in the bones of a rachitic kitten.³²⁰ It was later demonstrated by Wassjö and Eeg-Larsen³²¹ that, whereas in calcium starvation a stoichiometric reduction in the ash and citric acid occurred, a doubling of the ash citric acid ratio occurred in rachitic rats. When rickets was cured in the rat by increased phosphate, the amount of citric acid did not parallel the increase in ash content. On the other hand, the latter workers³²¹ showed that citric acid returned to normal in vitamin D-treated rachitic rats sooner than did the ash content. The fact that the citrate is a component directly related to vitamin D has also been proved recently by Steenbock and Bellin.³²² When vitamin D was administered to rats on normal or low-phosphorus rachitogenic diets, the citrate content of blood, bone, kidney, heart, and small intestine was increased; however, physiologic doses of vitamin D did not affect liver citrate. Vitamin D was effective in increasing blood and bone citrate both in the presence and in the absence of dietary bicarbonate. It was shown that an increase in urinary citrate reflects an augmentation of its accumulation in certain tissues.

Although the addition of citrate or of citric acid to rachitic diets in

³¹⁶ C. I. Reed and B. P. Reed, *Am. J. Physiol.*, **138**, 34-41 (1943).

³¹⁷ C. I. Reed and B. P. Reed, *Am. J. Physiol.*, **143**, 413-419 (1945).

³¹⁸ V. Ramalingaswami, S. Sriramachari, P. K. Dikshit, P. G. Tulpule, and V. N. Patwardhan, *Indian J. Med. Sci.*, **8**, 509-516 (1954).

³¹⁹ F. Dickens, *Biochem. J.*, **35**, 1011-1023 (1941).

³²⁰ R. Nicolaysen and R. Nordbø, *Acta Physiol. Scand.*, **5**, 212-214 (1943).

³²¹ E. Wassjö and N. Eeg-Larsen, *Acta Physiol. Scand.*, **25**, Suppl. No. 89, 84 (1951).

³²² H. Steenbock and S. A. Bellin, *J. Biol. Chem.*, **205**, 985-991 (1953).

proportions of 10% and 15% of the daily ration has been reported to have an antirachitic effect,¹ injections of citrate were found by Nicolaysen and Nordbø³²⁰ to be entirely ineffective in such cases. The positive effect of oral doses is probably to be ascribed to the formation of a soluble complex with calcium in the intestine. The most recent review of vitamin D and its relation to bone is that of Harris.²⁵⁷

(5) *Vitamin D and the Formation of Teeth*

According to the classical work of Mellanby and King,³²³ vitamin D functions in the metabolism of enamel. The development of enamel hypoplasia as a result of vitamin D deficiency has been demonstrated.¹ Moreover, the fat-soluble vitamins were shown to be concerned with the formation of secondary dentine. These results were established from a study of the structural relations of the teeth of children and from experimental work with dogs.¹

The relation between the growth of the tooth and the presence of vitamin D has been followed, and the tooth has been shown to require vitamin D just as is the case with bone. According to Irving,^{324,325} who studied the growth of teeth in rats from the histologic standpoint, the nature of the change differs with the Ca:P ratio in the diet and with dietary vitamin D. Following the administration of a good Ca:P diet without vitamin D, spherulities were precipitated in the predentin area which varied greatly in size and character from the finely granulated precipitate produced on the same diet with vitamin D. The incisor teeth of rats were shown by sodium fluoride injections to be a center of high activity. This tooth provided a very sensitive measure of vitamin D deficiency.

There also appears to be some relationship between dental decay (caries) and vitamin D level. Thus, Mellanby and Mellanby³²⁶ demonstrated that a relationship exists between hypoplasia in the tooth and the development of caries. However, the dietary regimen employed appears to be of considerable importance. Marshall-Day³²⁷ noted the presence of marked hypoplasia in the teeth of Indian children, while the incidence of caries was extremely low. Rickets is frequently associated with caries. The dietary factor is important.²⁵⁷ Vitamin D, by promoting adequate calcification of the teeth, insures a normal "dental architecture" and reduces the tendency to caries.²⁵⁷

³²³ M. Mellanby and J. D. King, *Ergeb. Vitamin- u. Hormonforsch.*, 2, 1-54 (1939).

³²⁴ J. T. Irving, *J. Physiol.*, 103, 9-26 (1944).

³²⁵ J. T. Irving, *J. Physiol.*, 104, 253-265 (1946).

³²⁶ M. Mellanby and H. Mellanby, *Brit. Med. J.*, 1948, II, 409-413.

³²⁷ C. D. Marshall-Day, *Brit. Dent. J.*, ser. 2, 76, 115-122, 143-147 (1944).

(6) *Vitamin D in Relation to the Intestinal and Fecal pH*

A considerable amount of experimental evidence has been amassed which indicates that a deficiency in vitamin D causes an increase of the fecal and intestinal pH in a number of species. Zucker and Matzner³²⁸ reported that the pH of the feces of rats becomes higher when they become rachitic, and that it reverts to the original level when cod liver oil is given. Similar results were obtained by Jephcott and Bacharach,³²⁹ who found that cod liver oil, irradiated cholesterol, and ultraviolet irradiation reduced the fecal pH to the acid side. Heller and Caskey³³⁰ observed the same response to vitamin D supplements.

A number of investigators³³¹⁻³³³ likewise noted a higher pH in the intestinal contents of rachitic rats than in normal controls. This phenomenon was apparently a general one, as it occurred throughout the small and large intestine. After the administration of cod liver oil, or of vitamin D itself, the pH of the intestinal contents decreased, *i.e.*, the acidity increased. Similar observations were made by Grayzel and Miller³³⁴ in the case of dogs, and by Kline and co-workers³³⁵ in chicks.

However, the relationship between the changes in intestinal pH and the cure of rickets is not clear. In the case of rachitic chicks, irradiation decreased the pH of the proximal portion of the intestines, but not that of the distal portion. Shohl and Bing³³⁶ observed a change in the feces from alkaline to acid when rats rendered rachitic by diet were cured by cod liver oil. However, this change did not occur in rats cured by irradiation of the food or by the administration of alkaline phosphates. Oser³³⁷ is of the opinion that the effect of vitamin D in increasing the pH of the feces is unreliable and non-specific. Moreover, Jones³³⁸ reported that increasing the acidity of the intestinal contents of rats by means other than the administration of vitamin D did not result in a consistent or

³²⁸ T. F. Zucker and M. J. Matzner, *Proc. Soc. Exptl. Biol. Med.*, **21**, 186-187 (1924).

³²⁹ H. Jephcott and A. L. Bacharach, *Biochem. J.*, **20**, 1351-1355 (1926).

³³⁰ V. G. Heller and C. Caskey, *J. Nutrition*, **2**, 59-65 (1929-1930).

³³¹ E. M. Abrahamson and E. G. Miller, Jr., *Proc. Soc. Exptl. Biol. Med.*, **22**, 438-439 (1924-1925).

³³² L. Yoder, *J. Biol. Chem.*, **74**, 321-329 (1927).

³³³ T. Redman, S. G. Willimott, and F. Wokes, *Biochem. J.*, **21**, 589-605 (1927).

³³⁴ D. M. Grayzel and E. G. Miller, Jr., *Proc. Soc. Exptl. Biol. Med.*, **24**, 668-670 (1926-1927).

³³⁵ O. L. Kline, J. A. Keenan, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.*, **98**, 121-131 (1932).

³³⁶ A. T. Shohl and F. C. Bing, *J. Biol. Chem.*, **79**, 269-274 (1928).

³³⁷ B. L. Oser, *J. Biol. Chem.*, **80**, 487-497 (1928).

³³⁸ J. H. Jones, *J. Biol. Chem.*, **142**, 557-567 (1942).

uniform increase in calcification. Friedman³³⁹ observed that the increased acidity in the intestine during the healing of rickets was due to a change in the intestinal flora from non-acid-forming bacteria to those which produce acid.

Although the above-cited results might indicate that there is no constant relationship between vitamin D and intestinal and fecal hydrogen ion concentration, later work of the Steenbock group has revived interest in this field. Bellin, Steenbock and Wiest³⁴⁰ reported not only a reduction in the intestinal pH in rats following the administration of vitamin D as a supplement to various diets, but also a concomitant rise in the urinary pH. An increase in citrate in the urine was likewise observed.³⁴¹ Steenbock and Bellin³⁴¹ suggest that these effects are secondary to that on calcium absorption, and are thus a result of the primary action of vitamin D.

(γ) *Vitamin D and Respiratory Metabolism*

Landelius and Ljungkvist³⁴² reported, in 1934, that vitamin D exerts a stimulatory effect upon oxygen consumption. Except for this single positive finding, data have indicated that no relationship exists between these phenomena. According to Nicolaysen,³⁴³ D-avitaminotic rats do have a lower respiratory metabolism than do normal animals, but this is because they are less active than the controls. When the animals were narcotized, or when the experiments were prolonged until the normal rats became quiescent, no differences in metabolism were noted between the rachitic and the normal animals. Presnell³⁴⁴ also reported a 30% reduction in Q_{O_2} in the skin of rachitic rats, as compared with that of normal controls, but this finding was not substantiated by Klungsöyr and Pihl.³⁴⁵ The latter workers observed that the oxygen consumption was in the same range in the skin of animals of the two groups, and that it was not affected by vitamin D. It would therefore appear to be very doubtful whether or not vitamin D functions directly in any way to regulate the respiratory metabolism.

³³⁹ H. Friedman, *J. Nutrition*, **12**, 165-172 (1936).

³⁴⁰ H. Steenbock, S. A. Bellin, and W. G. Wiest, *J. Biol. Chem.*, **193**, 843-849 (1951).

³⁴¹ S. A. Bellin and H. Steenbock, *J. Biol. Chem.*, **194**, 311-316 (1952).

³⁴² E. Landelius and G. Ljungkvist, *Skand. Arch. Physiol.*, **68**, 252-270 (1934).

³⁴³ R. Nicolaysen, *Skand. Arch. Physiol.*, **82**, 79-85 (1939).

³⁴⁴ A. K. Presnell, *J. Biol. Chem.*, **121**, 5-8 (1937).

³⁴⁵ L. Klungsöyr and A. Pihl, *Acta Physiol. Scand.*, **29**, No. 5, 377-380 (1953).

. The Requirement for Vitamin D

(1) *In the Lower Animals*

Many factors affect the amount of vitamin D required in the diet. The foremost of these factors is the extent to which the animal is exposed to sunlight, and the intensity of the ultraviolet light so received. In the case of babies, the amount of clothing is also of importance, inasmuch as the greater the area of exposure to sunlight, or to other sources of ultraviolet light, the greater will be the amount of synthesis of vitamin D. In some animals, particularly the rodents, the ratio of calcium to phosphorus in the diet influences the amount of vitamin D required for optimum nutrition. Another factor which causes a variation in the quantity of vitamin D required is the extent to which phosphate is available. Zucker and co-workers³⁴⁶ observed that the greater the acidity of the diet the less rachitogenic it becomes. This effect is presumably ascribable to the resultant increase in solubility of calcium and of phosphate with increasing acidity. However, Shohl *et al.*³⁴⁷ were unable to demonstrate any difference in the action of phosphate on rickets in rats, regardless of whether it was included in the diet as the acid, neutral, or alkaline salt. Analysis of bones showed the greatest ash deposition following neutral diets, less deposition with alkaline diets and least with acid diets.

Although the average requirement for vitamin D in the case of various domestic animals can only be a matter of conjecture, the Committee on Animal Nutrition of the National Research Council (U.S.)³⁴⁸ has set up tentative standards.

a. Chickens and Poultry. The amount of vitamin D recommended varies with age. The following amounts of vitamin D (AOAC units/lb. of feed) are set for chickens of these different groups: starting chicks (0 to 8 weeks), 180; growing chicks (8 to 18 weeks), 180; laying hens, 450; and breeding hens, 450. Table 7 gives a more exact evaluation of the requirement of chickens of different sizes and breeds.^{348,349}

³⁴⁶ T. F. Zucker, W. C. Johnson, and M. Barnett, *Proc. Soc. Exptl. Biol. Med.*, 20, 20-22 (1922-1923).

³⁴⁷ A. T. Shohl, H. B. Bennett, and K. L. Weed, *J. Biol. Chem.*, 78, 181-190 (1928).

³⁴⁸ Anonymous, Nat. Research Council, Comm. Animal Nutrition, *Nutrient Requirements for Domestic Animals*, No. 1. *Nutrient Requirements for Poultry*, revised, 1954; No. 2. *Nutrient Requirements for Swine*, revised, 1953; No. 3. *Recommended Nutrient Allowances for Dairy Cattle*, revised, 1950; No. 4. *Recommended Nutrient Allowances for Beef Cattle*, revised, 1950; No. 5. *Recommended Nutrient Allowances for Sheep*, revised, 1949; No. 6. *Recommended Nutrient Allowances for Horses*, 1949; No. 7. *Nutrient Requirements for Foxes and Minks*, 1953; No. 8. *Nutrient Requirements for Dogs*, 1953; No. 9. *Nutrient Requirements for Rabbits*, 1954.

³⁴⁹ J. H. Jones, *Vitamin D. Group. VIII. Requirements. A. Of Animals*, in W. H. Sebrell and R. S. Harris, *The Vitamins*, Vol. II, Acad. Press, New York, 1954, pp. 253-256.

TABLE 7
RECOMMENDED DAILY ALLOWANCES (I.U./ANIMAL) OF VITAMIN D FOR THE MAINTENANCE OF CHICKENS
OF DIFFERENT WEIGHTS, AND FOR LAYING AND BREEDING^{a,b}

	Maintenance, lb.											
	0.5	1.0	1.5	2.0	2.5	3.0	4.0	4.5	5.5 (?)		Laying	Breeding
<i>White Leghorn and similar breeds</i>												
11.88		18.5	23.2	26.1	28.3	31.1	28.4	—	—		92.7	92.7
<i>Heavy breeds</i>												
12.6		18.7	25.7	30.1	—	36.0	—	34.2	36.0 (?)		111.6	111.6

White Leghorn and similar breeds

Heavy breeds

^a Adapted from Natl. Research Council, Comm. Animal Nutrition, *Nutrient Requirements for Domestic Animals*, No. 1. *Nutrient Requirements for Poultry*, Washington, 1954.
^b Adapted by J. H. Jones, *Vitamin D*, Group. VIII. *Requirements of Animals*, in W. H. Sebrell and R. S. Harris, *The Vitamins*, Vol. II, 253-256 (1953).

In the case of turkeys, a diet containing 400 A.O.A.C. International chick units of vitamin D per pound of feed is recommended for turkey poults, for growing turkeys, and also for breeding turkeys.³⁴⁸

b. Swine and Dairy Cattle. The recommended amounts of vitamin D for swine and for dairy cattle are based upon the weight of the animal. These figures are summarized in Table 8.^{348,349}

TABLE 8
RECOMMENDED DAILY ALLOWANCES OF VITAMIN D FOR SWINE
(MARKET STOCK AND BREEDING) AND FOR DAIRY CATTLE (HEIFERS)
ACCORDING TO WEIGHT^{a,b}

Category	Swine		Dairy cattle (heifers)		
	Live weight, lb.	Vitamin D, I.U./animal	Live weight, lb.	Vitamin D I.U./animal I.U./lb. food	
Market stock	50	150	50	200	220
	100	265	100	400	200
	150	340	150	600	150
	200	375	200	800	130
	250	415			
Breeding stock					
Females and males					
Young	300	300			
Adults	500	375			
Lactating females					
Gilts	350	550			
Adults	450	625			

^a Adapted from Nat. Research Council, Comm. Animal Nutrition, *Nutrient Requirements for Domestic Animals*, No. 2. *Nutrient Requirements for Swine* (revised 1953); and No. 3. *Recommended Nutrient Allowances for Dairy Cattle* (revised 1950).

^b Adapted by J. H. Jones, *Vitamin D. Group. VIII. Requirements of Animals*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. II, 253-256 (1953).

c. Other Species. Beef cattle usually receive enough vitamin D through irradiation of the skin by sunlight while they are on the range. Jones,³⁴⁹ and Bechtel *et al.*,³⁵⁰ found that the requirement for young beeves approximated 300 I.U. of vitamin D/100 lb. live weight. Sheep and horses also spend a considerable proportion of their time on the range, and do not require supplementary vitamin D. When lambs are away from sunlight, 250 to 300 I.U. of vitamin D per 100 lb. body weight are recommended. It is also suggested that horses kept indoors be given supplements of 300

³⁵⁰ H. E. Bechtel, E. T. Hallman, C. F. Huffman, and C. W. Duncan, *Mich. Agr. Exptl. Sta. Tech. Bull. No. 150* (1936).

I.U. 100 lb. body weight.³⁴⁹ Harris and co-workers³⁵¹ found that 0.82 I.U. g. dry food was as satisfactory for foxes as it was for minks.³⁵²

(2) *In Man*

The same factors which affect the requirement of vitamin D in animals also apply to human subjects. It is known that the vitamin D is especially necessary in infancy during the period of growth. If insufficient vitamin D is present in the infant's diet, rickets is likely to develop. On the other hand, in older children and adults, when growth is slower, vitamin D deficiency has not been demonstrated.

According to the recommended daily allowances of the Food Committee of the National Research Council,³⁵³ the requirement for infants and for young children is established as 300 to 400 I.U./day when the calcium intake is adequate. Similar figures are proposed for adults, except that to maintain pregnancy and lactation, the suggested amount required is doubled (800 I.U./day). It is recognized that, in the case of an adult man spending a reasonable amount of his time out of doors, no supplementary vitamin D may be required. For a further discussion of vitamin D requirements in man, the reader is referred to the section by Kramer and Kanof³⁵⁴ in Sebrell and Harris, *The Vitamins*.

7. Hypervitaminosis D

The first report that vitamin D may be toxic in large doses resulted from the use of a product known as Vigantol, which was first prepared by the I. G. Farbenindustrie.^{355, 356} This compound contained a toxic product, toxisterol, along with vitamin D. It was given its name by Laquer and Linsert.³⁵⁷ Windaus and co-workers,³⁵⁸ and Hoyle³⁵⁹ found that, when vitamin D was moderately over-irradiated, a toxic property

³⁵¹ L. E. Harris, C. F. Bassett, and C. F. Wilke, *J. Nutrition*, **43**, 153-165 (1951).

³⁵² C. F. Bassett, L. E. Harris, and C. F. Wilke, *J. Nutrition*, **44**, 433-442 (1951).

³⁵³ Anonymous, *Recommended Dietary Allowances*, Nat. Research Council, Publ. No. 302, Washington, 1953.

³⁵⁴ B. Kramer and A. Kanof, *Vitamin D. Group. VIII. Requirements. B. of Human Beings*, in W. H. Sebrell and R. S. Harris, *The Vitamins*, Vol. II, Acad. Press, New York, 1954, pp. 257-266.

³⁵⁵ I. G. Farbenindustrie, *Brit. Patent No. 296,093* (Nov. 15, 1928).

³⁵⁶ J. Y. Johnson (to I. G. Farbenindustrie), *Brit. Patent No. 316,803* (Sept. 25, 1929); I. G. Farbenindustrie, *German Patent, No. 499,524* (Aug. 28, 1930).

³⁵⁷ E. Laquer and O. Linsert, *Klin. Wochschr.*, **12**, 753-754 (1933).

³⁵⁸ A. Windaus, A. Lüttringhaus, and P. Busse, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse, III*, 150-159 (1932).

³⁵⁹ J. C. Hoyle, *J. Pharm.*, **40**, 351-372 (1930).

causing calcification developed out of all proportion to its antirachitic property. This so-called toxisterol possessed no vitamin D action.³⁶⁰ In addition to toxisterol, two other compounds without an antirachitic action and possessing some toxicity are formed by over-irradiation of vitamin D.³⁶¹ These are known as suprasterol I and suprasterol II.³⁴⁹ These earlier results, therefore, could not be regarded as proof of the toxicity of large doses of vitamin D *per se*. The toxicity was rather a result of impurities in the early preparations. At one time it was considered that the toxic effects of excessive amounts of vitamin D were due entirely to the presence of toxic by-products.³⁵⁷

However, Pfannenstiel³⁶² and Kreitmair and Moll³⁶³ described, almost simultaneously, toxic effects ascribable to the vitamin D itself when given in large doses to animals. Harris and co-workers³⁶⁴⁻³⁶⁷ reported that symptoms of hypervitaminosis D could be provoked even on a diet poor in calcium, but that the symptoms were aggravated when the diet was rich in this substance.³⁶⁶ Furthermore, in the latter case, less vitamin was required to elicit the manifestations of hypervitaminosis.

(1) *Symptoms of Hypervitaminosis D*

The chief feature of the hypervitaminotic state is the dissolution of bone already formed. In the early stages the bones may exhibit accelerated calcification of the so-called "provisional zone of calcification," together with a thickening of the periosteum. However, in more advanced stages of the hypervitaminosis D, a diffuse demineralization of the bones occurs.³⁶⁵ The type of osteoporosis resulting from hypervitaminosis D differs from that caused by an excess of parathormone (parathyroid extract), in that the resorbed areas are not replaced by fibrous tissue.³⁶⁵ The calcium content of the bone is reduced concomitantly with an increased concentration of calcium and phosphorus in the plasma.³⁶⁹ According to Levy, Sapir, and Mignon,³⁷⁰ the serum phosphate increases much more rapidly

³⁶⁰ C. E. Bills, E. M. Honeywell, and W. M. Cox, *J. Biol. Chem.*, **80**, 557-563 (1928).

³⁶¹ A. Windaus, J. Gaede, J. Köser, and G. Stein, *Ann.*, **483**, 17-30 (1930).

³⁶² W. Pfannenstiel, *Münch. med. Wochschr.*, **75**, 1113-1114 (1928).

³⁶³ H. Kreitmair and T. Moll, *Münch. med. Wochschr.*, **75**, 637-639 (1928).

³⁶⁴ L. J. Harris and T. Moore, *Biochem. J.*, **22**, 1461-1477 (1928).

³⁶⁵ L. J. Harris and T. Moore, *Biochem. J.*, **23**, 261-273, 1114-1121 (1929).

³⁶⁶ L. J. Harris and J. R. M. Innes, *Biochem. J.*, **25**, 367-390 (1931).

³⁶⁷ L. J. Harris and C. P. Stewart, *Biochem. J.*, **23**, 206-209 (1929).

³⁶⁸ D. H. Shelling, *The Parathyroids in Health and Disease*, Mosby, St. Louis, 1935, p. 271.

³⁶⁹ S. Freeman, P. S. Rhoads, and L. B. Yeager, *J. Am. Med. Assoc.*, **130**, 197-202 (1946).

³⁷⁰ M. Levy, M. Sapir, and S. Mignon, *Bull. soc. chim. Biol.*, **31**, 300-321 (1949).

after toxic doses of vitamin D are given orally to human adults than does the serum calcium. The results were approximately the same, irrespective of whether 15, 30, or 90 mg. of the vitamin were given. These investigators³⁷⁰ conclude that plasma phosphorus is a more sensitive index of hypervitaminosis D than is serum calcium. When an excess of vitamin D is administered to rachitic rats, the hypertrophic cartilage becomes densely calcified; this occurs simultaneously with a dissolution of the bone already formed.

(2) *The Effect on Specific Tissues*

Obviously a generalized effect, involving all tissues, occurs after the administration of toxic doses of vitamin D. There is a generalized calcinosis, which affects the joints, synovial membranes, kidneys, myocardium, pulmonary alveoli, parathyroid glands, pancreas, skin, lymph glands, large and medium-sized arteries, the conjunctiva and cornea, and also the acid-secreting portion of the stomach.³⁵⁴ The abnormal calcification in these tissues appears as a white chalky deposit.

According to Nicolaysen and Eeg-Larsen,¹ the most dominant toxic effect is noted in the kidney, due to the precipitation of calcium phosphate in the tubules. Guldager³⁷¹ states that no damage occurs in the kidneys until the precipitation of calcium salts occurs. Toxicity results because the blood is overloaded with minerals which the kidneys are unable to excrete with sufficient rapidity.

(3) *Levels of Vitamin D Found Toxic*

Although toxicity symptoms in man have usually resulted when dosages exceeded 100,000 I.U. daily, much lower amounts have been shown to be harmful under certain conditions. According to Kramer and Kanof³⁵⁴ as little as 400 I.U. daily have been known to produce fatal results. The shortest period of administration of vitamin D before demonstrable calcification was produced in fourteen days. On the basis of linear growth in childhood as an index of the action of vitamin D, Jeans and Stearns³⁷² reported that harmful effects could be produced by as small a dosage as 1800 I.U. daily. The tolerance of the human adult for vitamin D before toxicity developed was found to vary considerably in different individuals.

³⁷¹ A. Guldager, *Hypervitaminosis D.*, A. Busck, Nyt Nordisk Forlag. København, 1936; cited by R. Nicolaysen and N. Eeg-Larsen, *Vitamins and Hormones*, 11, 29-60 (1953), p. 53.

³⁷² P. C. Jeans and G. Stearns, *J. Pediat.*, 13, 730-740 (1938).

Harris³⁷³ considers that 5,000 I.U./kg. body weight is a safe daily dose. He found that vitamin D injected intramuscularly caused no injury, whereas the same dose given orally induced symptoms of toxicity. Albright and Reifenstein³⁷⁴ are of the opinion that all manifestations of hypervitaminosis are merely an exaggeration of the normal action of the vitamin.

In the case of rats on a good calcium and phosphorus diet, Guldager³⁷¹ observed that vitamin D toxicity occurred when the dosage was in the range of 300 to 700 I.U. daily. When the maximum dosage of 700 I.U. was given, calcium deposits were found in the kidneys, without exception. Some differences in the toxicity of vitamin D₂ (activated ergosterol) and natural vitamin D (vitamin D₃-activated 7-dehydrocholesterol) were reported by Morgan *et al.*^{206, 375} in rats, and by McChesney and Kocher,³⁷⁶ also in rats. Morgan *et al.*³⁷⁵ found that both the absorption and the excretion of vitamin D₃ by rats were more efficient than was the case with vitamin D₂. Vitamin D₃ appeared to produce more prolonged hypercalcemia than did vitamin D₂.³⁷⁶ However, Nicolaysen and Eeg-Jansen¹ conclude that little difference in toxicity can be demonstrated between the several types of vitamins D. Animals and man frequently recover from toxic doses of the vitamins D, but if the dosage is sufficiently high, death ensues.

³⁷³ P. L. Harris, *Ann. Rev. Biochem.*, **18**, 391-434 (1949).

³⁷⁴ E. Albright and E. C. Reifenstein, *Parathyroid Gland and Metabolic Bone Disease*, Williams & Wilkins, Baltimore, 1948, p. 95.

³⁷⁵ A. F. Morgan, N. Shimotori, and J. B. Hendricks, *J. Biol. Chem.*, **134**, 761-779 (1940).

³⁷⁶ E. W. McChesney and H. Kocher, *Proc. Soc. Exptl. Biol. Med.*, **47**, 156-159 (1941).

CHAPTER XI

THE METABOLISM AND NUTRITIONAL VALUE OF THE VITAMINS E (TOCOPHEROLS)

1. Introduction

As in the case of many biologically important compounds, vitamin E serves several different functions in the animal. It is active as an anti-sterility vitamin, and it may also act as an antioxidant to prevent the oxidation of fats. We now know that, although the same vitamin E can mediate several physiologic activities, different members of the group act most efficiently in aiding each of the several functions attributed to this vitamin.

It is now generally believed that there are seven members of the vitamin E group, all of which have the chroman ring, but which differ in the number and position of the side chains attached to it. In the original separation (in 1936) by Evans, Emerson, and Emerson,¹ of the members of the group which were biologically active, the α -tocopherol was separated as a crystalline allophanate. Two years later, this group prepared a second and third active principle from wheat germ and cottonseed oil, which were designated β - and α -tocopherol.²

Subsequently, the fourth member of the group was discovered, in 1947, by Stern and co-workers,³ and was called δ -tocopherol. It was found to possess a greater antioxidant activity than that possessed by the members of the group which were discovered earlier. On the other hand, the anti-sterility action of this newest member was found to be the weakest of that present in any of the four vitamin E compounds then known. Eggitt and

¹ H. M. Evans, O. H. Emerson, and G. A. Emerson, *J. Biol. Chem.*, 113, 319-332 (1936).

² O. H. Emerson, G. A. Emerson, A. Mohammad, and H. M. Evans, *J. Biol. Chem.*, 122, 99-107 (1938).

³ M. Stern, C. D. Robeson, L. Weisler, and J. G. Baxter, *J. Am. Chem. Soc.*, 69, 869-874 (1947).

¹¹ H. M. Evans and K. S. Bishop, *J. Metabolic Research*, 3, 201-231, 233-316 (1923).

published in 1922 and 1923. When rats were fed on diets devoid of this new fat-soluble vitamin, they failed to reproduce. This deficiency was overcome when an extract containing the missing principle was given. It was named "vitamin E" and was frequently referred to as the "anti-sterility vitamin." It is not entirely certain whether or not Mattill and Conklin¹² should have been given credit for the discovery of vitamin E, since they observed, as early as 1920, that disturbances in reproduction occurred in rats fed milk diets. In any event, the reports of Evans and Bishop⁷⁻¹¹ were soon confirmed by Mattill and co-workers.¹³ Mattill¹⁴ recorded the new type of sterility in male rats deprived of a dietary "X substance"; this deficiency in capacity to reproduce appeared more slowly in male rats than in females, but the damage to the testes was shown by Mason^{15,16} to be permanent and irreparable. Evans and Burr¹⁷ reported that weanling rats from mothers on a restricted diet, presumably also deficient in vitamin E, developed paralysis. The damage could be cured when the mothers were given a natural food mixture, or it could be prevented if the mothers received wheat germ oil or other substances known to be rich in vitamin E. The wide application of vitamin E was evident from the fact that it was concerned not only with the growth of young chicks but also with the development of the embryo,^{18,19} since eggs from hens on a low-vitamin E diet exhibited a reduced hatchability. Adamstone and Card²⁰ noted that a testicular degeneration occurred in adult male birds subjected to a vitamin D-free diet.

In addition to the effect on reproduction, Goettsch²¹ and Pappenheimer,²² working independently and together,²³ observed that vitamin E exerted an important effect upon the metabolism of muscle. When rats, rabbits, or guinea pigs were fed diets devoid of vitamin E, a specific type of muscular dystrophy developed. Another physiologic response attributable to vitamin E is its effect upon growth. Thus, when rats are raised on a vitamin E-low diet, a growth-promoting action can be observed when in-

¹² H. A. Mattill and R. E. Conklin, *J. Biol. Chem.*, **44**, 137-158 (1920).

¹³ H. A. Mattill, J. S. Carman, and M. M. Clayton, *J. Biol. Chem.*, **61**, 729-740 (1924).

¹⁴ H. A. Mattill, *Am. J. Physiol.*, **79**, 305-315 (1927).

¹⁵ K. E. Mason, *Proc. Nat. Acad. Sci., U. S.*, **11**, 377-382 (1925).

¹⁶ K. E. Mason, *J. Exptl. Zool.*, **45**, 159-229 (1926).

¹⁷ H. M. Evans and G. O. Burr, *J. Biol. Chem.*, **76**, 273-297 (1928).

¹⁸ F. B. Adamstone, *J. Morphol.*, **52**, 47-90 (1931).

¹⁹ F. B. Adamstone, *Anat. Record*, **60**, No. 4, Suppl., 36-37 (1934).

²⁰ F. B. Adamstone and L. E. Card, *J. Morphol.*, **56**, 339-359 (1934).

²¹ M. Goettsch, *Proc. Soc. Exptl. Biol. Med.*, **27**, 564-567 (1930).

²² A. M. Pappenheimer, *Proc. Soc. Exptl. Biol. Med.*, **27**, 567-568 (1930).

²³ M. Goettsch and A. M. Pappenheimer, *J. Exptl. Med.*, **54**, 145-165 (1931).

creased amounts of the vitamin are given to rats five to seven months of age.^{24,25} This effect occurs late in adolescence, and appears later in the female than in the male. Martin²⁶ was of the opinion that the antisterility and growth-promoting effects of vitamin E are due to different factors; he was able to effect a partial separation of the factors by fractional crystallization of vitamin E concentrates. This suggestion would seem plausible, in view of the demonstration by Adamstone²⁷ of the possible relation of this vitamin to cell division, and especially since the multiple nature of the group has become increasingly evident in recent years.

Still another property which has played an important role in the *in vitro* and *in vivo* action of vitamin E is its antioxidant activity. Simmonds *et al.*²⁸ noted that wheat germ oil prevented the destructive effect of ferrous sulfate on vitamin A.²⁹ Mattill³⁰ observed that diets which produced sterility were usually those subject to a rapid development of rancidity. The question which was then raised was whether or not the antioxidant and antisterility effect of wheat germ oil are to be ascribed to the same compound. Olcott and Mattill³¹ answered this question in the negative by fractionating the antisterility and antioxidant principles of wheat germ oil with petroleum ether and 92% methyl alcohol. These investigators³² later reported that, whereas acetylation destroyed the antioxidant action completely, it did not interfere with the effectiveness as an antisterility agent. Moreover, Stern *et al.*³ and Olcott and Emerson³³ proved that the antisterility activity and antioxidant action varied inversely in the several tocopherols available to them. Thus, α -tocopherol exerted the greatest antisterility effect, and δ -tocopherol was least effective in this respect, while the antioxidant action of δ -tocopherol was greatest, and that of α -tocopherol was poorest.

The separation and identification of the tocopherols has been elucidated by a brilliant series of investigations. As early as 1927, it was recognized that vitamin E occurs in the non-saponifiable fraction of fats.³⁴ Olcott

²⁴ H. M. Evans, *J. Nutrition*, **1**, 23-28 (1928).

²⁵ G. A. Emerson and H. M. Evans, *J. Nutrition*, **14**, 169-178 (1937).

²⁶ G. J. Martin, *J. Nutrition*, **13**, 679-685 (1937).

²⁷ F. B. Adamstone, *Science*, **80**, 450 (1934).

²⁸ N. Simmonds, J. E. Becker, and E. V. McCollum, *J. Am. Med. Assoc.*, **88**, 1047-1050 (1927).

²⁹ J. H. Jones, *J. Biol. Chem.*, **75**, 139-146 (1927).

³⁰ H. A. Mattill, *J. Am. Med. Assoc.*, **89**, 1505-1508 (1927).

³¹ H. S. Olcott and H. A. Mattill, *J. Biol. Chem.*, **93**, 59-70 (1931).

³² H. S. Olcott and H. A. Mattill, *J. Biol. Chem.*, **104**, 423-435 (1934).

³³ H. S. Olcott and O. H. Emerson, *J. Am. Chem. Soc.*, **59**, 1008-1009 (1937).

³⁴ H. M. Evans and G. O. Burr, *Mem. Univ. Calif.*, **8**, 1-158 (1927).

and Mattill^{31,32} observed that the vitamin could be prepared in high concentration by vacuum distillation. Drummond and co-workers³⁵ employed chromatographic adsorption on Brockmann alumina columns for their separation. Evans, Emerson and Emerson¹ and Emerson *et al.*³⁶ prepared esters of vitamin E with allophanic acid, and were able to separate these into two pure crystalline products which had different vitamin E activity. The more potent of these was called α -tocopherol, while the second one was later identified as β -tocopherol.² Todd, Bergel, Waldmann, and Work^{37,38} confirmed the data of Evans *et al.*;¹ they also prepared a new allophanate which they called β -tocopheryl allophanate. This compound melted at 143.5-144.5°C., and is apparently the same product separated by Emerson and co-workers³⁶ in impure form. The term "tocopherol" was employed because of its relation to reproduction and by virtue of the fact that these compounds were proved to be alcohols.^{35,39}

Emerson and co-workers² subsequently separated a third member of the group from cottonseed oil, as an allophanate. This was called γ -tocopherol. Its biologic activity (as an antisterility vitamin) was found to be only one-third of that of α -tocopherol.² As already noted, δ -tocopherol, which was prepared from soybean oil, was considered to have the least antisterility action but the greatest antioxidant capacity of any of the four tocopherols then known.³ The separation of the remaining members of the tocopherol group has been accomplished largely by the use of specially devised chromatographic procedures. Brown and Blaxter⁴⁰ and Brown alone^{41,42} employed the classical tocopherol purification sequence—extraction, saponification, and separation of the carotenoids by column chromatography—as well as an additional step which involved desterolation by freezing out from a methanol solution. As a result of this procedure which was followed by the use of paper chromatographic methods,⁴³ Brown,⁴⁴ and Eggitt and Ward,^{4,45} demonstrated the presence of a new tocopherol-like material in wheat, which Brown⁴⁴ considered to be

³⁵ J. C. Drummond, E. Singer, and R. J. MacWalter, *Biochem. J.*, **29**, 456-471 (1935).

³⁶ O. H. Emerson, G. A. Emerson, and H. M. Evans, *Science*, **83**, 421 (1936).

³⁷ A. R. Todd, F. Bergel, H. Waldmann, and T. S. Work, *Nature*, **140**, 361-362 (1937).

³⁸ A. R. Todd, F. Bergel, H. Waldmann, and T. S. Work, *Biochem. J.*, **31**, 2247-2256 (1937).

³⁹ H. S. Olcott, *J. Biol. Chem.*, **110**, 695-701 (1935).

⁴⁰ F. Brown and K. L. Blaxter, *Chemistry & Industry*, **1951**, 633-634.

⁴¹ F. Brown, *Biochem. J.*, **51**, 237-239 (1952).

⁴² F. Brown, *Biochem. J.*, **52**, 523-526 (1952).

⁴³ P. W. R. Eggitt and L. D. Ward, *J. Sci. Food Agr.*, **4**, 176-179 (1953).

⁴⁴ F. Brown, *J. Sci. Food Agr.*, **4**, 161-165 (1953).

⁴⁵ P. W. R. Eggitt and L. D. Ward, *J. Sci. Food Agr.*, **6**, 329-337 (1955).

an artifact; this was shown to be 5-methyl-tocol or ϵ -tocopherol.⁴ By an extension of the chromatographic procedure to a two-dimensional separation, Green *et al.*⁵ not only confirmed the existence of ϵ -tocopherol, but also identified a new member of the group, ζ -tocopherol, in many natural oils; this compound was shown to have properties identical with those of 5,7-dimethyl-tocol, which had previously been synthesized by Karrer and Fritzsche,⁴⁶ as well as by Bergel *et al.*,⁴⁷ and which is, in fact, this compound.

According to Green *et al.*,⁵ the biopotency of ϵ -tocopherol would be expected to approximate that of β -tocopherol, because of the similarity in structure. Jacob, Sutcliffe, and Todd⁴⁸ reported that the synthetic 5,7-dimethyl-tocol, or natural ζ -tocopherol, has a biologic activity almost equivalent to that of α -tocopherol.

The structural relationships of the tocopherols were worked out by Fernholz^{49,50} who demonstrated that they possessed a chroman ring with one, two or three substituted methyl groups. Karrer and associates⁵¹⁻⁵³ first accomplished the synthesis of DL- α -tocopherol in 1938, and their work was later confirmed by Smith, Ungnade, and Pritchard.⁵⁴ Baxter and co-workers⁵⁵ and Robeson⁵⁶ succeeded in crystallizing the natural tocopherols, α and γ , but they were unable to prepare crystalline β -tocopherol except in the form of the allophanate. For a more extensive discussion of the structure of the tocopherols, the reader is referred to pages 804-809 of *The Lipids*, Vol. I. The synthesis is discussed on pages 809-811, and the properties are described on pages 812-819 of the same volume. Extensive reviews of the physiology of vitamin E include those of Evans,⁵⁷ of Mattill,⁵⁸ and of Mason,⁵⁹ while Smith⁶⁰ is the author of an excellent

⁴⁶ P. Karrer and H. Fritzsche, *Helv. Chim. Acta*, **22**, 260-263 (1939).

⁴⁷ F. Bergel, A. M. Copping, A. Jacob, A. R. Todd, and T. S. Work, *J. Chem. Soc.*, **1938**, 1382-1383.

⁴⁸ A. Jacob, F. K. Sutcliffe, and A. R. Todd, *J. Chem. Soc.*, **1940**, 327-332.

⁴⁹ E. Fernholz, *J. Am. Chem. Soc.*, **59**, 1154-1155 (1937).

⁵⁰ E. Fernholz, *J. Am. Chem. Soc.*, **60**, 700-705 (1938).

⁵¹ P. Karrer, H. Fritzsche, B. H. Ringier, and H. Salomon, *Nature*, **141**, 1057 (1938).

⁵² P. Karrer, H. Fritzsche, B. H. Ringier, and H. Salomon, *Helv. Chim. Acta*, **21**, 520-525 (1938).

⁵³ P. Karrer, H. Fritzsche, B. H. Ringier, and H. Salomon, *Helv. Chim. Acta*, **21**, 820-825 (1938).

⁵⁴ L. I. Smith, H. E. Ungnade, and W. W. Prichard, *Science*, **88**, 37-38 (1938).

⁵⁵ J. G. Baxter, C. D. Robeson, J. D. Taylor, and R. W. Lehman, *J. Am. Chem. Soc.*, **65**, 918-924 (1943).

⁵⁶ C. D. Robeson, *J. Am. Chem. Soc.*, **65**, 1660 (1943).

⁵⁷ H. M. Evans, *J. Am. Med. Assoc.*, **99**, 469-476 (1932).

⁵⁸ H. A. Mattill, *J. Am. Med. Assoc.*, **110**, 1831-1837 (1938).

⁵⁹ K. E. Mason, *Vitamins and Hormones*, **2**, 107-153 (1944).

⁶⁰ L. I. Smith, *Chem. Revs.*, **27**, 287-329 (1940).

review of the chemical aspects of the field. The monograph of Sebrell and Harris on *The Vitamins* includes a comprehensive review of the tocopherols, principally the article by Harris, Mattill, and Mason.⁶¹ The newer discoveries in the field are to be included in the Report of the Third International Congress on Vitamin E held in Venice in September, 1955. Especially revealing are the papers of Harris and Mason⁶² on the metabolic aspects and that of Bolliger and Bolliger-Quaife⁶ on analytical methods.

3. Occurrence of the Vitamins E

(1) Distribution in Plant Tissues

The various types of tocopherols (vitamins E) which are found in nature occur largely in the plants; a minimum quantity is present in animal tissues. Although the data now available on the total tocopherols present in various plants and animal sources appear to be fairly reliable, those which report the distribution of the several types of tocopherols must all be re-evaluated in view of the fact that the more recently discovered tocopherols—*i.e.*, ϵ and ζ —were not considered in the analytical procedures.

All green plants contain certain demonstrable amounts of the tocopherols. Wheat germ oil⁶³ and other seed germ oils^{32,64} are generally considered to have the highest concentration of the vitamins E of any oils. However, according to Dam *et al.*,⁶⁵ green leafy vegetables and rose-hips have more extractable vitamin E on a dry-weight basis than does wheat germ.⁵⁹ The vegetable fats are the relatively most important sources of the tocopherols among the natural foods. The proportions of the several tocopherols in various vegetable fats and hydrogenated fats are listed in Table 1.

Green *et al.*⁵ reported that the distribution of tocopherols in a chick mash which contained a total of 2.5 mg. of tocopherols/100 g. was as follows: α , 25.4%; γ , 0%; α , 16.2%; δ , 0%; ϵ , 47.0%; and ζ , 14.7%. According to Lange,⁶⁶ hydrogenated vegetable shortenings have been found to have the following total tocopherol content (expressed in mg. %):

⁶¹ R. S. Harris, H. A. Mattill, and K. E. Mason, *The Tocopherols*, in W. H. Sebrell Jr., and R. S. Harris, *The Vitamins*, Acad. Press, New York, 1954, Vol. III, pp. 481–573.

⁶² P. L. Harris and K. E. Mason, *Vitamin E and Metabolic Processes*. *Proc. Third Intern. Congr. on Vitamin E*, Venice, Sept., 1955. Valdona, Verona, 1956, pp. 1–25, 26–29.

⁶³ H. S. Olcott, *J. Biol. Chem.*, 107, 471–474 (1934).

⁶⁴ H. M. Evans and G. O. Burr, *Proc. Nat. Acad. Sci., U. S.*, 11, 334–341 (1925).

⁶⁵ H. Dam, J. Glavind, I. Prange, and J. Ottesen, *Kgl. Danske Videnskab. Selskab. Biol. Medd.*, 16, No. 7, 1–39 (1941).

⁶⁶ W. Lange, *J. Am. Oil Chemists' Soc.*, 27, 414–422 (1950).

TABLE 1
TOCOPHEROL (VITAMIN E) CONTENT OF SOME VEGETABLE OILS^a

Oil	Tocopherol content, mg. per 100 g.						
	Total	α-	β-	γ-	δ-	ε-	ζ-
Babassu, crude ^b	3	—	—	—	—	—	—
Barley	238	36.4	0	14.3	0	81.4	105.9
Bran (wheat)	320	35.2	17.6	0	0	217.6	49.6
Cacao butter ^{b,c}	3-13	—	—	—	—	—	—
Carrot ^{b,c}	162;500	—	—	0	0	—	—
Castor ^b	50	—	—	—	—	—	—
Coconut ^{b,c}	3;8.3	3.6	—	—	4.7 ^d	—	—
Corn ^c	87-250;91	10.0	0	81.0	0	0	0
Cottonseed	83-110;81	47.0	0	34.0	0	0	0
Linseed, crude ^b	113	—	—	—	—	—	—
Mustard	32	8.6	0	17.6	5.8	0	0
Oats	61	17.1	0	22.0	6.1	2.4	13.4
Okraseed, crude ^b	74	31	—	43	—	—	—
Olive ^b	3-30	—	—	—	—	—	—
Palm ^{b,c}	3-50;56	30	—	—	26 ^d	—	—
Peanut	22-59;19.5	6.9	0	12.6	0	0	0
Pecan, refined ^{b,c}	45;42	20	—	22	—	—	—
Poppyseed, crude ^b	44	—	—	—	—	—	—
Rapeseed	56	15.1	0	40.9	0	0	0
Rice bran ^{b,c}	55-100;91	58	—	33	—	—	—
Rye	248	96.7	0	12.4	0	79.4	59.5
Safflower, crude ^b	80	—	—	—	—	—	—
Soybean ^c	92-280;148	15.9	0	69.6	32.5	0	0
Soybean phosphatide ^a	200 ^c	—	—	—	—	—	—
Sunflowerseed, crude ^b	70	—	—	—	—	—	—
Wheat germ ^c	140-550;255	142.8	85.4	0	0	26.8	0

^a Adapted from H. J. Deuel, Jr., *The Lipids*, Vol. I, p. 798, but corrected to include data on ε- and δ-tocopherols according to J. Green, S. Marcinkiewicz, and P. R. Watt, *J. Sci. Food Agr.*, 6, 274-282 (1955).

^b Original data, not corrected for ε- and ζ-tocopherols.

^c Values following the semicolon are for the sample on which the fractionation is based.

^d Sum of γ- and δ-tocopherols.

^e Data of J. L. Jensen, K. C. D. Hickman, and P. L. Harris, *Proc. Soc. Exptl. Biol. Med.*, 54, 294-296 (1943).

Crisco, 104.2; margarine base stock, 100.5; Primex, 98.4; and Sweetex, 101.0. No distribution figures are given for the types of tocopherol present in the hydrogenated fats, but in all probability the distribution is the same as that in the limpid oil used for hydrogenation. There is no evidence that hydrogenation destroys vitamins E.

Olive oil, and especially coconut oil, are extremely low in tocopherols.

In fact, Bacharach *et al.*⁶⁷ reported the total absence of vitamin E in olive oil and its low concentration in peanut oil. Rosenberg⁶⁸ notes that lettuce and alfalfa contain considerable amounts of tocopherols, while bananas and oranges are low in this vitamin. Mason⁶⁹ is of the opinion that vitamin E is absent from such lower plant forms as algae, fungi, liverworts (*Hepaticae*), mosses, ferns, and primitive seed plants. In fact, Schopfer and Bloomer⁶⁹ definitely proved the absence of tocopherols from the saprophytic mold, *Phycomyces*, which is a fungus.

The tocopherols are present in highest concentration in seed oils in which they occur in unesterified (or free) form.^{70,71} It is interesting that the oils from wheat germ and from wheat bran are the only ones which contain an appreciable amount of β -tocopherol. β -Tocopherol is the principal form found in European wheat, in conjunction with a smaller or approximately equal amount of α -tocopherol,⁷¹ and with a smaller content of γ -tocopherol. According to Emerson,⁷² the oil from the germ of the California-grown wheat contains twice as much α - as β -tocopherol, with only a trace of the γ -type. γ -Tocopherol is especially high in corn and palm oils,⁷³ while δ -tocopherol is present chiefly in soybean oil.⁷⁴ ϵ -Tocopherol has been reported to have an especially high concentration in wheat bran oil and rye oil; it is comparatively low in wheat germ oil. Barley is especially rich in ζ -tocopherol, as is rye oil. Halden⁷⁰ observed that a total of 1050 mg. % may occur in rye germ oil.

(2) Distribution in Animal Tissues

In contradistinction to most vegetable fats, animal fats have an exceedingly low tocopherol content.⁷⁵ Thus, the values recorded for the several animal fats examined ranged between 0.2 to 4.2 mg. per 100 g. fat.⁷⁵ This level appears exceedingly low when compared with one of 400 mg. % for wheat germ oil or 100 mg. % for cottonseed oil. Oleo oil is reported

⁶⁷ A. L. Bacharach, E. Allchorne, and H. E. Glynn, *Biochem. J.*, **31**, 2287-2292 (1932).

⁶⁸ H. R. Rosenberg, *Chemistry and Physiology of the Vitamins*, Interscience, New York, 1945.

⁶⁹ W. H. Schopfer and S. Bloomer, *Z. Vitaminforsch.*, **9**, 344-349 (1939).

⁷⁰ W. Halden, *Monatsh.*, **77**, 197-205 (1947).

⁷¹ A. R. Moss and J. C. Drummond, *Biochem. J.*, **32**, 1953-1956 (1938).

⁷² O. H. Emerson, *J. Am. Chem. Soc.*, **60**, 1741-1742 (1938).

⁷³ O. H. Emerson, G. A. Emerson, and H. M. Evans, *Science*, **89**, 183 (1939).

⁷⁴ L. Weisler, C. D. Robeson, and J. G. Baxter, *Ind. Eng. Chem., Anal. Ed.*, **19**, 906-909 (1947).

⁷⁵ H. J. Deuel, Jr., *Nutrition Aspects of Cottonseed Oil Utilization*. Chap. XIX, in A. E. Bailey, *Cottonseed and Cottonseed Products*, Interscience, New York, 1948, 763-811, p. 772.

to have a tocopherol content of only 2 mg. %, 75 while Kofler⁷⁶ cites the value for tocopherol in beef tallow as 1 mg. %. In the case of butter, the results of numerous assays have shown figures varying between 1.7 and 4.2 mg. %.⁷⁷⁻⁸¹ Lard is reported to have similar low values;^{76,79} Harris *et al.*⁷⁹ observed a total tocopherol content in one sample of lard of 2.7 mg. %, of which 2.3 mg. was found to be α -tocopherol and <0.4 mg. % consisted of γ -tocopherol. Lange⁶⁶ compiled an exceedingly comprehensive and useful report of the tocopherol content of a large number of animal and vegetable fats, as well as of other tissues.

The amount of tocopherols present in animal fats is directly related to the amount taken in the diet. Barnes, Lundberg, Hanson, and Burr⁸² suggested that the antioxidants responsible for the normal stability of body fat in the rat are entirely of dietary origin, and consist solely of vitamins E. Thus, it was shown that when rats were raised from the time of weaning to 100 days of age on a diet containing no vitamin E or fat, their rendered body fats were abnormally susceptible to oxidation by atmospheric oxygen at the C=C linkages. The oxygen absorption measurements at 100°C. were characterized by an almost complete absence of an induction period. When the rats on the vitamin E-free diet were given a single dose of 200 mg. of α -tocopherol in the diet before they were sacrificed, the stability of the body fats was restored. In a later study by this group, Lundberg *et al.*⁸³ found that the maximum deposition of α -tocopherol was not achieved in the rat until seven to ten days after the feeding of a single 50 mg. dose. The concentration of tocopherol decreased slowly thereafter if no additional quantities were given; the level reached about one-half of the maximum value after two months. On the other hand, when varying amounts of α -tocopherol up to 500 mg. were given in single doses, increasing amounts were noted in the abdominal fats. Under such conditions, the fat depots may become the major sites of storage of vitamin

⁷⁶ M. Kofler, *Helv. Chim. Acta*, **26**, 2166-2176 (1943).

⁷⁷ M. L. Quaife, *J. Biol. Chem.*, **169**, 513-514 (1947).

⁷⁸ A. Emmerie and C. Engel, *Z. Vitaminforsch.*, **13**, 259-266 (1943); *Chem. Zentr.*, **1943**, II, 1974. Also cited by W. Lange, *J. Am. Oil Chemists' Soc.*, **27**, 414-422 (1950).

⁷⁹ P. L. Harris, M. L. Quaife, and W. J. Swanson, *J. Nutrition*, **40**, 367-381 (1950).

⁸⁰ P. L. Harris, W. J. Swanson, and K. C. D. Hickman, *J. Nutrition*, **33**, 411-427 (1947).

⁸¹ H. Lieck and H. Willstaedt, *Svensk Kem. Tid.*, **57**, 134-139 (1945); *Chem. Abst.*, **40**, 4759 (1946).

⁸² R. H. Barnes, W. O. Lundberg, H. T. Hanson, and G. O. Burr, *J. Biol. Chem.*, **149**, 313-322 (1943).

⁸³ W. O. Lundberg, R. H. Barnes, M. Clausen, and G. O. Burr, *J. Biol. Chem.*, **153**, 265-274 (1944).

E. In confirmation of their earlier work,⁸² Hanson and co-workers⁸⁴ found that only dietary α -tocopherol could restore the stability of the body fat of rats previously maintained on a vitamin E-free diet. The addition to the diet of other well-known antioxidants, including ascorbic acid (vitamin C), hydroquinone, α -naphthol, nordihydroguaiaretic acid (ND-GA), or lecithin was found to be ineffective in increasing the keeping qualities of the body fat. For a discussion of these and other antioxidants, the reader is referred to *The Lipids*, Vol. I, pages 288 to 299. It is believed that the only compounds deposited in the adipose tissues which are effective antioxidants are the vitamins E.

α -, β -, and γ -Tocopherols were all found to be capable of deposition in animal fats when fed in the diet to rats previously on a vitamin E-free diet.⁸⁵ The amounts of α - and β -compounds deposited were quite similar, as judged from the resulting stability of the fats; on the other hand, the amount of γ -tocopherol laid down in the adipose tissue was much less. In all cases the storage required seven days or more to reach the maximum. No differences were noted between the ability of natural and that of synthetic tocopherols to serve in this capacity.⁸⁵

With the possible exception of a crustacean, the water flea (*Daphnia magna*),⁸⁹ there is no evidence that the tocopherols are required by the invertebrates or, in fact, that they are stored in the tissues of these animals. Although many fish liver oils may contain appreciable amounts of the vitamins E, cod liver oil is generally considered to be completely devoid of these substances.⁵⁹ However, Quackenbush and co-workers⁸⁶ recorded an appreciable content of 26 mg. % of cod liver oil. Bocchi⁸⁷ observed some vitamin E in tuna liver oil, and Robeson and Baxter⁸⁸ noted small amounts in the liver oils of the shark (*Mangona*) (10 mg. % oil), and of the soupfin shark (*Galeorhinus zyopterus*) (4 mg. % oil).

In the case of the rat, the highest concentration of tocopherol was found in the pituitary gland (hypophysis), while the next highest concentration occurred in the suprarenal glands.⁸⁹ The liver, which is the usual storage organ for fat-soluble vitamins and other physiologic compounds, has been

⁸⁴ H. T. Hanson, R. H. Barnes, W. O. Lundburg, and G. O. Burr, *J. Biol. Chem.*, **156**, 673-677 (1944).

⁸⁵ W. O. Lundberg, R. H. Barnes, M. Clausen, N. Larson, and G. O. Burr, *J. Biol. Chem.*, **168**, 379-389 (1947).

⁸⁶ F. W. Quackenbush, H. L. Gottlieb, and H. Steenbock, *Ind. Eng. Chem.*, **33**, 1276-1278 (1941).

⁸⁷ L. Bocchi, *Ateneo parmense*, **10**, 107-126 (1938); *Chem. Abst.*, **33**, 2945 (1939).

⁸⁸ C. D. Robeson and J. G. Baxter, *J. Am. Chem. Soc.*, **65**, 940-943 (1943).

⁸⁹ M. L. Quaife, W. J. Swanson, M. Y. Dju, and P. L. Harris, *Ann. New York Acad. Sci.*, **52**, 300-305 (1949).

reported to have a low tocopherol content,^{90,91} although Mason⁹² demonstrated that this value is dependent upon the intake of vitamin E in the case of the rat. When the vitamin E intake was increased 100 times, the liver storage was augmented fourteen-fold, in contrast to a rise of only 4 to 4.5 times in most other tissues. When the tocopherol intake was increased to 10,000 times the daily minimum requirement, liver storage was further augmented to 150 times and that in the muscle to twelve times that noted when the minimal dosage was given. Thus, the liver provides a site for tocopherol storage in the rat, and this reserve serves to replenish the need of other tissues for vitamin E when the intake of this dietary constituent is less than is required. Mason⁵⁹ has recorded the quantity of vitamin E in the several tissues of the rat on a low or a high vitamin E intake. The content in the liver was increased from 8.3 to 1,000 mg./kg. tissue, that in the muscle from 16.6 to 200 mg./kg. tissue and that in the body fat from 20 to 125 mg./kg. tissue on the high level of intake of vitamin E. Chevallier *et al.*⁹³ found that, during fasting, vitamin E did not decrease in the same proportion as did other lipids, in the rat. Thus it appears that the relative amount of vitamin E and its antioxidant properties increase when the fat content of the organism decreases as a result of inanition. The distribution of vitamin E in the tissues of the adult rat after the feeding of supplementary α -tocopherol, as observed by Quaife and her co-workers⁸⁹ and by Mason,⁵⁹ is detailed in *The Lipids*, Vol. I, pages 799 and 800.

Vitamin E also occurs in the tissues and eggs of chickens and other birds. Although a detailed study of the distribution of vitamin E in the tissues of the hen similar to that carried out on the rat is not available, it is known that vitamin E occurs in the egg, and that the amount becomes proportionally higher with progressively increasing doses.⁹⁴ α -Tocopherol is absorbed and deposited in the hen, as in the cow and human, in preference to the other tocopherols; therefore this variety predominates in the tissues and eggs of chickens.⁸⁹ The efficiency of transfer of the tocopherols to the egg of the hen was reported by Quaife *et al.*⁸⁹ as 22.1% for the α -compound, 3.6% for γ -tocopherol, and 2.0% for δ -tocopherol. There is no evidence in any of these studies of an *in vivo* transformation of one tocopherol into another.

⁹⁰ W. F. J. Cuthbertson, R. R. Ridgeway, and J. C. Drummond, *Biochem. J.*, **34**, 34-39 (1940).

⁹¹ T. Moore, A. J. P. Martin, and K. R. Rajagopal, *Vitamin E Symposium*, April 22, 1939, *Soc. Chem. Ind. Food Group*, 1940, 41-43.

⁹² K. E. Mason, *J. Nutrition*, **23**, 71-81 (1942).

⁹³ A. Chevallier, R. Schneider, and C. Burg, *Compt. rend. soc. biol.*, **144**, 1394-1396 (1950).

⁹⁴ G. L. Barnum, *J. Nutrition*, **9**, 621-635 (1935).

The effect of the administration of tocopherols to turkeys on the composition of their tissues was carefully studied by Criddle and Morgan.⁹⁵ When a preparation containing about 50% α -tocopherol, and 50% γ -tocopherol was fed to female turkeys in doses of 0.01, 0.1, 1, or 2% of the feed for two to thirty-five days prior to sacrifice, significant increases in the tocopherol content of the liver, heart, gizzard, and muscle occurred. The liver usually contained the most tocopherol, followed by heart, gizzard, leg and breast muscle. The most efficient transfer of vitamin E to the tissues occurred when the dietary regimen containing 0.1% tocopherol was fed for thirty-five days. It was noted that the peroxide numbers of the subcutaneous fat and abdominal fat were greater after frozen storage and with decreasing amounts of tissue tocopherols.

According to Karrer and co-workers,⁹⁶ the livers of cattle and horses are rich in tocopherols. Mason⁹² has recorded appreciable amounts of this vitamin in the livers of monkeys and of man. Cow's milk has been found to be an exceedingly poor source of vitamin E, with an average figure of only 0.061 mg. %.⁹⁷ Quaife *et al.*⁸⁹ reported that, when the fodder of the cow was supplemented with α -tocopherol, increased amounts were secreted in the milk. On the other hand, when the added supplement consisted of γ - and δ -tocopherols, only slight increases in milk tocopherol were observed.

Krukovsky and Loosli⁹⁸ found that the tocopherol content of the milk fat obtained from cows on a standard winter ration gradually decreased throughout the season. Furthermore, the milk showed practically no resistance to oxidative deterioration, and this resulted in the development of "oxidized flavors." When the basal rations were supplemented with 1 to 2 g. of mixed tocopherols at the start of the test, both the tocopherol level of the fat and its ability to resist those reactions which cause "oxidized flavors" were improved. However, no such improvement was observed when the test was carried out with the cows at the end of the winter season. Thus, although the tocopherol supplement functioned satisfactorily in one set of tests, these investigators state that it cannot be depended upon to maintain the tocopherol content of the fat and to prevent or to correct "oxidized flavors" under all conditions.

Tocopherols also comprise an important constituent of the tissues of man. Quaife and Dju⁹⁹ estimated a total of 3.4 g. of tocopherols in the tissues of a

⁹⁵ J. E. Criddle and A. F. Morgan, *Proc. Soc. Exptl. Biol. Med.*, **78**, 41-46 (1951).

⁹⁶ P. Karrer, W. Jaeger, and H. Keller, *Helv. Chim. Acta*, **23**, 464-465 (1940).

⁹⁷ R. Abderhalden, *Biochem. Z.*, **318**, 47-53 (1948).

⁹⁸ V. N. Krukovsky and J. K. Loosli, *J. Dairy Sci.*, **35**, 843-838 (1952).

⁹⁹ M. L. Quaife and M. Y. Dju, *J. Biol. Chem.*, **180**, 263-272 (1949).

normal man; the following proportions (in mg.) were found in the several organs: fat, 1,885; muscle, 285; blood, 64; liver, 45; pancreas, 7; spleen, 4; heart, 3; kidney, 2; lung, 12; and testis, 2 (total, 2,309; total on the basis of 70 kg. weight, 3,440). In the case of a woman, the total was estimated at 8.12 g. for a normal 50 kg. woman. The values of tocopherol recorded in the several tissues (in mg.) were as follows: fat, 6,180; muscle, 269; blood, 45; liver, 33; pancreas, 10; spleen, 7; heart, 4; kidney, 10; uterus, 2 (total, 6,560; total on the basis of 50 kg. weight, 8,120). Although these data are for only one subject of each sex, it would appear that a marked sex difference exists in line with variations in the content of other lipids. There was a fifty-fold variation in the concentration of tocopherols in human tissues on the wet basis, but only an eight-fold variability when the tocopherol content was based upon the fat present; in this case the values varied from 0.2 to 1.2 mg./g. lipid. The tocopherol consisted primarily of α -tocopherol (91% in the case of the man and 88% for the woman). Human muscle (and also rat muscle) has been reported to contain from 17 to 30 mg. tocopherol/kg. wet muscle.¹⁰⁰ Mason, Dju, and Chapin¹⁰¹ noted that the total tocopherols present in eight different muscles examined, of eight visceral organs, and of adipose tissue from a twenty-one-year-old patient with advanced progressive muscular dystrophy, were in the normal range; however, these investigators¹⁰² suggest that this does not necessarily rule out the possibility of a congenital or acquired metabolic defect in the utilization of tocopherols by skeletal muscle.

Human milk has a much higher tocopherol content than does cow's milk. Quaife⁷⁷ gives a figure of 0.14 mg. % for the proportion of tocopherol in human milk, although a value as high as 3.6 mg. % has been reported for the colostrum of women during the first week following parturition. In a later study, Harris, Quaife and O'Grady¹⁰² recorded a somewhat higher mean value for tocopherol in human milk, namely 0.24 mg. % (range 0.10 to 0.48%), or about 80 μ g./g. fat. Tocopherol values did not vary with the stage of lactation, but could be correlated with the fat content of the milk. Tocopherol in human milk was found to be stable on pasteurization. Neuweiler¹⁰³ states that, in the human mother, the administration of vitamin E increased the content of this vitamin in the milk only if the milk was previously deficient in tocopherol. In the case of the

¹⁰⁰ H. Kaunitz and J. J. Beaver, *J. Biol. Chem.*, **166**, 205-217 (1946).

¹⁰¹ K. E. Mason, M. Y. Dju, and S. J. Chapin, *Federation Proc.*, **12**, 422 (1953).

¹⁰² P. L. Harris, M. L. Quaife, and P. O'Grady, *J. Nutrition*, **46**, 459-466 (1952).

¹⁰³ W. Neuweiler, *Z. Vitaminforsch.*, **20**, 108-116 (1948).

cow, Harris *et al.*⁸⁰ noted that vitamin E supplementation increased milk fat concentration (27%) and total milk production (21%), but that it did not result in an augmented vitamin E excretion into the milk.

4. The Digestion and Absorption of the Tocopherols

When the tocopherols are given, even in moderate doses, their absorption is quite inefficient. Juhász-Schäffer¹⁰⁴ proved that definite amounts of this vitamin are lost in the feces. After feeding C¹⁴-tagged tocopherol in oil solution to rats, Shantz¹⁰⁵ could account for an absorption of only 20%, the remainder being excreted *via* the intestine. Simon, Gross, and Milhorat¹⁰⁶ reported that, when rabbits were given 10 to 15 mg. of C¹⁴-labeled *d*- α -tocopheryl succinate orally, about 74% could be recovered from the feces in three days, and as much as 80% after six days. Engel and Heins¹⁰⁷ recorded a coefficient of digestibility of 60 for vitamin E from wheat germ oil, and a figure of only 13 when the source of the vitamin was dried grass. Pindborg¹⁰⁸ was of the opinion that a biosynthesis of vitamin E occurs in the intestine, but Harris¹⁰⁹ opposes this view and believes that the fecal vitamin E merely represents undigested tocopherol from the ingested food. The relatively low tocopherol content of muscles, body fat, and blood of rats suggests either that the absorption is very inefficient, or that the vitamin may be destroyed in the gastrointestinal tract, or both. It may also be considered to be the result of a rapid destruction within the body, or may be attributed to an inefficient capacity for storage.^{90,110} Dju and co-workers¹¹¹ observed that, when a high level of α -tocopherol was given to domestic hens, large amounts of unchanged tocopherol were excreted in the feces. Klatskin and Molander^{112,113} found that, in the case of healthy men on average diets, fecal excretion accounted for about two-thirds of the calculated dietary intake. However, it is not known how much of the

¹⁰⁴ A. Juhász-Schäffer, *Virchow's Arch. path. Anat. u. Physiol.*, **281**, 53-65 (1931).

¹⁰⁵ E. M. Shantz, Unpublished data cited by P. L. Harris, *Ann. Rev. Biochem.*, **18**, 391-394 (1949), p. 410.

¹⁰⁶ E. J. Simon, C. S. Gross, and A. T. Milhorat, *J. Biol. Chem.*, **221**, 797-805 (1956).

^{106a} E. J. Simon, A. Eisengart, L. Sundheim, and A. T. Milhorat, *J. Biol. Chem.*, **221**, 807-817 (1956).

¹⁰⁷ C. Engel and J. T. Heins, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **13**, 37 (1943).

¹⁰⁸ J. J. Pindborg, *Nature* **164**, 493 (1949).

¹⁰⁹ P. L. Harris, *Nature*, **165**, 572 (1950).

¹¹⁰ T. Moore and K. R. Rajagopal, *Biochem. J.*, **34**, 335-342 (1940).

¹¹¹ M. Y. Dju, M. L. Quaife, and P. L. Harris, *Am. J. Physiol.*, **160**, 259-263 (1950).

¹¹² G. Klatskin and D. W. Molander, *J. Clin. Invest.*, **31**, 159-170 (1952).

¹¹³ G. Klatskin and D. W. Molander, *J. Lab. Clin. Med.*, **39**, 802-814 (1952).

fecal tocopherol represents an unabsorbed fraction, and how much can be accounted for by reëxcretion of absorbed tocopherol through the bile, since it is known that the latter secretion has a vitamin E content similar to that of the plasma.⁶²

Bile is a requirement for the absorption of vitamin E, just as it is for the carotenoids, vitamin A, vitamin D, vitamin K, and the fats. Thus, Greaves and Schmidt¹¹⁴ found that vitamin E was not absorbed when fed to choledochocolostomized rats. Moreover, Brinkhous and Warner¹¹⁵ noted that dogs with bile fistulas were incapable of absorbing this vitamin. No information is available as to whether or not the bile acids form co-ordination compounds with vitamin E, or in what manner they mediate their action.

Tocopherol is absorbed relatively rapidly by the rat. Swick and Baumann¹¹⁶ noted the highest concentration of α -tocopherol in the gut wall (which presumably would coincide with the time of maximum absorption) five hours after the ingestion of free α -tocopherol and eight hours after the feeding of α -tocopheryl acetate. In the case of man, Quaife *et al.*⁸⁹ reported that maximum serum levels (which also should coincide roughly with the period of maximum absorption) were reached four hours after a 500 mg. dose of α -tocopherol had been given. This value amounted to 1.75 mg./100 ml. serum.

The ease of absorption of the tocopherols varies with the several members of the vitamin E group. Thus, in the case of the human subject studied by Quaife *et al.*,⁸⁹ although the maximum serum tocopherol level was reached after about the same interval when γ -tocopherol had been fed as after the feeding of α -tocopherol, namely four hours, the peak figure was only 1.35 mg. % in the former case, as compared with 1.75 mg. % for the α -tocopherol. Cows and hens have also been shown to exhibit a preferential absorption of α -tocopherol over the γ - and δ -tocopherols. Thus, Quaife and co-workers⁸⁹ reported that a considerable rise in the milk tocopherol level of cows occurred when the α -tocopherol was fed. However, in sharp contradistinction to this result, a much less pronounced effect on the milk tocopherol followed the administration of a mixture of tocopherols, 98% of which consisted of γ - and δ -tocopherols.

In the case of chickens, the most sensitive indices of the efficiency of tocopherol absorption are the tocopherol content in the egg,⁹⁴ and the type

¹¹⁴ J. D. Greaves and C. L. A. Schmidt, *Proc. Soc. Exptl. Biol. Med.*, **37**, 40-42 (1937).

¹¹⁵ K. M. Brinkhous and E. D. Warner, *Am. J. Pathol.*, **17**, 81-86 (1941).

¹¹⁶ R. W. Swick and C. A. Baumann, *Arch. Biochem. Biophys.*, **36**, 120-126 (1952).

found. Thus, when doses of α -, γ -, or δ -tocopherol were fed to hens in amounts of 100 to 4,000 mg./week, progressively increasing amounts of the dietary tocopherols were found to be transferred to the egg;⁸⁹ the amount of tocopherol varied according to the type fed. When the dosage was 400 mg./week, the relative efficiency of transfer to the egg was 22.1% for the α -tocopherol, 3.6% for the γ - variety, and 2.0% for the δ -type.^{89,111} There is no current information concerning the absorbability of the newer members of the tocopherol family, *i.e.*, ϵ - and ζ -tocopherols, but one would expect it to be comparable to that of other members of the group.

5. The Transport of Tocopherols from the Intestine to the Tissues

The data are extremely limited on the pathway of transport of absorbed tocopherols from the gut to the blood-stream and the tissues. There is no reason to suppose that the method of transport differs from that of the other fat-soluble vitamins. However, there does not appear to be any information in the literature in regard to the presence of vitamins E in the lymph, or any evaluation of the proportion of these vitamins carried *via* the lymph and by the portal system. Harris¹¹⁷ stated that the rates of absorption or orally-administered vitamins A and E are sufficiently similar to constitute presumptive evidence that the two vitamins travel from the intestine to the blood-stream by the same pathway. If this is the case, the vitamins E would be carried from the intestinal lacteals in the chyle, and would ultimately be poured into the blood-stream from the thoracic duct. None would be carried *via* the portal route.

6. The Metabolism of Absorbed Tocopherols

(1) *The Presence of Tocopherols in Blood*

According to Rosenberg,⁶⁸ the total blood tocopherol averages 5.6 $\mu\text{g.}/10$ c.c. of serum in female rats and 6.4 $\mu\text{g.}/10$ c.c. serum in male rats when a normal diet is given; however, when tocopherol is administered, the blood level can be increased to 100 $\mu\text{g.}$ Vitamin E occurs in the blood as the free alcohol, even after the ester has been administered. Quaife *et al.*⁸⁹ reported that 75% of the blood tocopherol in man consisted of the α -variety while other types comprised the balance. The total tocopherol contained

¹¹⁷ P. L. Harris, *Personal communication* to the author (March 1954). Cited in Vol. II, *The Lipids*, p. 336.

in the entire blood of a man was found to amount to 64 mg. while that in a woman was 45 mg.⁹⁹

The earlier values of Rosenberg⁶⁹ are much lower than the more recent figures obtained by using more modern technics based upon plasma rather than upon whole blood. Ames and Harris¹¹⁸ recorded values ranging from 0.9 to 1.2 mg. %, although extreme variations of from 0.1 to 3.0 mg. % sometimes occur. Values of approximately 1 mg. % have also been recorded by a number of other workers, including Lemley *et al.*,¹¹⁹ Klatskin and Krehl,¹²⁰ Darby and co-workers,¹²¹ Engel,¹²² and Couperus.¹²³ On the other hand, the results of Varangot *et al.*¹²⁴ for plasma vitamin E are quite out of line with those quoted above, being 0.19 mg. % for men and 0.22 mg. % for women. There is no explanation for this divergence.

In the case of cows, somewhat lower values have been observed for the blood tocopherols. Thus, van der Kaay and co-workers¹²⁵ reported a mean level of 800 μ g. % (0.8 mg. %) for the plasma tocopherol of cows on pasture; this was reduced to 0.1 to 0.2 mg. % (100 to 200 μ g. %) during the winter, when green forage was not available. Meunier, Ferrando, and Chenavier¹²⁶ recorded an average range of 200 to 300 μ g. % (0.2 to 0.3 mg. %) for sheep. Quaife and associates⁸⁹ give the figure 0.7 mg. % as the plasma tocopherol value for male rats on a vitamin E-free diet but receiving 1 mg. of α -tocopherol daily. However, Quaife and Harris¹²⁷ noted serum tocopherol averages of 1.72 mg. % and 1.68 mg. % for rats whose rations were supplemented with vitamin E to the extent of 12 mg./week and which were either subjected to stress or were free from stress, respectively. In sharp contrast to these figures, the serum tocopherol was found to be zero in rats which were vitamin E-deficient. For a more complete exposition of the blood tocopherols, including the general physiologic factors to which

¹¹⁸ S. R. Ames and P. L. Harris, *Intern. Rev. Vitamin Research*, **22**, 26-34 (1950).

¹¹⁹ J. M. Lemley, R. G. Gale, R. H. Furman, M. E. Cherrington, W. J. Darby, and G. R. Meneely, *Am. Heart J.*, **37**, 1029-1034 (1949).

¹²⁰ G. Klatskin and W. A. Krehl, *J. Clin. Invest.*, **29**, 1528-1541 (1930).

¹²¹ W. J. Darby, M. E. Cherrington, and J. M. Ruffin, *Proc. Soc. Exptl. Biol. Med.*, **63**, 310-312 (1946).

¹²² C. Engel, *Ann. New York Acad. Sci.*, **52**, 292-299 (1949).

¹²³ J. Couperus, *Z. Vitaminforsch.*, **13**, 193-207 (1943).

¹²⁴ J. Varangot, H. Chailly, and N. Rieux, *Compt. rend. soc. biol.*, **137**, 210-211 (1943).

¹²⁵ F. C. van der Kaay, G. B. H. Teunissen, A. Emmerie, and M. van Eekelen, *Ann. New York Acad. Sci.*, **52**, 276-283 (1949).

¹²⁶ P. Meunier, R. Ferrando, and P. Chenavier, *Compt. rend. soc. biol.*, **142**, 525-527 (1948).

¹²⁷ M. L. Quaife and P. L. Harris, *Proc. Soc. Exptl. Biol. Med.*, **78**, 188-191 (1951).

they are related and the effect of pathologic conditions, the reader is referred to *The Lipids*, Vol. II, pages 509-520.

(2) *The Excretion of Absorbed Tocopherols*

Absorbed tocopherols can be removed from the body either by excretion into the bile or, to some extent, by elimination *via* the kidney. As has already been discussed, bile has about the same tocopherol content as that of the plasma.⁶²

There has been some question as to whether or not absorbed tocopherols are excreted in the urine. McArthur and Watson¹²⁸ were unable to find any evidence of tocopherols in the urine of cows. Moreover, Klatskin and Molander^{112,113} could not account for any significant amount of vitamin E in the urine of healthy humans, although the fecal excretion amounted to two-thirds of that ingested. Simon, Gross, and Milhorat¹⁰⁶ also reported that none of the C¹⁴-labeled α -tocopheryl succinate was excreted in the urine of the rabbit when this compound was administered orally. However, Harris⁶² states that under similar conditions some urinary excretion of C¹⁴ occurs.

When the tocopherols are administered by parenteral routes, vitamin E or vitamin E-like compounds are unquestionably excreted in the urine. When C¹⁴-labeled α -tocopheryl succinate was injected subcutaneously, Simon, Gros, and Milhorat¹⁰⁶ were able to recover about 14% of the original dose administered from the site of the injection after eleven days. After intravenous injection, 90% of the radioactivity in the blood stream was lost within twenty-four hours. In this case, about one-third of the administered tocopherol-C¹⁴ was excreted in the urine. All of the radioactive product in the urine and about one-half of that eliminated *via* the gut comprised a new metabolite which must be considered as an intermediate of α -tocopherol.⁶² It is evident from these results that the esterified tocopherols are hydrolyzed by the tissue fluids.

3. *The Catabolism of Tocopherols*

The first step in the catabolism of α -tocopherol is believed to be its conversion to α -tocopheryl quinone, with a possible further reduction to α -tocopheryl hydroquinone, as illustrated below:

¹²⁸ C. S. McArthur and E. M. Watson, *Can. Chem. Process Inds.*, **23**, 350-352 (1939).

α -Tocopherylquinone (α -Tocoquinone) α -Tocopherylhydroquinone

First Steps in the Catabolism of α -Tocopherol

Although these oxidation products probably do not function as components of an oxidation-reduction system, as was formerly believed, they still are of importance, inasmuch as they have been shown by Mackenzie and Mackenzie¹²⁹ to be active as antidystrophic agents.

Simon, Gross, and Milhorat¹⁰⁶ found another major intermediate in the urine and feces of rabbits given labeled tocopherol, and in the urine of human subjects ingesting large amounts of α -tocopherol.¹⁰⁷ This degradation product appears to be a derivative of α -tocopherol in which the side

¹²⁹ J. B. Mackenzie and C. G. Mackenzie, *Proc. Soc. Exptl. Biol. Med.*, **84**, 388-392 (1953).

chain has been broken down to a γ lactone. This intermediate has the structure shown below:

Derivative of α -Tocopherol Excreted in the Urine by Human Subjects and by Rabbits after the Feeding of α -Tocopherol 62, 106a

7. The Importance of Tocopherols (Vitamins E) in Various Metabolic Processes

(1) Introduction

The tocopherols play a unique role among the vitamins in that they are implicated in a wide variety of actions. This would suggest that they are concerned with some general key reaction which is common to most cells and tissues of the body. One reaction which is generally recognized as the most important single property of vitamin E is its antioxidant action, which functions both *in vivo* and *in vitro*. However, according to Harris and Mason,⁶² a number of functions of vitamin E cannot be ascribed to its antioxidant nature, and are quite specific in character for α -tocopherol as, for example, its function in assisting in the normal resistance of the red blood cells to hemolysis. Although there are no convincing data which prove that vitamin E is essential for the formation or maintenance of a specific enzyme or coenzyme, or that it functions as a coenzyme, either of these functions might well be considered to be an additional activity over and above its antioxidant action.

Many of the deficiency symptoms resulting from an absence of vitamin E from the diet are irreversible. The deficiency of vitamin E may bring about irreversible muscle degeneration. Vitamin E added to the diet does not result in any reparative or curative action on the muscle fibers which have started to die. Any beneficial effect which is noted under such conditions, due to vitamin E therapy, is attributable to the changes in the proportions of regenerating and degenerating fibers, and also to the alteration in the relative speed of regeneration and degeneration. Other examples of the irreversibility of damage due to vitamin E deficiency include testicular degeneration in the male vitamin E-deficient rat, the fatal effect of encephalomalacia in the chicken if vitamin E therapy is not initiated

sufficiently early, the deposition of pigment in various organs of vitamin E-deficient animals, the high mortality rate of turkey embryos, and the susceptibility of the kidneys to autolysis.⁶²

(2) *The Function of the Tocopherols in the Metabolism of the Individual Foodstuffs*

According to Harris and Mason,⁶² this method of classifying the action of the tocopherols is highly artificial, since no reactions in the body occur separately and exclusively. All reactions are highly interrelated and they must function with one another in a highly complicated manner.

a. Protein Metabolism. (a) *Efficiency of Protein Utilization.* Dam¹³⁰ and later Hove,¹³¹ proved that the efficiency of protein utilization was increased in animals on an inadequate protein intake when tocopherol was administered. This was accepted as evidence that α -tocopherol participates in the intermediary metabolism of protein. The fact that tocopherol accelerates the growth and replacement of liver tissue following partial hepatectomy^{132,133} is, in all probability, another similar manifestation of the action of vitamin E on protein utilization. Harris and Mason⁶² consider that these data may be interpreted as evidence of the antioxidant action of α -tocopherol, although they do suggest the possibility that the mode of action of the vitamin at the cellular level may be the result of its influence upon nucleic acid, upon phosphocreatine metabolism, or upon protein synthesis, by its action as a coenzyme.

(b) *Creatinuria and Phosphocreatine.* Another role of vitamin E in intermediary protein metabolism was discovered by a study of the products of urinary excretion of animals in a state of extreme vitamin E deficiency. Under such conditions, creatine appears in the urine at the expense of some of the creatinine. This is probably a reflection of the loss of some muscle components (creatine and creatinine), associated with the loss of muscle function, and with the resulting muscular dystrophy.¹³⁴⁻¹³⁸ Sutton and

¹³⁰ H. Dam, *Proc. Soc. Exptl. Biol. Med.*, **55**, 55-56 (1944).

¹³¹ E. L. Hove, *Proc. Soc. Exptl. Biol. Med.*, **63**, 508-511 (1946).

¹³² G. C. Biaggini and G. Malagamba, *Riv. patol. clin.*, **6**, 281-292 (1951).

¹³³ N. Pacilli, *Ospedale maggiore*, **41**, 324-331 (1953); *Abst. Reports Third Intern. Cong. on Vitamin E*, Venice, 1955, Vol. I, p. 30; *Chem. Abst.*, **49**, 13387 (1955).

¹³⁴ E. L. Hove and J. O. Hardin, *J. Nutrition*, **48**, 193-199 (1952).

¹³⁵ E. L. Hove and J. O. Hardin, *J. Pharmacol. Exptl. Therap.*, **106**, 88-93 (1952).

¹³⁶ A. E. Milman and A. T. Milhorat, *Proc. Soc. Exptl. Biol. Med.*, **84**, 654-660 (1953).

¹³⁷ C. G. Mackenzie, *Experimental Muscular Dystrophy*, in R. M. Herriott, *Symposium on Nutrition*, Johns Hopkins Press, Baltimore, 1953, pp. 136-178.

¹³⁸ T. Itai, *J. Physiol. Soc. Japan*, **15**, 564-569 (1953); *Chem. Abst.*, **48**, 5318 (1948).

co-workers¹³⁹ considered that the maintenance of a high level of creatine and of phosphocreatine in the heart muscle of patients who have suffered from cardiac disturbances is evidence that complete recovery has taken place. In this case there would be an adequate supply of phosphocreatine as a source of potential energy for the contraction of the heart muscle, and possibly the likelihood of resisting future coronary stresses and strains would be increased. Phosphocreatine levels in the cortex of the kidney of the rat,¹⁴⁰ and in the skeletal muscle of the calf,¹⁴¹ have been found to be reduced by vitamin E deficiency.

Harris and Mason¹⁴² found that, in dystrophic children, the degree of creatinuria increased with advancing age, and that there was no appreciable change in the excretion of creatinine. In normal children of comparable ages, a slight decrease in creatinuria and a marked increase in creatine excretion occurred. Thus, the ratio of creatine to creatinine excreted in the urine is an effective criterion for expressing this response, since children with muscular dystrophy exhibit an increasing ratio with age, whereas the ratio for normal children actually decreases.

(c) *Myoglobinuria*. The attacks of paroxysmal myoglobinuria which occur during progressive muscular dystrophy are definitely relieved, and the excessive myoglobin excretion in the urine is completely inhibited, when α -tocopherol is given.¹⁴³ In experimental nutritional muscular dystrophy in the rabbit, the turnover rate of myoglobin is markedly increased.^{144,145} Blaxter¹⁴¹ likewise observed that, in calves suffering from muscular degeneration, a marked decrease in myoglobin and in the contractile proteins obtains.

(d) *Muscle Proteins*. Marked alterations in the levels of muscle proteins occur in vitamin E-deficiency. Thus, a number of different groups of investigators¹⁴⁶⁻¹⁴⁹ have confirmed the fact that there is a definite decrease in the myosin content of vitamin E-deficient rabbits, associated with a

¹³⁹ D. C. Sutton, G. C. Sutton, G. F. Hinkens, W. B. Buckingham, and R. Rondinelli, *Amer. Heart J.*, **47**, 67-76 (1954).

¹⁴⁰ J. Frey, *Nawyn-Schmiedleberg's Arch. exptl. Pathol. Pharmacol.*, **221**, 482-488 (1954).

¹⁴¹ K. L. Blaxter, *Vet. Record*, **65**, 835-838 (1953).

¹⁴² P. L. Harris and K. E. Mason, *Am. J. Clin. Nutrition*, **4**, 402-407 (1956).

¹⁴³ D. Acheson and D. McAlpine, *Lancet* (265), 1953, II, 372-375.

¹⁴⁴ I. Indovina, *Am. J. Physiol.*, **165**, 352-355 (1951).

¹⁴⁵ I. Indovina, *Boll. soc. ital. biol. sper.*, **27**, 91-92 (1951).

¹⁴⁶ M. Aloisi, A. Ascenzi, and E. Bonetti, *Experientia*, **8**, 266-267 (1952).

¹⁴⁷ M. Aloisi, A. Ascenzi, and E. Bonetti, *J. Pathol. Bacteriol.*, **64**, 321-327 (1952).

¹⁴⁸ E. Bonetti, M. Aloisi, and P. Merucci, *Experientia*, **8**, 69-71 (1952).

¹⁴⁹ G. Feuer and A. Frigyes, *Acta Physiol. Acad. Sci. Hung.*, **3**, 1-13 (1952); *Chem. Abst.*, **47**, 4976 (1953).

reduction in adenosine triphosphate (ATP), and with an augmentation of adenosine triphosphatase.¹⁴⁹ Rumery and co-workers¹⁵⁰ observed a marked loss of actomyosin in the muscles of weanling rats in "late-lactation" paralysis, with a concomitant increase in the proportion of insoluble protein, and with a simultaneous loss of protein nitrogen and of non-protein nitrogen as paralysis developed. This occurred between the ages of eighteen and twenty-five days. When spontaneous recovery ensued, these abnormalities were corrected by the time the rats were thirty days old, even though a deficiency sometimes persisted. Essentially parallel changes have been noted in the hamster suffering from muscular dystrophy, as well as during its repair when α -tocopherol was administered.¹⁵¹ These changes were followed in great detail histologically, by West and Mason.¹⁵¹⁻¹⁵³ One is led to the conclusion that α -tocopherol in some way, either directly or indirectly, exerts a control over the protein synthesis in the muscle cell. Milman and her co-workers¹⁵⁴ observed that, in vitamin E-deficient hamsters, serum protein was increased (presumably as a result of the protein lost from the muscles), and that the rate of release of amino nitrogen was curtailed in tissue slices.

(e) *Allantoin*. Allantoin, which is the normal end-product of purine metabolism in animals lower in the scale than man and monkey, also appears to be under the control of α -tocopherol. Thus, Young and Dinning¹⁵⁵ proved that the excretion of allantoin increased in rabbits on a vitamin E-deficient diet prior to the onset of the muscular dystrophy. An increased excretion of allantoin was found to occur concomitantly with an augmented concentration of nucleic acids in the skeletal muscles. These data were interpreted as evidence that the increased elimination of allantoin was the result of an accelerated turnover rate of tissue nucleic acids,¹⁵⁵ rather than of a loss of nucleic acids from the muscle. Subsequently, Dinning *et al.*^{156,157} proved this hypothesis by the use of C¹⁴-labeled sodium formate, which was administered to rats and rabbits which

¹⁵⁰ R. E. Rumery, S. I. Mauer, and K. E. Mason, *J. Exptl. Zool.*, **129**, 495-503 (1955).

¹⁵¹ W. T. West and K. E. Mason, *Muscle Lesions in Vitamin E-Deficient Hamsters and Their Response to Antidystrophic Compounds. Abst. Report Third Intern. Congr. on Vitamin E*, Venice, 1955, Vol. I, p. 21.

¹⁵² W. T. West and K. E. Mason, *Am. J. Physical Med.*, **34**, 223-239 (1955).

¹⁵³ K. E. Mason and W. T. West, *Anat. Rec.*, **118**, 327-328 (1954).

¹⁵⁴ A. E. Milman, D. N. Silides, and A. T. Milhorat, *Proc. Soc. Exptl. Biol. Med.*, **83**, 637-638 (1953).

¹⁵⁵ J. M. Young and J. S. Dinning, *J. Biol. Chem.*, **193**, 743-747 (1951).

¹⁵⁶ J. S. Dinning, *Vitamin E and Nucleic Acid Metabolism, Abst. Report Third Intern. Congr. on Vitamin E*, Venice, 1955, Vol. I, p. 50.

¹⁵⁷ J. S. Dinning, *J. Biol. Chem.*, **212**, 735-739 (1955); J. S. Dinning, J. T. Sime, and P. L. Day, *Ibid.*, **217**, 205-211 (1955).

had been on diets free from vitamin E, or containing vitamin E, respectively, for the preceding five months. The incorporation of radiocarbon into the ribonucleic acid and desoxyribonucleic acid fractions was considerably greater in the tissues of the vitamin E-deprived rats than it was in the controls.¹⁵⁷ When 1 mg. of α -tocopheryl phosphate was administered to the vitamin E-deficient rats one hour before the C¹⁴-formate was given, the rate of incorporation of labeled carbon into the nucleic acids was significantly reduced. It was also found^{157a} that vitamin E deficiency in the rabbit resulted in a marked increase in the incorporation of radioactive phosphorus (P³²) into tissue nucleic acids. Even greater differences in the nucleic acid synthesis of skeletal muscle and bone marrow characterized vitamin E-deficient and vitamin E-fed rabbits.⁶² It is believed by Dinning¹⁵⁶ and by Dinning *et al.*^{157a} that α -tocopherol regulates the synthesis and degradation of nucleic acid as one of its primary functions.

b. Carbohydrate Metabolism. (a) *Pyruvic Acid Formation.* Bellotti and Mezzano¹⁵⁸ called attention to the fact that the concentration of pyruvic acid is decreased in the blood and liver of vitamin E-deficient rats. Since this intermediate substance is so intimately bound up with carbohydrate metabolism, it is evident that tocopherol must play a role in normal carbohydrate breakdown. However, it is not known which of the metabolic pathways is regulated by tocopherol.

(b) *Action on Cytochrome c Reductase.* Houchin¹⁵⁹ was the first to postulate that tocopherol functions as an inhibitor of cytochrome *c* reduction in the metabolism of skeletal muscle. This hypothesis was based upon the increased rate of succinic acid oxidation in dystrophic muscle of the hamster,¹⁵⁹ and the depressing effect upon oxygen consumption.¹⁶⁰ However, the succinic dehydrogenase system is apparently not directly involved in nutritional muscular dystrophy.¹⁶¹ More recently, on the basis of additional data, Nason and Lehman¹⁶² have come to the conclusion that the tocopherols specifically stimulate cytochrome *c* reductase. The stimulating effect was noted in the case of the particulate matter from several sources following extraction with isooctane, but the tocopherol was ineffective in augmenting the action of pure cytochrome *c* reductase. It is therefore believed that there may be a lipid co-factor in the crude cytochrome *c* reductase which is specifically related to the tocopherols.

^{157a} J. S. Dinning, J. T. Sime, and P. L. Day, *J. Biol. Chem.*, **222**, 215-217 (1956).

¹⁵⁸ R. Bellotti and M. Mezzano, *Inform. med. Sez. clin. sci.*, **6**, 238-241 (1952); *Chem. Abst.*, **47**, 3944 (1953).

¹⁵⁹ O. B. Houchin, *J. Biol. Chem.*, **146**, 313-321 (1942).

¹⁶⁰ O. B. Houchin and H. H. Mattill, *J. Biol. Chem.*, **146**, 301-307, 309-312 (1942).

¹⁶¹ D. H. Basinski and J. P. Hummel, *J. Biol. Chem.*, **167**, 339-343 (1947).

¹⁶² A. Nason and I. R. Lehman, *Science*, **122**, 19-22 (1955).

(c) *Effect on the Synthesis of Phosphoglucomutase.* One may criticize the use of α -tocopheryl phosphate rather than free tocopherol in the alcohol form or the tocopherol-protein complexes which Ames and Risley¹⁶³ have shown are the naturally-occurring forms of vitamin E. However, when the tocopherol is introduced by feeding or injecting α -tocopherol phosphate into the animal before sacrifice, the isolated skeletal muscle of the normal rat still exhibits a depressed oxygen consumption. This defect in carbohydrate metabolism is believed to reflect a depressed rate of synthesis of phosphoglucomutase, which like other enzymes contains a protein moiety.⁶²

(d) *Effect on Glycogen and Blood Glucose.* When tocopherol was administered to normal rats or to animals made diabetic with alloxan, no effect on the respiratory quotient, liver or muscle glycogen could be observed, and the carbohydrate metabolism was not affected, except that an increase in heart glycogen occurred.¹⁶⁴ On the other hand, when tocopherol was given to fasting diabetic human subjects, blood sugar was reduced.¹⁶⁵ It was also noted that tocopherol acts synergistically with insulin in decreasing the glycemia.¹⁶⁵ Gounelle *et al.*¹⁶⁵ also found that rabbits made diabetic with alloxan were relieved by α -tocopherol. Toxicity was reduced, and the blood sugar levels were 50% lower.

The administration of α -tocopherol was reported to be effective in reducing the blood sugar level of normal dogs as well.¹⁶⁶ It is suggested by Gray and De Luca¹⁶⁶ that tocopherol acts under such conditions to speed up the equilibrium, "blood glucose $\rightleftharpoons$ tissue glycogen." It was also suggested by the latter investigators¹⁶⁶ that α -tocopherol has an insulin-like effect. This hypothesis was supported by the results of Butturini and Baronchelli,¹⁶⁷ who found that tocopherol administered with insulin increased its effect. However, Gray and De Luca¹⁶⁸ were later unable to obtain confirmation for this hypothesis in *in vitro* tests with diaphragm. On the other hand, Milman and collaborators¹⁶⁹ observed deficient deposition of administered glycogen in the muscle and liver of vitamin E-deficient rabbits, although glucose absorption and the blood glucose levels were

¹⁶³ S. R. Ames and H. A. Risley, *Ann. New York Acad. Sci.*, **52**, 149-155 (1949).

¹⁶⁴ G. La Grutta and A. Cilento, *Boll. soc. ital. biol. sper.*, **29**, 408-410, 1084-1085, 1573-1575, 1575-1578 (1953).

¹⁶⁵ H. Gounelle, C. Marnay, and H. Rabbii, *Presse méd.*, **62**, 888-890 (1954).

¹⁶⁶ D. E. Gray and H. A. De Luca, *Rev. can. biol.*, **11**, 64 (1952).

¹⁶⁷ U. Butturini and A. Baronchelli, *Giorn. clin. med. (Parma)*, **32**, 917-934 (1951); *Chem. Abst.*, **47**, 7677 (1953).

¹⁶⁸ D. E. Gray and H. A. De Luca, *Canad. J. Biochem. Physiol.*, **32**, 491-497 (1954).

¹⁶⁹ A. E. Milman, A. M. Treacy, and A. T. Milhorat, *Federation Proc.*, **13**, 100-101 (1954).

normal. Koch¹⁷⁰ likewise noted that the injection of α -tocopherol caused some increase in the liver glycogen of normal rats.

c. Lipid Metabolism. The interrelations between dietary fat and the tocopherols have been most widely recognized. The tocopherols originate in plants; they are concentrated primarily in the plant lipids, where they serve as fat antioxidants. In animals, the tocopherols, obtained from dietary sources, are distributed to all fat depots, as well as to cell cytoplasmic lipids; here again they function as antioxidants. However, it is not certain that this is their only function in the cells.

The tocopherols function widely as antioxidants of fat, not only *in vivo* but also *in vitro*. They protect the body from substances susceptible to oxidation from which harmful products may originate. Just how the tocopherols bring about this protection is a moot question. Tappel and associates¹⁷¹ suggest the possibility that antioxidants act by preventing a biradical formation. The hypothesis that they provide a mechanism to interrupt a chain reaction is also an attractive one.

Although the antioxidant activity of the tocopherols is widespread in reactions both *in vitro* and *in vivo*, there are certain types of *in vitro* effects which Tappel¹⁷²⁻¹⁷⁵ and Hove¹⁷⁶ independently concluded are more comparable to the *in vivo* changes under the specific control and regulation of vitamin E. Thus, the autoxidation of unsaturated fatty acids is inhibited by tocopherol,¹⁷³ but to only a small extent by methylene blue.^{62,174,176} In this case, tocopherol does not behave *in vitro* as it does *in vivo*. When the oxidation of oleic acid is catalyzed by cytochrome *c*, it can be counteracted not only by tocopherol but also by methylene blue. Thus, both of these antioxidants possess vitamin E biopotency. As an example of this behavior, Lea¹⁷⁷ demonstrated that the brown discolored fat, which is high in hemoglobin, in pigs fed diets of whale oil, can be protected from this accompanying peroxidation and from the oxidized odor by α -tocopherol, despite an increased deposition of unsaturated fatty acids in the adipose tissue.

The interrelationship between the intake of rancid fats and the tocopherol requirement has also been recognized for a long time. The rate of onset

¹⁷⁰ R. Koch, *Intern. Z. Vitaminforsch.*, **24**, 68-75 (1952).

¹⁷¹ A. L. Tappel, P. D. Boyer, and W. O. Lundberg, *J. Biol. Chem.*, **199**, 267-281 (1952).

¹⁷² A. L. Tappel, *Food Research*, **18**, 560-573 (1953).

¹⁷³ A. L. Tappel, *Arch. Biochem. Biophys.*, **44**, 378-395 (1953); **47**, 223-225 (1953).

¹⁷⁴ A. L. Tappel, *Arch. Biochem. Biophys.*, **50**, 473-485 (1954).

¹⁷⁵ A. L. Tappel, *Arch. Biochem. Biophys.*, **54**, 266-280 (1955).

¹⁷⁶ E. L. Hove, *J. Nutrition*, **50**, 361-371; **51**, 609-622 (1953).

¹⁷⁷ C. H. Lea, *Chemistry and Industry*, **1953**, 1303-1309.

of sterility symptoms, and the rapidity with which muscular dystrophy develops, are related to oxidative changes in fat accompanying the onset of rancidity in the unsaturated component of lard or of cod liver oil. Only fats having a considerable proportion of polyunsaturated acids and a minimum quantity of α -tocopherol, which therefore readily become rancid, can increase the rate of development of vitamin E-deficiency symptoms. Cormier¹⁷⁸ reported that the prolonged administration of fish liver oil resulted in serious lesions in the genitalia and adrenal glands of adult rats; the growth of immature animals was retarded. It is suggested that this may be due to the decrease in vitamin E in the organism resulting from the toxic effects of highly unsaturated fatty acids in the oil.

Harris and Mason⁶² have suggested three possible ways by which such highly unsaturated fats can influence the onset of deficiency symptoms: (1) Autoxidation of dietary fats may inactivate, to a greater or lesser extent, the tocopherols present in the food. (2) Tocopherols may be destroyed by similar reactions in the gastrointestinal tract prior to the absorption of the tocopherols. It is suggested that this interaction is more characteristic of herbivorous than of omnivorous or carnivorous animals. (3) When unsaturated fats are incorporated in the storage depots as well as in the cytoplasmic lipids throughout the tissues (especially in the phospholipids), the intracellular store of tocopherols may be depleted in attempting to effect an adequate stabilization of the lipid. On the other hand, an added stress may be placed on the cell which, without destroying the tocopherol, results in abnormalities of function or structure of the cell.

Blaxter¹⁷⁹ has considered some of the above questions in studies of nutritional muscular dystrophy in calves. This investigator reported that, when dystrophy was invoked by feeding a diet containing the polyethenoid fatty acids of cod liver oil, no significant reduction of the tocopherol content of the muscle cell or of the liver occurred. There was no destruction of vitamin E in the diet or in the gut. Moreover, when triorthocresylphosphate was given, the absorption of tocopherol was decreased, and a concomitant reduction in serum and liver tocopherol resulted.⁶² However, no symptoms of dystrophy were observed. It is suggested⁶² that some reversible system involving tocopherol facilitates the dissimilation of highly unsaturated acids or, on the other hand, may increase their incorporation into triglycerides.

(a) *Vitamin E Deficiencies Unrelated to Dietary Fat.* Harris and

¹⁷⁸ M. Cormier, *Bull. soc. chim. biol.*, **30**, 921-940 (1948).

¹⁷⁹ K. L. Blaxter, *Vitamin E and Muscular Degeneration in Calves. Abst. Report Third Intern. Congr. on Vitamin E*, Venice, 1955, Vol. I., p. 79.

Mason⁶² state that only four of the numerous deficiency symptoms of vitamin E are independent of the dietary fat of the animal. These include (1) testis degeneration in the rat and hamster, (2) fetal resorption in the rat, (3) nutritional muscular dystrophy in the rat, rabbit, and chick, and (4) necrotic liver degeneration in the rat. However, the presence of oxidizable fats in the diet does accelerate, to a variable degree, these several deficiency states. These conditions are discussed further in a later section of this chapter (see page 720).

(b) *Vitamin E Deficiencies Directly Related to Dietary Fat.* A number of deficiency conditions are ascribed to vitamin E deficiency which are dependent upon the presence of moderate amounts of oxidizable fat in the diet, and which are accelerated by the presence of larger amounts of these oxidizable fats.⁶² These include the following abnormal conditions: (1) accumulation of an acid-fast brown pigment in smooth, cardiac, and skeletal muscle, in the sex glands, in the adrenal glands and in the central nervous system of the rat; (2) late lactation paralysis in the rat; (3) depigmentation of incisor teeth of the rat; (4) increased susceptibility of red blood cells to hemolysis in the human and in other species; (5) encephalomalacia in chicks; and (6) exudative diathesis in chicks. These several conditions are discussed at some length in a later section (see page 725).

(c) *Vitamin E Deficiencies Requiring Excessively Large Amounts of Unsaturated Fats.* There is also a third set of conditions which are necessary before certain avitaminosis E symptoms appear. Thus, Harris and Mason⁶² reported that unphysiologically large amounts of highly unsaturated acids such as those contained in cod liver oil or linseed oil are prerequisites in the accompanying diet before these deficiencies can develop. This category includes: (1) brown pigmentation of adipose tissue in the mouse, rat, hamster, and pig; and (2) kidney autolysis in the rat. These conditions are described in detail in a later section (see page 713).

d. Mineral Metabolism. The relationship of vitamin E to mineral metabolism is somewhat more complicated than is that of the vitamin to the other foodstuffs. Harris and Mason⁶² do not commit themselves to a positive or negative view, although they appear to accept the hypothesis that vitamin E does play a role in mineral metabolism.

A number of observations can be interpreted as furnishing positive evidence that a relationship does exist between vitamin E and the metabolism of certain minerals. Burger and Hauge¹⁸⁰ reported that the vitamin E concentration of plants is directly related to the manganese status. It has

¹⁸⁰ O. J. Burger and S. M. Hauge, *Soil Sci.*, 72, 301-313 (1951).

been recorded by Maske and co-workers¹⁸¹ that the elevated level of zinc in the serum of dogs which occurs during alloxan diabetes is restored to normal by the administration of vitamin E. Lee and associates¹⁸² have described reproductive failure in mice induced by feeding a diet containing a combination of cobalt, copper, manganese, and zinc. This failure in reproduction was counteracted by the administration of vitamin E. The reason for the reversal of the symptoms by vitamin E is not entirely clear. It is probably similar to the muscle paralysis induced in mice when a similar salt mixture was employed in the diet.¹⁸³ On the other hand, Berzinš¹⁸⁴ reported a reverse effect. Thus, when cobalt salts were administered to pigs, the deposition of tocopherol in the tissues was increased.

There are likewise a number of reports in the literature which tend to show that vitamin E is related to the metabolism of calcium, sodium, potassium, and phosphate. Thus, in studies on muscle dystrophy in cattle, Blaxter and Wood¹⁸⁵ noted that the ash content of the muscle in the abnormal animals was greatly increased; calcium, sodium, and phosphate concentrations were increased, while that of potassium was reduced. Highman and Daft¹⁸⁶ observed a widespread calcification in arteries of the heart, kidneys, skeletal muscle, and lungs of mice fed diets deficient in vitamin E and protein. A decrease in the incidence of the muscle lesions occurred when cod liver oil was omitted from the diet, and an increased amount of tocopherol was administered simultaneously. Elevated calcium levels in the skeletal muscle, and increased calcium and phosphorus concentrations in the heart muscle have been noted in vitamin E-deficient lambs.¹⁸⁷ However, Cohen *et al.*¹⁸⁸ were unable to detect any influence of administered tocopherol on the rate of disappearance of radioactive sodium or phosphorus from the blood stream. Casuccio¹⁸⁹ noted that bone fractures in young, vitamin E-deficient rats exhibited a delayed callus formation, which did not occur in rats receiving adequate doses of vitamin E. Finally, there are two clinical reports which indicate that α -tocopherol

¹⁸¹ H. Maske, H. Wolff, B. Stampfl, and F. Baumgarten, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **216**, 457-472 (1952).

¹⁸² Y. C. P. Lee, J. T. King, and M. B. Visscher, *Am. J. Physiol.*, **173**, 456-458 (1953).

¹⁸³ J. T. King, Y. C. P. Lee, and M. B. Visscher, *Proc. Soc. Exptl. Biol. Med.*, **88**, 406-409 (1955).

¹⁸⁴ J. Berzinš, *Latvijas P S R Zinatnu Akad. Vestis*, **1951**, 415-420; *Chem. Abst.*, **47**, 12550 (1953).

¹⁸⁵ K. L. Blaxter and W. A. Wood, *Brit. J. Nutrition*, **6**, 144-163 (1952).

¹⁸⁶ B. Highman and F. S. Daft, *Am. J. Pathol.*, **29**, 582 (1953).

¹⁸⁷ F. A. Bacigalupo and R. W. Luecke, *J. Animal Sci.*, **13**, 245-248 (1954).

¹⁸⁸ J. A. Cohen, C. van der Meer, and M. G. P. J. Warringa, *Acta Physiol. et Pharmacol. Neerl.*, **2**, 270-275 (1951).

¹⁸⁹ C. Casuccio, *J. Bone and Joint Surg.*, **33 A**, 1035 (1951).

has an effect upon calcium metabolism. In the case of a patient with calcinosis associated with scleroderma, resorption of the calcified masses occurred after treatment with vitamin E.¹⁹⁰ Shute¹⁹¹ recorded two cases of *fragilitas ossium* in children which were benefited by the administration of vitamin E. These results could be interpreted as indicating a normalizing effect of tocopherol on bone metabolism. The evidence that vitamin E plays a role in the metabolism of certain minerals appears to be too widespread and convincing for this interrelationship to be denied. However, more experimental data are needed to further explain the relationship.

(3) *The Importance of Tocopherols in Metabolism as Influenced by Various Deficiency Symptoms*

a. Introduction. In the excellent 1954 review on the effects of a deficiency of vitamin E by Harris and co-workers in the Sebrell-Harris monograph on *The Vitamins*,⁶¹ a table is given summarizing the references which describe histopathologic changes following vitamin E deficiency in nineteen species of animals. These include those which occur in the reproductive system (testis degeneration or fetal resorption), those affecting the muscular system (classified according to skeletal, cardiac, and smooth), those in the nervous system, and finally those in the vascular system. These histopathologic lesions show a wide variation in character, and they represent morphologic alterations in completely unrelated tissues. According to Harris *et al.*,⁶¹ they cannot be proved to be related to dysfunction in any specific kind of cell or tissue. The nature of the abnormality is such that it can rarely be changed by tocopherol therapy, although the chemical and physiologic manifestations of the vitamin E deficiency may be corrected by such therapy. The species distribution of these lesions is included here in the discussion of each type of deficiency.

b. The Acid-Fast Pigment Associated with Vitamin E Deficiency. Martin and Moore¹⁹² were the first to describe the occurrence of an acid-fast pigment in uterine smooth muscle, skeletal muscle, and sex glands, as well as in other organs of rats maintained for long periods on a vitamin E-deficient diet. The pigment was characterized by its insolubility, its inertness, and by the fact that it was free from iron. According to Moore and Wang,^{193,194} it exhibits a brownish-yellow fluorescence when ex-

¹⁹⁰ H. A. Freund, *J. Michigan State Med. Soc.*, 50, 1233-1235 (1951).

¹⁹¹ W. E. Shute, *Summary*, 5, 1-2 (1953).

¹⁹² A. J. P. Martin and T. Moore, *J. Hyg.*, 39, 643-650 (1939).

¹⁹³ T. Moore and Y. L. Wang, *Biochem. J.*, 37, i (1943).

¹⁹⁴ T. Moore and Y. L. Wang, *Brit. J. Nutrition*, 1, 53-64 (1947).

posed to ultraviolet irradiation. The pigment has considerable resemblance to the so-called "ceroid" which was first characterized by Lillie *et al.*¹⁹⁵ It has been observed in association with nutritional cirrhosis in the rat. It is believed to originate^{196,197} through autoxidation of unsaturated lipids which have accumulated pathologically in the cells in which there is an insufficient supply of biologic antioxidants. Ceroid accumulates in animals fed on cirrhosis-producing diets when cod liver oil is given and vitamin E is deficient. Tocopherol supplements inhibit its formation.¹⁹⁸ Mason, Dam, and Granados¹⁹⁹ demonstrated that the accumulation of a ceroid-like pigment in the adipose tissue of rats on a low-vitamin E regimen, which is the closest morphologic counterpart to ceroid formation in infiltrated liver, occurs only when dienoic acids having at least eighteen carbons are present.²⁰⁰ Dam and Granados²⁰¹ are of the opinion that the presence of peroxides in the adipose tissue probably plays a role in pigment formation. Harris *et al.*⁶¹ calls attention to the fact that this acid-fast pigment accumulates in the same sites, and has many of the same characteristics, as the so-called "wear and tear" pigment normally found, according to Mason and Emmel,^{202,203} in the adrenal cortex, sex glands, and other tissues in the body. For this reason care should be exercised in relating this pigment formation solely to avitaminosis E.

c. The Role of Vitamin E in Reproduction. The discovery of vitamin E (α -tocopherol) was based upon its requirement for reproduction, and hence it was first referred to as the antisterility vitamin. It was soon recognized that vitamin E was necessary not only to prevent the resorption of the fetuses in the pregnant female, but also to prevent the degeneration of the testes in the male. While the effect of a vitamin E-deficiency in females is not a permanent one, and can be avoided in a subsequent pregnancy if sufficient vitamin E is available, the damage to the male is irreparable once it has occurred.

(a) *Testicular Degeneration in Avitaminosis E.* When young male rats are maintained on a vitamin E-free diet, no injury to the seminiferous epi-

¹⁹⁵ R. D. Lillie, L. L. Ashburn, W. H. Sebrell, F. S. Daft, and J. V. Lowry, *U. S. Pub. Health Repts.*, 57, 502-508 (1942).

¹⁹⁶ R. D. Lillie, *Stain Technol.*, 27, 37-45 (1952).

¹⁹⁷ W. G. B. Casselman, *J. Exptl. Med.*, 94, 549-562 (1951).

¹⁹⁸ J. Victor and A. M. Pappenheimer, *J. Exptl. Med.*, 82, 375-383 (1945).

¹⁹⁹ K. E. Mason, H. Dam, and H. Granados, *Anat. Record*, 94, 265-287 (1946).

²⁰⁰ L. J. Filer, Jr., R. E. Rumery, and K. E. Mason, *Trans. 1st Conf. on Biol. Antioxidants*, Josiah Macy, Jr., Foundation, New York, Oct., 1946, pp. 67-77.

²⁰¹ H. Dam and H. Granados, *Acta Physiol. Scand.*, 10, 162-171 (1945).

²⁰² K. E. Mason and A. F. Emmel, *Yale J. Biol. Med.*, 17, 189-202 (1944).

²⁰³ K. E. Mason and A. F. Emmel, *Anat. Record*, 92, 33-50 (1945).

thelium occurs until active spermatogenesis begins, that is, at about three months of age, after which a progressive and relatively rapid degeneration of the epithelium ensues. Although a number of investigators^{16, 61, 204-208} are in close agreement on the distinct character and the irreparable nature of the histopathologic changes, there is no satisfactory explanation of the nature of the underlying metabolic upset which is responsible for engendering this condition. Vitamin E therapy must be initiated within ten to fifteen days of the first appearance of injury to provide full protection. Mason²⁰⁹ reported that, when therapy is started later, certain seminiferous tubules may be protected, while progressive degeneration occurs in others. When vitamin E is withheld too long, degeneration of all of the seminiferous tubules may ensue. According to Kaunitz *et al.*,²¹⁰ as small an amount of tocopherol as 0.5 to 1.0 mg., when fed on the fifteenth day of life, will considerably delay postpubertal degeneration. However, Evans and Emerson²¹¹ noted that daily doses of 0.5 to 0.75 mg. of vitamin E are required to prevent testicular degeneration in rats reared for sixteen to seventeen months on vitamin E-low diets. The weight of the accessory sex glands is not affected by prolonged vitamin E deficiency;²¹¹ this indicates that the hormonal functions of these glands are not destroyed.⁶¹

Testicular degeneration has been noted following vitamin E deficiency in many other species, in addition to the rat.^{16, 204, 211} Strangely enough, the mouse is extremely resistant to this degeneration, and several investigators²¹²⁻²¹⁵ were unable to observe any injury to this tissue after deficiency periods of as long as fourteen months. However, Menschik and co-workers²¹⁶ did report marked atrophy of the germinal epithelium

²⁰⁴ K. E. Mason, *Am. J. Anat.*, **52**, 153-239 (1933).

²⁰⁵ A. Juhasz-Shäffer, *Virchow's Arch. pathol. Anat. u. Physiol.*, **281**, 3-34 (1931).

²⁰⁶ A. Juhasz-Shäffer, *Virchow's Arch. pathol. Anat. u. Physiol.*, **286**, 834-863 (1932).

²⁰⁷ A. Ringsted, Dissertation, *Undersøgelser over testis' histopathologi ved-E-avitaminose; en eksperimentelmorfologisk studie*. Nyt Nordisk Forlag, Copenhagen, 1936; cited by R. S. Harris, H. A. Mattill, and K. E. Mason, *The Tocopherols*, in W. H. Sebrell, Jr. and R. S. Harris, *The Vitamins*, Vol. III, Acad. Press, 1954, p. 517.

²⁰⁸ C. Engel and L. H. Britschneider, *Z. Vitaminforsch.*, **13**, 58-77 (1943).

²⁰⁹ K. E. Mason, *Am. J. Physiol.*, **131**, 268-280 (1940).

²¹⁰ H. Kaunitz, A. M. Pappenheimer, and C. Schogoleff, *Am. J. Pathol.*, **20**, 247-257 (1944).

²¹¹ H. M. Evans and G. A. Emerson, *J. Nutrition*, **26**, 555-567 (1943).

²¹² W. L. Bryan and K. E. Mason, *Am. J. Physiol.*, **131**, 263-267 (1940).

²¹³ M. Goettsch, *J. Nutrition*, **23**, 513-523 (1942).

²¹⁴ A. M. Pappenheimer, *Am. J. Pathol.*, **18**, 169-181 (1942).

²¹⁵ C. E. Tobin, *Arch. Pathol.*, **50**, 385-392 (1950).

²¹⁶ Z. Menschik, M. K. Munk, T. Rogalski, O. Rymaszewski, and T. J. Szczesniak, *Ann. New York Acad. Sci.*, **52**, 94-103 (1949).

after subjecting the animals to a vitamin E-free diet for eighteen months. In the case of the rabbit,^{217,218} the dog,¹¹⁵ and the monkey,²¹⁹ testicular damage comparable to that in the rat has been observed. Adamstone *et al.*²²⁰ reported that the germinal epithelium of young pigs is sensitive to vitamin E deficiency. In the case of the hamster,²¹⁹ the degeneration of the testicular tissue takes place much more slowly than in that of the rat, and for a considerable period of time the germinal epithelium can be restored by vitamin E therapy. The guinea pig²²¹ and the cockerel^{20,222-224} are susceptible to vitamin E deficiency. A most unusual finding is that of Cumings,^{225,226} who discovered that the testis of the male guppy fish (*Lebistes reticulatus*) also requires vitamin E. Mason²¹⁹ noted that the cotton rat (*Sigmodon hispidus*) has a resistance to injury from vitamin E similar to that of the mouse. Marked muscular dystrophy was observed to develop in this species without degeneration of the testis having occurred.

(b) *Fetal Resorption in Avitaminosis E.* The histopathologic investigations of the difficulty in reproduction characteristic of the female rat on a vitamin E-free diet, and the very rapid recovery of the survivors, were first described in the classical monograph of Evans and Burr³⁴ in 1927. A later and more complete description of this condition is given by Uerner,²²⁷ who compiled the facts on reproduction in the vitamin E-deficient albino rat (*Rattus norvegicus albinus*).

When young adult females are subjected to a vitamin E-free regimen, they are still able to conceive normally. All events proceed according to schedule up to the time of the implantation of the ovum, which usually occurs in the rat on the seventh day after insemination. Fetal development begins to slow down, the hemopoietic activity of the yolk sac and of the liver is decreased, and a rarefaction of the allantois and of the mesen-

²¹⁷ M. L. Chevrel and M. Cormier, *Compt. rend.*, 226, 1854-1855 (1948).

²¹⁸ M. L. Chevrel-Bodin and M. Cormier, *Ann. endocrinol. (Paris)*, 10, 19-30 (1949).

²¹⁹ K. E. Mason, Unpublished studies cited by R. S. Harris *et al.*, *The Tocopherols*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Academic Press, Inc., New York, 1954, Vol. III, pp. 481-573.

²²⁰ F. B. Adamstone, J. L. Krider, and M. F. James, *Ann. New York Acad. Sci.*, 52, 260-268 (1949).

²²¹ A. M. Pappenheimer and C. Schogoleff, *Am. J. Pathol.*, 20, 239-245 (1944).

²²² F. B. Adamstone and L. E. Card, *J. Morphol.*, 56, 325-337 (1934).

²²³ F. B. Adamstone, *Anat. Record*, 84, Abst., 499 (1942).

²²⁴ E. H. Herrick, I. M. Eide, and M. R. Snow, *Proc. Soc. Exptl. Biol. Med.*, 79, 441-444 (1952).

²²⁵ H. W. Cumings, Jr., Dissertation, University of Illinois (1940), cited by R. S. Harris *et al.*, *The Tocopherols*, in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Acad. Press, New York, 1954, Vol. III, pp. 481-573, p. 519.

²²⁶ H. W. Cumings, Jr., *Anat. Record*, 84, Abst., 499-500 (1942).

²²⁷ J. A. Uerner, *Anat. Record*, 50, 175-187 (1931).

chymal tissues of the embryo proper develops. The vascular relationship between the fetal and maternal components of the placenta becomes impaired, resulting in asphyxia, starvation, and death of the fetuses. Rapid necrosis and resorption of the fetuses occurs almost immediately, followed by a gradual regression of the placenta until only a blood clot remains at term.

When vitamin E is administered to the pregnant rat at any time during the first trimester (seven days), fetal resorption can be prevented, regardless of the nature of the earlier diet. When the dosage of tocopherol is critical, delivery of dead as well as of viable fetuses occurs, and none survives more than a few days. When the dosages of α -tocopherol are inadequate, fetal death and resorption occur later than the twelfth day. Mason^{228,229} observed that, under such conditions, fetuses exhibit changes in the vascular system on about the sixteenth day of gestation, which involve stasis, vasodilation, congestion, and local hemorrhages; there is a general ischemia prior to death.

When female rats are maintained on a low-vitamin E intake after the early months of life, a progressive increase in the vitamin E requirements for successful pregnancies ensues. However, the female is able to complete a satisfactory pregnancy when adequate amounts of vitamin E are available, even if fetal resorption has occurred in the earlier pregnancies as a result of an inadequate intake of vitamin E. With increasing age, there is a progressive interference with implantation of the ovum.²³⁰⁻²³² Harris and co-workers⁶¹ agree with the opinion of Blandau *et al.*²³³ that the dysfunction in avitaminosis E is uterine rather than ovarian.

Fetal death and resorption occur in a number of different species when the vitamin E intake is too restricted. The results in the mouse^{212,213} and those in the hamster²¹⁹ are quite comparable to those in rats. Suomalainen²³⁴ attributed the results in female mice to an impaired production of histotrophe. Pappenheimer and Goettsch,²³⁵ who observed resorption in the case of guinea pigs, considered the condition to be due to necrosis of

²²⁸ K. E. Mason, *Yale J. Biol. Med.*, **14**, 605-617 (1942).

²²⁹ K. E. Mason, *A Hemorrhagic State in the Vitamin E-Deficient Fetus of the Rat, Essays in Biology* (in honor of H. M. Evans), Univ. of California Press (1943), pp. 401-407.

²³⁰ G. A. Emerson and H. M. Evans, *J. Nutrition*, **18**, 501-506 (1939).

²³¹ H. Kaunitz and C. A. Slanetz, *Proc. Soc. Exptl. Biol. Med.*, **66**, 334-337 (1947).

²³² H. Kaunitz and C. A. Slanetz, *J. Nutrition*, **86**, 331-338 (1948).

²³³ R. J. Blandau, H. Kaunitz, and C. A. Slanetz, *J. Nutrition*, **38**, 97-104 (1949).

²³⁴ P. Suomalainen, *Nature*, **165**, 364 (1950).

²³⁵ A. M. Pappenheimer and M. Goettsch, *Proc. Soc. Exptl. Biol. Med.*, **47**, 268-270 (1941).

the placenta and fetal death. Ingelman-Sundberg²³⁶ suggested that it can be explained as an abortion due to the premature separation of the placenta.

When vitamin E is lacking, fetal death occurs in the incubating hen's egg on about the fourth day. Adamstone^{18,237} considers this to be the result of disintegration of the blood vessels of the blastoderm, and hemorrhage into the coelom and exocoelom, followed by cellular proliferations in the blastoderm, which cause an interruption in vitelline circulation. If this critical period does not develop in the embryo, a spontaneous rupture of the vascular vessels at different sites within the embryo may take place later.

d. The Role of Vitamin E in Relation to Skeletal Muscle. The role of vitamin E in muscle metabolism is the most widely recognized of any of its functions.^{152,238} The effects of an avitaminosis E on muscle metabolism and morphology have been reported in nineteen of the species investigated,⁶¹ and no instances are recorded in which the requirement is not evident. Although the histopathologic patterns of skeletal muscle, subject to nutritional muscular dystrophy due to lack of sufficient vitamin E, vary from species to species, and, in fact, even in the same species at different ages, two types of abnormality are quite consistent in the various animals, namely an acute type of reaction in young animals (as exemplified by late-lactation paralysis) and a chronic type in older animals (as seen in nutritional muscular dystrophy). These histologic deviations from the normal muscle structure undoubtedly reflect a vitally important function of tocopherol, but the nature of this metabolic effect is not understood. It occurs in the young weanling rat as "late-lactation" paralysis due to the inadequate storage of α -tocopherol. This results from the fact that the combined placental and mammary transfer of vitamin E is so small that it is barely able to satisfy the ordinary needs of the growing mammal. Deficiency is likely to occur during late lactation, both because of the rapid growth in this period, and because of the decreasing concentration of vitamin E present in the milk as lactation progresses.

(a) *Late-Lactation Paralysis.* Evans and Burr¹⁷ first called attention to the generalized paralysis in suckling rats, usually occurring between the eighteenth and the twenty-fifth days of life. The onset of the paralysis is usually quite sudden. This involves a flexor spasm of the forepaws,

²³⁶ A. Ingelman-Sundberg, *Acta Endocrinol.*, **2**, 335-346 (1949).

²³⁷ F. B. Adamstone, *Arch. Pathol.*, **31**, 622-626 (1941).

²³⁸ W. T. West and K. E. Mason, *Proc. Third Med. Conf. Muscular Dystrophy*, Musc. Dyst. Assoc., New York, 1954, pp. 223-239.

weakness, and dragging of the extremities; the animals are unable to regain their posture when placed on their backs. Death follows a diminution of body temperature and of respiration.²³⁹ In some cases the animals recover when the deficiency symptoms are mild. When vitamin E is given as late as the fifteenth day of the lactation period, the occurrence of the deficiency symptoms can be prevented. However, vitamin E therapy is ineffective once the symptoms have appeared. According to Evans,^{17,239} spontaneous recovery sometimes occurs, with a retention of the paralysis. Lipshutz²⁴⁰ and Gutiérrez-Mahoney²⁴¹ are of the opinion that lesions of the brain and spinal cord develop preceding the muscle abnormality. It is believed that the dystrophic process is purely of myogenic origin; thus, when the nerve supplying the muscle is resected prior to the seventeenth day, the incidence of dystrophy is greatly reduced.^{242,243} When death occurs, it is usually attributable to paralysis of the respiratory muscles, but Harris *et al.*⁶¹ are of the opinion that other metabolic dysfunctions may be involved. There are a number of excellent treatises on the muscular symptoms in this condition, including the article by Olcott,²⁴⁴ by Telford and co-workers,²⁴⁵ by Pappenheimer,^{242,243,246,247} by Harris *et al.*,⁶¹ and that by Mason *et al.*,²⁴⁸ to which the reader is referred for more detailed data.

Similar muscle changes, together with the external symptoms, have been observed in a number of species, including newborn rabbits,²⁴⁷ prepubertal mice,²¹⁴ young pigs,²²⁰ young lambs with "stiff-lamb" disease,²⁴⁹⁻²⁵² young calves with "white muscle disease";²⁵³ a comparable condition has

²³⁹ H. M. Evans, *J. Mt. Sinai Hosp. (New York)*, 6, 233-244 (1940).

²⁴⁰ M. D. Lipshutz, *Rev. Neurol.*, 65, 221-233 (1936).

²⁴¹ W. de Gutiérrez-Mahoney, *Southern Med. J.*, 34, 389-394 (1941).

²⁴² A. M. Pappenheimer, *J. Mt. Sinai Hosp. (New York)*, 7, 65-76 (1940).

²⁴³ A. M. Pappenheimer, *Physiol. Revs.*, 23, 37-50 (1943).

²⁴⁴ H. S. Olcott, *J. Nutrition*, 15, 221-227 (1938).

²⁴⁵ I. R. Telford, G. A. Emerson, and H. M. Evans, *Proc. Soc. Exptl. Biol. Med.*, 41, 291-295 (1939); 45, 135-136 (1940).

²⁴⁶ A. M. Pappenheimer, *Am. J. Pathol.*, 15, 179-184 (1939).

²⁴⁷ A. M. Pappenheimer, *On Certain Aspects of Vitamin E Deficiency, American Lecture Series No. 17*, Charles C Thomas, Springfield, Ill., 1948, pp. 3-33, p. 22.

²⁴⁸ K. E. Mason, M. Y. Dju, and S. J. Chapin, *Proc. 1st. and 2nd Med. Conf.* (1951-1952), *Muscular Dystrophy Assocs. Amer.*, New York, pp. 94-100.

²⁴⁹ J. P. Willman, S. A. Asdell, and P. Olafson, *Cornell Univ. Agr. Expt. Sta. Bull.*, 603, 3-20 (1934).

²⁵⁰ J. P. Willman, J. K. Loosli, S. A. Asdell, F. B. Morrison, and P. Olafson, *J. Animal Sci.*, 4, 128-132 (1945).

²⁵¹ J. P. Willman, J. K. Loosli, S. A. Asdell, F. B. Morrison, and J. P. Olafson, *Cornell Vet.*, 36, 200-204 (1946).

²⁵² H. H. Draper, M. F. James, and B. C. Johnson, *J. Nutrition*, 47, 583-597 (1952).

²⁵³ L. R. Vawter and E. Records, *J. Am. Vet. Med. Assoc.*, 110, 152-157 (1947).

been noted in the foal.²⁵⁴ Other young animals which are susceptible to this deficiency are puppies,²⁵⁵ mink,²⁵⁶ kids,^{257,258} chicks,²⁵⁹ and ducklings.²⁶⁰ The guppy fish has also been found to develop similar deficiency symptoms.^{225,226} Weanling rats on a diet low in vitamin E but exhibiting no paralysis, and rats which have recovered spontaneously from paralysis, usually present considerable muscle damage on histologic examination.^{242,243,245}

In a study of late-lactation paralysis, which can be produced experimentally in the calf, and which compares with the so-called "white muscle" disease of this species, Blaxter and associates^{185,261-264} considered the various factors which influence the development of this condition. It was found to be accentuated by adding cod liver oil to the diet, and it was prevented by treatment with α -tocopherol. This is also true for other herbivora such as the guinea pig, rabbit, and goat. For a detailed review, the reader is referred to the paper of Blaxter and Brown.²⁶⁵

(b) *Adult or Chronic Muscle Dystrophy.* Goettsch and Pappenheimer²³ were the first to produce nutritional dystrophy experimentally, and to recognize the nature of the disorder. They used guinea pigs and rabbits as their experimental animals. These species, in common with other herbivorous animals, are especially sensitive to this condition. It was not until ten years later that the Mackenzies and McCollum²⁶⁶⁻²⁶⁹ es-

²⁵⁴ T. C. Jones and W. O. Reed, *J. Am. Vet. Med. Assoc.*, **113**, 170-175 (1948).

²⁵⁵ H. D. Anderson, C. A. Elvehjem, and J. E. Gonce, Jr., *Proc. Soc. Exptl. Biol. Med.*, **42**, 750-755 (1939).

²⁵⁶ K. E. Mason and G. R. Hartsough, *J. Am. Vet. Med. Assoc.*, **119**, 72-75 (1951).

²⁵⁷ L. L. Madsen, C. M. McCay, and L. A. Maynard, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1434-1438 (1933).

²⁵⁸ L. L. Madsen, C. M. McCay, and L. A. Maynard, *Cornell Univ. Agr. Expt. Sta. Mem.*, **178**, 3-53 (1935).

²⁵⁹ H. Dam, I. Prange, and E. Sondergaard, *Acta Pathol. Microbiol. Scand.*, **31**, 172-184 (1952).

²⁶⁰ A. M. Pappenheimer and M. Goettsch, *J. Exptl. Med.*, **59**, 35-42 (1934).

²⁶¹ K. L. Blaxter, P. S. Watts, and W. A. Wood, *Brit. J. Nutrition*, **6**, 125-144 (1952).

²⁶² A. M. MacDonald, K. L. Blaxter, P. S. Watts, and W. A. Wood, *Brit. J. Nutrition*, **6**, 164-169 (1952).

²⁶³ K. L. Blaxter, W. A. Wood, and A. M. MacDonald, *Brit. J. Nutrition*, **7**, 34-50 (1953).

²⁶⁴ K. L. Blaxter, F. Brown, and A. M. MacDonald, *Brit. J. Nutrition*, **7**, 105-123 (1953).

²⁶⁵ K. F. Blaxter and F. Brown, *Nutrition Abstr. & Revs.*, **22**, 1-21 (1952).

²⁶⁶ C. G. Mackenzie and E. V. McCollum, *Science*, **89**, 370-371 (1939).

²⁶⁷ C. G. Mackenzie and E. V. McCollum, *J. Nutrition*, **19**, 345-362 (1940).

²⁶⁸ C. G. Mackenzie, J. B. Mackenzie, and E. V. McCollum, *Science*, **94**, 216-217 (1941).

²⁶⁹ C. G. Mackenzie, J. B. Mackenzie, and E. V. McCollum, *J. Nutrition*, **21**, 225-234 (1941).

tablished the fact that vitamin E deficiency was the causative agent. Because of the ease with which muscular dystrophy can be induced in the rabbit by diet and cured by α -tocopherol, much of the experimental work has been carried out on this species.²⁷⁰ According to Harris *et al.*,⁶¹ the type of dystrophy observed in the rabbit is the acute or explosive type, which is exemplified by late-lactation paralysis, rather than the chronic type exhibited by the adult rat. However, it is believed that the basic alterations which occur in the muscle fibers are fundamentally similar in both types. Considerable experimental work on the subject of muscular dystrophy due to vitamin E deficiency has been carried out on the rat. The gross and microscopic details of dystrophy in young adults and in mature animals of this species have been recorded by Ringsted,²⁷¹ by Einarson and Ringsted,²⁷² Evans *et al.*,²⁷³ Knowlton and associates,²⁷⁴ Mackenzie and co-workers,²⁷⁵ Pappenheimer,²⁴⁶ Martin and Moore,¹⁹² Monnier,^{276,277} and by Mason and Emmel.²⁰³ The paresis is characterized by a waddling and slightly incoördinated gait.²⁷⁵ This is followed by straddling of the hind legs, inability to walk or to stand, hyperflexion of the toes, flabbiness and weakness of the muscles, involving the adductor thigh muscles, dragging of the hind legs, distinct ataxia, pronounced muscle atrophy, and deformation of the feet. The animals are hump-backed, unkempt, and frequently develop skin ulcerations. Once the paresis is well established, vitamin E therapy can only arrest it. The growth and well-being of the rat are improved by α -tocopherol, but the paralysis remains permanent.^{203,272,275} The result of vitamin E differs from that in the rabbit; in the latter species vitamin therapy results in the disappearance of the paralysis.²⁷⁰

In addition to the rabbit, guinea pig, and rat, the young mouse is also subject to nutritional muscular dystrophy,²¹⁴ as are also the hamster,²⁴⁸ the cotton rat,²¹⁹ the dog,¹¹⁵ and the monkey.²⁷⁸

²⁷⁰ C. G. Mackenzie, *Proc. Soc. Exptl. Biol. Med.*, **49**, 313-317 (1942).

²⁷¹ A. Ringsted, *Biochem. J.*, **29**, 788-795 (1935).

²⁷² L. Einarson and A. Ringsted, *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats*. Levin and Munksgaard, Copenhagen, 1938. Cited by L. Einarson, *Acta Psychiat. Neurol. Scand.*, Suppl. No. 78, 8-76 (1952).

²⁷³ H. M. Evans, G. A. Emerson, and I. R. Telford, *Proc. Soc. Exptl. Biol. Med.*, **38**, 625-627 (1938).

²⁷⁴ G. C. Knowlton, H. M. Hines, and K. M. Brinkhous, *Proc. Soc. Exptl. Biol. Med.*, **41**, 453-456 (1939); **42**, 804-809 (1939).

²⁷⁵ C. G. Mackenzie, J. B. Mackenzie, and E. V. McCollum, *Proc. Soc. Exptl. Biol. Med.*, **44**, 95-98 (1940).

²⁷⁶ M. Monnier, *Compt. rend. soc. phys. et hist. nat. (Genève)*, **57**, 252-257 (1940).

²⁷⁷ M. Monnier, *Z. Vitaminforsch.*, **11**, 235-258 (1941).

²⁷⁸ K. E. Mason and I. R. Telford, *Arch. Pathol.*, **43**, 363-373 (1947).

a'. The Involvement of the Nervous System in Chronic Muscular Dystrophy: Most authorities agree that the dystrophic muscle lesions are of myogenic rather than neurogenic origin. The early onset of the symptoms, their biochemical nature, and their ready response to vitamin E therapy, reported by Houchin and Mattill,^{159,160} lend support to this conclusion. However, in the case of the adult rat kept for some months on a low-vitamin E diet, Einarson and Ringsted²⁷² observed lesions in the dorsal columns, dorsal nerve roots, and ventral horns of the spinal cord which, they state, may partly account for the muscular changes. Monnier^{276,277} is in agreement with this viewpoint. Luttrell and Mason²⁷⁹ and Malamud *et al.*²⁸⁰ confirmed the demyelination and gliosis in the posterior columns and dorsal nerve roots, but not the changes in the ventral horn and pyramidal tracts.

Telford²⁸¹ noted a loss of motor and plastics, in the case of young rats, secondary to degeneration of the muscle fibers. On the other hand, these structures and the peripheral nerves have been reported to be normal in nutritional myodegeneration of ducklings,²⁸² guinea pigs,^{283,284} and young rats.²⁸⁰

b'. Changes in Muscle Enzyme Systems in Chronic Muscular Dystrophy: Since tocopherols are believed to act in general as inhibitors in most enzyme systems, it is easy to explain why an increased rate of succinic acid oxidation occurs in dystrophic hamster muscle, as shown by Houchin.¹⁵⁹ On the other hand, the succinic dehydrogenase system is apparently unaffected under the same conditions.¹⁶¹ Although there are general disturbances in the phosphorylation mechanism in muscular dystrophy, as reflected by a decreased creatine level^{160,285} and a reduction in adenosine triphosphatase activity,²⁸⁶ Rabinovitz and Boyer²⁸⁷ did not find any decrease in the level of oxidative phosphorylation of heart muscle of dystrophic rabbits. In the absence of vitamin E, cholinesterase is decreased in liver, brain and serum.²⁸⁸

²⁷⁹ C. N. Luttrell and K. E. Mason, *Ann. New York Acad. Sci.*, **52**, 113-120 (1949).

²⁸⁰ N. Malamud, M. M. Nelson, and H. M. Evans, *Ann. New York Acad. Sci.*, **52**, 135-138 (1949).

²⁸¹ I. R. Telford, *Anat. Record*, **81**, 171-181 (1941).

²⁸² A. M. Pappenheimer, M. Goettsch, and E. Jungherr, *Storrs Agr. Exptl. Sta. Bull.*, **229** (1939); *Chem. Abst.*, **33**, 9374 (1939).

²⁸³ W. M. Rogers, A. M. Pappenheimer, and M. Goettsch, *J. Exptl. Med.*, **54**, 167-169 (1931).

²⁸⁴ H. Chor and R. E. Dolkart, *Arch. Pathol.*, **27**, 497-509 (1939).

²⁸⁵ M. Goettsch and E. F. Brown, *J. Biol. Chem.*, **97**, 549-561 (1932).

²⁸⁶ J. P. Hummel, *J. Biol. Chem.*, **172**, 421-429 (1948).

²⁸⁷ M. Rabinovitz and P. D. Boyer, *Proc. Soc. Exptl. Biol. Med.*, **77**, 103-105 (1951).

²⁸⁸ H. Bloch, *Helv. Chim. Acta*, **25**, 793-797 (1942).

c'. Antidystrophic Agents: α -Tocopherol has long been recognized because of its ability to correct muscular dystrophy, and two derivatives also function in this capacity. Thus, α -tocopherylhydroquinone, which is believed to be an intermediate of α -tocopherol, is a potent antidystrophic compound, but has no antisterility activity.^{129,289} Mackenzie and Mackenzie¹²⁹ demonstrated that it undergoes little or no conversion to α -tocopherol *in vivo*. A second compound, also recognized as an intermediate of α -tocopherol and as closely related to α -tocopherylhydroquinone, namely, α -tocopherylquinone (or α -tocoquinone) has been found to be active as an antidystrophy agent; it possesses no antisterility effect.²⁸⁹ The hydroxyquinone of α -tocopherol was found by Wright and Drummond²⁹⁰ to be without biologic effect in the case of pregnant rats on a vitamin E-deficient diet. In addition to the results of the Mackenzie group, which were obtained on the rabbit, and which have been confirmed by Farber, Milman, and Milhorat²⁹¹ on this species, a similar antidystrophic effect is exerted by the hydroquinone or quinone, or both, in the case of the hamster¹⁵² and the calf.¹⁷⁹ Blaxter *et al.*²⁹² found that α -tocopherol acetate also prevented muscular dystrophy in the calf. It is suggested^{61,137} that α -tocopherol, the antisterility vitamin, may at the same time be a provitamin for other compounds which function as antidystrophic agents. For the structure of these compounds, see page 702. In addition to the tocopherol intermediates, methylene blue has been found to be effective in counteracting the effects of muscular dystrophy. For a summary of this interesting action of methylene blue and of other redox components, the reader is referred to page 740.

e. **The Role of Vitamin E in Relation to Smooth Muscle.** When rats are deprived of vitamin E for several months, organs containing smooth muscle develop a chestnut brown coloration which becomes darker as the deficiency progresses. This discoloration is due to the deposition of acid-fast pigment granules in the smooth muscle cells, and of macrophages in the

²⁸⁹ A. Issidorides and H. A. Mattill, *J. Biol. Chem.*, 188, 313-316 (1951).

²⁹⁰ M. D. Wright and J. C. Drummond, *Biochem. J.*, 34, 32-33 (1940).

²⁹¹ M. C. Farber, A. E. Milman, and A. T. Milhorat, *Z. physiol. Chem.*, 295, 318-323 (1953).

²⁹² K. L. Blaxter, F. Brown, W. A. Wood, and A. W. MacDonald, *Brit. J. Nutrition*, 7, 337-349 (1953).

myometrium.^{192,193,203,293-298} Similar but less intense coloration occurs in the smooth muscle of other genital tissues, especially that of the fallopian tubes, cervix, vagina, in the female, and that of the seminal vesicle, prostate, and vas deferens in the male. The smooth muscle of the ureter, trabeculae, and capsule of the spleen, liver, lymph-nodes, small intestine, bronchi, and uterine and pulmonary veins are likewise colored.^{192,203,293,295a,296}

The deposition of the pigment can be prevented if the females are ovariectomized before pregnancy, but it will occur even in operated animals if they are given estrogens.^{299,300} When the pigment is once established, it cannot be influenced to any extent by prolonged tocopherol therapy or by pregnancy, castration or hormone treatment.

Species appears to be the most important factor in the determination of the locus of the smooth muscle affected in vitamin E-deficiency. In contradistinction to the results on the rat which are listed above, Mason and Telford²⁷⁸ report that, in the monkey, the pigment changes are especially marked in the vascular smooth muscle, but that they also occur in the smooth musculature of the small intestine, gall-bladder, urinary bladder, and bronchi. In the case of hamsters, the lesions are largely limited to the urinary bladder, blood vessels, and the small intestine.²¹⁹ In the dog only the small intestine is involved, while in the cotton rat, no lesions of this nature were noted as a result of vitamin E deficiency.²¹⁹ Only a brownish discoloration is present in the uterus of the mouse.²¹⁶ In the fowl no pigmentation in the smooth muscle has been observed as a result of vitamin E deficiency. Turkey poultts exhibit patches of hyaline necrosis of the smooth muscle in the gizzard, together with inflammatory reactions and fibrosis.³⁰¹ Chicks exhibit a milder type of reaction.³⁰²

f. The Role of Vitamin E in Relation to Cardiac Muscle. Changes

²⁹³ W. Ruppel, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.*, **206**, 584-601 (1949).

²⁹⁴ W. Hessler, *Z. Vitaminforsch.*, **11**, 9-29 (1941).

²⁹⁵ M. M. O. Barrie, *Biochem. J.*, **32**, 2134-2137 (1938).

^{295a} A. J. P. Martin and T. Moore, *Chemistry & Industry*, **55**, 236 (1935); **57**, 973-974 (1938).

²⁹⁶ V. Demole, *Schweiz. med. Wochschr.*, **71**, 1251-1253 (1941).

²⁹⁷ J. C. Radice and M. L. Herraiz, *Ann. New York Acad. Sci.*, **52**, 126-128 (1949).

²⁹⁸ H. Elftman, H. Kaunitz, and C. A. Slanetz, *Ann. New York Acad. Sci.*, **52**, 72-79 (1949).

²⁹⁹ W. B. Atkinson, H. Kaunitz, and C. A. Slanetz, *Ann. New York Acad. Sci.*, **52**, 68-71 (1949).

³⁰⁰ W. B. Atkinson, H. Kaunitz, and C. A. Slanetz, *Federation Proc.*, **8**, 349-350 (1949).

³⁰¹ E. Jungherr and A. M. Pappenheimer, *Proc. Soc. Exptl. Biol. Med.*, **37**, 520-526 (1937).

³⁰² E. L. Jungherr, *Ann. New York Acad. Sci.*, **52**, 104-112 (1949).

closely akin to those observed in skeletal muscle as a result of vitamin E deficiency have been recorded in the case of cardiac muscle. These include hyaline necrosis and replacement fibrosis. A remarkable species variation is evident as regards the onset of the condition and its severity. In the case of herbivorous animals such as the rabbit, guinea pig, sheep, and cattle, the condition is characterized by a rapid onset without pigment deposition. It is frequently the cause of death due to myocardial failure. In sharp contradistinction to the herbivora, the laboratory animals (rat, hamster, cotton rat) may develop extensive focal necrosis and scarring of the myocardium, with increasing pigmentation, but they survive without serious complications for many months. The effect of vitamin E deficiency upon cardiac muscle is evident in the rabbit,^{257,258,303,304} guinea pig,^{257,258,305} young calf,^{185,261,262} cow,^{306,307} sheep,^{250,251,308} goat,^{257,258} rat,^{203,293,309,310} mouse,²¹⁵ hamster,²¹⁹ and cotton rat.²¹⁹

g. The Role of Vitamin E in the Vascular System. Vitamin E deficiency affects the vascular system under certain conditions; it may bring about profound abnormalities. The chick and other fowl are particularly susceptible to this deficiency, but some instances of vascular changes have been noted in mammals, as a result of an insufficiency in vitamin E. Exudative diathesis and nutritional encephalomalacia constitute the two principal disorders in the chick which result from an inadequate intake of vitamin E. Both of these conditions usually occur during the first two months of life, just as lesions of the skeletal muscle in the duck, the chick, and the pheasant,^{247,259,260,302} and the gizzard necrosis in the chick and the turkey,^{282,302} occur in the young birds. As discussed earlier, a lack of vitamin E in the egg may also result in mortality of the chick embryo¹⁸ about the fourth day of incubation, as a result of vascular difficulties.

(a) *Exudative Diathesis.* Dam and Glavind³¹¹ were the first to describe a symptom which could be produced in chicks on an artificial diet in which the protein had been thoroughly extracted with alcohol. This condition, known as exudative diathesis, is characterized by the presence of large

³⁰³ A. J. Gatz and O. B. Houchin, *Anat. Record*, **110**, 249-260 (1951).

³⁰⁴ J. H. Bragdon and H. D. Levine, *Am. J. Pathol.*, **25**, 265-271 (1949).

³⁰⁵ S. Americano Freire and B. Figueriredo Magalhães, *Rev. brasil. biol.*, **3**, 91-98 (1943).

³⁰⁶ T. W. Gullickson and C. E. Calverley, *Science*, **104**, 312-313 (1946).

³⁰⁷ T. W. Gullickson, *Ann. New York Acad. Sci.*, **52**, 256-259 (1949).

³⁰⁸ R. Culik, F. A. Bacigalupo, F. Thorp, Jr., R. W. Luecke, and R. H. Nelson, *J. Animal Sci.*, **10**, 1006-1016 (1951).

³⁰⁹ S. Americano Freire, *Brasil-méd.*, **55**, 308-310 (1941).

³¹⁰ G. J. Martin and F. B. Faust, *Exptl. Med. and Surg.*, **5**, 405-410 (1947).

³¹¹ H. Dam and J. Glavind, *Nature*, **142**, 1077-1078 (1938).

patches of subcutaneous edema on the breast and abdomen of the young chicks.^{312,313} It sometimes occurs on the neck, legs, or wings as well. There is a local accumulation of a plasma-like fluid which is tinged green by decomposed hemoglobin. The affected tissues present increased capillary permeability. Dam³¹⁴ observed that the subcutaneous tissue at the site of the lesions remained buff-colored for a long period after this condition had been cured. A more severe type of exudative diathesis which involves the brain and lungs as well as the heart and pericardium and other organs was described by Bird and Culton.³¹⁵ This severe form of the condition usually results in death. According to Dam,³¹⁴ exudative diathesis rarely occurs in chicks on purified vitamin E-free diets in the absence of dietary fats.

(b) *Nutritional Encephalomalacia*. This deficiency is characterized by a disorder of the nervous system. It has been described by Pappenheimer and co-workers,^{247,282,316,317} and also by Adamstone.^{318,319} The symptoms involved include motor incoördination, ataxia, head retraction and opisthotonos, tremors, prostration, with legs spastic and claws strongly flexed, somnolence, stupor, and death. Postmortem examination reveals blister-like swellings on the nerve-endings, flattening and irregular distortion of the cerebral convolutions; there is frequently a greenish-brown discoloration in the cerebellum, associated with hemorrhage and edema. In some cases, the discoloration also occurs in the cerebrum, midbrain, and even in the medulla. Not only can this condition be produced experimentally on vitamin E-low diets, but it frequently occurs spontaneously in commercial flocks; under such conditions, it has been referred to as the "crazy chick disease."^{282,302} Jungherr³⁰² demonstrated that this disease is associated with extensive fibrosis of the cerebellum. Large areas of increased vascularity are often present in other parts of the central nervous system, specifically the medulla, the midbrain, and the thalamus.³⁰²

Dam³¹⁴ stated that unsaturated fats in the diet increased the severity of both the exudative diathesis and encephalomalacia. It is possible, by varying the fats, to produce either condition, or both simultaneously. Considerable protection from the deficiency symptoms in chickens can be

³¹² H. Dam and H. Glavind, *Nature*, 143, 810-811 (1939).

³¹³ H. Dam and H. Glavind, *Skand. Arch. Physiol.*, 82, 299-316 (1939).

³¹⁴ H. Dam, *J. Nutrition*, 27, 193-211 (1944).

³¹⁵ H. R. Bird and T. G. Culton, *Proc. Soc. Exptl. Biol. Med.*, 44, 543-547 (1940).

³¹⁶ A. M. Pappenheimer and M. Goettsch, *J. Exptl. Med.*, 53, 11-26 (1931).

³¹⁷ A. Wolf and A. M. Pappenheimer, *J. Exptl. Med.*, 54, 399-405 (1931).

³¹⁸ F. B. Adamstone, *Arch. Pathol.*, 31, 603-612 (1941).

³¹⁹ F. B. Adamstone, *Arch. Pathol.*, 43, 301-312 (1947).

obtained from such compounds as xanthophyll,³²⁰ ascorbic acid,³²¹ or various redox systems,³²² which may act as antioxidants in the absence of tocopherol. Methylene blue, thionine, thiodiphenylamine, Antabuse, and nordihydroguaiaretic acid (NDGA) afforded considerable, although not complete, protection against the exudative diathesis.³²²

(c) *Hemorrhage in Mammals.* The vascular changes in rats which have been raised on diets low in vitamin E and high in the unsaturated fatty acids of cod liver oil somewhat resemble those which occur in the chick. The lesions in the rat also include interfascial, subcutaneous, and thymic hemorrhages.³²³ Elvehjem and co-workers³²⁴ also described vascular changes in puppies born of mothers receiving a mineralized milk diet low in vitamin E; in the latter case, hemorrhages into cranial and visceral cavities, the lungs, kidneys, and intestines were noted.

Holman^{325,326} observed that necrotizing arteritis in dogs, occurring as a consequence of induced renal insufficiency following a high-fat diet (cod liver oil), could be retarded or completely prevented by feeding tocopherol. Skelton and associates³²⁷ are of the opinion that vitamin E protects dogs from thrombocytopenic purpura induced by parenterally administered stilbestrol. However, Richtsmeier *et al.*³²⁸ concluded that α -tocopherol did not exert any antipurpuric effect in the hemorrhagic diathesis produced by stilbestrol. Tocopherols were found by Ames *et al.*³²⁹ to protect rats from an increased capillary fragility due to α -irradiation by means of radon ointment containing 90% α -particles, the remainder β - and γ -particles. This vitamin has likewise been shown to reduce the injuries due to x-irradiation,³³⁰ provided the dosage does not exceed 700 r. However, Haley *et al.*³³¹ observed that doses of water-soluble vitamin E as high as 2 to 3.18

³²⁰ P. Goldhaber, L. Zacharias, and V. E. Kinsey, *J. Nutrition*, **42**, 453-462 (1950).

³²¹ L. Zacharias, P. Goldhaber, and V. E. Kinsey, *J. Nutrition*, **42**, 359-373 (1950).

³²² H. Dam, I. Kruse, I. Prange, and E. Sndergaard, *Acta Physiol. Scand.*, **22**, 299-310 (1951).

³²³ R. E. Rumery, Thesis, *Histological and Biochemical Reactions of the Immature Rat to Diets Low in Tocopherols (Vitamin E) and High in Unsaturated Fatty Acids*. University of Rochester (N. Y.), 1952; cited by R. S. Harris, H. A. Mattill, and K. E. Mason, *The Tocopherols*, in W. H. Sebrell, Jr. and R. S. Harris, *The Vitamins*, Vol. III, Acad. Press, New York, 1954, p. 536.

³²⁴ C. A. Elvehjem, J. E. Gonce, Jr., and G. W. Newell, *J. Pediat.*, **24**, 436-441 (1944).

³²⁵ R. L. Holman, *Proc. Soc. Exptl. Biol. Med.*, **66**, 307-309 (1947).

³²⁶ R. L. Holman, *Southern Med. J.*, **42**, 108-114 (1949).

³²⁷ F. Skelton, E. Shute, H. G. Skinner, and R. A. Waud, *Science*, **103**, 762 (1946).

³²⁸ A. J. Richtsmeier, M. Spooner, and O. O. Meyer, *Proc. Soc. Exptl. Biol. Med.*, **65**, 298-301 (1947).

³²⁹ S. R. Ames, J. G. Baxter, and J. Q. Griffith, *Z. Vitamin-forsch.*, **22**, 401-407 (1951).

³³⁰ Z. M. Bacq, *Experientia*, **7**, 11-19 (1951).

³³¹ T. J. Haley, S. F. McCulloh, and W. G. McCormick, *Science*, **119**, 126-127 (1954).

mg. daily per animal increased the rate of mortality and the total mortality of x-irradiated mice.

(d) *Hemolysis*. György and Rose³³² were the first to demonstrate that hemoglobinuria associated with intravascular hemolysis occurred in alloxan-treated rats. Death usually followed. When tocopherols were included in the diet, the hemolysis disappeared, although the diabetic symptoms were not relieved.³³³ These investigators later found that dialuric acid, which is related to alloxan, and similar compounds (alloxantin, ninhydrin), also caused hemolysis of the red blood cells.^{334,335} α -Tocopherol is believed to prevent the red blood cells from injury by acting as an antioxidant, under which condition it probably counteracts a free radicle or peroxide formed as an intermediate in the oxidation/reduction system of dialuric acid and alloxan.^{334,335} Gordon and co-workers³³⁶ noted that, in the case of both premature and full-term infants, α -tocopherol reversed the susceptibility of the erythrocytes to hydrogen peroxide and consequent hemolysis. In children suffering from faulty fat absorption, a marked susceptibility of the erythrocytes to H_2O_2 was observed, which could be reversed by feeding D- α -tocopherol acetate. György *et al.*³³⁷ demonstrated that the blood cells of newborn infants were hemolyzed by dilute solutions of hydrogen peroxide *in vitro* as well as *in vivo*; this hemolysis could be prevented in either case by tocopherols. However, when mixed tocopherols were given to the mother in a large dose (500 mg. daily) during the last weeks of pregnancy, there was no alteration in the hemolysis reaction of the newborn. An increased tendency to hemolysis of the red blood cells when exposed to hydrogen peroxide was observed by Mason²¹⁹ in vitamin E-deficient monkeys, in premature infants,³³⁸ in full-term infants,³³⁹ and also in new born rats.³³⁹ Rose and György³⁴⁰ pointed out that there is a specific relationship between the vitamin E-activity of a substance and its ability to protect the blood cells of vitamin E-deficient rats from hemolysis as a result of dialuric acid. The most effective of the tocopherols in affording protection was found to be α -tocopherol.³⁴⁰ In spite of these effects of the tocopherols upon hemolysis, it is not believed that the vitamin

³³² P. György and C. S. Rose, *Science*, 108, 716-718 (1948).

³³³ C. S. Rose and P. György, *J. Nutrition*, 39, 529-541 (1949).

³³⁴ C. S. Rose and P. György, *Blood*, 5, 1062-1074 (1950).

³³⁵ P. György and C. S. Rose, *Ann. New York Acad. Sci.*, 52, 231-239 (1949).

³³⁶ H. H. Gordon, R. H. Davis, and K. Morganstein, *Federation Proc.*, 12, 415 (1953).

³³⁷ P. György, G. Cogan, and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.*, 81, 536-538 (1952).

³³⁸ H. H. Gordon and J. P. De Metry, *Proc. Soc. Exptl. Biol. Med.*, 79, 446-450 (1952).

³³⁹ P. György, G. M. Cogan, and C. S. Rose, *Am. J. Diseases Children*, 82, 237-238 (1951).

³⁴⁰ C. S. Rose and P. György, *Am. J. Physiol.*, 168, 414-420 (1952).

exerts any effect upon the hematopoietic functions of blood.⁶¹ Vitamin E does counteract the leucocytosis which occurs in vitamin E-deficient rabbits.³⁴¹ However, Dinning³⁴¹ does not consider this to be proof that the leucocytosis exhibited by rabbits suffering from nutritional muscular dystrophy is a direct result of vitamin E-deficiency.

h. The Role of Vitamin E in the Deposition of Adipose Tissue. When vitamin E is deficient in the diet over a prolonged period, the proportion of adipose tissue is decreased; this accounts for the plateau in the body weight occurring during early adult life, and also for the emaciated appearance of rats in advanced stages of vitamin E-deficiency. As a result of the study on mice, Menschik and co-workers^{216,342} concluded that vitamin E deficiency reduces the ability of the animal to deposit fat, both that ingested in the diet and that synthesized in the body.

When a large proportion of polyunsaturated acids are contained in the dietary fat fed to animals on vitamin E-deficient diets, a brownish discoloration of the subcutaneous and intraperitoneal adipose tissue occurs. This result was demonstrated by Mason *et al.*¹⁹⁹ when the diet contained 20% of cod liver oil, and by Granados, Mason, and Dam³⁴³ when the highly unsaturated fractions of cod liver oil were fed. Filer *et al.*³⁰⁰ likewise proved that the brown pigmentation results when the methyl esters of linseed, corn, and soybean oils are added to the diet. Presumably the changes do not occur with the corresponding unsaponified oils, because of their high tocopherol content. In the case of cod liver oil, the tocopherol content is too low to counteract these changes; therefore the pigmentation occurs both when the whole fat is given and when the highly unsaturated fractions are used. These fat depots, which presumably contain a considerable portion of highly unsaturated fats, have a large content of peroxides, which may be demonstrated both chemically²⁰¹ and by histochemical procedures.³⁴⁴⁻³⁴⁶ Although tocopherol therapy will inhibit further pigmentation, it does not appreciably decrease the coloration already existing in the tissues. This condition is widely distributed among different species, including the mouse,²¹⁵ the hamster,²¹⁹ the pig,^{347,348} and the mink^{256,349}

³⁴¹ J. S. Dinning, *Proc. Soc. Exptl. Biol. Med.*, **79**, 231-232 (1952).

³⁴² Z. Menschik, *Edinburgh Med. J.*, **51**, 486-489 (1944).

³⁴³ H. Granados, K. E. Mason, and H. Dam, *Acta Pathol. Microbiol. Scand.*, **24**, 86-95 (1947).

³⁴⁴ H. Granados and H. Dam, *Acta Pathol. Microbiol. Scand.*, **27**, 591-596 (1950).

³⁴⁵ J. Glavind, H. Granados, S. Hartmann, and H. Dam, *Experientia*, **5**, 84-85 (1949).

³⁴⁶ H. Dam, *Ann. New York Acad. Sci.*, **52**, 195-199 (1949).

³⁴⁷ K. L. Robinson and W. E. Coey, *Nature*, **168**, 997-998 (1951).

³⁴⁸ J. R. Gorham, N. Boe, and G. A. Baker, *Cornell Vet.*, **41**, 332-338 (1951).

³⁴⁹ J. R. Gorham, G. A. Baker, and N. Boe, *Vet. Med.*, **46**, 100-102 (1951).

("yellow fat disease" or "steatitis"). Harris *et al.*⁶¹ suggest that the best explanation for these phenomena is that the unsaturated dietary fats become incorporated into adipose tissue cells which do not have enough tocopherol to stabilize them or to counteract peroxide development; polymerization takes place, or a combination with cell proteins, or both, to produce acid-fast pigment.

According to Jessen *et al.*,³⁵⁰ peroxidation of human adipose tissue occurs in peripheral venous diseases in the case of vitamin E-deficient patients, as demonstrated by the low but positive peroxide values. In *ulcus cruris* (indolent ulcer of the leg), in which local changes in adipose tissue occur, vitamin E exerts a beneficial effect, according to Burgess and Pritchard.³⁵¹ For a further discussion of this pigment, the reader is referred to page 713.

i. The Role of Vitamin E in Liver Cirrhosis. Most types of liver damage are the result of the simultaneous operation of several factors, which may or may not include vitamin E. Menschik and co-workers^{216,352} reported the occurrence of a pathologic condition in the liver of mice after fourteen months on a high-fat diet low in vitamin E. Lipoproteic globules were formed which were believed to result from abnormal metabolic changes in the liver fat.

In the case of nutritional cirrhosis, which can be produced readily in rats fed diets lacking in the lipotropic factors, methionine or choline, the usual pathologic changes are more severe when the deficient diet is combined with a low vitamin E regimen. Under these conditions, the livers become extremely brownish yellow in color, and accumulations of ceroid pigment can be observed in the cells, on histologic examination.^{195,353,354} This pigment appears to be the same as the acid-fast pigment laid down in many tissues on a diet low in vitamin E. Although the tocopherols do not influence the fibrotic changes which result from the lack of lipotropic agents, they do retard or entirely prevent ceroid formation.¹⁹⁸ Highly unsaturated dietary fats accentuate the severity of the abnormality.³⁵⁵

A third type of liver injury involves a deficiency of vitamin E combined with a low cystine intake and a deficiency of the so-called "Factor 3."³⁵⁶ This abnormality occurs on certain diets low in vitamin E and lacking in

³⁵⁰ K. E. Jessen, J. Glavind, S. Hartmann, and H. Dam, *Acta Pathol. Microbiol. Scand.*, **29**, 73-76 (1951).

³⁵¹ J. F. Burgess and J. E. Pritchard, *J. Canad. Med. Ass. c.*, **59**, 242-247 (1948).

³⁵² Z. Menschik and T. J. Szczesniak, *Anat. Record*, **103**, 349-363 (1949).

³⁵³ P. György, *Am. J. Clin. Pathol.*, **14**, 67-88 (1944).

³⁵⁴ H. Popper, P. György, and H. Goldblatt, *Arch. Pathol.*, **37**, 161-168 (1944).

³⁵⁵ M. Wachstein, *Proc. Soc. Exptl. Biol. Med.*, **59**, 73-77 (1945).

³⁵⁶ K. Schwarz, *Proc. Soc. Exptl. Biol. Med.*, **77**, 818-823 (1951); **78**, 852-856 (1951).

the sulfur-containing amino acids,³⁵⁷⁻³⁶² or on diets containing certain yeasts,^{356, 362, 363} which are lacking in "Factor 3" as, for example, American torula yeast.³⁵⁶ In the absence of these several components, a sudden massive necrosis occurs in the liver of the rat, together with hemorrhage,^{356, 357, 361-363} or with lung hemorrhages³⁶⁰ which are the cause of death. Schwarz, Chernick, *et al.*³⁶⁴⁻³⁶⁶ described the necrotic liver degeneration which occurs several weeks after a metabolic lesion characterized by a rapid decline in oxygen consumption by liver slices after a thirty-minute period of normal respiration. This metabolic upset is believed to be due to an interference in oxidative phosphorylation in some way related to the Krebs cycle.³⁶⁴⁻³⁶⁶ Both the preliminary metabolic phase and the succeeding morphologic changes can be prevented by α -tocopherol, while the metabolic changes can be counteracted either by α -tocopherol or by α -tocopherylhydroquinone.

Hove¹⁷⁶ described a somewhat simpler form of liver injury, which occurs in rats on low-protein diets deficient in the sulfur-containing amino acids, when hepatotoxic agents such as triorthocresyl phosphate, carbon tetrachloride, or pyridine are given; this condition can be alleviated by increasing either the protein or the tocopherol intake. It is the opinion of Hove¹⁷⁶ that the hepatotoxic agents function as prooxidants in unsaturated lipid systems; this results in the formation of peroxides and in a reduction of the tocopherol in the tissues. If this hypothesis is correct, tocopherol would act by alleviating the state of tissue peroxidation. Sellers *et al.*³⁶⁷ suggest that tocopherol may protect —SH groups, so that they are available for detoxication reactions. Olson and co-workers³⁶⁸ postulate that

³⁵⁷ P. György and H. Goldblatt, *J. Exptl. Med.*, **89**, 245-268 (1949).

³⁵⁸ K. Schwarz, *Z. physiol. Chem.*, **281**, 101-108 (1944); **283**, 106-112 (1948).

³⁵⁹ K. Schwarz, *Ann. New York Acad. Sci.*, **52**, 225-230 (1949).

³⁶⁰ E. L. Hove, D. H. Copeland, and W. D. Salmon, *J. Nutrition*, **39**, 397-467 (1949).

³⁶¹ M. Goettsch, *J. Nutrition*, **44**, 443-454 (1951).

³⁶² O. Lindan and H. P. Himsworth, *Brit. J. Exptl. Pathol.*, **31**, 651-663 (1950).

³⁶³ P. György, C. S. Rose, R. M. Tomarelli, and H. Goldblatt, *J. Nutrition*, **41**, 265-278 (1950).

³⁶⁴ K. Schwarz, S. S. Chernick, C. P. Rodnan, J. G. Moe, and W. Mertz, *Dietary Necrotic Liver Degeneration: Occurrence of a Specific Defect Reversible by Intraportal Vitamin E*, *Abst. Reports Third Intern. Congr. on Vitamin E*, Venice, 1955, Vol. I, p. 30.

³⁶⁵ K. Schwarz, *Ann. New York Acad. Sci.*, **57**, 878-888 (1954).

³⁶⁶ S. S. Chernick, C. P. Rodnan, and K. Schwarz, *Federation Proc.*, **13**, 191 (1954); S. S. Chernick, J. G. Moe, and K. Schwarz, *Ibid.*, **14**, 191-192; G. P. Rodnan and K. Schwarz, 270 (1955).

³⁶⁷ E. A. Sellers, R. W. You, and C. C. Lucas, *Proc. Soc. Exptl. Biol. Med.*, **75**, 118-121 (1950).

³⁶⁸ R. E. Olson, C. S. Yang, M. Riegl, and B. Stewart, *Federation Proc.*, **14**, 447 (1955).

the tocopherol causes an enhancement in the synthesis or in the turnover rate of CoA.

D- α -Tocopherol was found by Hove³⁶⁹ to decrease markedly the toxicity of carbon tetrachloride for rats on a 10% casein, vitamin E-free diet. The α -form exhibited a much better protective action than did D- γ -tocopherol. Other compounds which exerted a similar or moderate protective action included theophylline (1,3-dimethylxanthine), xanthine, and guanine, while hypoxanthine and theobromine (3,7-dimethylxanthine) were inactive. DL-Methionine was likewise active, while negative results were obtained with glycine and arginine.

j. The Role of Vitamin E in Kidney Nephrosis. Martin and Moore¹⁹² were the first to call attention to the nephrotic syndrome which develops in the outer cortex of the kidneys of rats receiving a low-vitamin E diet, after three or four months of deficiency, and which becomes generalized after ten months. Although the glomeruli show no changes, the tubular epithelia become coarsely granular, irregular in contour, and are separated from the basement membrane. Acid-fast pigment is laid down in the rat,^{199,203} monkey,²⁷⁸ and mink²⁵⁶ in the absence of nephrotic changes. Emmel^{370,371} suggested that the kidneys of rats raised on low vitamin E diets which are high in long-chain unsaturated acids (such as those in cod-liver oil, or the fatty acids of linseed oil) are subject to much more rapid postmortem autolysis than are kidneys of rats which have received dietary tocopherol. The tissue tocopherol levels of these rats,³⁷² as in the case of the tissues from calves¹⁷⁹ having a cod-liver oil-induced dystrophy, is not lower than in tissues from control animals on a lard diet. Although tocopherol therapy over a relatively short period will not reverse the rapid kidney autolysis, supplementation with this vitamin over the whole period will effectively prevent the development of this abnormal condition.

k. The Role of Vitamin E in Preventing Stomach Ulcers. Hyperplastic ulcer-like lesions can be produced in the rumen of the rat under a wide assortment of nutritional disorders; these were reviewed by Zucker *et al.*³⁷³ It was suggested that in most cases a low protein level in the diet appeared to be a critical factor in producing this disorder. Zucker and co-workers³⁷³

³⁶⁹ E. L. Hove, *Arch. Biochem.*, **17**, 467-474 (1948).

³⁷⁰ V. M. Emmel, *Anat. Record*, **118**, 384-385 (1954); **121**, 289-280 (1955).

³⁷¹ V. M. Emmel, *Kidney Autolysis in Vitamin E-Deficient Rats Receiving a Diet Rich in Unsaturated Fatty Acids*. *Abst. Reports Third Intern. Congr. on Vitamin E*, Venice, 1955, Vol. I, p. 31.

³⁷² V. M. Emmel, personal communication cited by P. L. Harris and K. E. Mason in *Proc. Third Intern. Congr. on Vitamin E*, Venice, 1955, Valdona, 1956, p. 13.

³⁷³ T. F. Zucker, B. N. Berg, and L. M. Zucker, *J. Nutrition*, **30**, 319-331 (1945).

voiced the opinion that the factor important for the prevention of the lesions "may be either an amino acid labile to commonly employed methods of purifying casein, or an unknown factor associated with crude casein."

Some data indicate that vitamin E may be at least one essential factor in preventing ulcer formation. Jensen³⁷⁴ reported that the administration of α -tocopherol decreased the incidence of ulcer formation in rats previously deficient in vitamin A, and therefore predisposed to ulcer. Harris and co-workers³⁷⁵ proved that the beneficial action of α -tocopherol was not due to its sparing action on vitamin A (thus making more of the latter available to the tissues); even with high intakes of vitamin A, tocopherol was still necessary to improve the ulcer condition. In a later study by Hove and Harris,³⁷⁶ indisputable evidence was presented that α -tocopherol increased the rate of cure of stomach lesions produced in rats by total starvation for eight days when repletion was carried on with 40 Calories daily of a vitamin E-free diet. α -Tocopherol had beneficial effects upon the incidence and severity of rumen lesions produced by semistarvation (20 Calories daily for forty days) when lard was in the diet, but it did not improve the condition when the ulcers were produced by hydrogenated coconut oil. The latter results only partially confirm an earlier report of Miller *et al.*³⁷⁷ It is suggested that both α -tocopherol and the essential fatty acids (which are absent from hydrogenated coconut oil) are necessary to produce the beneficial action.

1. The Role of Vitamin E in Depigmentation of the Teeth. Moore^{378,379} and Davies and Moore³⁸⁰ pointed out the relationship of vitamin E deficiency to depigmentation of the maxillary incisors of the rat. When the dietary protein was an additional limiting factor, the pigmentation of the mandibular incisors was also less than normal. The depigmentation resulted as a secondary effect of atrophic changes in the enamel organs.³⁸¹⁻³⁸³

Under ordinary conditions, the pigment is being continuously formed and deposited in the enamel organ of the incisor, to replace that worn

³⁷⁴ J. L. Jensen, *Science*, 103, 586-587 (1946).

³⁷⁵ P. L. Harris, E. L. Hove, M. Mellott, and K. Hickman, *Proc. Soc. Exptl. Biol. Med.*, 64, 273-277 (1947).

³⁷⁶ E. L. Hove and P. L. Harris, *J. Nutrition*, 40, 177-191 (1950).

³⁷⁷ J. A. Miller, B. E. Kline, H. P. Ruseh, and C. A. Baumann, *Cancer Research*, 4, 153-158, 756-761 (1944).

³⁷⁸ T. Moore, *Biochem. J.*, 37, 112-115 (1943).

³⁷⁹ T. Moore, *Ann. New York Acad. Sci.*, 52, 206-216 (1949).

³⁸⁰ A. W. Davies and T. Moore, *Nature*, 147, 794-796 (1941).

³⁸¹ J. T. Irving, *Nature*, 150, 122-123 (1942).

³⁸² H. Granados, K. E. Mason, and H. Dam, *J. Dental Research*, 24, 197 (1945).

³⁸³ H. Granados, K. E. Mason, and H. Dam, *J. Dental Research*, 25, 179 (1946).

away by attrition. Vitamin E therapy restores the pigmentation of the teeth, not by forming more pigment, but by correcting the function of the enamel organ so that pigment can be properly deposited.

Granados and co-workers^{354,355} reported that, when manganese was incorporated in the salt mixture, depigmentation of the enamel of vitamin E-deficient rats was inhibited. A possible explanation for this result might be that the manganese acts upon the alkaline phosphatase in the enamel organ of the depigmented incisors of rats on vitamin E-deficient diets. However, Glavind *et al.*³⁵⁶ found that the alkaline phosphatase activity in the enamel organ of the depigmented incisors of vitamin E-deficient rats was not significantly different from that in the controls which were fed vitamin E-rich diets. These results are difficult to correlate with those of Dam, Granados and Maltesen,³⁵⁷ who noted that manganese was greatly increased in both the enamel and the dentine of vitamin E-deficient rats, as compared with that present in these tissues when vitamin E was supplied. On the other hand, iron was found to be greatly diminished in both the dentine and the enamel of the vitamin E-deficient animals. Hamsters also exhibited depigmentation of the maxillary incisors when subjected to a vitamin E-deficient diet.³⁵³

m. The Role of Tocopherol as a Component of Various Enzyme Systems.

There are a number of reports on investigations of the action of tocopherol, particularly as its phosphate ester, and of its effect on different enzyme systems. D- α -Tocopheryl phosphate was employed in these tests, because of its greater solubility in water as compared with the free vitamin, and because of the implied behavior of the phosphate group. The phosphate ester was found to inhibit practically every *in vitro* enzyme system on which it was tested. Houchin¹⁵⁹ was the first to observe the inhibition of the succinoxidase by α -tocopherol phosphate; this result has been confirmed by a number of investigators.³⁵⁸⁻³⁵⁹ Jacobi *et al.*³⁵⁹ reported that, when calcium and aluminum were omitted from the medium, an extremely low

³⁵⁴ H. Granados, E. Aaes-Jørgensen, and H. Dam, *Brit. J. Nutrition*, **3**, 320-334 (1949).

³⁵⁵ H. Granados, E. Aaes-Jørgensen, and H. Dam, *Acta pathol. et microbiol. Scand.*, **27**, 304-312 (1950).

³⁵⁶ J. Glavind, E. Aaes-Jørgensen, H. Granados, and H. Dam, *J. Dental Research*, **29**, 689 (1950).

³⁵⁷ H. Dam, H. Granados, and L. Maltesen, *Acta Physiol. Scand.*, **21**, 124-130 (1950).

³⁵⁸ W. M. Govier, V. Bergmann, and K. H. Beyer, *J. Pharmacol. Exptl. Therap.*, **85**, 143-149 (1945).

³⁵⁹ H. P. Jacobi, S. Rosenblatt, J. W. Chappell, and S. Morgulis, *Arch. Biochem.*, **27**, 9-18 (1950).

³⁶⁰ P. D. Boyer, M. Rabinovitz, and E. Liebe, *Ann. New York Acad. Sci.*, **52**, 188-194 (1949).

³⁶¹ S. R. Ames, *J. Biol. Chem.*, **169**, 503-512 (1947).

concentration of tocopherol phosphate produced inhibition. Tocopheryl phosphate has both a specific action and a secondary effect involving the removal of calcium.³⁹¹ Other enzyme systems which have been shown to be inhibited by vitamin E phosphate include trypsin,³⁹² liver acid phosphatase,³⁹³ hyaluronidase,³⁹⁴ liver esterase,^{394a} lipoxidase^{395,396} (only temporarily) and a malic oxidase system employing cytochrome *c* and cholinesterase.³⁹⁷ On the other hand, Jacobi *et al.*³⁹⁷ reported that the α -tocopheryl phosphate has little or no effect upon a malic oxidase-methylene blue system, a lactic oxidase-methylene blue system, D-amino acid oxidase, uricase, an adenosinetriphosphatase system containing calcium, and liver acid phosphatase. Catalase activity was somewhat increased. According to Rabinovitz and Boyer,³⁹⁸ the observed effects of D- α -tocopheryl phosphate on the several enzyme systems, in which the enzyme systems were inhibited, may be the result of its properties as an anion with a large non-polar group; these effects are not necessarily related to its activity as a vitamin.

n. Miscellaneous Factors Related to the Role of Tocopherol. Since vitamin E improves the efficiency of utilization of protein, it is reasonable to postulate that the symptoms of protein deficiency would be accentuated on a vitamin E-free diet, and *vice versa*. Moore³⁷⁹ was the first to show that rats reared on low-protein and low-vitamin E diets differed from those which received adequate vitamin E. When vitamin E was absent, there was a shorter survival time, a decreased efficiency in protein utilization at certain levels of protein intake, the occurrence of hepatic lesions, and a depigmentation of the incisors. Holman³²⁶ found that after dogs had been fed for two months on low-protein, low-vitamin E diets, they developed a pronounced necrotizing arteritis when subjected to induced renal insufficiency. This condition could be counteracted by vitamin E.

When regimens adequate in protein but low in vitamin E were fed to rats, it was found that the onset of muscular dystrophy could be hastened by the hepatotoxic agent, triorthocresyl phosphate.³⁹⁹ Thompson⁴⁰⁰ reported a similar result on the rabbit, while Draper *et al.*²⁵² noted an ac-

³⁹² K. L. Zierler, D. Grob, and J. L. Lilienthal, Jr., *Am. J. Physiol.*, **153**, 127-132 (1948).

³⁹³ H. P. Jacobi, J. W. Chappell, and S. Morgulis, *Federation Proc.*, **6**, 163 (1947).

³⁹⁴ W. H. Miller and A. M. Dessert, *Ann. New York Acad. Sci.*, **52**, 167-179 (1949).

^{394a} C. Van der Meer and H. T. M. Nieuwerkerk, *Biochim. et Biophys. Acta*, **7**, 263-271 (1951).

³⁹⁵ R. T. Holman, *Arch. Biochem.*, **15**, 403-413 (1947).

³⁹⁶ H. O. Kunkel, *Arch. Biochem.*, **30**, 306-316 (1951).

³⁹⁷ H. P. Jacobi, J. W. Chappell, and S. Morgulis, *Arch. Biochem.*, **27**, 22-28 (1950).

³⁹⁸ M. Rabinovitz and P. D. Boyer, *J. Biol. Chem.*, **183**, 111-121 (1950).

celeration of dystrophy symptoms in the lamb when this drug was given. An increased rate of degeneration of the testis, and irreversible sterility, have also been observed in the rat under such conditions.^{399,400}

There is some indication that the excretion of creatine and of allantoin is increased in rats and rabbits deficient in both vitamin B⁶ and vitamin E.^{155,401} Supplementation of the diet with either of these vitamins prevents this increase. A similar relationship is said to exist between vitamins E and K.⁴⁰² Absence of these two vitamins produced heart muscle lesions in mice fed purified diets supplemented with sulfaguanidine. Moore *et al.*⁴⁰³ observed that the rapid and pronounced hemoglobinuria produced in rats on a vitamin E-deficient diet by the intramuscular injection of *Synkavit* (tetra-sodium-2-methyl-1,4-naphthohydroquinone diphosphate) could be counteracted by vitamin E.

The toxicity of thyroid in chicks⁴⁰⁴ and in rats⁴⁰⁵ in accelerating the onset of vitamin E-deficiency symptoms can be prevented with α -tocopherol. Moreover, this vitamin also inhibits the goiter-producing action of methylthiouracil.⁴⁰⁶ Vitamin E is also of use in counteracting the hypoxic factor in rabbits on low-vitamin E diets.⁴⁰⁷ Likewise, Taylor⁴⁰⁸ and Gerschman *et al.*⁴⁰⁹ reported that the resistance to hyperoxia, (high oxygen pressures) is reduced in vitamin E deficiency. Presumably in the latter case, vitamin E counteracts some abnormal cell metabolite which is formed under such conditions. It is therefore evident that the various physiologic actions of vitamin E are extremely widespread.

(4) *The Importance of Tocopherols as Related to Their Antioxidant Action*

The ability of the tocopherols to act as antioxidants both *in vivo* and *in vitro* was recognized at an early date. Many of the deficiencies resulting

³⁹⁹ D. K. Myers, H. E. W. Mulder, and E. G. L. Simons, *Biochem. J.*, **55**, i (1953).

⁴⁰⁰ R. H. S. Thompson, *Chemistry and Industry*, 1954, 749-751.

⁴⁰¹ J. M. Young, Jr., J. S. Dinning, and P. L. Day, *Proc. Soc. Exptl. Biol. Med.*, **89**, 216-217 (1955).

⁴⁰² F. I. Dessau, L. Lipchuck, and S. Klein, *Proc. Soc. Exptl. Biol. Med.*, **87**, 522-524 (1954).

⁴⁰³ T. Moore, I. M. Sharman, and R. F. Ward, *Experimental Studies on Vitamin E, Abst. Reports Third Intern. Congr. on Vitamin E*, Venice, 1955, Vol. I., pp. 47-48.

⁴⁰⁴ R. S. Wheeler and J. D. Perkinson, *Am. J. Physiol.*, **159**, 287-290 (1949).

⁴⁰⁵ L. Tentori, G. Toschi, and G. Vivaldi, *Boll. soc. ital. biol. sper.*, **29**, 90-91 (1953).

⁴⁰⁶ E. Gitsch and H. Tulzer, *Wien. Med. Wochschr.*, **103**, 124-125 (1933).

⁴⁰⁷ I. R. Telford, O. B. Wiswell, and E. L. Smith, *Proc. Soc. Exptl. Biol. Med.*, **87**, 162-164 (1954).

⁴⁰⁸ D. W. Taylor, *J. Physiol.*, **121**, 47P-48P (1953).

⁴⁰⁹ R. Gerschman, D. L. Gilbert, S. W. Nye, and W. O. Fenn, *Federation Proc.*, **14**, 56 (1955).

from a vitamin E-free diet are associated with a lack of this vitamin in the adipose tissue or in other fat-storing organs so pronounced that there is an insufficient content of antioxidants to prevent the development of peroxides in the tissues. This latter condition occurs especially if there are unusually large amounts of highly unsaturated fats in the diet.

a. The *in vivo* Action of Vitamin E as an Antioxidant. Barnes, Lundberg, Hanson, and Burr⁸² were the first to note that, although the fats of rats fed for 100 days on a vitamin E-free diet were extremely susceptible to rancidity, the administration of a single dose of α -tocopherol in the diet restored the stability of the body fats to their normal level. The maximum deposition of the tocopherol took place in seven to ten days, after which the quantities in the fat gradually decreased to one-half of the maximum value during the course of two months.⁸⁵ α -Tocopherol was found to be slightly more effective in the subsequent protection of the body fats than was γ -tocopherol; this effect is exactly opposite to that obtained when the tocopherols are added directly to the fats *in vitro*. β -Tocopherol was found to have a potency intermediate between that of the α - and the γ -types. Since the quantities of α -, β -, and γ -tocopherol present in the fats varied directly with their ability to stabilize fat, variations in the extent of absorption are believed to be a partial explanation for the different antioxygenic action of the three types of tocopherols when fed to the animals. Further studies on the *in vivo* effect of tocopherols on fat stability have been recorded by the Burr-Lundberg group.^{82,85,410}

In addition to rats, other animals use tocopherol to stabilize their body fats against peroxidation. Thus, Major and Watts⁴¹¹ recorded a similar protective effect in the case of rabbits on a purified diet and fed or injected with tocopherols at relatively high levels. Carpenter and Lundberg⁴¹² were able to obtain increased protection of the fat of baby pigs when the tocopherol supplements were continued over a period of twelve weeks before the animals were killed. The storage of turkeys was improved, and the peroxide values of the fat were lower, when increased amounts of tocopherol were fed prior to the killing.⁹⁵

The effect of tocopherol feeding upon the stability of milk is still somewhat open to question, due to the conflicting reports of different workers. Burr, Lundberg, and Chipault⁴¹⁰ observed that, when a vitamin E-free diet was fed to cows, the effect upon the stability of the butterfat was

⁴¹⁰ G. O. Burr, W. O. Lundberg, and J. R. Chipault, *Oil & Soap*, 23, 382-384 (1946).

⁴¹¹ R. Major and B. M. Watts, *J. Nutrition*, 35, 103-116 (1948).

⁴¹² L. E. Carpenter and W. O. Lundberg, *Ann. New York Acad. Sci.*, 52, 269-275 (1949).

unfavorable, and oxidation of the fat was accelerated. Moreover, Krukovsky and Loosli⁹⁸ noted that supplementation of the diet of cows with 1 to 2 g. of mixed tocopherols per cow raised the tocopherol content of the milk-fat and increased its resistance to oxidative deterioration. The prevention of oxidized flavors in milks was later shown to be correlated with the increased tocopherol content produced by feeding the vitamin.⁴¹³ However, no improvement occurred when tocopherol supplements were fed to cows at the end of the winter season. It was concluded that tocopherol cannot be used to maintain the tocopherol content of milk fat or to prevent the oxidized flavor under all conditions. Moreover, Nielsen and co-workers⁴¹⁴ in Denmark were unable to increase the keeping quality of butter made from the milk of cows given 2 g. of DL- α -tocopheryl acetate daily, although the tocopherol content of the butter was increased from 10 to 12 $\mu\text{g./g.}$, in the unsupplemented control group, to 23 to 26 $\mu\text{g./g.}$ in the supplemented group. However, the experimental details of the two conflicting reports differ. It seems probable that, under certain conditions, the tocopherols can reduce the oxidized flavor in milk, and increase its stability against deterioration.

b. The *in vitro* Action of Vitamin E as an Antioxidant. Olcott and Mattill,^{31,32} and Bradway and Mattill⁴¹⁵ first concentrated the natural antioxidants in oils by the procedures useful for the preparation of vitamin E. It had previously been impossible to separate these antioxidants (or inhibitols) from vitamin E. Since the isolation¹ of several forms of vitamin E (the tocopherols) it has been found that α -tocopherol is most effective as an antisterility agent, while β - and γ -tocopherols excel as antioxidants.³³ δ -Tocopherol has been found to have the highest antioxidant activity of any of the tocopherols.³ The addition of α -tocopherol or of mixed tocopherols to fats or to fat-containing products is now widely used as an *in vitro* method of preventing rancidity. A number of products such as ascorbic, citric, and phosphoric acid may act as synergists, while vitamin A, carotene, cephalin, methionine, sulfhydryl groups, and crude sources of the vitamin B complex also serve in that capacity. For a more complete exposition of the role of tocopherols as antioxidants, and of their relationships to the synergists, the reader is referred to *The Lipids*, Vol. I, pp. 281-300.

c. The Sparing Action of Vitamin E on Vitamin A and the Carotenoids.

⁴¹³ V. N. Krukovsky, J. K. Loosli, and F. Whiting, *J. Dairy Sci.*, **32**, 196-201 (1949).

⁴¹⁴ J. Nielsen, A. N. Fisker, A. H. Pedersen, I. Prange, E. Sndergaard, and H. Dam, *J. Dairy Research*, **20**, 333-339 (1953).

⁴¹⁵ E. M. Bradway and H. A. Mattill, *J. Am. Chem. Soc.*, **56**, 2405-2408 (1934).

Moore⁴¹⁶ and Bacharach⁴¹⁷ are generally credited with the discovery that vitamin E potentiates the action of vitamin A. It was found that the vitamin A reserves in the livers of rats kept for a long interval on vitamin E-deficient diets had a much lower concentration of vitamin A than occurred in this organ when the rats received adequate vitamin E. Quackenbush and associates⁴¹⁸ demonstrated that α -tocopherol prevented the autoxidation of carotene *in vitro*. It was likewise observed that tocopherol augmented the growth response of vitamin A-deficient rats to minimum quantities of carotene. Hickman and co-workers⁴¹⁹ also arrived at the same conclusion, namely that α -tocopherol has the power to augment the A vitamins. The interaction was referred to by this group⁴²⁰ as a "sparing" or "synergistic" action, or better as "co-vitamin E" activity. Hickman *et al.*⁴²⁰ reported that the natural vitamin E (mixed tocopherols) enhanced the growth-promoting action of vitamin A alcohol, vitamin A acetate, and U.S.P. vitamin A reference oil. Moreover, it was observed that a longer interval was required to deplete young rats of vitamin A in the presence of tocopherols. The survival time was also increased when a given dosage of vitamin A was administered to vitamin A-deficient rats if they also received vitamin E. Harris and co-workers⁴²¹ reported that α -tocopherol potentiated the vitamin A-like action of carotene; approximately 0.5 mg. of the natural mixed tocopherols was found to afford maximum protection. In a further simultaneous report of this group,⁴²² it was further recorded that the three tocopherols (α -, β -, and γ -) were equally effective in sparing vitamin A. This is in contradistinction to their effect as antisterility agents; in this respect the α -form was the most potent and γ -tocopherol was the weakest. Their activity was augmented slightly by mutual admixture, and especially by the addition of selected antioxidants. Tococquinone is as effective as tocopherol in sparing carotene. Since the carotene content of the feces was markedly increased when tocopherols were given, it is postulated that tocopherols exert their beneficial effect in controlling the oxidative changes in the intestine.⁴²² The ineffective-

⁴¹⁶ T. Moore, *Biochem. J.*, **34**, 1321-1328 (1940).

⁴¹⁷ A. L. Bacharach, *Quart. J. Pharm. Pharmacol.*, **13**, 138-149 (1940).

⁴¹⁸ F. W. Quackenbush, R. P. Cox, and H. Steenbock, *J. Biol. Chem.*, **145**, 169-177 (1942).

⁴¹⁹ K. C. D. Hickman, P. L. Harris, and M. R. Woodside, *Nature*, **150**, 91-92 (1942).

⁴²⁰ K. C. D. Hickman, M. W. Kaley, and P. L. Harris, *J. Biol. Chem.*, **152**, 303-311 (1944).

⁴²¹ P. L. Harris, M. W. Kaley, and K. C. D. Hickman, *J. Biol. Chem.*, **152**, 313-320 (1944).

⁴²² K. C. D. Hickman, M. W. Kaley, and P. L. Harris, *J. Biol. Chem.*, **152**, 321-328 (1944).

ness of DL-tocopheryl acetate in protecting carotene and vitamin A (as determined by weight gains),⁴²² which has been confirmed,^{417,423} would also suggest that the antioxygenic action of tocopherol takes place within or near the alimentary tract rather than in the liver as was suggested by Popper *et al.*⁴²⁴

Although α -, β -, and γ -tocopherols were first reported to be equally effective in protecting vitamin A,⁴²² the later studies of Stern *et al.*,³ which also included δ -tocopherol, demonstrated that the antioxidant potency of the tocopherols in protecting vitamin A or carotene was, in order of potency: δ , γ -, β -, and α - (proceeding from the strongest to the weakest). The order of effectiveness of the tocopherols as antioxidants, as demonstrated by Olcott and Emerson,³³ was γ -, β -, α -tocopherol (in the ratio of 4.3:1.7:1). Hove and Hove,⁴²⁵ who used a different experimental method, found that at low temperatures the three compounds had approximately equal antioxidant activity, while at high temperatures γ -tocopherol was several times more active than the β - and α -forms (5.10:2.-63:1). Dam and co-workers^{426,427} showed that, in addition to vitamin E, methylene blue, thionine, thiodiphenylamine, and Antabuse (tetraethylthiuram disulfide) caused increased deposition of vitamin A in the livers of chicks and rats when vitamin E was deficient. However, when cod liver oil was replaced by lard, or when fat was omitted from the diet, methylene blue was found to have no effect upon the storage of vitamin A. For a further discussion, the reader is referred to the following section.

d. The Vitamin E-Like Action of Methylene Blue and of Related Antioxidants. (a) *Protection against Resorption Gestation.* A number of non-tocopherol antioxidants have been shown to produce a vitamin E-like action when tested by the classical method employed for vitamin E, but the results are somewhat inconsistent. Thus, Dam and Granados⁴²⁸ reported that methylene blue improved the reproductive capacity of female rats, raised on vitamin E-deficient diets which were "fat-free" or which contained cod liver oil, to about the same extent as did vitamin E. These investigators are of the opinion that methylene blue acts in the same manner as vitamin E to maintain the integrity of certain enzymatic redox

⁴²³ M. C. Miles, E. M. Erickson, and H. A. Mattill, *Proc. Soc. Exptl. Biol. Med.*, **70**, 162-165 (1949).

⁴²⁴ H. Popper, F. Steigmann, and H. A. Dyniewicz, *Gastro-enterology*, **10**, 987-1000 (1948).

⁴²⁵ E. L. Hove and Z. Hove, *J. Biol. Chem.*, **156**, 611-621, 623-632 (1944).

⁴²⁶ H. Dam, I. Prange, and E. Sndergaard, *Experientia*, **7**, 184-185 (1951).

⁴²⁷ H. Dam, I. Prange, and E. Sndergaard, *Acta Pharmacol. Toxicol.*, **8**, 1-22, 23-29 (1952).

⁴²⁸ H. Dam and H. Granados, *Acta Pharmacol. Toxicol.*, **8**, 47-54 (1952).

systems. Markees⁴²⁹ confirmed the results of Dam and Granados.⁴²⁸ Diphenyl-*p*-phenylenediamine (DPPD) has also produced a positive result as regards its ability to replace vitamin E in the resorption-gestation mechanism.⁴³⁰ However, since the effect of DPPD in supporting pregnancy and lactation in rats became succeedingly less in successive generations, Johnson and Goodyear⁴³¹ are now of the opinion that the positive effect of DPPD is due to the fact that it protects the minimum quantities of vitamin E in the diet and in the gut, rather than that it substitutes for it in the metabolic functions *in vivo*. The reported effectiveness of DPPD in preventing vitamin E-deficiency symptoms (encephalomalacia) in chicks, as recorded by Singsen and co-workers,⁴³² is attributed by Scott and Norris⁴³³ to its protective action upon traces of vitamin E in the presence of the unsaturated fats of the diet. Granados *et al.*⁴³⁴ demonstrated that sulfaguanidine also possesses a vitamin E-like effect upon vitamin E-deficient rats. Thus, when this compound was fed to the extent of 1% in the diet, the color of the lumbar fat, and the peroxide value for the fats were similar to those obtained when the animals were supplemented with tocopherol. However, the degree of incisor pigmentation was not appreciably affected.

On the other hand, Moore, Sharman, and Ward^{435,436} were unable to confirm the results of the Dam group indicating that methylene blue can substitute for tocopherol in the reproductive process. Moreover, the atrophy of the testes in male rats on a vitamin E-free diet could not be prevented by methylene blue.^{435,436} Such divergent results leave one somewhat in doubt about the effectiveness of these non-tocopherol antioxidants in substituting for vitamin E to prevent the resorption-gestation syndrome. Moreover, the Mackenzies¹²⁹ and Harris⁴³⁷ reported a negative response to

⁴²⁹ S. Markees, *Intern. Z. Vitaminforsch.*, **25**, 316-317 (1954).

⁴³⁰ B. C. Johnson and S. Goodyear, *The Role of Antioxidants in Vitamin E Nutrition*, Report cited by P. L. Harris and K. E. Mason, *Proc. Third Intern. Congr. on Vitamin E*, Venice, 1955, Valdonega, Verona, 1956, pp. 20, 21.

⁴³¹ B. C. Johnson and S. Goodyear, Personal communication, 1955, cited by P. L. Harris and K. E. Mason, *Proc. Third Intern. Congr. on Vitamin E*, Venice, 1955, Valdonega, Verona, 1956, p. 21.

⁴³² E. P. Singsen, R. H. Bunnell, A. Kozeff, L. D. Matterson, and E. L. Jungherr, *Poultry Sci.*, **32**, 924-925 (1953).

⁴³³ M. L. Scott and L. C. Norris, *Studies on Vitamin E in Chick Nutrition*, cited by P. L. Harris and K. E. Mason, *Proc. Third Intern. Congr. on Vitamin E*, Venice, 1955, Valdonega, Verona, 1956, p. 21.

⁴³⁴ H. Granados, E. Aaes-Jørgensen and H. Dam, *Experientia*, **6**, 150-156 (1950).

⁴³⁵ T. Moore, I. M. Sharman, and R. J. Ward, *Biochem. J.*, **53**, xxxi (1953).

⁴³⁶ T. Moore, I. M. Sharman, and R. J. Ward, *Biochem. J.*, **58**, vii (1954).

⁴³⁷ P. L. Harris, Unpublished observations, 1955; cited by P. L. Harris and K. E. Mason, *Proc. Third Intern. Congr. on Vitamin E*, Venice, 1955, Valdonega, Verona, 1956, p. 21.

the resorption-gestation syndrome when α -tocohydroquinone was substituted for vitamin E.

(b) *Protection against Brown Pigmentation.* In spite of the fact that Moore *et al.*^{435,436} failed to demonstrate that methylene blue could substitute for vitamin E in rats in so far as the reproductive function is concerned, it was found to be fully active against brown discoloration of the uterus.⁴³⁶ In addition to methylene blue, thiodiphenylamine, Bind-schedler's green, malachite green, leucomalachite green, and new methylene blue, were also found to be potent^{436,438} in preventing discoloration of the uterus. When rosaniline was present in the diet to the extent of 1% of the diet, or methyl violet in the proportion of 0.126%, the brown discoloration of the uterus was also prevented.⁴³⁶ However, no protection against brown discoloration was obtained with *p*-aminobenzoic acid (0.1%), or with sulfa-pyridine (0.1%).⁴³⁶ Ascorbic acid, cystine, and a group of purine derivatives were likewise found to give negative results.⁴³⁹ Aaes-Jørgensen, Dam, and Granados⁴⁴⁰ reported that methylene blue and Antabuse protected fairly well against yellow-brown discoloration of the fat, and incisor depigmentation.

(c) *Protection against Muscular Dystrophy.* Although Dam and co-workers²⁵⁹ failed to obtain any relief by means of methylene blue in the treatment of muscular dystrophy in the chick, Moore and co-workers⁴³⁶ did report a positive effect in the case of rats, while Markees⁴²⁹ observed a similar result in rabbits, and Blaxter alone¹⁷⁹ and with associates²⁹² noted a curative effect upon muscular dystrophy in the calf. α -Tocoquinone and α -tocohydroquinone have been reported to be effective in the treatment of muscular dystrophy in rabbits,^{129,291} in the hamster,¹⁵² and in calves.¹⁷⁹

(d) *Protection against Hemolysis of Red Blood Cells.* Dam and Granados⁴⁴¹ reported that methylene blue, when fed at a level of 0.126% in the diet, offers complete protection against the massive liver necrosis and liver hemorrhage which usually develops in animals on a low-protein, vitamin E-deficient, fat-containing diet. In an earlier study from this laboratory, Christensen and Dam⁴⁴² had observed that the feeding of methylene blue at a level of 0.126% in the basal diet provided a marked though not complete protection against hemolysis of erythrocytes in a dialuric acid solution. The authors suggest that the action may be due to a non-specific

⁴³⁸ T. Moore, I. M. Sharman, and R. J. Ward, *Biochem. J.*, **54**, xvi-xvii (1953).

⁴³⁹ T. Moore, I. M. Sharman, and R. J. Ward, *Proc. Nutr. Soc.*, **12**, v (1953).

⁴⁴⁰ K. Aaes-Jørgensen, H. Dam, and H. Granados, *Acta Pharmacol. Toxicol.*, **7**, 171-180 (1951).

⁴⁴¹ H. Dam and H. Granados, *Acta Pharmacol. Toxicol.*, **7**, 181-188 (1947).

⁴⁴² F. Christensen and H. Dam, *Acta Pharmacol. Toxicol.*, **7**, 167-170 (1951).

antioxidant effect of leucomethylene blue, and they consider that a series of other substances may have a similar effect. In contradistinction to these findings, Moore and co-workers^{436,443} noted that the red blood cells of rats were not protected by methylene blue from hemolysis by dialuric acid. Moreover, Johnson and Goodyear⁴³⁰ reported that DPPD gave a negative response in restoring this function. Rose and György³⁴⁰ also obtained negative results with fat-soluble alkyl hydroquinones. Because of the decisiveness of the positive results of the Dam group, on the one hand, and that of the negative results of Moore and co-workers on the other hand, one is forced to reserve judgment at the present time as to which effect is the more usual physiologic response.

(e) *Protection against Exudative Diathesis and Encephalomalacia in Chicks.* Dam and co-workers,³²² and Miller *et al.*⁴⁴⁴ both reported that methylene blue cured both exudative diathesis and encephalomalacia in the chick. DPPD also gave positive results in these chick deficiencies according to Singsen *et al.*,⁴³² and Bunnell and co-workers,⁴⁴⁵ but Scott *et al.*^{445a} failed to confirm this result. Bunnell and associates⁴⁴⁵ also obtained a positive effect with fat-soluble alkyl hydroquinone, but Scott and Norris⁴³³ reported a negative result. Neither the Dam group³²² nor Scott and Norris⁴³³ were able to demonstrate any positive effect exerted by α -tocoquinone or by α -tocohydroquinone on the chick deficiency syndrome. The industrially important antioxidant, nordihydroguaiaretic acid (NDGA) was found to afford some protection against exudative diathesis in chicks.^{322,446} Antabuse was found to provide some protection against exudative diathesis, but it was not effective against encephalomalacia in the chick.³²²

(f) *Miscellaneous Effects of Methylene Blue and of Related Antioxidants in Counteracting Vitamin E Deficiency.* There appears to be unanimity of opinion that methylene blue feeding increases the retention of vitamin A in the livers of rats and of chicks on a vitamin E-deficient diet.^{426,427,435} Antabuse has likewise been shown by the Dam group⁴²⁷ to protect liver vitamin A. Aaes-Jørgensen *et al.*⁴⁴⁰ reported that methylene blue and Antabuse protect against the depigmentation of the incisors of rats which

⁴⁴³ D. H. Heard, T. Moore, and I. M. Sharman, *Biochem. J.*, **52**, i (1952).

⁴⁴⁴ R. F. Miller, G. Small, and L. C. Norris, *J. Nutrition*, **55**, 81-95 (1955).

⁴⁴⁵ R. H. Bunnell, L. D. Matterson, E. P. Singsen, L. M. Potter, A. Kozeff, and E. L. Jungherr, *Poultry Sci.*, **33**, 1046 (1954).

^{445a} M. L. Scott, F. W. Hill, L. C. Norris, D. C. Dobson, and T. S. Nelson, *J. Nutrition*, **56**, 387-402 (1955).

⁴⁴⁶ H. Dam, I. Kruse, I. Prange, and E. Søndergaard, *Biochem. Biophys. Acta*, **2**, 501-513 (1948).

otherwise occurs on vitamin E-free diets. In 1953 Dam⁴⁴⁷ reviewed the subject of vitamin E as an *in vivo* antioxidant.

8. The Biopotency of Various Forms of Vitamin E

(1) *Comparative Biopotency of α -Tocopherol as Ester and Free Alcohol*

Although Joffe and Harris,⁴⁴⁸ in 1943, reported that free α -tocopherol had the same biopotency against sterility as it did in the form of an ester (α -tocopheryl acid succinate), Demole *et al.*⁴⁴⁹ and Mason and Harris⁴⁵⁰ reported that, under standard conditions, the tocopherol esters, both natural and synthetic, showed a consistent superiority in vitamin E potency as compared with the respective tocopherols in the active form. In a later, more thorough, evaluation, Harris and Ludwig,⁴⁵¹ basing their calculations upon an experimentally determined equivalency of natural D- α -tocopheryl acetate, 1 mg. = 1.36 I.U., exhibited 62% more vitamin E potency than it did as the free alcohol.⁴⁵² This relationship was observed both for the natural tocopherols, *i.e.*, D- α esterified *versus* D- α free, and for synthetic tocopherols, DL- α -esterified *versus* DL- α free. Harris and Ludwig⁴⁵² explain the greater biopotency of the tocopherol esters over the free tocopherol on the basis that the former are more resistant to oxidative destruction such as might occur prior to absorption in the intestinal tract of the assay rats. Thus, when antioxidants were added to the tocopherol solutions fed, free α -tocopherol was preserved sufficiently to induce a response equivalent to that of an equimolecular dose of the esterified α -tocopherol.

(2) *Comparative Biopotency of DL- and D- α -Tocopherols*

Since position 2 involves a center of asymmetry in the tocopherol molecule, it is optically active. The natural form has been found to be D (or *d*)- α -tocopherol. Although the optical activity is very slight, Harris and co-workers⁴⁵³ reported that synthetic DL- α -tocopherol has only two-thirds of the biopotency of natural D- α -tocopherol, while synthetic DL- β -

⁴⁴⁷ H. Dam, *Experientia*, Suppl. I, 1953, 195-207.

⁴⁴⁸ M. Joffe and P. L. Harris, *J. Am. Chem. Soc.*, **65**, 925-927 (1943).

⁴⁴⁹ V. Demole, O. Isler, B. H. Ringier, H. Salomon, and P. Karrer, *Helv. Chim. Acta*, **22**, 65-68 (1930).

⁴⁵⁰ K. E. Mason and P. L. Harris, *Biol. Symposia*, **12**, 459-483 (1947).

⁴⁵¹ P. L. Harris and M. I. Ludwig, *J. Biol. Chem.*, **179**, 1111-1115 (1949).

⁴⁵² P. L. Harris and M. I. Ludwig, *J. Biol. Chem.*, **180**, 611-614 (1949).

⁴⁵³ P. L. Harris, J. L. Jensen, M. Joffe, and K. E. Mason, *J. Biol. Chem.*, **156**, 491-498 (1944).

tocopherol exhibits only one-half of the activity of the natural D- β -tocopherol.

(3) Comparative Biopotency of α -, β -, γ -, and δ -Tocopherols

Bacharach,⁴⁵⁴ who reviewed the subject in 1938, concluded that no difference in relative biopotency could be established for the several types of tocopherols. However, Mason²²⁸ reported, several years later, that α -, β -, and γ -tocopherols had relative biopotencies of one, one-half, and one-quarter, respectively. Karrer and co-workers,⁴⁵⁵ using synthetic materials, noted that α -tocopherol (5,7,8-trimethyltolcol) had the highest potency of all of the tocols. Joffe and Harris⁴⁴⁸ reported that the M.F.D. (mean fertility dose) of β -tocopherol was 1.9 mg., and that of γ -tocopherol 9.0 mg., as compared with a value of 0.75 mg. for α -tocopherol. This would make the β -form four-tenths as potent, while γ -tocopherol would be one-twelfth as potent as the α -form. Weisler *et al.*⁴⁵⁶ observed a similar relationship. Gottlieb *et al.*⁴⁵⁷ reported that the biopotencies of synthetic α -, β -, and γ -tocopherols and α -tocopheryl acetate were in the ratio 100:25:19:100. When the cure of muscle dystrophy in rabbits was the basis for comparison of biopotency, the potency ratios for α , β , and γ types of natural tocopherols⁴⁵⁸ were 100:30:20. The natural D- γ -tocopherol was almost three times as active as the synthetic DL- γ -tocopherol in curing dystrophy, and the natural D- β -tocopherol exhibited about 30% of the activity of D- α -tocopherol.⁴⁵⁸ For a review of the biopotency of various synthetic compounds related to the tocopherols, the reader is referred to *The Lipids*, Vol. I, pp. 820-827. Harris⁴⁵⁹ has also given a short review of the relative biologic potency of various tocopherols. For a discussion of the antioxygenic activity of the several types of tocopherols, see *The Lipids*, Vol. I, pp. 816-819.

9. The Requirements for Vitamin E

(1) Normal Requirements of Animals for Vitamin E

A large number of standards have been set up for vitamin E, but they are to some extent arbitrary. The International Unit adopted by the

⁴⁵⁴ A. L. Bacharach, *Biochem. J.*, **32**, 2017-2023 (1938).

⁴⁵⁵ P. Karrer, H. Koenig, B. H. Ringier, and H. Salomon, *Helv. Chim. Acta*, **22**, 1139-1145 (1939).

⁴⁵⁶ L. Weisler, J. G. Baxter, and M. I. Ludwig, *J. Am. Chem. Soc.*, **67**, 1230-1231 (1945).

⁴⁵⁷ H. Gottlieb, F. W. Quackenbush, and H. Steenbock, *J. Nutrition*, **25**, 433-440 (1943).

⁴⁵⁸ E. L. Hove and P. L. Harris, *J. Nutrition*, **33**, 95-106 (1947).

⁴⁵⁹ P. L. Harris, *Ann. New York Acad. Sci.*, **52**, 342-344 (1949).

League of Nations is defined as the specific activity of 1 mg. of synthetic racemic tocopherol acetate. The international standard contains one mg. of a standard α -tocopheryl acetate in 100 mg. of olive oil. This is the amount which, when administered orally, prevents resorption-gestation in rats deprived of vitamin E.^{460,460a} On the other hand, the M.F.D. (mean fertility dose), which is more widely employed, amounts to 0.75 mg. of α -tocopherol. For a further discussion of vitamin E standards, the reader is referred to *The Lipids*, Vol. I, pp. 819 and 820.

It is impossible to make categorical statements about the vitamin E requirements without setting up precisely the conditions of the diet, age, sex, type of fat, etc. For example, a number of investigators, including Gottlieb *et al.*,⁴⁵⁷ Emerson and Evans,⁴⁶¹ Dam *et al.*,⁴⁶² and Bruce⁴⁶³ have observed that the vitamin E requirement is increased by high-fat diets, particularly when they are composed of highly unsaturated fats.⁴⁶² Although the daily requirement of the two sexes is about the same in mature rats, Mason²⁰⁹ states that the male requirement to prevent irreversible disturbances of the germinal epithelium begins at the age of forty to fifty days, while that of the females to ensure reproduction does not become critical until after conception and implantation, which may occur at any stage of the reproductive period.

Goettsch and Pappenheimer⁴⁶⁴ reported that the amounts of vitamin E necessary to prevent sterility and to inhibit dystrophy are within the same range in the rat (2.5 mg. *vs.* 0.5 mg.). However, the amount of vitamin E necessary to protect against "late lactation" paralysis in young rats from vitamin E-deficient mothers depends upon the time of initiation of the vitamin E therapy. Thus, when it is delayed, in the case of suckling rats from the tenth to the seventeenth day, an increased dosage is required for their protection. Evans and Emerson²¹¹ noted that, when the daily dosage of tocopherol was decreased below 0.75 mg./day, the length of the period of fertility was reduced. McCay and co-workers⁴⁶⁵ reported that the curtailment of the fertility period in the male and female, as the result of an insufficient intake of vitamin E, did not influence longevity.

The amount of vitamin E required daily by rabbits is estimated as 0.2 to 0.4 mg. per kg. body weight.⁴⁶⁶ The total requirement for the guinea

⁴⁶⁰ E. M. Hume, *Nature*, 148, 472-473 (1941).

^{460a} Anonymous, *An International Standard for Vitamin E*, *Nature*, 148, 473 (1941).

⁴⁶¹ G. A. Emerson and H. M. Evans, *J. Nutrition*, 27, 469-476 (1944).

⁴⁶² H. Dam, H. Granados, and L. Prange, *Acta Physiol. Scand.*, 18, 161-170 (1949).

⁴⁶³ H. M. Bruce, *J. Hyg.*, 48, 171-183 (1950).

⁴⁶⁴ M. Goettsch and A. M. Pappenheimer, *J. Nutrition*, 22, 463-476 (1941).

⁴⁶⁵ C. M. McCay, G. Sperling, and L. L. Barnes, *Arch. Biochem.*, 2, 469-479 (1943).

⁴⁶⁶ S. H. Eppstein and S. Morgulis, *J. Nutrition*, 22, 415-424 (1941).

pig is given as 6 mg. per animal every other day.⁴⁶⁷ Dju and co-workers¹¹¹ reported that the laying hen requires 1.2 mg. vitamin E per day. It seems probable that the requirement for vitamin E, like that of other vitamins, can best be related to some physiologic function rather than to that of the whole body weight. Harris⁴⁶⁸ plotted the vitamin E requirements for various species of animals, on the basis of adapted values cited for: rats;^{209,211,213,464} mice;^{212,213} chicks;^{282,314,469} ducklings;⁴⁷⁰ guinea pigs;^{467,471} chickens;⁴⁷² lambs;²⁵⁰ dogs;³²⁴ calves;⁴⁷³ rabbits;^{267,466,474,475} and goats.⁴⁷⁶

It is known that the absorption of fats from the gastrointestinal tract is best related to surface area⁴⁷⁷ (See *The Lipids*, Vol. II, pp. 128, 129). This value may be obtained by multiplying weight to the 0.67 power by a constant which varies with each species. MacKay and Bergman⁴⁷⁸ reported that surface area is the most satisfactory constant for determining absorption of glucose. Brody⁴⁷⁹ stated that various functions and reactions of the body are related, not to body weight itself, but to the 0.7 power of the body weight. He designated this function of the body weight ($\text{wgt}^{0.7}$) as the "physiological weight," as contrasted with $\text{wgt}^{1.0}$, which is referred to as "physical" or "gravitational" weight. Factors such as basal metabolism, endogenous nitrogen excretion, milk energy production, egg energy production and related phenomena all appear to vary as the 0.7 power of body weight.⁴⁶⁸

Harris⁴⁶⁸ found that, when the log dose of the daily requirement of vitamin E was plotted against the log of body weight, the points fell along a relatively straight line, the slope of which was 0.73. This value is not significantly different from Brody's value of 0.7. It is therefore believed that the requirement of different species of lower animals for vitamin E can be deduced from the weight.^{0.73}

⁴⁶⁷ F. A. Farmer, B. C. Mutch, J. M. Bell, L. D. Woolsey, and E. W. Crampton, *J. Nutrition*, **42**, 309-318 (1950).

⁴⁶⁸ P. L. Harris, *Ann. New York Acad. Sci.*, **52**, 240-242 (1949).

⁴⁶⁹ H. Patrick and C. L. Morgan, *Poultry Sci.*, **23**, 525-528 (1944).

⁴⁷⁰ A. M. Pappenheimer, *Proc. Soc. Exptl. Biol. Med.*, **45**, 457-459 (1940).

⁴⁷¹ N. Shimotori, G. A. Emerson, and H. M. Evans, *Science*, **90**, 89 (1939).

⁴⁷² M. Y. Dju, Unpublished results cited by P. L. Harris, *Ann. New York Acad. Sci.*, **52**, 240-242 (1949).

⁴⁷³ D. B. Parrish, G. H. Wise, and J. S. Hughes, *J. Dairy Sci.*, **30**, 849-860 (1947).

⁴⁷⁴ E. L. Hove and P. L. Harris, *J. Nutrition*, **33**, 95-106 (1947).

⁴⁷⁵ S. H. Eppstein and S. Morgulis, *J. Nutrition*, **23**, 473-482 (1942).

⁴⁷⁶ D. A. Spencer, *Food & Life*, U. S. Printing Office House Doc., **28**, 758-762 (1939); cited by P. L. Harris, *Ann. New York Acad. Sci.*, **52**, 240-242 (1949), p. 241.

⁴⁷⁷ H. J. Deuel, Jr., L. Hallman, and A. Leonard, *J. Nutrition*, **20**, 215-226 (1940).

⁴⁷⁸ E. M. MacKay and H. C. Bergman, *J. Biol. Chem.*, **101**, 453-462 (1933).

⁴⁷⁹ S. Brody, *Bioenergetics and Growth*, Reinhold, New York, 1945.

Figure 2 indicates the relationship between the tocopherol requirement and body weight in eleven species of animals, as adapted from values based upon various standards of measurement.

Fig. 2. The vitamin E requirement of different species, expressed in I.U. per day, plotted against the average body weight in kg. (The line joining the points has an inflection of 0.73.)⁴⁶⁸

(2) Normal Requirements of Man for Vitamin E

There are no reliable figures for the vitamin E requirement of man. When one calculates an intermediate value from comparative body weights of the lower animals and of man, one arrives at a figure of 60 mg./day. Harris⁴⁶⁸ considers this value inordinately large. On the other hand, when the interpolation is made from the relation to the 0.7 power of the body weight, the figure for a 70 kg. man would be 12 mg. daily. Harris⁴⁶⁸ considers that the requirement of man probably falls in the range of 12 to 60 mg. per day, and a figure of 30 mg. is arbitrarily chosen.

Dietary surveys in Holland¹²² and in the United States⁴⁸⁰ have given the

⁴⁸⁰ L. E. Booher and I. T. Behan, *J. Nutrition*, 39, 495-515 (1949).

vitamin E intake as 6 mg./1000 Calories or 14 mg. (13 I.U.) daily per capita.⁷¹ Hickman⁴⁸¹ suggests that diets considered as low, average, and high in vitamin E should provide the following daily quantities of α -tocopherol for persons of different ages, respectively: infants, 1, 4, and 10 mg.; children, 4, 10, and 20 mg.; adults, 5, 15, and 35 mg.; and aged, 3, 10 and, and 15 mg. The lower values suggested for aged individuals are based upon reduced tissue storage, which is normally noted during this period.^{482,483} Mason and Dju⁴⁸³ observed suboptimal vitamin E values during the early postnatal period as well. Neuweiler¹⁰³ placed the requirement of the newborn infant for vitamin E as 5 mg. per day; it has practically no vitamin E reserve, and is therefore dependent upon that in the milk to fulfil the requirement.

There is no evidence that a state of hypervitaminosis E can be produced by excessive dosages of this vitamin. Thus, Demole⁴⁸⁴ reported that mice will tolerate oral doses of 50 g./kg., while rats tolerate 4 g./kg. over a period of two months. Human adults have taken daily doses of 1 g. orally for months with no undesirable symptoms. For a discussion of the therapeutic use of vitamin E in heart disease, peripheral vascular disease, menopausal syndrome, primary fibrositis and related disorders, and in diabetes mellitus, the reader is referred to the review by Harris, Mattill, and Mason,⁶¹ pp. 564-570.

⁴⁸¹ K. C. D. Hickman, *Record Chem. Prog.*, 9, 104-121 (1948).

⁴⁸² K. E. Mason, M. Y. Dju, and I. J. Filer, Jr., *Federation Proc.*, 11, 449-450 (1952).

⁴⁸³ K. E. Mason and M. Y. Dju, *Nat. Vitamin Found., New York, Nutrition Symp. Ser.*, No. 7, 1-19 (1953).

⁴⁸⁴ V. Demole, *Z. Vitaminforsch.*, 8, 338-341 (1939).

CHAPTER XII

THE METABOLISM AND NUTRITIONAL VALUE OF THE VITAMINS K

1. Introduction

The vitamins K represent the most recently discovered major group of the fat-soluble vitamins. As early as 1929, Dam¹ reported that certain symptoms, including subcutaneous and intramuscular hemorrhages, could be produced in chicks by an artificial diet low in lipids. The deficiency failed to respond to any of the known vitamins. Dam² later found that the disease was associated with prolonged blood clotting time. It was first postulated in 1934, by Dam and Schönheyder,³ that the hemorrhagic disease in chicks resulted from an avitaminosis. The essential vitamin concerned was found to be present in green leaves and in certain vegetables,⁴ and was named *vitamin K*,^{4,5} inasmuch as Dam and others had most frequently referred to it as *koagulationsvitamin*, in the German and Danish literature. Others, who reported before 1935 that modifications in the diet led to hemorrhagic symptoms in the chick, or to changes in blood clotting time, included Holst and Halbrook,⁶ Horvath,⁷ and McFarlane *et al.*^{8,9}

The first convincing chemical evidence that vitamin K occurs in the fat-soluble, non-saponifiable, non-sterol fraction of fats was reported in 1935 by Dam^{4,5} and by Almquist and Stokstad.^{10,11} Dam, Schönheyder, and Tage-Hansen¹² demonstrated that vitamin K is of importance in regulating

¹ H. Dam, *Biochem. Z.*, **215**, 475-492 (1929).

² H. Dam, *Biochem. Z.*, **220**, 158-163 (1930).

³ H. Dam and F. Schönheyder, *Biochem. J.*, **28**, 1355-1359 (1934).

⁴ H. Dam, *Biochem. J.*, **29**, 1273-1285 (1935).

⁵ H. Dam, *Nature*, **135**, 652-653 (1935).

⁶ W. F. Holst and E. R. Halbrook, *Science*, **77**, 354 (1933).

⁷ A. A. Horvath, *Am. J. Physiol.*, **94**, 65-68 (1930).

⁸ W. D. McFarlane, W. R. Graham, Jr., and G. E. Hall, *J. Nutrition*, **4**, 331-349 (1931).

⁹ W. D. McFarlane, W. R. Graham, Jr., and F. Richardson, *Biochem. J.*, **25**, 358-366 (1931).

¹⁰ H. J. Almquist and E. L. R. Stokstad, *Nature*, **136**, 31⁺ (1935).

¹¹ H. J. Almquist and E. L. R. Stokstad, *J. Biol. Chem.*, **111**, 105-113 (1935).

¹² H. Dam, F. Schönheyder and E. Tage-Hansen, *Biochem. J.*, **30**, 1075-1079 (1936).

and maintaining the normal level of prothrombin in the blood. It was recognized^{13,14} that subnormal levels of prothrombin occur in vitamin K avitaminosis. This may well account for the retardation or complete absence of blood clotting. The formation of vitamin K in foodstuffs relatively free from the vitamin was shown to be initiated by certain micro-organisms.^{4,5,10,11} In 1938, the importance of vitamin K deficiency in explaining some types of hemorrhagic disease in man was first recognized and proved by several groups of workers in the United States,^{15,16} as well as by Dam and Glavind^{17,18} in Denmark.

Pure vitamin K was first isolated almost simultaneously, in 1939, by Dam and associates¹⁹ and by MacCorquodale and co-workers.²⁰ The product prepared from alfalfa by Dam *et al.*¹⁹ and by MacCorquodale *et al.*²⁰ differed in both physical and chemical properties from the preparation of McKee and co-workers,^{21,22} who employed putrified fish meal as the starting material. Both preparations were highly active as antihemorrhagic agents. This indicated that more than one product possesses vitamin K activity. The one present in alfalfa was named vitamin K₁, while that occurring in putrified fish was designated as vitamin K₂.

The structural formulae of the K vitamins were elucidated in record time after they had been separated in pure form. Thus, that for vitamin K₁ was announced almost simultaneously in 1939 by Almquist and Klose,²³ by Binkley *et al.*,²⁴ and by Fieser *et al.*²⁵ The proof of the postulated structure was in all cases supported by evidence of synthesis. Binkley, McKee,

¹³ F. Schönheyder, *Nature*, **135**, 653 (1935).

¹⁴ F. Schönheyder, *Biochem. J.*, **30**, 890-896 (1936).

¹⁵ E. D. Warner, K. M. Brinkhous, and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, **37**, 628-630 (1938).

¹⁶ A. M. Snell, T. B. Magath, E. W. Boland, A. E. Osterberg, H. R. Butt, J. L. Bollman, and W. Walters, *Proc. Staff Meetings, Mayo Clinic*, **13**, 65-80 (1938).

¹⁷ H. Dam and H. Glavind, *Lancet*, **234**, 720-721 (1938).

¹⁸ H. Dam and H. Glavind, *Acta Med. Scand.*, **96**, 108-128 (1938).

¹⁹ H. Dam, E. Geiger, J. Glavind, P. Karrer, W. Karrer, E. Rothschild, and H. Salomon, *Helv. Chim. Acta*, **22**, 310-313 (1939).

²⁰ D. W. MacCorquodale, S. B. Binkley, R. W. McKee, S. A. Thayer, and E. A. Doisy, *Proc. Soc. Exptl. Biol. Med.*, **40**, 482-483 (1939).

²¹ R. W. McKee, S. B. Binkley, D. W. MacCorquodale, S. A. Thayer, and E. A. Doisy, *J. Am. Chem. Soc.*, **61**, 1295 (1939).

²² R. W. McKee, S. B. Binkley, S. A. Thayer, D. W. MacCorquodale, and E. A. Doisy, *J. Biol. Chem.*, **131**, 327-344 (1939).

²³ H. J. Almquist and A. A. Klose, *J. Am. Chem. Soc.*, **61**, 2157-2158 (1939).

²⁴ S. B. Binkley, L. C. Cheney, W. F. Holcomb, R. W. McKee, S. A. Thayer, D. W. MacCorquodale, and E. A. Doisy, *J. Am. Chem. Soc.*, **61**, 2158-2159 (1939).

²⁵ L. F. Fieser, D. M. Bowen, W. P. Campbell, E. M. Fry, and M. D. Gates, Jr., *J. Am. Chem. Soc.*, **61**, 1926-1927 (1939).

Thayer, and Doisy²⁶ reported the structure of vitamin K₂ the following year.

The active group for vitamin K activity was shown by Ansbacher and Fernholz²⁷ to be 2-methyl-1,4-naphthoquinone. This grouping occurs in both vitamins K₁ and K₂, and their biopotency was found to be proportional to the amount of 2-methyl-1,4-naphthoquinone which could be derived from their molecules. Fieser, Tishler, and Sampson²⁸ made a thorough and comprehensive study of the biologic activity of a wide variety of compounds including the naphthoquinones, naphthohydroquinones, esters and ethers of the hydroquinones and of many related compounds. For a more complete discussion of the structural relationships of vitamin K, the reader is referred to *The Lipids*, Vol. I, pages 832–859. Excellent reviews on vitamin K include those of Wöhlisch,²⁹ Riegel,³⁰ Grossman,³¹ Brinkhous,³² and Rosenberg.³³ The more recent reviews by the authorities in the field include those of Almquist,³⁴ Harris *et al.*,³⁵ Dam,^{36,37} and Doisy *et al.*³⁸

The formulas of the vitamin K-active compounds are shown below.

Vitamin K₁

(2-Methyl-3-phytyl-1,4-naphthoquinone)

²⁶ S. B. Binkley, R. W. McKee, S. A. Thayer, and E. A. Doisy, *J. Biol. Chem.*, **133**, 721–729 (1940).

²⁷ S. Ansbacher and E. Fernholz, *J. Am. Chem. Soc.*, **61**, 1924–1925 (1939).

²⁸ L. F. Fieser, M. Tishler, and W. L. Sampson, *J. Biol. Chem.*, **137**, 659–692 (1941).

²⁹ E. Wöhlisch, *Ergeb. Physiol. biol. Chem. exptl. Pharmacol.*, **43**, 174–370 (1940).

³⁰ B. Riegel, *Ergeb. Physiol. biol. Chem. exptl. Pharmacol.*, **43**, 133–173 (1940).

³¹ A. M. Grossman, *J. Pediat.*, **16**, 239–253 (1940).

³² K. M. Brinkhous, *Medicine*, **19**, 329–416 (1940).

³³ H. R. Rosenberg, *Chemistry and Physiology of the Vitamins*, Interscience, New York, 1945.

³⁴ H. J. Almquist, *Physiol. Revs.*, **21**, 194–216 (1941).

³⁵ R. L. Harris, H. J. Almquist, and C. A. Owen, Jr., *Vitamin K. Group*, in W. H. Sebrell, Jr. and R. S. Harris, *The Vitamins*, Vol. II, Academic Press, Inc., New York, 1954, pp. 387–448.

³⁶ H. Dam, *Advances in Enzymol.*, **2**, 285–324 (1942).

³⁷ H. Dam, *Vitamins and Hormones*, **6**, 27–53 (1948).

³⁸ E. A. Doisy, S. B. Binkley, and S. A. Thayer, *Chem. Revs.*, **28**, 477–517 (1941).

Vitamin K₂

(2-Methyl-3-difarnesyl-1,4-naphthoquinone)

Menadione

(2-Methyl-1,4-naphthoquinone)

2. The Occurrence of the Vitamins K

(1) Occurrence in Plants

The chief natural sources of vitamin K₁ are the green leafy plants such as alfalfa,^{11,39,40} spinach,^{29,40} cabbage,^{4,5,10,40} kale, cauliflower, nettle, and chestnut leaves.⁴⁰ The inner leaves of the cabbage have been shown to have only about one-fourth of the vitamin K content of the outer leaves.⁴⁰ The tops of carrots are a good source of this vitamin, but very little is present in the roots.³⁵ Dam and Glavind⁴⁰ noted the presence of vitamin K in tomatoes, hempseed, and seaweed, while Almquist and Stokstad⁴¹ found that it was a component of soybean oil. More vitamin K occurs in peas sprouted in the light than in those kept in the dark.⁴⁰ According to Shinowara *et al.*,⁴² the berries of the European mountain ash (*Sorbus aucuparia*, L.) are good sources of the vitamin. Vitamin K has also been found as a component of oat shoots, and to a limited extent in

³⁹ A. J. Quick, *Am. J. Physiol.*, **118**, 260-271 (1937).

⁴⁰ H. Dam and J. Glavind, *Biochem., J.*, **32**, 485-487 (1938).

⁴¹ H. J. Almquist and E. L. R. Stokstad, *J. Nutrition*, **14**, 235-240 (1937).

⁴² G. Y. Shinowara, J. C. DeLor, and J. W. Means, *J. Lab. Clin. Med.*, **27**, 897-907 (1942).

cereals.^{16, 29, 40, 43, 44} Dam, Glavind, and Gabrielson⁴⁵ reviewed the general subject of the occurrence of vitamin K in plants. They concluded that the parts of the plant from which chlorophyll is absent are largely devoid of vitamin K. However, yellow-spotted leaf areas were found to contain as much vitamin K as did the green areas. Variations in the intensity of green in the leaves of different varieties of plants could not be correlated with a similar difference in vitamin K content. The natural changes in chlorophyll which occur on the death of the leaves in the autumn do not bring about a corresponding destruction of the vitamin K. Finally, no relationship has been established between the carotene or xanthophyll content and the amount of vitamin K.⁴⁵ Vitamin K present in the pressjuice of spinach leaves was stable after standing overnight. Most of the vitamin was found in the chloroplasts.⁴⁶

(2) Occurrence in Microorganisms

According to Almquist, Pentler, and Mecchi,⁴⁷ most bacteria contain vitamin K₂. Antihemorrhagic activity was shown to develop in foodstuffs during bacterial spoilage,⁴¹ and also to be present in the feces of chicks which did not receive vitamin K in their diet.⁴⁸ In a further study of this problem, Almquist³⁴ cultivated various pure strains of bacteria on a vitamin K-free medium, and determined the vitamin K activity of the bacterial cultures. The vitamin K activities (expressed as 2-methyl-1,4-naphthoquinone units per g.) were as follows:⁴⁵ *Bacillus subtilis*, 190; *Bacillus mycoides*, 155; *Bacillus cereus*, 115; *Sarcina lutea*, 100; *Proteus vulgaris* (*Bacterium proteus*), 75; *Micrococcus pyogenes* var. *aureus*, 60; *Mycobacterium tuberculosis*, 55; *Shigella flexneri*, 30; *Aerobacter aerogenes*, 20; *Serratia marcescens* (*Erythrobacillus prodigiosus*), 20; *Salmonella typhosa*, 15; and *Escherichia coli*, 15.

Intestinal bacteria, in general, can readily synthesize vitamin K. For this reason the vitamin appears in feces of the chick⁴⁸ and of human subjects. It is also present in the rumen of the cow, even when the animal is on a vitamin K-deficient diet.⁴⁹ In this case the vitamin is synthesized by the microorganisms in the rumen. Molds, yeasts, and fungi contain practically no vitamin K₁ or K₂.

⁴³ H. Dam, *Angew. Chem.*, 50, 807-811 (1937).

⁴⁴ E. M. Nelson and C. D. Tolle, *Ann. Rev. Biochem.*, 8, 415-434 (1939).

⁴⁵ H. Dam, J. Glavind, and E. K. Gabrielson, *Acta Physiol. Scand.*, 13, 9-19 (1947).

⁴⁶ H. Dam, E. Hjorth, and I. Kruse, *Physiologia Plantarum*, 1, 379-381 (1948).

⁴⁷ J. Almquist, C. F. Pentler, and E. Mecchi, *Proc. Soc. Exptl. Biol. Med.*, 38, 336-338 (1938).

⁴⁸ H. J. Almquist and E. L. R. Stokstad, *J. Nutrition*, 12, 329-335 (1936).

⁴⁹ L. W. McElroy and H. Goss, *J. Nutrition*, 20, 527-540 (1940).

a. The Role of Vitamin K in Bacterial Growth. At least one microorganism, John's bacillus (*Mycobacterium paratuberculosis*), which causes chronic dysentery in cattle, responds with increased growth when vitamin K, phthiocol, or methyl naphthoquinone is added to the synthetic medium upon which the bacteria are grown.⁵⁰ On the other hand, Glavind and Dam⁵¹ were unable to confirm the growth-stimulating effect of methyl-naphthoquinone or of phthiocol on a related (Danish) strain of this bacterium.

Vitamin K was found by Iland⁵² to exert a growth-depressing action on *Mycobacterium tuberculosis*. Methyl-naphthohydroquinone diphosphate, also, was reported to exhibit a bacteriostatic effect against this organism, as were 2-methyl-4-amino-1-naphthol and the bisulfite addition compound.⁵³ Dam⁵⁴ reviewed the various effect of vitamin K on caries, tumors, and microorganisms.

b. The Effect of Antibiotics. When animals are treated with sulfonamides, bacterial action is decreased and there is a concomitant reduction in the synthesis of vitamin K. Thus, when sulfaguanidine or sulfasuxidine was added to a synthetic diet free from vitamin K, a relatively rapid onset of prothrombin deficiency was noted;⁵⁵ this could be prevented by the administration of vitamin K.^{56,57}

(3) Occurrence in Animals

Most animal products contain very little vitamin K, especially if it has not been included in the diet. Both vitamins K₁ and K₂ may be stored in the tissues; the type which is present will depend upon the source of the vitamin. Thus, when abundant amounts of vitamin K₁ are taken in the food, vitamin K₁ will be the main form found in the tissues. On the other hand, when the bulk of vitamin K stored can be traced to that originating in the gut, vitamin K₂ predominates in the tissues. Dam and Schönheyder⁵⁸ reported that hog liver is the main source of vitamin K in animal products, while chicken liver^{48,58} and rat liver⁵⁸ contain very little

⁵⁰ D. W. Woolley and J. R. McCarter, *Proc. Soc. Exptl. Biol. Med.*, **45**, 357-360 (1940).

⁵¹ J. Glavind and H. Dam, *Physiologia Plantarum*, **1**, 1-4 (1948).

⁵² C. N. Iland, *Nature*, **161**, 1010 (1948).

⁵³ A. Kimler, *J. Bacteriol.*, **60**, 469-472 (1950).

⁵⁴ H. Dam, *Ann. Rev. Biochem.*, **20**, 265-304 (1951).

⁵⁵ S. Black, R. S. Overman, C. A. Elvehjem, and K. P. Link, *J. Biol. Chem.*, **145**, 137-143 (1942).

⁵⁶ A. D. Welch and L. D. Wright, *J. Nutrition*, **25**, 555-570 (1943).

⁵⁷ H. G. Day, K. G. Wakim, M. M. Krider, and E. E. O'Banion, *J. Nutrition*, **26**, 585-600 (1943).

⁵⁸ H. Dam and F. Schönheyder, *Biochem., J.*, **30**, 897-901 (1936).

vitamin K. Almquist and Stokstad⁴⁸ demonstrated that egg-yolk contains considerable amounts of the vitamin when the hens are given a vitamin K-rich diet. Rosenberg³³ reported that cow's milk is fairly rich in vitamin K when the cow has received adequate amounts of the vitamin in her rations.

3. The Digestion and Absorption of the Vitamins K

Vitamin K is available for absorption from the gastrointestinal tract from two sources, namely that present in the diet and that synthesized *in situ*. The natural forms of vitamin K are insoluble in water, and are soluble only in fats, by virtue of the property conferred upon them as a result of the long aliphatic side chains. However, menadione (2-methyl-1,4-naphthoquinone), which is the most potent compound with vitamin K activity, is slightly soluble in water; it does not possess the aliphatic side chain. It is therefore evident that the factors involved in the absorption of the natural forms of vitamin K differ considerably from those of importance in the menadione absorption.

No enzymes are required to prepare either the natural or the synthetic K vitamins for absorption. However, as in the case of the other fat-soluble vitamins and of fat itself, bile is essential to insure the absorption of the vitamins K. Greaves and Schmidt⁵⁹ were the first to prove that bile is necessary to enable rats to utilize vitamin K satisfactorily. The importance of bile in the absorption of the vitamin has also been demonstrated in chicks¹⁸ and in dogs.^{60,61} Manifestations of vitamin K deficiency such as loss of coagulability of the blood, which was associated with a low prothrombin content, occurred when the bile duct was ligated. It was observed that the symptoms of vitamin K deficiency in rats with occluded bile ducts could be partially relieved when vitamin K was given orally. However, efficient recovery took place only when bile or bile salts were administered along with the vitamin. According to Quick,³⁹ bile salts have the same relationship to the prothrombin level as to vitamin K.

Schmidt⁶² suggested that desoxycholic acid is responsible for bringing about the absorption of vitamin K from the gut. Almquist and Klose⁶³ reported the preparation of a vitamin K-choleic acid coördination complex

⁵⁹ J. D. Greaves and C. L. A. Schmidt, *Proc. Soc. Exptl. Biol. Med.*, **37**, 43-45 (1937).

⁶⁰ W. B. Hawkins and K. M. Brinkhous, *J. Exptl. Med.*, **63**, 795-801 (1936).

⁶¹ H. P. Smith, E. D. Warner, K. M. Brinkhous, and W. H. Seegers, *J. Exptl. Med.*, **67**, 911-920 (1938).

⁶² C. L. A. Schmidt, *Pacific Coast Med.*, **5**, No. 3, 7-10 (1938).

⁶³ H. J. Almquist and A. A. Klose, *J. Am. Chem. Soc.*, **61**, 745-746 (1939).

which was effective in curing vitamin K deficiency in bile-fistula rats. Dam *et al.*⁶⁴ confirmed these results by demonstrating that a crude vitamin K solution in desoxycholate cured vitamin K deficiency in chicks when given subcutaneously; a water emulsion was also found to be effective when administered by the intramuscular route, but not when injected subcutaneously.

Several observers^{59,61,65} have demonstrated that the administration of bile to animals with ligated bile ducts results in an increased prothrombin level in the blood, in spite of the fact that bile contains practically none of this vitamin.^{66,67} The interpretation of the beneficial effect of bile under these conditions has been that it assists in the absorption of the vitamin K which has been newly-synthesized in the intestine, as well as of any vitamin K in the food.

The results of a deficiency of vitamin K in animals are mirrored by those of lack of vitamin K in man. However, vitamin K deficiency does not occur in man only when the vitamin is lacking in the diet, inasmuch as adequate quantities are synthesized in the gut. On the other hand, when the absorption of vitamin K is prevented by the absence of bile in the gastrointestinal tract, characteristic deficiency symptoms rapidly develop. Thus Quick and co-workers⁶⁸ noted that subnormal prothrombin values obtained in jaundice, while a number of workers^{15,16,69} reported that the hemorrhagic tendency in obstructive jaundice could be overcome when vitamin K was administered with bile or with bile salts. Water-soluble preparations of vitamin K can be absorbed in the absence of bile.⁷⁰

Several factors other than bile may influence the absorption of vitamin K. Thus, when mineral oil is fed, the appearance of vitamin K deficiency is accelerated, in mice⁷¹ and in rats.^{72,73} According to Javert and Maeri,⁷⁴

⁶⁴ H. Dam, J. Glavind, L. Lewis, and E. Tage-Hansen, *Skand. Arch. Physiol.*, **79**, 121-133 (1938).

⁶⁵ J. D. Greaves, *Am. J. Physiol.*, **125**, 423-428 (1939).

⁶⁶ J. D. Greaves, *Am. J. Physiol.*, **125**, 429-436 (1939).

⁶⁷ H. J. Almquist, *Science*, **87**, 538 (1938).

⁶⁸ A. J. Quick, M. Stanley-Brown, and F. W. Bancroft, *Am. J. Med. Sci.*, **190**, 501-511 (1935).

⁶⁹ K. M. Brinkhous, H. P. Smith, and E. D. Warner, *Am. J. Med. Sci.*, **196**, 50-57 (1938).

⁷⁰ E. D. Warner and J. E. Flynn, *Proc. Soc. Exptl. Biol. Med.*, **44**, 607-608 (1940).

⁷¹ W. A. Barnes, *Proc. Soc. Exptl. Biol. Med.*, **49**, 15-19 (1942).

⁷² M. C. Elliott, B. Isaacs, and A. C. Ivy, *Proc. Soc. Exptl. Biol. Med.*, **43**, 240-245 (1940).

⁷³ E. K. Bacon, S. Lassen, S. M. Greenberg, J. W. Mehl, and H. J. Deuel, Jr., *J. Nutrition*, **47**, 383-398 (1952).

⁷⁴ C. T. Javert and C. Maeri, *Am. J. Obstet. Gynecol.*, **42**, 409-414 (1941).

the prothrombin time is increased in man after the administration of petroleum oil. It is believed that the mineral oil prevents the absorption of the endogenous vitamin K which is synthesized in the intestine, and also of that of exogenous origin. Almquist³⁴ reviewed the subject of bile in relation to the absorption of vitamin K, in his excellent treatise on this vitamin.

4. The Synthesis of Vitamin K in the Gastrointestinal Tract

It has already been mentioned that most bacteria are capable of synthesizing vitamin K when grown on a vitamin K-free medium. Many of the bacteria which belong in this category are normal inhabitants of the gastrointestinal tract. When these bacteria are prevented from functioning in their normal manner, and when vitamin K is also withheld from the diet, symptoms of vitamin K deficiency develop very rapidly, in experimental animals as well as in man.

The sulfa drugs exert a powerful action in the intestine, where they prevent normal bacterial growth. Black *et al.*^{55,75} were the first to demonstrate that sulfaguanidine decreased the rate of growth in rats, and resulted in hypoprothrombinemia. These effects could be prevented when vitamin K was administered. Kornberg *et al.*⁷⁶ later reported that sulfapyrazine, sulfadiazine, and sulfathiazole were more effective in producing a deficiency of vitamin K than were sulfaguanidine, succinyl sulfathiazole, or sulfanilamide. The coliform organisms are known to produce vitamin K when incubated *in vitro*.⁷⁷ These are the organisms which appear in reduced amounts in the feces of rats after treatment with sulfaguanidine or succinyl sulfathiazole.⁷⁸ Thus, the action of sulfaguanidine in producing vitamin K deficiency is explained by its inhibiting effect upon the synthesis of vitamin K by the intestinal flora.⁷⁵ Support for this theory is given by the results of Kornberg and co-workers,⁷⁹ who showed that the cecal contents and the collected feces of rats treated with sulfonamides possessed only a slight vitamin K activity. According to Day *et al.*,⁵⁷ cecectomy increases the deficiency of vitamin K in rats fed succinyl sulfathiazole.

In the case of newborn babies, a vitamin K deficiency sometimes occurs

⁷⁵ S. Black, J. M. McKibbin, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **47**, 308-310 (1941).

⁷⁶ A. Kornberg, F. S. Daft, and W. H. Sebrell, *Pub. Health Reports, U. S. Pub. Health Service*, **59**, 832-844 (1944).

⁷⁷ S. Orla-Jensen, A. D. Orla-Jensen, H. Dam, and J. Glavind, *Zentr. Bacteriol., Abl.*, **2**, 104, 202-204 (1941); *Chem. Zentr.*, **113**, 1155 (1942).

⁷⁸ O. K. Gant, B. Ransone, E. McCoy, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **52**, 276-279 (1943).

⁷⁹ A. Kornberg, F. S. Daft, and W. H. Sebrell, *J. Biol. Chem.*, **155**, 193-200 (1944).

which may be defined as alimentary. A number of investigators reported that the prothrombin level is low at birth,^{80,81} or, if normal, ^{82,83} rapidly drops during the early days of life,⁸⁴ presumably because of the lack of vitamin K in the diet, and of any alimentary source, since vitamin K cannot be synthesized in the sterile meconium of the gastrointestinal tract. When food is taken, recovery may occur spontaneously, presumably due to the establishment of suitable intestinal flora.⁸⁵⁻⁸⁷

5. The Transport of Vitamin K from the Intestine to the Tissues

There is little direct information as to the mechanism by which the vitamins K are distributed to the various tissues of the animal. Since increased amounts of the vitamin occur in the liver and other tissues after their administration, this must mean that they are transported either by the blood stream or by the lymphatics, or both. Since the natural forms are insoluble in water, and are soluble only in fat, it would seem probable that the pathway of transport from the intestine would be the same as that for other fat-soluble vitamins and for fat itself, namely the lacteals, the intestinal lymphatics, and ultimately the thoracic duct, from which it is emptied into the blood stream.

Evidence for the lymphatic route has recently been adduced by the use of a new operative procedure devised by Bollman, Cain, and Grindley⁸⁸ for the collection of lymph from the liver. When the intestinal lymph was drained, a vitamin K deficiency could be produced within a short period.⁸⁹ One must interpret these data as evidence that the natural forms of vitamin K are transported *via* the lymph, and that an insufficient quantity is carried *via* the portal system to counteract the hypoprothrombinemia. Millar *et al.*,⁹⁰ who used radioactive vitamin K₁, reported the presence of the vitamin in the intestinal lymph of rats and dogs. Radioactive products were also

⁸⁰ K. M. Brinkhous, H. P. Smith, and E. D. Warner, *Am. J. Med. Sci.*, 193, 475-480 (1937).

⁸¹ K. Kato and H. G. Poncher, *J. Am. Med. Assoc.*, 114, 749-753 (1940).

⁸² K. K. Nygaard, *Acta Obstet. Gynecol. Scand.*, 19, 361-370 (1939).

⁸³ C. A. Owen, G. R. Hoffman, S. E. Ziffren, and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, 41, 181-185 (1939).

⁸⁴ A. J. Quick, *Wisconsin Med. J.*, 38, 746 (1939).

⁸⁵ A. J. Quick and A. M. Grossman, *Am. J. Med. Sci.*, 199, 1-9 (1940).

⁸⁶ A. J. Quick and A. M. Grossman, *Proc. Soc. Exptl. Biol. Med.*, 40, 647-648 (1939).

⁸⁷ A. J. Quick and A. M. Grossman, *Proc. Soc. Exptl. Biol. Med.*, 41, 227-228 (1939).

⁸⁸ J. L. Bollman, J. C. Cain, and J. H. Grindley, *J. Lab. Clin. Med.*, 33, 1349-1352 (1948).

⁸⁹ J. D. Mann, F. D. Mann, and J. L. Bollman, *Am. J. Physiol.*, 158, 311-314 (1949).

⁹⁰ G. J. Millar, J. A. Leddy, and L. Fisher, *XIX International Physiological Congress*, Montreal, 1953, *Abstracts of Communications*, pp. 618-619.

excreted in the urine. Although the results on the pathway of absorption are convincing for the natural forms of vitamin K, they do not furnish information concerning the synthetic water-soluble forms such as menadione. It would appear highly probable that a portion if not all of such simpler preparations might be transported from the gut by the portal route in the blood stream. Solvonuk *et al.*^{91,92} found that, when labeled menadione was injected intramuscularly into mice and cholecystonephrostomized dogs, the material disappeared from the injection site, and that only traces of radioactivity were found in the blood; 60% of the injected amount was excreted in the urine within three hours. On the other hand, when Dam and co-workers⁹³ injected vitamin K₁ intravenously into chicks, they found that it disappeared from the blood stream, and was deposited in the liver, spleen, and lungs. No vitamin K₁ could be detected in the bile. After massive doses of vitamin K₁ were injected, 16% could still be recovered in the liver plus spleen after forty-two days. Apparently it is possible for the tissues to store considerable amounts of vitamin K under some conditions.

Richert⁹⁴ reported that 4-amino-2-methyl-1-naphthol, 2-methyl-1,4-naphthohydroquinone diphosphate, and 2-methyl-1-tetralone, which are all powerful antihemorrhagic agents, are extensively converted to methyl-naphthoquinone in the organism, and are excreted in combined form in the urine.

6. The Physiologic Role of Vitamin K

(1) *The Importance in Blood Coagulation*

The discovery of vitamin K and, in fact, most of the information concerning this vitamin, have been based upon its requirement in the maintenance of a normal blood-clotting mechanism. When vitamin K is withheld from the diet, and when that synthesized in the gut cannot be absorbed, an immediate and precipitous drop occurs in the prothrombin level in the blood. It is evident that vitamin K in some way controls the synthesis of this blood protein, which is necessary in the clotting mechanism as a source of thrombin. Hypoprothrombinemia is the immediate result of its absence from the body.

⁹¹ P. F. Solvonuk, L. B. Jaques, J. E. Ieddy, L. W. Trevoy, and J. W. T. Spinks, *Proc. Soc. Exptl. Biol. Med.*, **79**, 597-604 (1952).

⁹² P. F. Solvonuk, J. M. Pepper, and G. J. Millar, *Rev. can. biol.*, **11**, 83 (1952).

⁹³ H. Dam, I. Prange, and E. Søndergaard, *Acta pharmacol. et toxicol.*, **10**, 58-68 (1954).

⁹⁴ D. A. Richert, *J. Biol. Chem.*, **154**, 1-8 (1944).

On the other hand, vitamin K cannot effect blood clotting when added directly to the blood. Thus, Dam *et al.*^{12,64} reported that the vitamin in contact with prothrombin-deficient chick blood did not cause an acceleration in clotting time when tested *in vitro*. Even water-soluble forms such as methylnaphthoquinone, phthiocol, and the diphosphoric acid ester do not influence the rate of clotting when added directly to the blood.³⁴ When vitamin K is given in an emulsion to deficient chicks, no increase in prothrombin occurs immediately; at least five hours are required to restore the prothrombin level to normal.⁶⁴ It is believed that this precludes the possibility that vitamin K serves as a prosthetic group in combination with any of the blood elements.

a. The Liver as the Site of Action of Vitamin K. Since vitamin K is without activity in producing clotting *in vitro*, and can act only in relation to the animal as a whole, it is believed that it is required in the normal *in vivo* synthesis of prothrombin. There is considerable evidence that prothrombin is synthesized in the liver. It is now agreed that vitamin K must act in this organ to aid in the normal synthetic processes.

The proof that the liver is the site of prothrombin synthesis is based upon a number of different observations. Thus, Andrus and co-workers,⁹⁵ and Warren and Rhoads,⁹⁶ found that hepatectomy in dogs was followed by a decrease in the blood prothrombin level. When two-thirds of the liver was removed, in rats, a decreased prothrombin formation was likewise noted.⁹⁷ An injury to the functional activity of liver tissue has also been shown to reduce the ability to synthesize prothrombin. For example, prothrombin formation is impaired after chloroform poisoning,^{98,99} and after traumatic injury to this organ.¹⁰⁰ In none of these latter cases was vitamin K effective in stimulating the synthesis of prothrombin. Human patients with severe liver damage failed to respond to the administration of substrates containing vitamin K and bile salts.⁹⁶ Brinkhous and Walker¹⁰¹ found that the prothrombin concentration in portal lymph is similar to that in the blood, whereas lymph from other sources has lower concentrations. This result would support the hypothesis that plasma prothrombin

⁹⁵ W. D. Andrus, J. W. Lord, Jr., and R. A. Moore, *Surgery*, **6**, 899-900 (1939).

⁹⁶ R. Warren and J. E. Rhoads, *Am. J. Med. Sci.*, **198**, 193-197 (1939).

⁹⁷ E. D. Warner, *J. Exptl. Med.*, **68**, 831-835 (1938).

⁹⁸ K. D. Brinkhous and E. D. Warner, *Proc. Soc. Exptl. Biol. Med.*, **44**, 609-610 (1940).

⁹⁹ J. L. Bollman, H. R. Butt, and A. M. Snell, *J. Am. Med. Assoc.*, **115**, 1087-1091 (1940).

¹⁰⁰ J. W. Lord, *Surgery*, **6**, 896-898 (1939).

¹⁰¹ K. M. Brinkhous and S. A. Walker, *Am. J. Physiol.*, **132**, 666-669 (1941).

in synthesized in the liver or, at least, that the finishing touches are completed there on proteins originating elsewhere.

Dicumarol, the action of which can be counteracted by vitamin K, is displaced from the liver, according to Lee *et al.*¹⁰² and Spinks and Jaques.¹⁰³ Other data which indicate that the liver is the site of vitamin K action include the report of Nassi and Ragazzini,¹⁰⁴ who reported that relatively large doses of vitamin K reduced liver glycogen. Hueper¹⁰⁵ found that einchophen, which acts upon the liver, did not change the clotting time of the blood of dogs unless they had been on a diet free from vitamin K. All these data would seem to point to the liver as the site of action of vitamin K. For a further discussion of the relation of the liver damage and prothrombin deficiency, the reader is referred to the review of Ferguson.¹⁰⁶

b. The Mechanism of Action of Vitamin K in Prothrombin Synthesis.

Quick and Collentine^{107,108} postulated that vitamin K₁ is a prosthetic group of an enzyme in the liver, which participates in the formation of prothrombin. Another suggestion is that a part or all of the vitamin is incorporated into the prothrombin molecule. This hypothesis gains some support from the fact that Lyons¹⁰⁹ found evidence for a quinone structure in thrombin. However, the prevailing opinion is that vitamin K does not form a part of the prothrombin molecule. Dam and associates⁶⁴ reported that refined prothrombin preparations possess no vitamin K activity. Quick¹¹⁰ calculated that less than one molecule of vitamin K is required for each mole of prothrombin produced.¹²⁰

McCawley and Gurchot¹¹¹ have advanced the suggestion that quinones with vitamin K activity aid in the synthesis of prothrombin, by virtue of their oxidation-reduction powers. Thus, the redox potential values of three active compounds were found to be 458, 328, and 256 for 2-methyl-1,4-naphthoquinone, vitamin K₁, and phthiocol, respectively. This is in the same order as their biopotencies. The data of Trenner and Bacher¹¹²

¹⁰² C. C. Lee, L. W. Trevoy, J. W. T. Spinks, and L. B. Jaques, *Proc. Soc. Exptl. Biol. Med.*, **74**, 151-155 (1950).

¹⁰³ J. W. T. Spinks and L. B. Jaques, *Nature*, **166**, 184 (1950).

¹⁰⁴ L. Nassi and F. Ragazzini, *Boll. soc. ital. biol. sper.*, **24**, 703-705 (1948).

¹⁰⁵ W. C. Hueper, *Arch. Pathol.*, **41**, 592-600 (1946).

¹⁰⁶ J. H. Ferguson, *Ann. Rev. Physiol.*, **8**, 231-262 (1946).

¹⁰⁷ A. J. Quick and G. E. Collentine, *Am. J. Physiol.*, **164**, 716-721 (1951).

¹⁰⁸ A. J. Quick and G. E. Collentine, *J. Lab. Clin. Med.*, **36**, 976 (1950).

¹⁰⁹ R. N. Lyons, *Nature*, **155**, 633-634 (1945).

¹¹⁰ A. J. Quick, *The Physiology and Pathology of Hemostasis*. Lea and Febiger, Philadelphia, 1951.

¹¹¹ E. L. McCawley and C. Gurchot, *Univ. Calif. (Berkeley) Publ. Pharmacol.*, **1**, 325-338 (1940).

¹¹² N. R. Trenner and F. A. Bacher, *J. Biol. Chem.*, **137**, 745-755 (1945).

support these conclusions. However, redox potential is not the only important consideration, since some of the naphthoquinones which had no biopotency possessed appreciable redox potential values.

It is not known what enzyme or enzymes may be concerned in the synthesis of prothrombin. Naphthoquinones which possess biopotency have been found to inhibit heart muscle succinoxidase,¹¹³ choline acetylase,^{114,115} and lactic acid-producing bacterial enzymes.^{116,117} Schönberg *et al.*¹¹⁸ stated that the configuration of menadione and that of phytyl menadione are theoretically and actually adequate for the Strecker degradation of α -amino acids. Another suggestion is that the naphthoquinones may act as plant respiratory pigments, for example juglon in the walnut, and lawson in henna leaves.¹¹¹ This fits in with the finding that, in certain plant leaves, vitamin K could be synthesized only if exposed to sunlight.^{40,48} Erkama and Pettersson¹¹⁹ observed that vitamin K formed in pea seedlings in the absence of light, but to a much lesser degree than when they were exposed to light.

c. Vitamin K in Various Types of Bleeding. A tendency to bleeding is a rather common condition in some types of nutritional deficiency such as scurvy, in hereditary hemophilia, and in thrombocytopenia. However, vitamin K is of no value in any of these hemorrhagic conditions, except in vitamin K deficiency. Table 1 summarizes the main characteristics of several types of bleeding.

According to Sebrell and Harris,¹²⁰ serious abnormalities in blood coagulation may be present in the absence of hemorrhage. They suggest that, when the plasma prothrombin falls to 20% of the normal value, danger of bleeding exists. When the prothrombin level exceeds this figure, bleeding seldom occurs. However, Brinkhous and co-workers⁶⁹ have suggested that the danger level for prothrombin is 35 to 20%, and Koller and Frick¹²¹ recorded a figure as low as 5% of normal as the critical prothrombin level.

The bleeding in vitamin K deficiency may first be recognized by slow oozing from the mucous membranes of the nasopharynx, by the occurrence

¹¹³ E. G. Ball, C. B. Anfinsen, and O. Cooper, *J. Biol. Chem.*, **168**, 257-270 (1947).

¹¹⁴ C. Torda and H. G. Wolff, *Proc. Soc. Exptl. Biol. Med.*, **57**, 236-239 (1944).

¹¹⁵ C. Torda and H. G. Wolff, *Science*, **103**, 645-646 (1946).

¹¹⁶ W. D. Armstrong, W. W. Spink, and J. Kahnke, *Proc. Soc. Exptl. Biol. Med.*, **53**, 230-234 (1943).

¹¹⁷ P. Atkins and J. L. Ward, *Brit. J. Exptl. Pathol.*, **26**, 120-124 (1945).

¹¹⁸ A. Schönberg, R. Moubasher, and A. Said, *Nature*, **164**, 140 (1949).

¹¹⁹ J. Erkama and N. Pettersson, *Acta Chem. Scand.*, **4**, 922-925 (1950).

¹²⁰ W. H. Sebrell, Jr. and R. S. Harris, *The Vitamins*, Vol. II, Acad. Press, New York, 1954, pp. 387-448, p. 420.

¹²¹ F. Koller and P. Frick, *Helv. Chim. Acta*, **32**, 717-722 (1949).

TABLE 1
CHARACTERISTICS OF SEVERAL COMMON TYPES OF BLEEDING^a

Category	Type of disorder			
	Vitamin K deficiency	Scurvy	Hemophilia	Thrombocytopenia
Clot time	Long	Normal	Long	Normal
Plasma prothrombin	Low	Normal ^b	Normal	Normal
Bleeding time	Normal	Long	Normal	Long
Capillary fragility	Normal	Increased	Normal	Increased
Response to vitamin K	+	0	0	0
Response to vitamin C	0	+	0	0
Response to "antihemophilic globulin"	0	0	+	0
Response to transfusion of whole blood	+		+	+

^a Adapted from W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Volume II, Acad. Press, New York, 1954, p. 420.

^b Unless there is a coexisting vitamin K deficiency.

of ecchymoses associated with mild trauma and, in surgical cases, by hematomas and persistent bleeding at the site of the operation. Massive hemorrhage may occur within the muscles and beneath the skin, particularly in the extremities.¹²⁰ For an extensive study of the gross and microscopic features of experimental vitamin K deficiency, the reader is referred to the article of Ferraro and Roizin.¹²²

d. Various Factors Associated with Vitamin K and with the Clotting Mechanism. Dam and Sndergaard¹²³ discovered that, when chicken plasma from vitamin K-deficient chicks was added to dicumarol plasma, the prothrombin time of the latter was reduced. Conversely, the clotting time of the chicken plasma was shortened by the addition of dicumarol plasma. Srbye *et al.*¹²⁴ succeeded in concentrating the active factor from oxalated plasma of vitamin K-deficient chicks. It was called factor κ ; its adsorption characteristics resemble those of prothrombin, but it was found to be distinctly different from the latter protein. It also differs from the labile factor in chicken plasma corresponding to factor V of mammalian plasma. It is believed that the synthesis of this factor κ is independent of vitamin K.

In a later study, Srbye and co-workers¹²⁵ concentrated a factor in di-

¹²² A. Ferraro and L. Roizin, *Am. J. Pathol.*, 22, 1109-1179 (1946).

¹²³ H. Dam and E. Sndergaard, *Biochim. Biophys. Acta*, 2, 409-413 (1948).

¹²⁴ O. Srbye, I. Kruse, and H. Dam, *Acta Chem. Scand.*, 4, 549-550 (1950).

¹²⁵ O. Srbye, I. Kruse, and H. Dam, *Acta Chem. Scand.*, 4, 831-832 (1950).

cumarolized chicks which was capable of accelerating the clotting of vitamin K-deficient plasma. This new factor was called the δ -factor.¹²⁶ It differed from factor V, from prothrombin, and from factor κ .

It is assumed that, during blood coagulation, thromboplastin does not act directly on prothrombin, but on proconvertin, and that it activates the latter to convertin.¹²⁷ Convertin transforms prothrombin into thrombin, aided by *accelerin*, which is formed from *proaccelerin*.¹²⁷ The latter is believed to consist of several factors. Dam¹²⁷ reviewed this subject in 1953.

(2) *In Counteracting Hemorrhagic Conditions due to Dicumarol*

The understanding of the mode of action and source of some of the natural hemorrhagic agents constitutes one of the brilliant discoveries of recent years. It was recognized by Roderick¹²⁸ that the disease occurring in cattle as a result of feeding on spoiled sweet clover was associated with a prothrombin deficiency. Approximately twelve to twenty-four hours elapse after the feeding of spoiled sweet clover before the hypoprothrombinemia is established. It is believed that this period of time is required to catabolize the prothrombin present at the time of the feeding.^{129,130} Since no new prothrombin is synthesized after the toxic agent is ingested, the blood prothrombin falls as that present becomes exhausted. Apparently the animal requires a continuous supply of new prothrombin to replace that being used, since no storehouse exists for this protein.¹³¹

Campbell and Link¹³² and Stahmann *et al.*¹³³ were the first to establish the identity of the toxic agent in spoiled sweet clover as "dicoumarin" (dicumarol), which chemically is 3,3'-methylenebis(4-hydroxycumarin). When this compound was administered to various species of animals, it was found to reduce blood prothrombin, especially when the vitamin K intake was low. It was demonstrated that the hypoprothrombinemia caused by dicumarol could be reversed by vitamin K.¹³⁴ Since dicumarol

¹²⁶ O. Sørbye, I. Kruse, and H. Dam, *Acta Chem. Scand.*, **5**, 487-488 (1951).

¹²⁷ H. Dam, *Vitamins and Antivitamins in Blood Coagulation*, Consiglio Nazionale delle Ricerche, Convegno sulle Vitamine, Milan, April 12-16, 1953, pp. 1-11.

¹²⁸ L. M. Roderick, *Am. J. Physiol.*, **96**, 413-424 (1931).

¹²⁹ K. P. Link, R. S. Overman, W. R. Sullivan, C. F. Huebner, and L. D. Scheel, *J. Biol. Chem.*, **147**, 463-474 (1943).

¹³⁰ M. Stefanini and A. V. Pisciotta, *Science*, **111**, 364 (1950).

¹³¹ J. R. Carter, G. H. Chambers, and E. D. Warner, *Proc. Soc. Exptl. Biol. Med.*, **72** 52-57 (1949).

¹³² H. A. Campbell and K. P. Link, *J. Biol. Chem.*, **138**, 21-33 (1941).

¹³³ M. A. Stahmann, C. F. Huebner, and K. P. Link, *J. Biol. Chem.*, **138**, 513-527 (1941).

¹³⁴ A. J. Quick and M. Stefanini, *J. Biol. Chem.*, **175**, 945-952 (1948).

can readily be degraded to salicylic acid by chemical means, and since the administration of salicylic acid to rats on a low-vitamin K diet caused a hypoprothrombinemia similar to that occurring after the administration of dicumarol, or in the absence of vitamin K, it was suggested that salicylic acid might be an intermediate of the latter compound, which possesses the hemorrhagic action. However, like vitamin K, neither dicumarol nor salicylic acid influences the clotting time of blood when applied to it *in vitro*.¹²⁹ Presumably, the hemorrhagic agent acts by inhibiting the synthesis of prothrombin in the liver, possibly by some interference with essential enzyme systems.

In addition to dicumarol (also called dicumarin, dikumarin, dicoumarol, "A.P.," bis-hydroxycoumarin)¹³⁵⁻¹³⁷ other related compounds have been found to possess a similar anticoagulant action.¹²⁰ These include hydrodicumarol (3,3'-methylenebis(3,4-dihydro, 4-hydroxycoumarol));¹³⁸ troloxan (3,3'-carboxylenebis(4-hydroxycoumarin) ethyl ester acetic acid, or 4,4'-dioxycoumarin ethyl acetate);¹³⁹ pelentan, "B.O.E.A.," ethyl biscoumacetate;¹⁴⁰⁻¹⁴³ phenylindanedione (phenyl-2-indanedione-1,3, ("P.-I.D.")),¹⁴⁴⁻¹⁴⁶ "danilone," "hedulin";¹²⁰ and anticoagulant "63" (2-methyl-2-methoxy-4-phenyl-5-oxodihydropyrano (3,2-C) (1)-benzopyran, cumopyran).¹⁴⁷⁻¹⁵⁰ These anticoagulants have been widely employed in medicine to reduce the ability of the blood to clot under certain conditions, and so to lessen the chances of thromboses.

¹³⁵ R. S. Overman, M. A. Stahmann, W. R. Sullivan, C. F. Huebner, H. A. Campbell, and K. P. Link, *J. Biol. Chem.*, **142**, 941-955 (1942).

¹³⁶ H. R. Butt, E. V. Allen, and J. L. Bollman, *Proc. Staff Meetings Mayo Clinic*, **16**, 388-395 (1941).

¹³⁷ H. Dyckerhoff, *Biochem. Z.*, **316**, 397-403 (1944).

¹³⁸ F. Ericksen, E. Jacobsen, and C. M. Plum, *Acta Pharmacol. Toxicol.*, **1**, 379-393 (1945).

¹³⁹ G. E. Burke and I. S. Wright, *Trans. Conf. on Blood Clotting and Allied Problems, New York*, **3**, 57-67 (1950).

¹⁴⁰ K. N. von Kaulla and R. Pulver, *Schweiz. med. Wochschr.*, **78**, 806-810 (1948).

¹⁴¹ R. Della Santa, *Schweiz. med. Wochschr.*, **79**, 195-206 (1949).

¹⁴² C. Solomon, H. J. McNeile, and R. Lange, *J. Lab. Clin. Med.*, **36**, 19-22 (1950).

¹⁴³ N. W. Barker, J. E. Estes, Jr., and F. D. Mann, *Proc. Staff Meetings Mayo Clinic*, **26**, 162-168 (1951).

¹⁴⁴ L. B. Jaques, E. Lepp, and E. Gordon, *Trans. Conf. on Blood Clotting and Allied Problems, New York*, **3**, 11-28 (1950).

¹⁴⁵ P. Meunier, C. Mentzer, and D. Molho, *Compt. rend.*, **224**, 1666-1667 (1947).

¹⁴⁶ J. P. Soulier and J. Guéguen, *Rev. d. hématol.*, **3**, 180-184, 185-195 (1948).

¹⁴⁷ M. Ikawa, M. A. Stahmann, and K. P. Link, *J. Am. Chem. Soc.*, **66**, 902-906 (1944).

¹⁴⁸ W. D. Battle, R. T. Capps, O. S. Orth, and O. O. Meyer, *J. Lab. Clin. Med.*, **35**, 8-15 (1950).

¹⁴⁹ R. Rotler and O. O. Meyer, *J. Lab. Clin. Med.*, **36**, 981-982 (1950).

¹⁵⁰ H. H. Hanson, N. W. Barker, and F. D. Mann, *Circulation*, **4**, 844-853 (1951).

One interesting compound which has a powerful anticoagulant action is the so-called *coumachlor*, which is 3-(α -*p*-chlorophenyl- β -acetylethyl)-4-hydroxycumarol; it is useful as a rodenticide, because of its anticoagulant action. Reiff and Wiesmann¹⁵¹ discovered that this compound, when ingested, prolonged the prothrombin time and resulted in hemorrhage and death. This effect can be counteracted by vitamin K₁, but not by menadione.¹⁵² Dam and Sndergaard¹⁵³ reported that coumachlor is more effective as an anticoagulant than is dicumarol, in chicks and rats.

One mechanism of action of dicumarol and related compounds has been ascribed to an injury to the liver which prevents prothrombin synthesis. If such were the case, the injury could be counteracted by vitamin K. Evidence supporting the liver injury theory includes the observation that overdoses produce fatty livers in rabbits,¹⁵⁴ and central necrosis of the liver in rats;¹⁵⁵ in animals with damaged livers the effect of dicumarol is accentuated.¹⁵⁶ Moreover, when the liver is already damaged, smaller doses of dicumarol produce the same results as do larger doses in the normal animal.¹⁵⁷ Dicumarol in small doses stimulates fibrinogen production, while larger doses, as in the case of other hepatotoxic agents, depress fibrinogen synthesis.¹⁵⁸ Moreover, the fact that the amount of vitamin K necessary to reverse the action of dicumarol followed no fixed ratio led Glavind and Jansen¹⁵⁹ to assume that liver injury was the determining factor.

A second theory to explain the mechanism of the anticoagulants is that a competition exists between vitamin K and dicumarol. Collentine and Quick¹⁶⁰ postulated the existence of a mutual competition between vitamin K and dicumarol for the prothrombinogenic enzyme systems. They arrived at their conclusion because of the minuteness of the doses of vitamin K₁ or of dicumarol which were found to counteract each other in dogs depleted of vitamin K. Molho, Moraux, and Meunier¹⁶¹ base their opinion upon the similarity in structure between the hemorrhagic agents

¹⁵¹ M. Reiff and R. Wiesmann, *Acta Tropica*, **8**, 97-130 (1951)

¹⁵² C. Montigel and R. Pulver, II. *Congrs. International de Biochimie*, Paris, 1952, *Rsums des Communications*, p. 220.

¹⁵³ H. Dam and E. Sndergaard, *Acta pharmacol. et toxicol.*, **9**, 137-147 (1953).

¹⁵⁴ K. F. Jansen, *Nord. med.*, **20**, 1993-1997 (1943).

¹⁵⁵ C. L. Rose, P. N. Harris, and K. K. Chen, *Proc. Soc. Exptl. Biol. Med.*, **50**, 228-232 (1942).

¹⁵⁶ J. L. Bollman and F. W. Preston, *J. Am. Med. Assoc.*, **120**, 1021-1025 (1942).

¹⁵⁷ D. Roller and O. Mudrak, *Z. ges. exptl. Med.*, **114**, 75-90 (1944).

¹⁵⁸ U. D. Irish and L. B. Jaques, *Am. J. Physiol.*, **143**, 101-104 (1945).

¹⁵⁹ J. Glavind and K. F. Jansen, *Acta Physiol. Scand.*, **8**, 173-182 (1944).

¹⁶⁰ G. E. Collentine and A. J. Quick, *Am. J. Med. Sci.*, **222**, 7-12 (1951).

¹⁶¹ D. Molho, J. Moraux, and P. Meunier, *Bull. soc. chim. biol.*, **30**, 637-642 (1948); P. Meunier, *Ibid.*, 884-891 (1948).

and those which counteract the effect of these agents. The activity of menadione (A), which is the most potent antihemorrhagic agent, is reduced when OH is introduced into position 3 (phthiocol) (B); when the methyl group is replaced by an ethyl radical, the antihemorrhagic activity is further decreased (Compound C), while the combining of two molecules of phthiocol through their methyl groups (Compound D) actually produces antivitamin K activity.¹⁶² Similarly, when two menadione molecules are combined, the resulting compound is no longer an antihemorrhagic agent but possesses hemorrhagic properties.¹⁶³ The relationship of some of these compounds to dicumarol is shown in Figure 1.

Fig. 1. The effect of structural changes on the action of a compound as a hemorrhagic agent (D, E) or as an antihemorrhagic agent (A, B, C).¹²⁰

Several compounds which do not occur as "double molecules" possess hemorrhagic properties. Thus, in the compound shown below, the nature of the R group determines its activity. If R is a halide or a phenyl derivative, it is an antivitamin K.¹⁴⁵ If R is a methyl group, the compound has

¹⁶² P. Meunier and C. Mentzer, *Bull. soc. chim. biol.*, 25, 80-86; with N. P. Buu-Hoi and P. Cagniant, 384-390 (1943).

¹⁶³ C. Mentzer, *Bull. soc. chim. biol.*, 30, 872-884 (1948).

an antihemorrhagic action resembling that of vitamin K. Moreover, asymmetric phenylindanedione and several other single molecular compounds are active hemorrhagic agents.¹⁶⁴

It is impossible to determine definitely which is the best theory to explain the behavior of these anticoagulants. Woolley^{165,166} indicates that the dicumarol-vitamin K antagonism differs from characteristic analog competitions. Thus, vitamin K counteracts dicumarol over a limited dosage range, and the inhibition index is not constant within this range. Moreover, an index of less than 1.0 is much lower than is noted in the usual analog competitions. Actually an index as low as 0.4 occurs in the rat, in which case 10 mg. of dicumarol are inhibited by 25 mg. of vitamin K.

Lee *et al.*¹⁰² and Spinks and Jaques¹⁰³ were able to obtain further facts about the action of dicumarol by the use of dicumarol labeled in the methylene bridge with C¹⁴. When this product was fed, the isotope was found almost exclusively in the liver, gall-bladder, urine, and feces, while degradation products appeared in the urine. This would indicate that the hepatic cell was involved. The metabolism of dicumarol was not accelerated by the administration of vitamin K.¹⁶⁷ The active compounds are excreted by the kidneys, as is indicated by the report of Bollman and Preston¹⁶⁶ that the activity of the anticoagulant is prolonged when the treated animal is nephrectomized. When the hemorrhagic agent (phenylindanedione) is rapidly excreted, due to a decreased glomerular capillary resistance, the duration of the anticoagulant action is greatly reduced.^{168,169} Seegers¹⁷⁰ reviewed the effect of drugs on blood clotting.

A large number of investigators¹⁷¹⁻¹⁷⁹ have observed that the hypoprothrombinemia induced by the dicumarol type of anticoagulants can be

¹⁶⁴ P. Meunier, C. Mentzer, and A. Vinet, *Helv. Chim. Acta*, **29**, 1291-1297 (1946).

¹⁶⁵ D. W. Woolley, *Physiol. Revs.*, **27**, 308-333 (1947).

¹⁶⁶ D. W. Woolley, *Advances in Enzymol.*, **6**, 129-146 (1946).

¹⁶⁷ S. Shapiro, W. Weiner, and G. Simson, *New Engl. J. Med.*, **243**, 775-779 (1950).

¹⁶⁸ P. Gley and J. Delor, *Bull. soc. chim. biol.*, **30**, 891-896 (1948).

¹⁶⁹ J. Badin, C. Mentzer, J. Moraux, and P. Meunier, *Compt. rend. soc. biol.*, **144**, 871-872 (1950).

¹⁷⁰ W. H. Seegers, *Pharmacol. Revs.*, **3**, 278-344 (1951).

¹⁷¹ C. S. Davidson and H. MacDonald, *New Engl. J. Med.*, **229**, 353-355 (1943).

¹⁷² D. F. James, T. D. Johnson, and S. Burnett, *Am. J. Med.*, **11**, 244 (1951).

¹⁷³ T. B. Van Itallie, R. P. Geyer, and F. J. Stare, *J. Clin. Invest.*, **30**, 680 (1951).

¹⁷⁴ D. M. Watkin, T. B. Van Itallie, W. B. Logan, R. P. Geyer, C. S. Davidson, and F. J. Stare, *J. Lab. Clin. Med.*, **37**, 269-271 (1951).

¹⁷⁵ A. Rehbein, A. Jaretzki, and D. V. Habif, *Ann. Surg.*, **135**, 454-469 (1952).

¹⁷⁶ F. Koller, A. Looliger, and F. Duckert, *Rev. d'hémat.*, **7**, 156-167 (1952).

¹⁷⁷ F. Koller and H. Jakob, *Schweiz. med. Wochschr.*, **83**, 476-480 (1953).

¹⁷⁸ C. G. Guttas, W. C. Moloney, and H. S. Sise, *Blood*, **8**, 276-281 (1953).

¹⁷⁹ K. W. G. Brown and R. L. MacMillan, *Am. J. Med. Sci.*, **225**, 495-500 (1953).

counteracted by vitamin K₁ or by vitamin K₁ oxide, which is usually given intravenously as an emulsion. The results with menadione and with the simpler vitamin K-active compounds have been less convincing. Menadione and *Synkavit* are considered to have only a slight effect against anticoagulants such as the dicumarol group,¹⁸⁰⁻¹⁸⁶ although menadione bisulfite has been reported to be as effective when administered intravenously¹⁸⁷⁻¹⁸⁹ as it is intramuscularly and orally.¹⁹⁰ Geill and co-workers¹⁹¹ reported that doses of 10 to 20 mg. of vitamin K₁ given intravenously in the form of a sterile aqueous colloidal solution are recommended as a means of raising the proconvertin-prothrombin value in cases in which treatment with dicumarol anticoagulants has resulted in serious hemorrhage. Higher doses were found to be unnecessary, and were dangerous in that they increased the incidence of thrombosis. Menadione as such was of no value in counteracting the hypoprothrombinemia caused by the dicumarol anticoagulant.

(3) *Miscellaneous Effects of Vitamin K*

In addition to counteracting the effect of anticoagulants in which it acts as a stimulant to prothrombin production, vitamin K has been found to reduce the hemorrhagic tendency occurring when a condition of hypervitaminosis A obtains. Light, Alscher, and Frey¹⁹² reported that this defect in clotting responds to treatment with vitamin K. It is not certain what the mechanism is by which vitamin A produces the hemorrhagic tendency.

¹⁸⁰ D. F. James, I. L. Bennett, Jr., P. Scheinberg, and J. J. Butler, *Arch. Internal Med.*, **83**, 632-652 (1949).

¹⁸¹ R. Miller, W. P. Harvey, and C. A. Finch, *New Engl. J. Med.*, **242**, 211-215 (1950).

¹⁸² T. B. Van Itallie, R. P. Geyer, and F. J. Stare, *J. Am. Med. Assoc.*, **147**, 1652 (1951).

¹⁸³ N. W. Barker, H. H. Hanson, and F. D. Mann, *J. Am. Med. Assoc.*, **148**, 274-277 (1952).

¹⁸⁴ A. S. Douglas and A. Brown, *Brit. Med. J.*, **1952**, **I**, 412-415.

¹⁸⁵ W. C. Van Buskirk, *New Engl. J. Med.*, **248**, 57-58 (1953).

¹⁸⁶ W. G. Bannon, C. A. Owen, Jr., and N. W. Barker, *J. Lab. Clin. Med.*, **41**, 393-404 (1953).

¹⁸⁷ H. E. Cromer and N. W. Barker, *Proc. Staff Meetings, Mayo Clinic*, **19**, 217-223 (1944).

¹⁸⁸ C. J. Bjerkelund, *Scand. J. Clin. & Lab. Invest.*, **2**, 83-91 (1950).

¹⁸⁹ R. S. Overman, C. W. Sorenson, and I. S. Wright, *J. Am. Med. Assoc.*, **145**, 393-399 (1951).

¹⁹⁰ T. Hilden and O. Munck, *Scand. J. Clin. & Lab. Invest.*, **5**, 361-365 (1953).

¹⁹¹ T. Geill, E. Lund, H. Dam, and E. Søndergaard, *Scand. J. Clin. & Lab. Invest.*, **6**, 203-209 (1954).

¹⁹² R. F. Light, R. P. Alscher, and C. N. Frey, *Science*, **100**, 225-226 (1944).

Vitamin K is also able to counteract the action of α -tocopheryl-quinone in producing hemorrhagic symptoms in the reproductive system and the resultant resorption in pregnant mice.¹⁹³ Large doses of vitamin E were found to be ineffective in reversing this effect. Woolley¹⁹³ suggests that the similarity in structure of α -tocopheryl-quinone and vitamin K may account for the effectiveness of the latter.

7. Vitamin K Hypovitaminosis

(1) *Methods of Producing Deficiency*

a. Dietary Lack of Vitamin K. Although the classical method for producing vitamin deficiencies by feeding the animal on a vitamin-deficient diet is quite effective in the case of the chick, for vitamin K,¹⁹⁴ and in ducklings and young geese,¹⁹⁴ it is difficult to produce the deficiency in other animals solely by dietary means. This is due to the fact that, in the chick, although vitamin K is produced in the lower intestinal tract, it cannot be absorbed from this portion of the intestine.⁴⁸ Almquist and Stokstad⁴⁸ reported the presence of vitamin K in the droppings of vitamin K-deficient chicks. On the other hand, sufficient vitamin K is normally synthesized in some areas of the intestinal tract of other species as to allow the absorption of enough of the vitamin to prevent severe vitamin K-avitaminosis. One exception to the above is the development of vitamin K deficiency symptoms in the newborn. Thus, the newborn of man,^{80,195} as well as of sheep and goats,¹⁹⁶ and of the guinea pig,¹⁹⁷ are lacking in vitamin K at birth. Since the gastrointestinal tract is sterile at birth, no vitamin K is available from this source. Under such conditions, the prothrombin is reduced to dangerously low levels if vitamin K is not given by mouth or administered parenterally. There is even the possibility that death may result. As soon as food is taken and the intestinal flora are established, the synthesis of vitamin K begins, and prothrombin formation follows the absorption of the vitamin.

However, Dam and Glavind¹⁸ demonstrated that it was possible to produce a low grade avitaminosis K by feeding animals a diet low in vitamin K over a prolonged period. Murphy¹⁹⁸ confirmed these findings in

¹⁹³ D. W. Woolley, *J. Biol. Chem.*, **159**, 59-66 (1945).

¹⁹⁴ H. Dam, F. Schönheyder, and L. Lewis, *Biochem. J.*, **31**, 22-27 (1937).

¹⁹⁵ W. W. Waddell, Jr., D. Guerry, III, W. E. Bray, and O. R. Kelley, *Proc. Soc. Exptl. Biol. Med.*, **40**, 432-434 (1939).

¹⁹⁶ A. van Vyve, *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.*, **11**, 101-104 (1941).

¹⁹⁷ F. Widenbauer and U. Krebs, *Monatsschr. Kinderheilk.*, **91**, 223-225 (1942).

¹⁹⁸ R. Murphy, *Science*, **89**, 203-204 (1939).

mice by proving that a somewhat prolonged bleeding time existed in this species following a vitamin K-free diet. Oleson and co-workers¹⁹⁹ reported a vitamin K-like deficiency in rats fed synthetic diet apparently devoid of vitamin K. This condition was characterized by subcutaneous hemorrhages or by purpura in the paws and bleeding from the nose. Dam and Glavind²⁰⁰ later proved that this condition in rats was a true avitaminosis K. They were able to demonstrate a characteristic vitamin K deficiency in seven out of fifteen rats on a vitamin K-free diet, within fifteen to eighty days. The symptoms were alleviated by intravenous injections of vitamin K. Thus, it would appear certain that avitaminosis K can be provoked in rabbits, rats, and mice solely by dietary restriction of this food component. Plasma and tissue extracts from chickens on a diet devoid of vitamin K, tested²⁰¹ by the blood-clotting method, exhibited analogous results.

b. Inhibition of the Synthesis of Vitamin K in the Gastrointestinal Tract.

The method by which vitamin K deficiency is most readily established in all species is by the inhibition of normal synthesis of vitamin K in the gut, following the administration of a diet free from vitamin K. The intestinal bacteria constitute the largest and most steady source of vitamin K under most conditions.^{11,34} *Escherichia coli* is an important bacterial source of vitamin K.⁷⁷ According to Dam,²⁰² the farnesyl type of vitamin K (K₂) originates as a result of the action of intestinal bacteria. However, Taveira²⁰³ reported the recovery of phytyl-vitamin K (K_{c1}) from this source. Further investigation is needed to clarify these discordant views.

Sulfonamides produce a marked reduction of vitamin K synthesis in the gastrointestinal tract by virtue of their bacteriostatic and bactericidal action. The comparative effectiveness of the sulfonamides in depressing vitamin K synthesis²⁰⁴ (from the most potent to the weakest) is as follows: sulfapyrazine, sulfadiazine,⁷⁶ sulfathiazole,²⁰⁵ sulfasuxidine,⁵⁹ sulfanilamide,²⁰⁴ and sulfaguanidine.⁵⁸ When *p*-aminobenzoic acid is given, either orally or parenterally, it concentrates in the intestinal tract and prevents the depression of vitamin K synthesis by the intestinal flora after the administration of sulfonamides.⁷⁹ The effect of the sulfonamides upon

¹⁹⁹ J. J. Oleson, H. R. Bird, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.*, **127**, 23-42 (1939).

²⁰⁰ H. Dam and J. Glavind, *Z. Vitaminforsch.*, **9**, 71-74 (1939).

²⁰¹ H. Dam and J. Glavind, *Biochem. J.*, **32**, 1018-1023 (1938).

²⁰² H. Dam, *Med. Welt.*, **20**, 958 (1951).

²⁰³ M. Taveira, *Ann. pharm. franc.*, **9**, 344-347 (1951).

²⁰⁴ W. H. Sebrell, *Harvey Lectures*, **39**, 288-315 (1943-1944).

²⁰⁵ B. M. Braganca and M. V. Radhakrishna Rao, *Indian J. Med. Research*, **35**, 15-21 (1947).

vitamin K synthesis in the gastrointestinal tract has been discussed earlier (see page 759).

According to Lockhart, Sherman, and Harris,²⁰⁶ the administration to rats of a diet high in triglycerides containing dihydroxystearic acid produces a condition of hypoprothrombinemia. Since the intestinal bacteria are qualitatively and quantitatively normal without producing any vitamin K, it is assumed that the vitamin-synthesizing enzyme systems in the bacteria may be blocked.²⁰⁷ One result which is difficult to interpret in line with the thesis that vitamin K is synthesized in the gut by the intestinal bacteria is that of Luckey *et al.*,²⁰⁸ who worked with germ-free rats. The animals which were maintained in such a manner that the gastrointestinal tract remained sterile did not develop any abnormality in the blood-clotting mechanism when fed on a diet devoid of vitamin K. However, none of the vitamin was found in the liver, feces, bladder contents, or cecal contents.

c. Reduced Absorption of Vitamin K. Any condition which reduces the ability of the intestine to absorb vitamin K will tend to result in a vitamin K deficiency. Thus, one reason why poultry are readily susceptible to vitamin K-deficiency is that they have a short large intestine, which is believed to be inadequate for the absorption of bacterially-produced vitamin K.⁴⁸ The lower colon of mammals is probably also incapable of absorbing vitamin K effectively, even in the presence of bile; this is shown by the fact that retention enemas cannot supply vitamin K to the organism.²⁰⁹ Also Greaves⁶⁶ demonstrated that one could produce a vitamin K deficiency in rats if the bile duct was diverted into the colon. On the other hand, the cecum has been reported to be an important site of bacterial synthesis of vitamin K.⁵⁷ Dam and Glavind²¹⁰ reported that the clotting time was prolonged in chickens as a result of ligation of the bile duct.

The absence of bile from the gut prevents the absorption of exogenous as well as of endogenous vitamin K. This was discussed at some length in an earlier section. The condition can be corrected by the administration of bile or of bile salts. Water-soluble preparations of vitamin K are effectively absorbed when given orally in the absence of bile.^{70,211}

²⁰⁶ E. E. Lockhart, H. Sherman, and R. S. Harris, *Science*, **96**, 542-543 (1942).

²⁰⁷ G. Nightingale, E. E. Lockhart, and R. S. Harris, *Arch. Biochem.*, **12**, 381-387 (1947).

²⁰⁸ T. D. Luckey, J. R. Pleasants, M. Wagner, H. A. Gordon, and J. A. Reyniers, *J. Nutrition*, **57**, 169-182 (1955).

²⁰⁹ H. P. Smith, S. E. Ziffern, C. A. Owen, G. R. Hoffman, and J. E. Flynn, *J. Iowa State Med. Soc.*, **29**, 377-384 (1939).

²¹⁰ H. Dam and J. Glavind, *Z. Vitaminforsch.*, **10**, 71-79 (1940).

²¹¹ H. P. Smith and C. A. Owen, *J. Biol. Chem.*, **134**, 783-784 (1940).

Another condition in which inadequate absorption of vitamin K is found, resulting in vitamin K-deficiency, occurs in intestinal disease. Thus, in a number of pathologic conditions such as intestinal obstruction, gastrocolic fistula, external enterostomy, chronic ulcerative colitis, regional ileitis, intestinal polyposis,²¹²⁻²¹⁴ and tuberculous enteritis,²¹⁵ the absorption of vitamin K is mechanically prevented. In other diseases, such as tropical²¹⁶ and non-tropical²¹² sprue and in the prolonged diarrhea which sometimes occurs in pellagra,²¹⁷ a similar condition occurs.

When the intestinal lymph is diverted, there is a marked decrease in clotting time in the blood of the rat.²¹⁸ This condition can be corrected by the administration of vitamin K parenterally. Since the lymphatics are the normal pathway of absorption of vitamin K, the withdrawal of the intestinal or thoracic lymph would be expected to prevent the transfer of the vitamin from the intestinal wall to the liver and to other tissues.

d. The Importance of the Liver in Vitamin K Utilization. After hepatectomy, the clotting time of blood is prolonged.^{219,220} It has also been known for many years that a tendency to hemorrhage exists when there is severe liver damage.^{221,222} The reason for these findings was not understood until a number of investigators^{95,97-99,223} demonstrated that a lack of prothrombin in the blood was a prominent condition associated with hepatic disease, with occlusion of the liver,²²⁴ or following hepatectomy (evisceration).^{225,226} Although vitamin K stimulates prothrombin synthesis in normal livers, it is frequently unable to effect a similar formation of

²¹² H. R. Butt and A. M. Snell, *Vitamin K*, Saunders, Philadelphia, 1941, pp. 96 ff.

²¹³ R. L. Clark, Jr., C. F. Dixon, H. R. Butt, and A. M. Snell, *Proc. Staff Meetings Mayo Clinic*, 14, 407-416 (1939).

²¹⁴ T. T. Mackie, *New York State J. Med.*, 40, 987-995 (1940).

²¹⁵ E. Tanner and F. Suter, *Schweiz. med. Wochschr.*, 74, 552-554 (1944).

²¹⁶ R. S. Diaz y Rivera, *Puerto Rico J. Public Health Trop. Med.*, 17, 124-128 (1941).

²¹⁷ E. D. Warner, T. D. Spies, and C. A. Owen, *Southern Med. J.*, 34, 161-163 (1941).

²¹⁸ C. A. Owen, Jr., *Studies on the Conversion of Prothrombin to Thrombin; Effect of Conversion Variations on Prothrombin Tests*. Thesis, Graduate School, Univ. Minnesota, 1950; cited by W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. II, Acad. Press, New York, 1954, p. 426.

²¹⁹ M. Doyon and N. Kareff, *Compt. rend. soc. biol.*, 56, 612-613 (1904).

²²⁰ P. Nolf, *Arch. intern. physiol.*, 3, 1-43 (1905-1906).

²²¹ M. Doyon, *Compt. rend. soc. biol.*, 57, 30-31 (1905).

²²² M. Doyon, A. Morel, and N. Kareff, *Compt. rend. soc. biol.*, 57, 493-494 (1905).

²²³ H. P. Smith, E. D. Warner, and K. M. Brinkhous, *J. Exptl. Med.*, 66, 801-811 (1937).

²²⁴ B. Uvnas, *Acta Physiol. Scand.*, 3, 97-110 (1941).

²²⁵ D. J. Ingle, *Exptl. Med. & Surg.*, 7, 34-36 (1949).

²²⁶ D. J. Ingle, J. E. Nezamis, and M. C. Prestrud, *Am. J. Physiol.*, 161, 199-201 (1950).

prothrombin in damaged livers.²²⁷⁻²²⁹ On the other hand, when large doses of vitamin K are administered prior to exposure of animals to chloroform, a protective action on the liver can be noted.²³⁰ When the administration of vitamin K, parenterally or orally, is ineffective in causing a production of prothrombin, it is generally considered that the hypoprothrombinemia results from failure of the liver tissue to synthesize the prothrombin, rather than from failure of the supply of vitamin K.^{231,232} A mild hepatic insufficiency such as that resulting from fever,²³³ anesthesia,²³⁴ or traumatic injury of the liver,¹⁰⁰ may be sufficient to interfere with its ability to synthesize prothrombin. Anticoagulant drugs also exert their effect through a direct action upon the liver.

(2) *Symptoms of Avitaminosis K*

The effect of vitamin K deficiency on animals is simple, consisting in a single abnormality, namely a reduction of plasma prothrombin. As a result of this decrease, the clotting time of the blood is greatly prolonged. This condition frequently causes subcutaneous and intramuscular hemorrhage, which usually results in the death of the animal.

In the case of man the picture is somewhat similar. Certain complications, such as a poor intestinal absorption, due to intestinal diseases, to occlusion of the bile duct, or to liver injury, as well as a deficiency in vitamin K in the diet, may cause this condition. In fact, in the case of man and of animals, with the exception of the fowl, avitaminosis K seldom occurs solely as a result of lack of vitamin K in the diet. In the human, vitamin K deficiency is most frequently observed in the newborn and in adults with biliary obstruction.

8. Hypervitaminosis K

Although symptoms of a true hypervitaminosis K have not been demonstrated comparable to those occurring when excessive amounts of vitamins A or D are ingested, there is evidence of toxicity when large

²²⁷ S. J. Wilson, *Proc. Soc. Exptl. Biol. Med.*, **41**, 559-561 (1939).

²²⁸ F. J. Pohle and J. K. Stewart, *J. Clin. Invest.*, **19**, 365-372 (1940).

²²⁹ R. Kark and A. W. Souter, *Lancet*, **1940**, *I*, 1149-1153.

²³⁰ M. A. Pessagno, *Dia méd.*, **22**, 1264 (1950); cited by W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. II, Acad. Press, New York, 1954, p. 427.

²³¹ P. N. Unger, S. Shapiro, and S. Schwalb, *J. Clin. Invest.*, **27**, 39-47 (1948).

²³² P. N. Unger, M. Weiner, and S. Shapiro, *Am. J. Clin. Pathol.*, **18**, 835-851 (1948).

²³³ R. K. Richards, *Science*, **97**, 313 (1943).

²³⁴ S. C. Cullen, S. E. Ziffern, R. B. Gibson, and H. P. Smith, *J. Am. Med. Assoc.*, **115**, 991-994 (1940).

amounts of some vitamin K-active compounds are given. Although the usual daily dosages of vitamin K were formerly in the range of a few milligrams, since the advent of dicumarol several hundred milligrams of vitamin K₁ may be given in a single dose, without deleterious effects, to overcome the anticoagulant action of dicumarol. In the tests of Phelps and Jones,²³⁵ some nausea was observed after the intravenous administration of 150 mg. of *Synkavit*, while more severe reactions occurred in several cases in which the dose was increased to 600 mg. On the other hand, 1000 mg. of phytyl menadione (vitamin K₁) caused no symptoms, although a febrile response was noted when vitamin K₁ oxide was employed. Stagnell^{235a} obtained rapid reversal of the hypoprothrombinemia induced by dicumarol, by the intravenous administration of as little as 100 mg. of emulsified vitamin K₁. Koller *et al.*^{235b} found 500 mg. of vitamin K₁ effective.

Minimal lethal doses of menadione derivatives, for mice, have been shown to range from 200 and 800 mg./kg. body weight for subcutaneous or intramuscular administration, and from 250 to 450 mg./kg. body weight when the intravenous route was employed. The average LD₅₀ values for oral administration (in mg./kg. body weight) are as follows:¹²⁰ phthiocol, 200;²³⁶ menadione, 500;²³⁶ 620;²³⁷ 800;²³⁸ menadiol disuccinate, 400;²³⁹ and menadiol, 300;²³⁷ 400.²³⁸ For intramuscular or subcutaneous administration, the values were as follows: menadione, 138²³⁷ and 250;²³⁸ menadiol disuccinate, 300;²³⁹ and menadiol, 80²³⁸ and 100.²³⁷ The figures for intravenous dosing were recorded as follows: menadione bisulfite, 250;²⁴⁰ menadiol disuccinate, 275;²³⁹ and menadiol, 450.²⁴⁶

The data on the LD₅₀ of animals other than the mouse are limited. A figure of 840 mg./kg. body weight has been recorded for the chick for menadione given orally.²³⁷ In the case of the dog, LD₅₀ figures of 100 to 150²⁴⁰ and 100²³⁹ were given for menadione bisulfite and menadiol disuccinate, respectively. The rabbit appears to be quite sensitive to menadione; an LD₅₀ of 15 to 20 mg./kg. body weight was noted when the compound was given intravenously,²³⁹ although values as high as 100 and 120 mg. have been recorded for the intravenous route,²⁴⁰ and 250 mg.

²³⁵ E. T. Phelps and S. N. Jones, *Bull. Georgetown Univ. Med. Center*, **4**, 41-44 (1950).

^{235a} R. Stagnell, *Am. Heart J.*, **44**, 124-130 (1952).

^{235b} F. Koller, A. Loeliger, and P. Flückinger, *Helv. Med. Acta*, **19**, 411-416 (1952).

²³⁶ H. Molitor and H. J. Robinson, *Proc. Soc. Exptl. Biol. Med.*, **43**, 125-128 (1940).

²³⁷ S. Ansbacher, W. C. Corwin, and B. G. H. Thomas, *J. Pharmacol. Exptl. Therap.*, **75**, 111-124 (1942).

²³⁸ M. B. Shimkin, *J. Pharmacol. Exptl. Therap.*, **71**, 210-214 (1941).

²³⁹ K. Fromherz, *Z. Vitaminforsch.*, **11**, 65-75 (1941).

²⁴⁰ R. K. Richards and S. Shapiro, *J. Pharmacol. Exptl. Therap.*, **84**, 93-104 (1945).

for oral administration.²³⁷ When toxicity develops, death usually results from circulatory²³⁷ or respiratory failure.²⁴¹ In mice, also, respiratory failure is the cause of death.²³⁸

When menadione was given in a high dosage (0.3% of the diet), growth was found to be depressed;²⁴² when the proportion in the diet was further increased to 0.8%, the inhibition of growth was more severe. Anemia and splenomegaly also occurred. Anemia has been reported by a number of workers as a symptom of overdosage of menadione in several species, including the dog,²⁴³ the rat,²³⁶ and the rabbit.²⁴¹ Polycythemia has been observed in some cases in the same species.²³⁷ Other symptoms of deranged hemoglobin metabolism resulting from excessive doses of menadione include methemoglobinemia,²³⁹ porphyrinuria, albuminuria^{120, 243} and hemorrhagic extravasations into the liver and kidneys.²⁴³ Methemoglobin formation has been found to occur *in vitro*.²⁴⁴ Phytyl menadione (vitamin K₁) does not cause a similar toxicity.¹²⁰

9. Comparative Activity of Vitamin K-Active Compounds

The molecular structure required for maximum activity is that present in 2-methyl-1,4-naphthoquinone. This compound has been given the non-proprietary name of menadione, by the American Medical Association.²⁴⁵ Thayer *et al.*²⁴⁶ advised the employment of this compound as a standard of activity, 1 μ g. being defined as one unit. Ewing *et al.*²⁴⁷ suggested that 2-methyl-1,4-naphthohydroquinone diacetate might be preferable as a standard, in spite of the fact that it possesses only one-half of the biopotency of menadione. Vitamin K₁ is only 50% as active as menadione; it contains the heavy long aliphatic hydrocarbon side chain which contributes nothing to its biopotency. Since the difarnesyl side chain on vitamin K₂ is longer and heavier than the phytyl side chain on vitamin K₁, the biopotency of vitamin K₂ is appreciably less than that of vitamin K₁. For a more complete exposition of the vitamin K biopotency of related compounds, the reader is referred to the paper of Fieser, Tishler, and Sampson,²⁸ and to *The Lipids*, Volume I, pp. 844-859.

²⁴¹ R. H. K. Foster, *Proc. Soc. Exptl. Biol. Med.*, **45**, 412-415 (1940).

²⁴² E. H. Hatton, A. Dodds, H. C. Hodge, and L. S. Fosdick, *J. Dental Research*, **24**, 283-295 (1945).

²⁴³ F. Koller, *Schweiz. med. Wochschr.*, **69**, 1159-1161 (1939).

²⁴⁴ A. Cannavà, *Bull. soc. ital. biol. sper.*, **24**, 593-594 (1948).

²⁴⁵ Anonymous, Council on Pharmacy and Chemistry, *J. Am. Med. Assoc.*, **116**, 1054 (1941).

²⁴⁶ S. A. Thayer, S. B. Binkley, D. W. MacCorquodale, E. A. Doisy, A. D. Emmett, R. A. Brown, and O. D. Bird, *J. Am. Chem. Soc.*, **61**, 2563 (1939).

²⁴⁷ D. T. Ewing, J. M. Vandenbelt, and O. Kamm, *J. Biol. Chem.*, **131**, 345-356 (1939).

In a comparison of the rate of action of several vitamin K-active compounds, Dam and Sndergaard²⁴⁸ reported that vitamin K₁, menadione, and *Synkavit* (the tetrasodium or tetracalcium salt of 2-methyl-1,4-naphthoquinone diphosphate) had approximately equal biologic activities, on a molar basis, against vitamin K deficiency when tested by the usual assay method with chicks over a period of twenty hours. However, after the intravenous injection of vitamin K₁ the effect on prothrombin formation began sooner with vitamin K₁ than with the other related products.

In the case of the newborn, Dyggve, Dam, and Sndergaard²⁴⁹ found that vitamin K₁ and *Synkavit* gave approximately the same response when administered either intravenously or orally. On the other hand, when an aqueous colloidal solution of vitamin K₁ (*Konakion*) was given at dosages of 20 or 40 mg. one hour before delivery,²⁵⁰ it was as effective as *Synkavit*, as determined by the influence upon the prothrombin time of the newborn during the first six days after birth. At lower dosages, *Synkavit* was definitely superior. Vitamin K₁ dissolved in peanut oil was found to be much less effective than when given in an aqueous colloidal solution.²⁵⁰ Dam and Glavind²¹⁰ reported that vitamin K was equally active in raising the prothrombin content of the blood in chickens with avitaminosis K, whether the condition was produced by dietary restriction or was induced by ligation of the bile duct.

10. The Requirements for Vitamin K

(1) Requirement for the Chick

More data are available on the requirement of vitamin K for the chick than for other animals. The blood-clotting time, which is an index of the state of vitamin K reserves, depends upon the vitamin K intake of the parent hen. Almquist *et al.*²⁵¹ reported that a diet containing 2.5% of alfalfa meal provided protection for the chick. Cravens and co-workers²⁵² found that the blood-clotting time and prothrombin levels of day-old chicks were a reflection of the vitamin K content of the diet of the parent hen. One or two per cent of dried alfalfa or grass were found to be sufficient to provide the necessary protection for the newly-hatched chick.

²⁴⁸ H. Dam and E. Sndergaard, *Acta pharmacol. et toxicol.*, **9**, 131-136 (1953).

²⁴⁹ H. V. Dyggve, H. Dam, and E. Sndergaard, *Acta Paediatrica*, **43**, 27-31 (1954).

²⁵⁰ H. V. Dyggve, H. Dam, and E. Sndergaard, *Acta Obst. Gyn. Scandinav.*, **33**, 283-288 (1954).

²⁵¹ H. J. Almquist, E. Mecchi, and A. A. Klose, *Biochem. J.*, **32**, 1897-1903 (1938).

²⁵² W. W. Cravens, S. B. Randle, C. A. Elvehjem, and J. G. Halpin, *Poultry Sci.*, **20**, 313-316 (1941).

The vitamin K was found in the egg yolk but not in the white of the egg. It is suggested that the increase in prothrombin content, which may occur in chick blood for several days after hatching, may result from the assimilation of the last of the vitamin K stores from the yolk.²⁵³

When the amount of vitamin K required was based upon the quantity necessary to reduce the blood-clotting time to ten minutes or less, eighteen hours after dosage, several groups of workers^{28, 254, 255} reported a figure of 1 μ g. The quantity of methylnaphthoquinone that is needed to effect a similar improvement has been given as 0.3 μ g. by Fieser *et al.*²⁸ Values employing the six-hour assay were 1.5 μ g. for vitamin K₁ and 0.5 μ g. for menadione.²⁸

Different procedures have also been employed. Thus, Quick and Stefanini¹³⁴ reported that 5 μ g. of menadione would bring the level of prothrombin in the chick from 10% of normal to normal within four hours. Tidrick and associates²⁵⁶ observed a complete recovery of prothrombin in six hours when 16 μ g. were given, and a 50% recovery with 8 μ g. of menadione.

More precise information on the daily requirements for vitamin K in chickens can be obtained by determination of the amount necessary to maintain a normal blood-clotting time. When the assays were continued for five days or more, 2 μ g. of menadione daily were found by a number of groups of investigators^{134, 253, 257, 258} to be sufficient.

(2) Requirement for Other Animals

Flynn and Warner²⁵⁹ reported that two intraperitoneal doses of 1000 μ g. of phthioecol, or two daily doses of 2 μ g. of menadione are necessary to bring about a moderate recovery in bile-fistula rats. Warner and Flynn⁷⁰ observed that 8 μ g. of the disulfuric acid ester of 3-methyl-1,4-naphthoquinone are required daily to cure depleted rats. In the case of dogs rendered vitamin K-deficient by cholecystonephrostomy, Collentine and Quick¹⁶⁰ noted that the intravenous injection of 10 μ g. of phytylmenadione (vitamin K₁)/kg. body weight restored the clotting time of the blood to

²⁵³ F. W. Stamler, R. T. Tidrick, and E. D. Warner, *J. Nutrition*, **26**, 95-103 (1943).

²⁵⁴ E. Fernholz, S. Ansbacher, and H. B. MacPhillamy, *J. Am. Chem. Soc.*, **62**, 430-432 (1940).

²⁵⁵ A. D. Emmett, R. A. Brown, and O. Kamm, *J. Biol. Chem.*, **132**, 467-468 (1940).

²⁵⁶ R. T. Tidrick, F. T. Joyce, and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, **42**, 853-857 (1939).

²⁵⁷ H. J. Almquist and A. A. Klose, *J. Biol. Chem.*, **130**, 787-789 (1939).

²⁵⁸ H. J. Almquist and A. A. Klose, *Proc. Soc. Exptl. Biol. Med.*, **45**, 55-59 (1940).

²⁵⁹ J. E. Flynn and E. D. Warner, *Proc. Soc. Exptl. Biol. Med.*, **43**, 190-194 (1940).

normal within four hours, and maintained the normal level for twenty-four hours. Although these data do not give actual requirement levels, they are of value in determining minimal dosages. In all cases these are in the microgram range.

(3) Requirement for Man

No precise figures are available for the vitamin K requirement of man, but it appears to be in the microgram range. Thus, Sells *et al.*²⁶⁰ reported that a single intramuscular injection of 10 μ g. of 4-amino-2-methyl naphthol hydrochloric acid given at birth almost completely prevents the delay in the prothrombin time during the first week of life. Daily doses of 1 μ g. were found to be equally effective. Hardwicke²⁶¹ reported comparable results with the diphosphoric acid ester of menadiol, and Adler²⁶² also confirmed the effectiveness of dosages of 1 to 5 μ g. of vitamin K.

²⁶⁰ R. L. Sells, S. A. Walker, and C. A. Owen, *Proc. Soc. Exptl. Biol. Med.*, 47, 441-445 (1941).

²⁶¹ S. H. Hardwicke, *J. Pediat.*, 24, 259-269 (1944).

²⁶² B. Adler, *Monatschr. Geburtschülfe u. Gynakol.*, 118, 225-248 (1944).

CHAPTER XIII

THE PHYSIOLOGY AND BIOCHEMISTRY OF THE ESSENTIAL FATTY ACIDS

1. Introduction

A number of physiologic functions depend more or less upon the fat content of the organism for an efficient performance. Whether some of these functions depend upon the general fat molecule *per se* or rather upon certain fatty acid components is a moot question. In the present chapter the physiologic reactions which can be definitely traced to certain polyunsaturated fatty acids are discussed. The first investigation which proved that the rigid exclusion of fats from the diet results in a fat-deficiency syndrome was that of Evans and Burr in 1926.¹ This was followed shortly thereafter by a report of a similar syndrome observed by McAmis and her co-workers,² who demonstrated that rats receiving fats in their diets grew better than did animals on a fat-free regimen. The final proof that the lack of fat produces a deficiency disease was brought forward by Burr and Burr,³ who also demonstrated that the condition could not be ascribed to the lack of fat-soluble vitamins. In a further study of this new fat-deficiency disease, Burr and Burr⁴ showed that the deficiency symptoms disappeared immediately when lard, cod-liver oil, corn oil, or linseed oil was fed. The curative agents were found to be present in the saponifiable fraction. The highly unsaturated acids, linoleic, linolenic, and arachidonic acids, were excellent curative agents, while saturated acids such as stearic, palmitic, myristic, lauric, and those with shorter chain lengths were found to possess no biologic activity. Thus, coconut oil, hydrogenated coconut oil, and methyl stearate gave completely negative results.⁵ The fact that the polyunsaturated acids might be the active

¹ H. M. Evans and G. O. Burr, *Proc. Soc. Exptl. Biol. Med.*, **24**, 740-743 (1926-1927).

² A. J. McAmis, W. E. Anderson, and L. B. Mendel, *J. Biol. Chem.*, **82**, 247-262 (1929).

³ G. O. Burr and M. M. Burr, *J. Biol. Chem.*, **82**, 345-367 (1929).

⁴ G. O. Burr and M. M. Burr, *J. Biol. Chem.*, **86**, 587-621 (1930).

⁵ G. O. Burr, *Federation Proc.*, **1**, 224-233 (1942).

agents in preventing the fat-deficiency syndromes was indicated by the finding that a number of vegetable oils, such as corn, linseed, poppyseed, and olive oils were likewise potent prophylactic agents.⁴ Moreover, glycerol, which is the other component of the active fraction (saponifiable), was proved to be completely ineffective as a curative compound.³ Reviews of the nutritional significance of the essential fatty acids (hereafter abbreviated as EFA) include that of Burr,⁵ one by Sherman,⁶ one by Deuel and Greenberg,⁷ and another by Deuel alone.⁸

The polyunsaturated fatty acids were found by Klein and Johnson^{9,10} to be widely distributed in the cell. The proportion of the unsaturated fatty acids (EFA) in mitochondria, microsomes, and the poorly sedimentable layer of rat liver cells showed little fluctuation during growth and maturation. However, there was an accumulation of trienoic acid in senescent animals, which reached its highest concentration in the poorly sedimentable layer of the oldest animals. In EFA deficiency, mitochondria, microsomes, and the poorly sedimentable layer lost dienoic, tetraenoic, and pentaenoic acids at varying rates, while trienoic acid increased steadily in all fractions. The most pronounced losses of polyunsaturated acids occurred in the microsomes. Fat-deficient animals synthesized phospholipids at an increased rate (as determined by the rate of uptake of radiophosphorus), but the uptake in the acid-soluble organic P fraction of the liver was decreased. These changes were found to occur within six weeks on the fat-free diet, before any external symptoms of EFA deficiency appeared. It is suggested that the changes accompany the particulate lipid losses. Thus, the basic biochemical defect may occur within a short period of exclusion of dietary fat.

Earlier reviews of the significance of the essential fatty acids (EFA) included those of Deuel,¹¹ and of Holman and Greenberg.¹²

2. Chemical Relations of Polyunsaturated Fatty Acids

(1) *Polyunsaturated Acids Having Biologic Importance*

The polyunsaturated fatty acids which were first recognized by Burr and Burr⁴ as possessing biopotency in curing or preventing the fat-de-

⁶ H. Sherman, *Vitamins and Hormones*, 8, 55-68 (1950).

⁷ H. J. Deuel, Jr., and S. M. Greenberg, *Some Biochemical and Nutritional Aspects in Fat Chemistry*, *Fortschr. Chem. org. Naturstoffe*, 6, 1-86 (1950).

⁸ H. J. Deuel, Jr., *Federation Proc.*, 14, 639-649 (1955).

⁹ P. D. Klein and R. M. Johnson, *Federation Proc.*, 12, 231 (1953).

¹⁰ P. D. Klein and R. M. Johnson, *Arch. Biochem. Biophys.*, 48, 380-385 (1954).

¹¹ H. J. Deuel, Jr., *Progr. Chem. Fats and Other Lipids*, 2, 99-192 (1954).

¹² R. T. Holman and S. I. Greenberg, *Arch. Biochem. Biophys.*, 49, 49-57 (1954).

iciency disease produced by the rigid exclusion of fat from the diet, were *linoleic acid* (9,12-octadecadienoic acid) *linolenic acid* (9,12,15-octadecatrienoic acid) and *arachidonic acid* (5,8,11,14-eicosatetraenoic acid). Hume *et al.*¹³ reported that a docosahexaenoic acid of unknown structure possesses similar biologic activity. Moreover, methyl linoleate and linoleyl alcohol (9,12-octadecadienol) were found by Turpeinen^{14,15} to possess biopotency, presumably because they are converted to arachidonic acid in the body in the course of their intermediary metabolism.

On the other hand, several polyunsaturated acids have been found to be biologically inactive in respect to the fat-deficiency syndrome. Included in this category are the following acids: linolelaidic acid (*trans*-9-*trans*-12-octadecadienoic acid), as reported by Burr,⁵ 9,11-linoleic acid (9,11-octadecadienoic acid), as recorded both by Burr⁵ and by Thomasson,¹⁶ who employed a new bioassay procedure. 10,12-Octadecadienoic acid was likewise reported by the latter investigator to be biologically inactive. Other dienoic acids concerning which data are available include 2,6-phytadienoic,¹⁷ which is inactive; 10,13-nonadecadienoic acid, reported as inactive by Karrer and Koenig,¹⁷ and as possessing 9% of the activity of linoleic acid by Thomasson;¹⁶ and 11,14-eicosadienoic acid, also listed as practically inactive by Karrer and Koenig,¹⁷ but to which a value of 43% of that of linoleate was attributed by Thomasson.¹⁶ Sorbic acid (2,4-hexadienoic acid) has likewise been reported by Deuel, *et al.*¹⁸ to be biologically inactive.

Holman¹⁹ and Privett, Pusch, and Holman²⁰ have published a series of studies demonstrating the bioimpotence of *trans* isomers, and Holman and Greenberg¹² reported that of oxidized and conjugated linoleate.

Thomasson¹⁶ noted that ordinary linolenic acid (9,12,15-octadecatrienoic acid), formerly considered as possessing biopotency equal to that of linoleic acid, under certain conditions, is actually practically inactive (9%) when tested by the new bioassay method of the author; on the other hand, γ -linolenic acid (6,9,12-octadecatrienoic acid) is considered

¹³ E. M. Hume, L. C. A. Nunn, I. Smedley-MacLean, and H. H. Smith, *Biochem. J.*, **32**, 2162-2177 (1938).

¹⁴ O. Turpeinen, *Proc. Soc. Exptl. Biol. Med.*, **37**, 37-40 (1937).

¹⁵ O. Turpeinen, *J. Nutrition*, **15**, 351-366 (1938).

¹⁶ H. Thomasson, *Intern. Rev. Vitamin Research*, **25**, 62-82 (1953).

¹⁷ P. Karrer and H. Koenig, *Helv. Chim. Acta*, **26**, 619-626 (1943).

¹⁸ H. J. Deuel, Jr., R. B. Alfin-Slater, C. S. Weil, and H. F. Smyth, Jr., *Food Research*, **19**, 1-12 (1954).

¹⁹ R. T. Holman, *Proc. Soc. Exptl. Biol. Med.*, **76**, 100-102 (1951).

²⁰ O. S. Privett, F. J. Pusch, and R. T. Holman, *Arch. Biochem. Biophys.*, **57**, 156-162 (1955).

to possess 100% biopotency as compared with linoleate. The conjugated trienoic acid, α -elaeostearic acid (9,11,13-octadecatrienoic acid), was reported by Burr, Burr, and Miller²¹ to be ineffective in counteracting the fat-deficiency syndrome; confirmatory negative results have been recorded by Deuel *et al.*^{18,22} Tange²³ likewise noted that 4,8,12,15,19-docosapentaenoic acid gave a negative biologic response.

Thomasson¹⁶ is of the opinion that the presence of double bonds at the 6:7 and the 9:10 positions (counting from the terminal CH₃— group (or ω -carbon)) is essential for the biologic activity of the unsaturated fatty acids. When the number of double bonds is extended on the CH₃— side of these afore-mentioned positions, the bioactivity decreases, whereas dehydrogenation on the carboxyl side does not weaken the EFA activity, but may actually augment it.

(2) *Methods of Preparation of Polyunsaturated Acids*

a. Introduction. The preparation of pure polyunsaturated fatty acids still poses a very difficult problem. This is especially the situation in the case of arachidonic acid; variations in biologic response to this acid have been attributed to differences in the purity of the samples assayed.

If the source of the polyunsaturated acid is a tissue rather than a pure fat or oil, the total lipid must first be extracted by some method of wet extraction. For example, Holman and Greenberg²⁴ employ ethanol, or ethanol-ether, as a solvent, or separate the acid after digestion of the tissue with 30% potassium hydroxide. It is important to protect the sample as much as possible during all stages of preparation, and for this reason the reactions are generally carried out as far as is practicable under nitrogen. After isolation of the lipid mixture, the material is saponified (if this step has not been previously carried out), and the acid is freed, washed, and dried.

The saturated fatty acids may be removed from the mixture by the lead soap ether or the lead soap alcohol method; these methods depend upon the relative insolubility of the lead soaps of the saturated acids in organic solvents.

b. Low-Temperature Crystallization. The lead soap method for the separation of the saturated acids has been largely replaced by several newer

²¹ G. O. Burr, M. M. Burr, and E. S. Miller, *J. Biol. Chem.*, **97**, 1-9 (1932).

²² H. J. Deuel, Jr., R. B. Alfin-Slater, A. F. Wells, G. D. Kryder, and L. Aftergood, *J. Nutrition*, **55**, 337-346 (1955).

²³ U. Tange, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **20**, 13-28 (1932).

²⁴ R. T. Holman and S. E. Greenberg, *J. Am. Oil Chemists' Soc.*, **30**, 600-601 (1953).

procedures. One of these methods, which involves low-temperature crystallization from organic solvents, was developed in the laboratories of J. B. Brown twenty years ago. The features of the procedure were reviewed by Brown.²⁵ The low-temperature fractionation not only facilitates separation of saturated from unsaturated acids but also renders possible the preparation of samples of oleic and linoleic acid of high purity. However, highly purified linolenic acid cannot be prepared by this method.

c. The Urea-Adduct Method. The use of the urea inclusion compounds for the preparation of pure saturated and unsaturated compounds has proved to be a simple and highly effective method for the preparation of a number of saturated and unsaturated acids. According to Schlenk,²⁶ who recently published a comprehensive review on this subject, the compounds which form urea adducts must necessarily have more than four to six carbon atoms, and the carbons must be linked to form a straight chain. The reaction of urea to form compounds with fats was discovered by Bengen (as described by Schlenk^{27,28}) in the case of milk fats. It was later noted by Bengen and Schlenk²⁹ that urea inclusion compounds were formed with straight-chain but not with branched-chain molecules. Bengen³⁰ patented these findings under the title *Method for Separation of Aliphatic Oxygen-Containing Compounds and of Straight-Chain Hydrocarbons of at Least 6 Carbon Atoms from Mixtures Containing Them*.

Schlenk and Holman³¹ described the separation of urea inclusion compounds, of increasing degrees of unsaturation, by the fractional precipitation of a mixture of fatty acids with methanol solutions of urea. Reiser,³² in studies of fat absorption, employed a similar procedure for the preparation of conjugated linoleic acid. Swern and Parker³³ prepared linoleic acid of 85% to 95% purity from corn oil, in a 72% to 50% yield, and 87% to 89% pure linolenic acid, in a yield of 71% to 55%, from perilla oil. Achaya *et al.*³⁴ obtained linoleic acid with an iodine number of 172 to 175 (theory 181.4) in a single operation from safflowerseed fatty acids.

²⁵ J. B. Brown, *Chem. Revs.*, **29**, 333-354 (1941).

²⁶ H. Schlenk, *Progr. Chem. Fats and Other Lipids*, **2**, 243-267 (1954).

²⁷ W. Schlenk, Jr., *Ann.*, **565**, 204-240 (1949).

²⁸ W. Schlenk, Jr., *Angew. Chem.*, **62A**, 299-301 (1950).

²⁹ F. Bengen and W. Schlenk, Jr., *Experientia*, **5**, 200 (1949).

³⁰ F. Bengen, *German Patent appl.* (to I. G. Farbenindustrie, A. G.), **3**, 190, 197 IV d/12 (March 18, 1940).

³¹ H. Schlenk and R. T. Holman, *J. Am. Chem. Soc.*, **72**, 5001-5004 (1950).

³² R. Reiser, *Pro. Soc. Exptl. Biol. Med.*, **74**, 666-669 (1950).

³³ D. Swern and W. E. Parker, *J. Am. Oil Chemists' Soc.*, **30**, 5-7 (1953).

³⁴ K. T. Achaya, B. P. Baliga, S. A. Saletore, and S. H. Zaheer, Personal communication to the author (H. J. D.), 1954.

Abu-Nasr, Potts, and Holman³⁵ employed the urea inclusion technic in an effort to prepare fatty acids having four, five, or six double bonds from marine fish oil. Although they were unsuccessful, they did obtain fractions with iodine numbers as high as 356.

d. Preparation from Bromo-Derivatives. One of the most widely used procedures for the preparation of certain polyunsaturated acids is by decomposition of the corresponding bromo-derivatives. Under certain conditions, when the double bonds are saturated by bromination, the halogen compounds possess solubility differences in organic solvents which permit their isolation.³⁶

However, at least two drawbacks have been ascribed to the use of this procedure for the preparation of the EFA. Thus, in the first place, Frankel and Brown³⁷ showed that the unsaturated acids formed after debromination consist of mixtures of *cis*- and *trans*-isomers. Since the geometrical isomerization is an important factor in establishing whether or not a product may possess biologic activity, this criticism merits most serious consideration. A second difficulty associated with the use of the debromination method in the preparation of polyunsaturated acids is its limited application. Thus, the procedure cannot be applied to acids which have four or more double bonds, since these bromo-compounds are too insoluble to allow their separation in a satisfactory manner from a solvent; moreover, such polybromo-derivatives prove to be difficult to debrominate. Steinberg³⁸ has found tetrahydrofuran useful for this purpose.

e. Chromatographic and Related Methods. The only methods which permit certain highly unsaturated acids to be obtained in a high degree of purity in their natural form are the newer procedures of chromatography. Elution analysis, frontal analysis, displacement analysis, partition chromatography, and paper partition chromatography have all been employed.

Partition chromatography has been used over the past decade for the separation and analysis of volatile fatty acids, in which chloroform + 1% butanol, along with silica gel, was the resolving mixture.³⁹⁻⁴¹ Moyle *et al.*⁴² were able to effect the separation of a somewhat wider range of

³⁵ A. M. Abu-Nasr, W. M. Potts, and R. T. Holman, *J. Am. Oil Chemists' Soc.*, **31**, 16-20 (1954).

³⁶ K. S. Markley, *Fatty Acids*, Interscience, New York, 1947, pp. 603 ff.

³⁷ J. S. Frankel and J. B. Brown, *J. Am. Chem. Soc.*, **65**, 415-418 (1943).

³⁸ G. Steinberg, *The Metabolism of the Essential Fatty Acids*, Ph. D. Thesis, Univ. Calif. at Los Angeles, 1956.

³⁹ E. L. Smith, *Biochem. J.*, **36**, xxii-xxiii (1945).

⁴⁰ L. L. Ramsey and W. I. Patterson, *J. Assoc. Offic. Agr. Chemists*, **28**, 644-656 (1945).

⁴¹ S. R. Elsdon, *Biochem. J.*, **40**, 252-256 (1946).

⁴² V. Moyle, E. Baldwin, and R. Scarisbrick, *Biochem. J.*, **32**, 2178-2184 (1938).

saturated fatty acids (C_2 to C_8) by the additional precaution of buffering the silica gel tube. Peterson and Johnson⁴³ reported that the even-chain acids from C_2 to C_{10} , and also formic acid, could be separated by employing benzene-aqueous sulfuric acid in Celite-packed tubes. This procedure is the first which has found application in the fractionation of unsaturated fatty acids.⁴⁴ Oleic and linoleic acids could be effectively separated by this method to a degree not possible by other fractionation methods. Abu-Nasr and Holman⁴⁵ isolated docosahexaenoic acid from cod-liver oil by adsorption on charcoal in a modified Tiselius-Claesson apparatus, as described by Holman and Hagdahl.⁴⁶ Abu-Nasr and Holman⁴⁵ also reported the preparation of eicosapentaenoic acid of 91% purity by the preliminary concentration of the saturated acid on silicic acid, followed by its adsorption on charcoal in the modified Tiselius-Claesson apparatus. When silicic acid was used as the adsorbent and a petroleum ether-chloroform mixture as the eluant, these workers succeeded in preparing almost pure ethyl docosapentaenoate.

Some of the highly purified esters of the polyunsaturated acids were prepared by the workers at the Eastern Regional Research Laboratories of the United States Department of Agriculture, by adsorption on silicic acid followed by fractionation. The products prepared included the methyl esters of natural linoleic and linolenic acids,⁴⁷ almost pure methyl arachidonate,⁴⁸ methyl eicosapentaenoate and methyl docosapentaenoate.⁴⁹ In general, the procedure involved the separation of oleic acid from the mixture by crystallization from acetone at a low temperature, the subsequent removal of the nonsaponifiable matter, the preparation of the methyl esters of the fatty acids, followed by their adsorption on silicic acid. Elution was carried out with petroleum ether containing 0.25% ethyl ether.

Simmons and Quackenbush⁵⁰ employed a combined technic of chromatographic separation and elution of 2,4-dinitrobenzenesulfonyl chloride derivatives of unsaturated acids as a new "isolation" method of analysis.

⁴³ M. H. Peterson and M. S. Johnson, *J. Biol. Chem.*, **174**, 775-789 (1948).

⁴⁴ C. L. Reinhold and H. J. Dutton, *J. Am. Oil Chemists' Soc.*, **25**, 117-120, 120-124 (1948).

⁴⁵ A. M. Abu-Nasr and R. T. Holman, *J. Am. Oil Chemists' Soc.*, **31**, 41-45 (1954).

⁴⁶ R. T. Holman and L. Hagdahl, *Anal. Chem.*, **23**, 794-797 (1951).

⁴⁷ R. W. Riemenschneider, S. F. Herb, and P. L. Nichols, Jr., *J. Am. Oil Chemists' Soc.*, **37**, 371-374 (1949).

⁴⁸ S. F. Herb, R. W. Riemenschneider, and J. Donaldson, *J. Am. Oil Chemists' Soc.*, **28**, 55-58 (1951).

⁴⁹ S. F. Herb, L. P. Witnauer, and R. W. Riemenschneider, *J. Am. Oil Chemists' Soc.*, **28**, 505-507 (1951).

⁵⁰ R. O. Simmons and F. W. Quackenbush, *J. Am. Oil Chemists' Soc.*, **30**, 614-616 (1953).

The saturated acids do not react with 2,4-dinitrobenzenesulfonyl chloride, whereas the derivatives of the unsaturated acids were found to be separable into well-defined bands on MgSO_4 columns. They could be recovered as individual compounds in an eluate of benzene/ether (95/5), with yields above 95% in each case (oleic, linoleic, and linolenic acids). Zechmeister⁵¹ reviewed the subject of partition chromatography, and Holman^{51a} has more recently discussed the subject of separation of fatty acids from a somewhat broader viewpoint.

In 1955 Howton⁵² reported that the bromine derivatives of the methyl esters of polyunsaturated acids are "easily and cleanly separated by gradient elution from alumina chromatographic columns."

Crombie, *et al.*⁵³ approached this problem by reversed-phase chromatography. Alkaline permanganate oxidation was used to separate the saturated from the unsaturated acids, which were eluted at equal rates.

Inouye, Noda, and Hirayama⁵⁴ reported a reversed-phase paper chromatographic procedure for the determination of mercuric acetate addition compounds of unsaturated fatty acid esters. Silk and Hahn⁵⁵ have devised a method for the analysis of pilchard oil based upon preliminary separation of the unsaturated acids by the lithium soap-acetone technique, urea complex fractionation, and molecular distillation. The unsaturated acids were further resolved by reversed-phase partition chromatography, and the chain length by complete hydrogenation followed by reversed-phase partition chromatography.

(3) *Chemical and Physical Methods for the Analysis of Polyunsaturated Acids*

The qualitative and quantitative determinations of the polyunsaturated acids have posed exceedingly refractory problems, and they have only recently been partially resolved. The earlier methods were tedious, and of such uncertain interpretation that they were of little value. Frequently, they were resolved by the very unsatisfactory expedient of measuring changes of iodine value. It is obvious that only the simplest generalizations can be arrived at by such procedures.

a. Thiocyanogen Number. With the advent of the thiocyanogen num-

⁵¹ L. Zechmeister, *Progress in Chromatography, 1938-1947*, Chapman & Hall, London, 1950.

^{51a} R. T. Holman, *Progr. Chem. Fats and Other Lipids*, 1, 104-126 (1953).

⁵² D. R. Howton, *Science*, 121, 704-706 (1955).

⁵³ W. M. L. Crombie, R. Comber, and S. G. Boatman, *Biochem. J.*, 59, 309-316 (1955).

⁵⁴ Y. Inouye, M. Noda, and O. Hirayama, *J. Am. Oil Chemists' Soc.*, 32, 132-135 (1955).

⁵⁵ M. H. Silk and H. H. Hahn, *Biochem. J.*, 57, 577-582 (1954).

ber test for the analysis of fats evolved by Kaufmann,⁵⁶ it became possible to determine the unsaturated fatty acids, both qualitatively and quantitatively, with considerably more facility and precision than had hitherto been the case. Thiocyanogen reacts quantitatively with monoethenoid acids, but with only one unsaturated linkage of diethenoid acids, and with only two of the three double bonds of triethenoid acids. By combining the information obtained from the iodine value and from the thiocyanogen number, Kaufmann⁵⁶⁻⁵⁸ was able to determine the unsaturated fatty acid composition of fatty acid mixtures. The procedure has been standardized by Lambou and Dollear,^{59,60} and it is now an official method of the American Oil Chemists' Society (*Official and Tentative Methods*).⁶¹

b. Solubility of Bromide Derivatives. The relative solubilities of the bromides of the unsaturated acids were employed by Ault and Brown,⁶² Shinowara and Brown,⁶³ Brown and Frankel,⁶⁴ and White and Brown⁶⁵ for the estimation of polyunsaturated acids. However, because of substitution reactions, the partial solubilities of the bromides, the mutual solubility effects, the formation of isomers, which becomes more serious as unsaturation increases, and also the differences in the solubilities of the isomers, this procedure is much less satisfactory for quantitative assays than are some of those recently proposed.

c. Spectrophotometric Determination. Chemical and metabolic studies on the polyunsaturated acids received a tremendous impetus as a result of the observation of Moore⁶⁶ that methylene-interrupted double bonds in polyunsaturated fatty acids become partially conjugated upon prolonged saponification. Since conjugated double bonds absorb ultraviolet light of specific wave lengths, whereas methylene-interrupted double bonds do not possess this property, this discovery suggested a procedure which might be employed as a quantitative assay for the qualitative and quantitative determination of small amounts of polyunsaturated fats and oils.

⁵⁶ H. P. Kaufmann, *Z. Untersuch. Lebensm.*, **51**, 15-27 (1926).

⁵⁷ H. P. Kaufmann, *Arch. Pharm.*, 1925 (Sonderdruck), 1-47; *Chem. Abst.*, **20**, 3243 (1926).

⁵⁸ H. P. Kaufmann, *Analyst*, **51**, *Abst.*, 157-158, 264-265 (1926).

⁵⁹ M. G. Lambou and F. G. Dollear, *Oil & Soap*, **22**, 226-232 (1945).

⁶⁰ M. G. Lambou and F. G. Dollear, *Oil & Soap*, **23**, 97-101 (1946).

⁶¹ American Oil Chemists' Society, *Official and Tentative Methods*, 1946, Sect. C, Cd, 2-38, pp. 1-6.

⁶² W. C. Ault and J. B. Brown, *J. Biol. Chem.*, **107**, 615-622 (1934).

⁶³ S. Y. Shinowara and J. B. Brown, *J. Am. Chem. Soc.*, **60**, 2734-2738 (1938).

⁶⁴ J. B. Brown and J. Frankel, *J. Am. Chem. Soc.*, **60**, 54-56 (1938).

⁶⁵ M. F. White and J. B. Brown, *J. Am. Oil Chemists' Soc.*, **26**, 385-388 (1949).

⁶⁶ T. Moore, *Biochem. J.*, **31**, 138-154 (1937).

Mitchell, Kraybill, and Zscheile,⁶⁷ in 1943, proposed the first standardized empirical procedure for ascertaining the saturated, and mono-, di-, and triethenoid acids by the use of alkali isomerization and spectrophotometric measurements, combined with the determination of the iodine values. In 1944 the use of this procedure was extended to tetraenoic acids by Beadle and Kraybill,⁶⁸ who reported the extinction coefficient for arachidonic acid.

The alkali-isomerization method for the determination of the polyunsaturated acids now generally in use is essentially that described by Brice and co-workers,⁶⁹ who employed new standards for extinction coefficients. These standards were prepared from "natural" acids by chromatographic procedures, rather than those used by Brice and Swain⁷⁰ and by Brice *et al.*,⁷¹ which included bromination-debromination technics.

The Spectroscopy Committee of the American Oil Chemists' Society⁷² has developed a modification of the above method in which ethylene glycol is used for the saponification medium, and in which nitrogen gas is employed to prevent oxidation. With some modifications, the same procedure has been accepted by the American Oil Chemists' Society as a tentative method.

Although the AOCS method can be carried out with 50 to 100 mg. of sample, micromethods have been devised for use when limited amounts of fat are available. Thus, Herb and Riemenschneider⁷³ devised a procedure which requires only 1 to 10 mg. of fat, and microprocedures have been devised for the determination of polyunsaturated acids in blood by O'Connell *et al.*,⁷⁴ Wiese and Hansen.⁷⁵

d. Résumé of Method for the Analysis of Polyunsaturated Acids. The spectrophotometric method involving the alkali isomerization of polyunsaturated fatty acids is the most accurate and sensitive technic for the determination of these several unsaturated acids. This procedure, however, does have some limitations. Thus, in the determination of linoleic

⁶⁷ J. H. Mitchell, Jr., H. R. Kraybill, and F. P. Zscheile, *Ind. Eng. Chem., Anal. Ed.*, **15**, 1-3 (1943).

⁶⁸ B. W. Beadle and H. R. Kraybill, *J. Am. Chem. Soc.*, **66**, 1232 (1944).

⁶⁹ B. A. Brice, M. L. Swain, S. F. Herb, P. L. Nichols, Jr., and R. W. Riemenschneider, *J. Am. Oil Chemists' Soc.*, **29**, 279-287 (1952).

⁷⁰ B. A. Brice and M. L. Swain, *J. Opt. Soc. Amer.*, **35**, 532-544 (1945).

⁷¹ B. A. Brice, M. L. Swain, B. B. Schaeffer, and W. C. Ault, *Oil & Soap*, **22**, 219-224 (1945).

⁷² R. C. Stillman, Report of the Spectroscopy Committee, *J. Am. Oil Chemists' Soc.*, **26**, 399-404 (1949).

⁷³ S. F. Herb and R. W. Riemenschneider, *Anal. Chem.*, **25**, 953-955 (1953).

⁷⁴ P. W. O'Connell, E. Lipscomb, and B. F. Daubert, *Arch. Biochem. Biophys.*, **36**, 304-310 (1952).

⁷⁵ H. F. Wiese and A. E. Hansen, *J. Biol. Chem.*, **202**, 417-423 (1952).

or linolenic acids in cod-liver oil, it is practically impossible to correct for the presence of the highly unsaturated acids. The best results, with this procedure, are obtained with plant fats in which these highly unsaturated acids are not present. This method can be employed in studies of animal fat when the highly unsaturated acids are absent, or when they are present in a relatively insignificant amount as compared with the dienoic and trienoic acids. However, since the procedure is empirical, and the extinction coefficients are obtained from the literature, it is imperative that all conditions of the analysis be rigidly observed. The degree of alkali isomerization varies with the degree of alkalinity, the solvent, the temperature, and the time. When all conditions are scrupulously fulfilled, the agreement between different determinations is good. However, Deuel, Greenberg, Anisfeld, and Melnick⁷⁶ showed that, although there is usually agreement between the EFA content determined by the alkali-isomerization method and by the growth bioassay, this is not invariably true. In the case of a shortening prepared by non-selective hydrogenation, the bioassay method gave a value approximately twice that obtained by the spectrophotometric procedure. It is suggested that there may be isomers of unsaturated fatty acid which are biologically active but in which conjugation cannot be effected by the alkali treatment. It would therefore appear to the authors that the absolute index of EFA content can be obtained only when the sample is tested by bioassay procedure.

(4) *Synthesis of Polyunsaturated Acids*

Although the polyunsaturated acids appear superficially to be rather simple organic compounds, only the dienoic acid, linoleic acid, has been satisfactorily prepared synthetically. The synthesis of this acid was recently reported almost simultaneously by workers in three different laboratories, *i.e.*, Walborsky *et al.*,⁷⁷ Gensler and Thomas,⁷⁸ and Raphael and Sondheimer.⁷⁹ The procedures employed were essentially the same and depended upon the synthesis of a long-chain acetylenic acid (9,12 octadecadiynoic acid) by the method of Ahmad, Bumpus, and Strong,^{79a} followed by partial hydrogenation to produce the corresponding *cis*-ethylenic acid.

⁷⁶ H. J. Deuel, Jr., S. M. Greenberg, L. Anisfeld, and D. Melnick, *J. Nutrition*, **45**, 535-549 (1951).

⁷⁷ H. M. Walborsky, R. H. Davis, and D. R. Howton, *J. Am. Chem. Soc.*, **73**, 2590-2594 (1951).

⁷⁸ W. J. Gensler and G. R. Thomas, *J. Am. Chem. Soc.*, **73**, 4601-4604 (1951).

⁷⁹ R. A. Raphael and F. Sondheimer, *J. Chem. Soc.*, 1950, 2100-2103.

^{79a} K. Ahmad, F. M. Bumpus, and S. M. Strong, *J. Am. Chem. Soc.*, **70**, 3391-3394 (1948).

A method of synthesis which should prove useful for the preparation of carboxyl-labeled linoleic acid has been recently announced by Howton and co-workers.^{79b} In this procedure tetrabromstearic acid was produced by bromination of natural linoleic acid. After decarboxylation of the silver salt of the tetrabrom-acid with bromine, the compound was debrominated with zinc, and the linoleic acid was regenerated with a new carboxyl group by means of the Grignard reagent with CO₂.

Howton, Davis, and Nevenzel⁸⁰ synthesized 1-C¹⁴-linoleic acid as well as 1-C¹⁴-linolenic acid.

3. Bioassay of Essential Fatty Acids

(1) *Growth Method of Greenberg and Co-Workers*

When weanling rats are fed diets complete in all essential dietary components with the exception of the EFA, retardation of the growth of the animals begins within several weeks, and the body weight reaches a plateau after nine to ten weeks, according to Deuel *et al.*⁸¹ From then on, the body weight may remain fairly constant over an extended period of time, or it may gradually decline, and the rats may succumb. If EFA are fed to the depleted rats on a basal fat-free diet after a constant body weight has been established, an increase in body weight promptly occurs. Within certain limits, the increase is proportional to the amount and potency of the active compound employed. Greenberg and associates⁸² reported that, when the log dose of linoleate fed was plotted against the gain-in-weight of male rats for doses of linoleate of 5, 10, 20, or 50 mg. per day, a straight-line function occurred promptly at three weeks, and continued for as long as twelve weeks, at which time the experiment was terminated. By comparing the gain-in-weight induced by a test substance fed at several levels with that noted when standardized doses of linoleate are used, it is possible to calculate the linoleate equivalent of the unknown.

Although the rationale of the growth method is a sound one, since it is based upon the same procedure as the one suggested by Coward⁸³ for

^{79b} D. R. Howton, R. H. Davis, and J. C. Nevenzel, *J. Am. Chem. Soc.*, **74**, 1179 (1952).

⁸⁰ D. R. Howton, R. H. Davis, and J. C. Nevenzel, *J. Am. Chem. Soc.*, **76**, 4970-4974 (1954); also unpublished data, 1954.

⁸¹ H. J. Deuel, Jr., S. M. Greenberg, C. E. Calbert, E. E. Savage, and T. Fukui, *J. Nutrition*, **40**, 351-366 (1950).

⁸² S. M. Greenberg, C. E. Calbert, E. E. Savage, and H. J. Deuel, Jr., *J. Nutrition*, **41**, 473-486 (1950).

⁸³ K. H. Coward, *The Biological Standardization of the Vitamins*, 2nd ed., Williams & Wilkins, Baltimore, 1947, pp. 35 ff.

the bioassay of vitamin A, there are several difficulties in the application of the test to the EFA. In the first place, the period of twenty weeks required for the depletion and assay periods is excessive. The diet, which contains vitamin-test casein, is expensive. Finally, the amount of the test substance which must be administered to ensure that a sufficient dose of EFA will be given may be so great that it constitutes a serious difficulty in the feeding. Deuel *et al.*⁷⁶ fed the margarine and butter supplements in daily doses of 250 and 500 mg. The administration of this amount of fat in the test groups was compensated for in the control linoleate tests by giving proportionate doses of hydrogenated coconut oil. Since the hydrogenated coconut oil was found to decrease the period of depletion, although the growth effect during the assay period was counteracted by linoleate, Deuel and co-workers²² consider that the absolute results from the assay may be open to some question. It would now seem preferable to use linoleate-free, partially hydrogenated triolein as the compensating fat in these bioassays.

(2) *Growth Method of Thomasson Based upon Rationing of Water*

The new weight increase method proposed by Thomasson¹⁶ is based upon the disturbed water metabolism which occurs in EFA deficiency. As early as 1930, Burr and Burr⁴ reported that rats on fat-free diets drink more water than normal animals do. In the new bioassay procedure, only weanling male rats are used. Animals weighing approximately 40 gm. at weaning, from mothers who had received the Sherman diet, were transferred to a fat-free diet. During the first two weeks on the diet, the water intake of the rats was not limited; after that the intake of water was limited to 14 ml. per rat per day for the rest of the depletion period and during the period of bioassay. The preparatory depletion period lasted for five weeks (three weeks with water rationing); after this, some groups of animals were dosed with appropriate levels of linoleate, and other groups received the test substance five times per week during the four-week bioassay period. The linoleate and test substance were given in doses made up to a total volume of 0.2 ml. with hydrogenated coconut oil. The determination of the quantity of essential fatty acids was based upon the slope of the gain-in-weight/log dose curve obtained, compared with that for the standard. Instead of using linoleate directly as a standard, Thomasson¹⁶ preferred to employ sunflowerseed oil in doses of 10 and 50 mg. One Unit of EFA (also referred to by Thomasson as vitamin F) is defined as the activity of 10 mg. of linoleic acid. Thus, one Unit corresponds almost

exactly to a 1% value of EFA. Thomasson¹⁶ reported numerous bioassays with this new procedure which are, in general, consistent with other available data. This procedure has the distinct advantage that the total period required is only nine weeks.

4. Comparative Biopotencies of the Essential Fatty Acids

(1) *Introduction*

Linoleic, linolenic, and arachidonic acids have been recognized as the chief polyunsaturated fatty acids which possess an appreciable biopotency in counteracting the fat-deficiency symptoms in rats, and in allowing a normal growth and metabolism in animals on a fat-free diet. Of these acids, linoleic and linolenic occur as components of many vegetable oils, whereas arachidonic acid is present exclusively in animal fats. Linoleic acid and, in some cases, linolenic acid, are likewise present in the blood and tissue fat of some animals, but it is usually considered that arachidonic acid represents the active biologic form of the EFA. There is excellent evidence that linoleic acid can be transformed to arachidonic acid in the animal body. In the past it was generally considered that the linolenate molecule was convertible into arachidonate, but more recent work shows that if this takes place at all it does so at a much lower order of efficiency than is the case with the linoleate molecule.

(2) *Proof of the Inability of Animals to Effect a Biosynthesis of the Polyunsaturated Acids*

The necessity of polyunsaturated fatty acids as dietary components has been based upon the observation that they appeared not to be synthesized by the animal *de novo*. If they are absent from the diet, tissues in which they play an integral role and enzyme systems in which they may participate become depleted. They then function less effectively, or they may disappear entirely.

It has long been recognized that the animal can synthesize saturated fatty acids from carbohydrate; it is now known that two-carbon intermediates, which originate from carbohydrates, participate in this biosynthesis, together with Coenzyme A. Moreover, it was convincingly demonstrated by Schoenheimer and Rittenberg,⁸⁴ by the use of tagged stearate molecules, that the mouse is able to bring about the desaturation of stearic acid into oleic acid. Palmitoleic acid has likewise been shown to originate from pal-

⁸⁴ R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **113**, 505-510 (1936).

mitic acid, according to Stetten and Schoenheimer,⁸⁵ while a similar desaturation of myristic acid was shown to occur, resulting in the formation of the monoethenoid acid, myristoleic acid, described by Anker.⁸⁶

In sharp contrast to the ease of synthesis of the monoethenoid acids from the corresponding saturated fatty acids, Bernhard and Schoenheimer⁸⁷ demonstrated that the rat is unable to synthesize either the diethenoid acid, linoleic, or the triethenoid acid, linolenic, from carbohydrate, although large amounts of stearic and palmitic acids were shown to originate concomitantly from this foodstuff. Thus, saturated and monoethenoid acids, which have most important functions in the animal, are considered to be non-essential from a dietary standpoint, since they can be synthesized even on nonfat diets. On the other hand, linoleic, linolenic, and arachidonic acids are regarded as essential.

The requirements of different animals for essential fatty acids differ considerably. Since tissue lipids, especially phospholipids, invariably contain significant amounts of EFA, one may conclude that some animals must be able to synthesize the compounds, but that the degree of this ability varies not only between phyla class and species, but also between different age groups within the same species. There is direct evidence of the ability of adult animals to synthesize "essential" fatty acids. Barki and associates⁸⁸ reported that the adult rat may not only recover from fatty acid deficiency on a fat-free diet, but may even increase its content of linoleic, linolenic, and arachidonic acids. Reiser, Gibson, Carr, and Lamp⁸⁹ showed that hens on a fat-free ration for one year continued to lay eggs containing appreciable quantities of linoleic acid.

(3) *The Question of the Possible Biopotency of Oleic Acid and of Other Monoethenoid Acids*

Burr and Burr² were originally of the opinion that oleic acid might possess some activity in counteracting the fat-deficiency syndrome. Burr *et al.*^{4,21} later reported that the monoethenoid acid was completely devoid of bioactivity. Evans and Lepkovsky⁹⁰ also reported the negative effect of oleic acid in preventing fat deficiency. Moreover, it was recently (1954)

⁸⁵ De W. Stetten, Jr., and R. Schoenheimer, *J. Biol. Chem.*, **133**, 329-345 (1940).

⁸⁶ H. S. Anker, *J. Biol. Chem.*, **194**, 177-182 (1952).

⁸⁷ K. Bernhard and R. Schoenheimer, *J. Biol. Chem.*, **133**, 707-712, 713-720 (1940).

⁸⁸ V. H. Barki, R. A. Collins, E. B. Hart, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **71**, 694-696 (1949).

⁸⁹ R. Reiser, B. Gibson, M. J. Carr, and B. G. Lamp, *J. Nutrition*, **44**, 159-176 (1951).

⁹⁰ H. M. Evans and S. Lepkovsky, *J. Biol. Chem.*, **96**, 157-164 (1932).

observed by Cheng⁹¹ that triolein affords no protection against x-irradiation injury. Alfin-Slater *et al.*^{92,93} found the administration of methyl oleate ineffective in counteracting the abnormal cholesterol accumulation in the liver which occurs in fat deficiency. These results leave no doubt that oleic acid is entirely ineffective in preventing or curing the deficiency brought about by the exclusion of EFA from the diet.

In addition to the negative response to oleic acid, Thomasson¹⁶ reported that the following closely related monoethenoid acids likewise lack any biopotency: 11-octadecenoic acid; 12-octadecenoic acid; 13-docosenoic acid (erucic); and 12-hydroxy-9-octadecenoic acid (ricinoleic). Turpeinen¹⁵ also noted that 12-octadecenoic acid, erucic, and ricinoleic acids, as well as chaulmoogric acid, are without biologic activity. Elaidic acid, the *trans*-isomer of oleic acid, has likewise been reported to be without curative effect in fat deficiency. It is thus obvious that none of the monoethenoid acids affords protection from fat deficiency.

(4) *The Biopotency of Linolenic Acid vs. Linoleic Acid*

Burr and co-workers²¹ stated that linoleic and linolenic acids possess about equal potency in protecting against the fat-deficiency syndrome. However, a number of workers have disagreed with these original observations. For example, Martin⁹⁴ obtained negative results with the triethenoid acid, and Hume and collaborators¹³ stated that linolenic acid has only one-sixth of the biopotency of linoleic acid. On the other hand, Tange²³ noted that these acids have the same biologic activity. More recently, Greenberg *et al.*⁸² reported that linolenic acid, when given alone, was practically without activity to restore growth in fat-deficient rats. However, when the triethenoid acid was administered together with linoleic acid, the effect of linolenic acid appeared to be equivalent to that of linoleic acid, and the effects of the two acids were additive. This was confirmed by Deuel and co-workers.⁹⁵ Another interpretation is that linolenate "protects" linoleate during transport and storage, thus making more linoleate available for metabolic purposes.

Thomasson,¹⁶ using his bioassay technic for the EFA, reported a new

⁹¹ A. L. S. Cheng, Personal communication to the author (H. J. D.), 1954.

⁹² R. B. Alfin-Slater, L. Aftergood, A. F. Wells, and H. J. Deuel, Jr., *Federation Proc.*, **13**, 174 (1954).

⁹³ R. B. Alfin-Slater, L. Aftergood, A. F. Wells, and H. J. Deuel, Jr., *Arch. Biochem. Biophys.*, **52**, 180-185 (1954).

⁹⁴ G. J. Martin, *J. Nutrition*, **17**, 127-141 (1939).

⁹⁵ H. J. Deuel, Jr., C. R. Martin, and R. B. Alfin-Slater, *J. Nutrition*, **57**, 297-302 (1955).

finding which may possibly explain the divergent results of the several laboratories in regard to the biologic activity of ordinary linolenic acid. Thus, it was observed that ordinary linolenic acid (9,12,15-octadecatrienoic acid) possesses practically no EFA activity (only 9%). On the other hand, isolinolenic acid (the so-called γ -linolenic acid, which is chemically 6,9,12-octadecatrienoic acid) has 100% of the biopotency of linoleic acid. Although γ -linolenic acid is known to occur in the seeds of the evening primrose (*Oenothera biennis*), its distribution is limited. It is uncertain whether or not ordinary linolenate may be activated by being transformed to the γ -isomer. More studies are needed to clarify the relationship of the several linolenic acids as possible components of the EFA. Finally, Burr *et al.*²¹ and more recently Deuel and associates¹⁸ proved that another natural trienoic acid, *i.e.*, α -claeostearic acid (9,11,13-octadecatrienoic acid), which is present in tung oil, is completely devoid of biologic potency.

(5) *The Biopotency of Arachidonic Acid vs. Linoleic Acid*

In the original report of Burr and collaborators,²¹ methyl arachidonate was listed as being somewhat inferior to linoleate as a source of EFA. These workers were unable to rationalize this observation, since the arachidonate content is known to be high in liver fat and in lard, both of which possess a high curative action against the fat-deficiency syndrome. Later workers, Turpeinen,¹⁵ Hume *et al.*,⁹⁶ and Greenberg and co-workers,⁹⁷ however, assigned to arachidonic acid potencies two or three times that of linoleic acid. On the other hand, Thomasson¹⁶ has ascribed a value of only 131% to the tetraenoic acid, as compared with that of linoleate.

The reasons for the divergence in bioactivity reported by the different groups of workers are not entirely clear. In the original tests of Burr *et al.*,²¹ the low value for arachidonate may have been due to the fact that "some of the purified arachidonic acid which we have fed has been altered in the process of preparation." Even so, Burr *et al.*⁹⁸ were unable to confirm the high potency of arachidonate reported by Turpeinen¹⁵ when they used the same sample of acid. However, in spite of this single negative finding, the consensus is that arachidonic acid is the most active member of the EFA.

On the basis of the experimental findings, there appears to be little doubt

⁹⁶ E. M. Hume, L. C. A. Nunn, I. Smedley-MacLean, and H. H. Smith, *Biochem. J.*, **34**, 879-883 (1940).

⁹⁷ S. M. Greenberg, C. E. Calbert, H. J. Deuel, Jr., and J. B. Brown, *J. Nutrition*, **45**, 521-533 (1951).

⁹⁸ G. O. Burr, J. B. Brown, J. P. Kass, and W. O. Lundberg, *Proc. Soc. Exptl. Biol. Med.*, **44**, 242-244 (1940).

that linoleic acid can be converted to arachidonic acid in the animal body,⁹⁹ and that some synthesis and interconversion of fatty acids occur in the body.^{88, 100, 101} The transformation of linoleic acid involves the extension of the carbon chain by two carbons, and a double desaturation at the carboxyl side of the original double bonds. Mead and co-workers¹⁰² proved that isotopic acetate can be demonstrated in the arachidonic acid molecules synthesized from linoleate.

Barki *et al.*¹⁰³ observed that mature rats could be maintained on a fat-free diet for a prolonged period, without presenting deficiency symptoms, except for emaciation. Moreover, when the depleted animals were fed the fat-free diet *ad libitum* for a sufficient period, the EFA deficiency symptoms disappeared spontaneously. This suggests that some synthesis of the EFA may occur.

Greenberg¹⁰⁴ suggested that one mole of arachidonic acid can arise from two molecules of linoleic acid. This viewpoint is supported by the findings of Widmer and Holman,¹⁰⁵ Reiser,^{101, 106} and Holman,¹⁹ who demonstrated that, when dienoic and trienoic acids were fed, tetraenoic, pentaenoic, and hexaenoic acids, respectively, were present in the organs and body fat of rats,^{19, 105} in egg yolk,¹⁰¹ and in the body fat of chicks.¹⁰⁶

(6) *The Biologic Activity of Related Compounds*

In addition to the three main EFA, linoleyl alcohol,¹⁵ docosahexaenoic acid, and two hexahydroxystearic acids (linusic and isolinusic)¹³ have been reported to exhibit low degrees of activity in protecting against fat-deficiency symptoms. However, Thomasson¹⁶ observed that linusic acid possessed no bioactivity, and that the potency of isolinusic acid was only slight. In addition, Thomasson¹⁶ recorded a biopotency of 43% for 11,14-eicosadienoic acid and one of 9% for 10,13-nonadecadienoic acid. According to this author, the presence of double bonds on the 6:7 and 9:10 positions (counting from the terminal CH₃ group, or ω -carbon) is required for EFA activity. An increase in the number of double bonds on the CH₃-

⁹⁹ L. C. A. Nunn and I. Smedley-MacLean, *Biochem. J.*, **32**, 2178-2184 (1938).

¹⁰⁰ I. G. Rieckehoff, R. T. Holman, and G. O. Burr, *Arch. Biochem.*, **20**, 331-340 (1949).

¹⁰¹ R. Reiser, *J. Nutrition*, **42**, 325-336 (1950).

¹⁰² J. F. Mead, G. Steinberg, and D. R. Howton, *Federation Proc.*, **12**, 244 (1953).

¹⁰³ V. H. Barki, H. Nath, E. B. Hart, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **66**, 474-478 (1947).

¹⁰⁴ S. M. Greenberg, *A Nutritional Evaluation of the Essential Fatty Acids in the Rat*. Dissertation, Univ. Southern Calif., Dept. Biochem. Nutrition, 1951.

¹⁰⁵ C. Widmer, Jr., and R. T. Holman, *Arch. Biochem.*, **25**, 1-12 (1950).

¹⁰⁶ R. Reiser, *J. Nutrition*, **44**, 159-175 (1951).

side appears to decrease biopotency, whereas increased unsaturation on the carboxyl side may potentiate it.

5. Factors Affecting the Requirement for Essential Fatty Acids

(1) *The Effect of Species*

Although the discovery of the requirement for EFA as a dietary constituent was first made on the rat, and considerable information has been obtained by the study of this species, the requirement for these acids has been found to be very widespread. Fraenkel and Blewett¹⁰⁷ demonstrated that the EFA are required by certain insects, such as the Mediterranean flour moth (*Ephestia kuehniella*), the tobacco moth (*E. elutella*), the fig moth (*E. cautella*), and the Indian-meal moth (*Plodia interpunctella* Lep.). This is also true of the pink boll-worm (*Pectinophora gossypiella* Saund.) studied by Reiser, Kerur, and Vanderzant.¹⁰⁸

According to White and associates,¹⁰⁹ mice develop a deficiency similar to that of rats on regimens devoid of EFA. In fact, this species has recently been used by Decker *et al.*¹¹⁰ for a comprehensive study of EFA. Although Russell and co-workers¹¹¹ were unable to provoke a fat deficiency in chickens, Reiser¹¹² was later successful in producing the deficiency in this species. In the latter tests, sucrose was used in the diet in place of the ground yellow corn employed by Russell *et al.*¹¹¹ It was suggested that, in the earlier tests, there was a sufficient supply of EFA, chemically combined with the starch particles and not removed by the usual methods of extraction, to prevent EFA deficiency.

The hog represents a species relatively refractory to EFA deficiency. The failure to induce a fat deficiency in this species may be related to the high content of EFA frequently stored in its tissues. Thus, Ellis and Isbell^{113,114} noted that lard from hogs on a high-soybean diet sometimes had a linoleate content as high as 31.9%. Ellis and Zeller¹¹⁵ found that, when the animals were subjected to a fat-free diet, the linoleate content decreased to as little as 1.3%. It would appear that fat deficiency has not been

¹⁰⁷ G. Fraenkel and M. Blewett, *J. Exptl. Biol.*, **22**, 172-190 (1946).

¹⁰⁸ R. Reiser, D. R. Kerur, and E. S. Vanderzant, unpublished data, 1955.

¹⁰⁹ E. A. White, J. R. Foy, and L. R. Cerecedo, *Proc. Soc. Exptl. Biol. Med.*, **54**, 301-302 (1943).

¹¹⁰ A. B. Decker, D. L. Fillerup, and J. F. Mead, *J. Nutrition*, **41**, 507-521 (1950).

¹¹¹ W. C. Russell, M. W. Taylor, and L. J. Polskin, *J. Nutrition*, **19**, 555-562 (1940).

¹¹² R. Reiser, *J. Nutrition*, **42**, 319-323 (1950).

¹¹³ N. R. Ellis and H. S. Isbell, *J. Biol. Chem.*, **69**, 219-238 (1926).

¹¹⁴ N. R. Ellis and H. S. Isbell, *J. Biol. Chem.*, **69**, 239-248 (1926).

¹¹⁵ N. R. Ellis and J. H. Zeller, *J. Biol. Chem.*, **89**, 185-197 (1930).

observed in the hog because the tissue linoleate is sufficient to protect the animal on a fat-free diet for an extended period. It is also possible that swine synthesize more linoleic acid than do other species. On the other hand, Witz and Beeson¹¹⁶ described a fatty acid deficiency in the pig.

Definite evidence of the role of the EFA in calves has only recently become available. In fact, Gullickson *et al.*¹¹⁷ were unable to demonstrate that essential fatty acids were required by calves; when vegetable oils were homogenized into skimmed milk, the resulting product could not be tolerated by the calves. Most of the animals did poorly, and a number of them died, in contradistinction to the relatively satisfactory results when whole milk was used. Although Gullickson and co-workers¹¹⁸ indicated more recently that the replacement of butterfat with vegetable oils caused some symptoms characteristic of vitamin E deficiency, they have likewise reported the normal development of a calf maintained for over forty-five days on a skim-milk diet into which partially hydrogenated vegetable oil was homogenized. It has also been impossible to produce fat deficiency in cows, according to Gibson and Huffman,¹¹⁹ and Maynard *et al.*,¹²⁰ although a decrease in the iodine value of the blood lipids was shown to occur when the animals were continued on a fat-free diet over a prolonged period. The recent studies of Lambert *et al.*¹²¹ have left no doubt that the calf is susceptible to EFA deficiency. Thus, these workers observed a marked retardation of growth of 50% in the case of calves on a fat-deficient regimen. Scaly dandruff was also noted, together with an excessive loss of hair on the back, shoulders, and tail. These characteristic symptoms of fat deficiency were promptly alleviated when sources of the EFA were administered.

The dog represents a species which is readily susceptible to the fat-deficiency syndrome. Because of the convenient size of this species, which will enable a clinical study to be made on one animal over a period of time, considerable work has been reported on EFA deficiency in these animals. Hansen and Wiese¹²² demonstrated, in 1943, that the symptoms of fat

¹¹⁶ W. M. Witz and W. M. Beeson, *J. Animal Sci.*, **10**, 112-128 (1951).

¹¹⁷ T. W. Gullickson, F. C. Fountaine, and J. B. Fitch, *J. Dairy Sci.*, **24A**, 315-316 (1941).

¹¹⁸ T. W. Gullickson, R. S. Adams, J. Gander, and J. H. Sautter, *J. Dairy Sci.*, **36**, 599-600 (1953).

¹¹⁹ G. Gibson and C. F. Huffman, *Michigan State Coll., Agr., Appl. Sci., Agr. Exptl. Sta. Quart. Bull.*, **21**, 258-264 (1939).

¹²⁰ L. A. Maynard, K. E. Gardner, and A. Hodson, *New York (Cornell Univ.) Agr. Exptl. Sta. Bull. No. 722* (March 10, 1939), pp. 1-30.

¹²¹ M. R. Lambert, N. L. Jacobson, R. S. Allen, and J. H. Zaletel, *J. Nutrition*, **52**, 259-272 (1954).

¹²² A. E. Hansen and H. F. Wiese, *Proc. Soc. Exptl. Biol. Med.*, **52**, 205-208 (1943).

deficiency in the dog were similar to those produced by fat-free diets in other species. It has been suggested that dietary fat may supply a factor necessary for the maturation of epithelial, sebaceous, and sudoriparous cells. The fat-deficiency symptoms in the dog were found to respond quickly to a diet containing fresh lard to the extent of 29% of the total calories.^{122,123}

There is less absolute proof that the EFA are required by man than is the case for other species. v. Gröer¹²⁴ and Hansen¹²⁵ reported that a characteristic eczema develops in infants on a fat-low diet which rapidly responds when fat is added to the restricted regimen. Although neither Taub and Zakon¹²⁶ nor Ginsberg *et al.*¹²⁷ were able to confirm the above results on infants, it is possible that the latter workers may have been dealing with a non-specific type of skin disorder.

Faber and Roberts,¹²⁸ and Cornbleet and Pace¹²⁹ correlated the appearance of eczematous symptoms in adult subjects with the reduction in the level of unsaturated fatty acids in the blood, and especially with that of linoleate and arachidonate. Finnerud *et al.*¹³⁰ found that, when the skin condition was relieved by the administration of lard, the improved clinical condition was reflected by an increased plasma linoleate, although the plasma arachidonate was not necessarily augmented. Brown and Hansen¹³¹ reported the following average values for linoleate in per cent of total acids in young and in adult eczematous patients, respectively: *young patients*, 4.80% and 3.20%; *adult patients*, 5.20% and 4.20%. The mean blood arachidonate of these groups also was lower in eczema, being as follows: *young patients*, 2.83% and 1.34%; *adult patients*, 2.90% and 1.60%. In a comprehensive study of the polyunsaturated fatty acid levels in the blood of ninety-three well-nourished infants and children, Wiese and her co-workers¹³² observed a slightly lower value for dienoic and tetraenoic acids in the infants than in children two to fifteen years of age. When 3% of the total calories were ingested in the form of linoleate, 30.3%, 1.5% and 10.2%

¹²³ A. E. Hansen and H. F. Wiese, *Texas Repts. Biol. Med.*, **9**, 491-515 (1951).

¹²⁴ F. von Gröer, *Biochem. Z.*, **97**, 311-329 (1919).

¹²⁵ A. E. Hansen, *Am. J. Diseases Children*, **53**, 933-946 (1937).

¹²⁶ S. J. Taub and S. J. Zakon, *J. Am. Med. Assoc.*, **105**, 1675 (1935).

¹²⁷ J. E. Ginsberg, C. Bernstein, Jr., and L. V. Iob, *Arch. Dermatol. Syphilol.*, **36**, 1033-1038 (1937).

¹²⁸ H. K. Faber and D. B. Roberts, *J. Pediat.*, **6**, 490-493 (1935).

¹²⁹ T. Cornbleet and E. R. Pace, *Arch. Dermatol. Syphilol.*, **31**, 224-226 (1935).

¹³⁰ C. W. Finnerud, R. L. Kesler, and H. F. Wiese, *Arch. Dermatol. Syphilol.*, **44**, 849-861 (1941).

¹³¹ W. R. Brown and A. E. Hansen, *Proc. Soc. Exptl. Biol. Med.*, **36**, 113-117 (1937).

¹³² H. F. Wiese, R. H. Gibbs, and A. E. Hansen, *J. Nutrition*, **52**, 355-365 (1954).

of the total plasma fatty acids consisted of dienoic, trienoic, and tetraenoic acids, respectively. Hansen, Sinclair, and Wiese¹³³ reported that no significant differences in the values of the total plasma fatty acids were to be noted in the case of fifty-seven poorly nourished infants and children, as compared with the figures obtained for well-nourished subjects. However, it was observed that the levels of dienoic, tetraenoic, and hexaenoic acids were significantly lower in the poorly nourished individuals than in the well-nourished group. There would thus appear to be a correlation between the EFA content of the blood and the occurrence of eczema, not only in the case of infants but also in that of young children and adults.

In spite of the unequivocal relationship between the skin symptoms and the blood level of EFA in man, it has not been possible to produce the fat-deficiency syndrome in man experimentally by diet. In a single experiment on a normal male subject maintained on a fat-free regimen for six months, Brown *et al.*¹³⁴ were unable to observe any of the classical symptoms of fat deficiency. It would have been surprising if any such symptoms had appeared, since these conditions do not produce deficiency, even in rats. However, a 50% reduction in plasma linoleate and plasma arachidonate occurred, which was completely out of proportion to the extent of decrease of other blood lipids. In spite of this single negative result on man, it would seem that the proof of the requirement of EFA by man is unequivocal. Because of the greater life span, and the difficulties of experimenting with humans, the fat-deficiency syndrome cannot be initiated as easily in man as in the lower animals. Possibly man should be classified with the hog as being less susceptible to fat deficiency, in contrast to the ready production of the fat-deficiency syndrome in the mouse, rat, chicken, dog, and calf.

(2) *The Effect of Sex*

It is well known that a sex difference exists in the metabolism of fats. This variation is reflected not only by the higher level of ketonuria which obtains during fasting in women, as compared with men, as demonstrated by Deuel and Gulick,¹³⁵ but also in the increased levels of urine ketone bodies in the female rat in exogenous ketonuria, reported by Butts and Deuel¹³⁶ and in endogenous ketonuria by Deuel *et al.*,¹³⁷ as compared with

¹³³ A. E. Hansen, J. G. Sinclair, and H. F. Wiese, *J. Nutrition*, **52**, 541-554 (1954).

¹³⁴ W. R. Brown, A. E. Hansen, G. O. Burr, and I. McQuarrie, *J. Nutrition*, **16**, 511-524 (1938).

¹³⁵ H. J. Deuel, Jr., and M. Gulick, *J. Biol. Chem.*, **96**, 25-34 (1932).

¹³⁶ J. S. Butts and H. J. Deuel, Jr., *J. Biol. Chem.*, **100**, 415-428 (1933).

¹³⁷ H. J. Deuel, Jr., L. F. Hallman, and S. Murray, *J. Biol. Chem.*, **119**, 257-268 (1937).

the levels in male rats. For a further discussion of these and other aspects of sex difference in fat metabolism, the reader is referred to other publications of Deuel.^{11,138}

It is not surprising therefore to note that the optimum requirement for linoleate varies with sex. Data on the requirement of EFA for growth are available only for the rat. If one accepts maximum gain-in-weight during the assay period as an index of optimum nutrition, then one must conclude that the daily requirement for male rats probably exceeds 200 mg. This conclusion was reached on the basis that the curve obtained, when the gain-in-weight is plotted against log dose, is a straight line not only for several daily dosages up to 100 mg. as observed by Greenberg *et al.*,^{52,97} but even for 200 mg., according to Deuel, Greenberg, Anisfeld, and Melnick.⁷⁶

In contradistinction to these results, on the basis of similar experimental data, the daily requirement of the female rat for EFA does not exceed 100 mg. and probably varies between 20 and 50 mg., as stated by Greenberg *et al.*⁸² and by Anisfeld *et al.*¹³⁹ Anisfeld and her co-workers¹³⁹ likewise reported that the reduced growth response of the female rat to increasing doses of linoleate is not related to variations in the tocopherol requirement of the sexes. Cheng, Kryder *et al.*¹⁴⁰ also noted that sex plays a role in the protective effect of fats against x-irradiation injury. Thus, the male can be protected by fats from x-irradiation under conditions which afford no protection for the female. Although all of the data concerning sex differences in requirement for EFA have been obtained on the rat, there is no reason to believe that the sex factor is unique for this species. One must come to this conclusion if one accepts the fact that this is only one of the expressions of sex variation in fat metabolism, a phenomenon which has been shown to be shared by many species, including man.

(3) *The Effect of Substances Fed Concomitantly*

a. Fatty Acids. The fat-deficiency syndrome has been found to develop much more rapidly when saturated fatty acids are fed to the rat along with the EFA-deficient diet. Evans and Lepkovsky⁹⁰ reported, as early as 1932, that the fat-deficiency syndrome developed more rapidly in rats on an EFA-free diet than in animals on a similar diet devoid of the saturated fat. These findings were confirmed by Sinclair¹⁴¹ and by Deuel *et al.*⁷⁶ In a later

¹³⁸ H. J. Deuel, Jr., *The Wholesomeness and Nutritional Value of Fats and Oils*, Special brochure prepared for the Institute of Shortening and Edible Oils, Inc., 1954.

¹³⁹ L. Anisfeld, S. M. Greenberg, and H. J. Deuel, Jr., *J. Nutrition*, **45**, 599-607 (1951).

¹⁴⁰ A. L. S. Cheng, G. D. Kryder, L. Bergquist, and H. J. Deuel, Jr., *J. Nutrition*, **48**, 161-182 (1952).

¹⁴¹ R. O. Sinclair, *J. Biol. Chem.*, **114**, xciv (1936).

study by Deuel and associates,²² it was noted that not only was the time of depletion appreciably shortened when hydrogenated coconut oil was included in the EFA-free diet, but also the level at which the body weight of the rats reached a plateau was considerably lower when hydrogenated coconut oil was present in the diet. However, the continued presence of hydrogenated coconut oil in the diet did not appear to alter the growth response to linoleate, although the animals depleted with the hydrogenated coconut oil diet had a greater growth potential than did those depleted on the fat-free regimen. The intensity of the effect with hydrogenated coconut fat varied with the proportion of this foodstuff included in the diet. Thus, the effect of a 15% addition exceeded that of 5%, and a much more pronounced effect was noted when the fat was included at the 30% level. On the other hand, Wells and Deuel¹⁴² found that partially hydrogenated triolein did not influence the length of the depletion period or the body weight at which the fat-deficiency syndrome was established. Moreover, triolein did not change in any way the effect of linoleate in providing a gain-in-weight in fat-deficient animals. Finally, triolein has been found by Cheng⁹¹ to have no beneficial or deleterious influence upon the resistance to x-irradiation injury in the rat. It is therefore apparent that hydrogenated coconut oil and partially hydrogenated triolein behave differently in the rat insofar as their relationship to EFA is concerned. It is possible that the effect of the hydrogenated coconut oil may be related to the large proportion of lauric acid and shorter chain acids in its molecule, as compared with the more physiologic triolein.

b. Mineral Oil. Bacon and co-workers¹⁴³ demonstrated that a fat-deficiency syndrome could be precipitated in weanling rats within a relatively short period if mineral oil was incorporated into a fat-low diet. When the level of added mineral oil was less than 5%, the cessation of growth was noted at the same time as in the control tests (at eleven to twelve weeks); however, when 7.5% or 10% of mineral oil was included in the regimen fed to the weanling rats, the classical fat-deficiency symptoms appeared within two or three weeks. In addition to growth failure, an increased water consumption was likewise noted.

Proof that the deficiency produced by the inclusion of mineral oil in the diet is a fat deficiency was based not only upon the typical symptoms which developed but also upon the fact that the inclusion of 50 mg. of linoleate per day in the diet prevented them. Moreover, there was an increased

¹⁴² A. F. Wells and H. J. Deuel, Jr., Unpublished observations, 1954.

¹⁴³ E. K. Bacon, S. Lassen, S. M. Greenberg, J. W. Mehl, and H. J. Deuel, Jr., *J. Nutrition*, **47**, 383-398 (1952).

excretion of fatty acids in the feces of rats fed mineral oil. In addition, the fecal fatty acids were found to have an increased iodine value after the ingestion of mineral oil. However, it is believed that this effect cannot be ascribed solely to the solvent action of the hydrocarbon on EFA, since the deficiency symptoms were prevented by the administration of linoleate either orally or intraperitoneally.

(4) *The Effects of Vitamins and Hormones*

Although the metabolism of most of the water-soluble B vitamins is interrelated with that of fat in general, it was pointed out by Salmon¹⁴⁴ that the deficiencies in pantothenic acid, pyridoxine, and EFA, respectively, result in the production of forms of dermatitis which are similar in appearance. The closest relationship appears to exist between the requirement for fat and that of pyridoxine, according to Hogan and Richardson,¹⁴⁵ and Birch and György.¹⁴⁶ The dermatitis which occurs on a diet low in pyridoxine but containing 10% of fat can be cured when a small amount of lard is added to the diet.¹⁴⁶ Birch¹⁴⁷ later showed that the onset of dermatitis due to a pyridoxine deficiency was delayed by the addition of fat to the diet; in some cases, the abnormal skin condition was completely prevented.

Most of the evidence points to the fact that the effectiveness of fats in counteracting the dermatitis caused by pyridoxine deficiency is proportional to the unsaturation, *i.e.*, presumably to the EFA content. This explanation would account for the fact that lard is effective in alleviating pyridoxine deficiency, since it is ordinarily relatively high in arachidonic and linoleic acids, whereas butter, which has a relatively low content of EFA, is somewhat less efficacious. Schneider¹⁴⁸ ascribed the low antidermatitis potency of rancid butter to the destruction of the antidermatitis factor (later identified as linoleic acid) during the course of rancidification.

All of the principal components of the EFA are capable of curing pyridoxine deficiency. According to Quackenbush *et al.*¹⁴⁹ linoleic acid is the most effective of the three principal EFA in alleviating the deficiency symptoms due to the lack of pyridoxine. Salmon¹⁵⁰ found that the methyl esters of linoleic and linolenic acid are less effective than the oils or their

¹⁴⁴ W. D. Salmon, *J. Biol. Chem.*, **140**, cix-cx (1941).

¹⁴⁵ A. G. Hogan and L. R. Richardson, *Nature*, **136**, 186 (1935).

¹⁴⁶ T. W. Birch and P. György, *Biochem. J.*, **30**, 304-315 (1936).

¹⁴⁷ T. W. Birch, *J. Biol. Chem.*, **124**, 775-793 (1938).

¹⁴⁸ H. A. Schneider, *Proc. Soc. Exptl. Biol. Med.*, **44**, 266-267 (1940).

¹⁴⁹ F. W. Quackenbush, F. A. Kummerow, and H. Steenbock, *J. Nutrition*, **24**, 213-224 (1942).

¹⁵⁰ W. D. Salmon, *J. Biol. Chem.*, **133**, civ-cv (1938).

total fatty acids. Schneider and co-workers¹⁵¹ state that linoleic acid possesses a better curative action in pyridoxine deficiency than does linolenic acid; it has also been found that the effectiveness of several natural fats in counteracting aerodyspnea is in proportion to their linoleate content. Corn oil was shown to be superior to linseed oil, while Salmon¹⁵⁰ found cod-liver oil almost completely ineffective. Richardson and associates¹⁵² reported that both methyl arachidonate and linoleic acid resulted in good growth, but that neither afforded permanent protection against the dermatitis caused by pyridoxine deficiency. These workers suggest that the EFA do not replace pyridoxine but simply delay the onset of the skin symptoms.

The question naturally arises as to whether or not the skin condition resulting from EFA and from pyridoxine deficiency is the same. Medes *et al.*¹⁵³ emphasized the fact that an interrelation exists between the two types of deficiency; however, their results might be interpreted to mean that two different factors are involved. When the diet was lacking in both factors, a relief from deficiency symptoms was obtained when either ethyl linoleate or pyridoxine was administered. On the other hand, the effectiveness of ethyl linoleate was less than it was when optimum doses of pyridoxine were given. Best results were obtained when both of these components were included in the diet simultaneously.

The relationship between pyridoxine and oleic acid appears to be opposite to that of pyridoxine and the EFA. Thus, Sarma *et al.*¹⁵⁴ observed that the growth inhibition in rats caused by feeding diets deficient in pyridoxine or pyridoxal was accentuated when oleic acid was added to the diet. However, the inhibitory action of oleate could be counteracted by the administration of additional quantities of vitamin B₆. Sinclair¹⁵⁵ reviewed the relationship between pyridoxine and the EFA.

Fat is an effective agent in thyrotoxicosis. Thus, Greenberg and Deuel¹⁵⁶ observed that, on diets with a high cottonseed oil content, not only did rats lose weight when thyroid powder was also given, but no mortality occurred. In sharp contrast to this finding, the inclusion of thyroid hormone in a low-

¹⁵¹ H. Schneider, H. Steenbock, and B. R. Platz, *J. Biol. Chem.*, **132**, 539-551 (1940).

¹⁵² L. R. Richardson, A. G. Hogan, and K. F. Itschner, *Univ. Missouri Agr. Expt. Sta., Research Bull.* **333**, 3-12 (1941); *Chem. Abst.*, **36**, 2591 (1942).

¹⁵³ G. Medes, D. C. Keller, and A. Kurkjian, *Arch. Biochem.*, **15**, 19-29 (1947).

¹⁵⁴ P. S. Sarma, E. E. Snell, and C. A. Elvehjem, *J. Nutrition*, **33**, 121-128 (1947).

¹⁵⁵ H. M. Sinclair, *Essential Fatty Acids and Their Relation to Pyridoxine*, in R. T. Williams, *Lipid Metabolism*, Biochem. Soc. Symposia No. 9, 80-99, Cambridge Univ. Press, 1952.

¹⁵⁶ S. M. Greenberg and H. J. Deuel, Jr., *J. Nutrition*, **42**, 279-284 (1950).

¹⁵⁷ E. Keeser, *Klin. Wochschr.*, **17**, 1100-1103 (1938).

fat diet caused a marked depression in the growth of the rats, and also a mortality rate as high as 83% was observed.

One explanation for the protective effect of fats in thyrotoxicosis is that they furnish the EFA. However, oleic acid and linoleic acid were found by Keeser¹⁵⁷ to prevent the increase in metabolism following the administration of thyroid powder. On the other hand, Zain^{158,159} reported that linoleic acid, but not stearic acid, prevented the loss of liver glycogen after massive doses of the thyroid hormone. It is of course possible that the failure of stearic acid to prevent thyrotoxicosis may be related to the fact that the acid cannot be absorbed. Since a practically fat-free liver preparation has been shown to have a beneficial effect in the fat-deficiency syndrome, Ershoff¹⁶⁰ is of the opinion that the EFA are not necessarily the specific agents in counteracting thyrotoxicosis. On the other hand, Greenberg¹⁶¹ reported that both methyl linoleate and cottonseed oil have a protective effect against thyrotoxicosis.

6. Interconversions of the Polyunsaturated Fatty Acids

(1) *Interconversions of Linoleic Acid*

a. Conversion to Arachidonic Acid. (a) *Experimental Evidence of the Reaction.* Although there are abundant data to indicate that the animal cannot synthesize significant amounts of the so-called EFA *de novo*, there is also considerable evidence that a certain degree of interconversion is possible between the several members of the EFA.

One indication of the interconversion of the polyunsaturated acids is the fact that one acid may prevent the deficiency caused by the exclusion of other essential fatty acids from the diet. Thus, Turpeinen¹⁵ and Smedley-MacLean and Nunn¹⁶² are of the opinion that linoleic acid is the precursor of arachidonic acid. The higher biopotency of arachidonic acid as compared with linoleic acid (see Section 4(5)) is explained as due to the fact that linoleate is only partially converted to arachidonic acid; the comparative biopotency of linoleate is a reflection of the amount of arachidonate formed from given amounts of linoleate.

Convincing proof of the linoleate $\rightarrow$ arachidonate conversion is found in balance experiments involving the content of arachidonate in the tissues

¹⁵⁸ H. Zain, *Klin. Wochschr.*, **15**, 1722 (1936).

¹⁵⁹ H. Zain, *Arch. exptl. Pathol. Pharmacol.*, **187**, 302-323 (1937).

¹⁶⁰ B. H. Ershoff, *J. Nutrition*, **39**, 259-281 (1949).

¹⁶¹ S. M. Greenberg, *J. Nutrition*, **47**, 31-39 (1952).

¹⁶² J. Smedley-MacLean and L. C. A. Nunn, *Biochem. J.*, **34**, 884-902 (1940).

when linoleate is fed, as compared with the values in control experiments. The supplementation of fat-deficient rats with corn oil (which contains linoleate but no arachidonate) markedly increased the tetraenoic acid content of liver, kidney, heart, and brain in the experiments of Rieckehoff *et al.*¹⁰⁰ Even before the spectrophotometric method of analysis for the polyunsaturated acids was available, Ellis and Isbell^{113,114} found evidence of the appearance of arachidonic acid in the pig upon the ingestion of linoleic acid. Nunn and Smedley-MacLean,⁹⁹ and also Smedley-MacLean and Hume¹⁶³ presented additional evidence of the appearance of arachidonate in fat-deficient rats following the administration of linoleate. Widmer and Holman,¹⁰⁵ in a study of the effects of fatty acids in the diet on the synthesis of the EFA, confirmed the transformation of linoleate to arachidonate in the rat.

Evidence for the synthesis of arachidonic acid from linoleic acid has likewise been adduced from experiments on chickens. Thus, Reiser and Gibson¹⁶⁴ reported an increase of tetraenoic acid in the tissues of growing chicks on a fat-free diet when cottonseed oil or ethyl linoleate was fed. The increases were least in the neutral fat of the carcass, more pronounced in the organ neutral fat, and increasingly greater in the carcass and organ phospholipids. In other studies by Reiser and Gibson¹⁶⁵ and Reiser *et al.*,⁸⁹ it was shown that the polyunsaturated fatty acids reached a minimum value in the yolk of eggs obtained from hens on a fat-free diet. When single unsaturated acids were fed, their interconversion to other unsaturated acids in the egg-yolk fat could be followed. It was found that tetraenoic acid (arachidonic acid) made its appearance in the yolk after ethyl linoleate or cottonseed oil had been fed to the hens. It must be pointed out that spectral evidence for the interconversions of polyunsaturated acids should be interpreted with caution. The presence of dienoic, trienoic, or tetraenoic acids does not necessarily mean that biologically potent acids have been produced. However, Holman¹⁶⁶ states that the fact that arachidonic acid is not a constituent of plants but is usually a component of animal tissues, and that it may substitute for linoleate in the nutrition of the animal, leads one on *a priori* grounds to conclude that the transformation of linoleate to arachidonate can actually occur. Finally, probably the most convincing evidence of the change of linoleate to arachidonate is found in the experiments with tagged acids described in the next section.

¹⁶³ I. Smedley-MacLean and E. M. Hume, *Biochem., J.*, **35**, 996-1002 (1941).

¹⁶⁴ R. Reiser and B. Gibson, *J. Nutrition*, **42**, 325-336 (1950).

¹⁶⁵ R. Reiser and B. Gibson, *J. Nutrition*, **40**, 429-440 (1950).

¹⁶⁶ R. T. Holman, Personal communication to the author (H. J. D.), 1955.

(b) *Mechanism of the Conversion.* The reactions involved in the change of one unsaturated acid to another have been largely a matter of conjecture. Reiser¹⁶⁷ suggested that "fragments of the ingested acids containing the double bonds might combine to form the more highly unsaturated members of the series." Thomasson¹⁶ is in agreement with Greenberg,¹⁰⁴ who suggests that two molecules of linoleate give rise to one arachidonic acid molecule. Sinclair¹⁵⁵ was likewise convinced of the synthesis of arachidonic acid from linoleic acid, "despite the improbability of a change involving the addition of two carbon atoms and desaturation at the 5:6 and 8:9 positions." Although the mechanism of these changes is not entirely understood, Mead *et al.*¹⁶⁸ demonstrated it in the intact animal. These workers found that acetate was incorporated into arachidonic acid by addition to an exogenous (unlabeled) C₁₈ compound. The nature of this compound was demonstrated by the fact that the carboxy group of linoleic acid appeared as the third carbon in the arachidonic acid, as demonstrated by Steinberg and associates.¹⁶⁹ Lack of activity beyond the third carbon demonstrated that linoleate or an intact C₁₈ derivative was indeed the precursor of arachidonic acid. As was expected, acetate derived by degradation of the active linoleate appeared in the carboxyl group of the arachidonate. Following the feeding of carboxyl-labeled linolenate to rats, no appreciable amount of activity was found in the linoleate isolated from the rats, as pointed out by Mead, Steinberg *et al.*^{169,170} The activity did appear in the arachidic acid derived from the ether-insoluble polybromides, however, predominantly in the first and third carbons. This demonstrated that linolenate was incorporated into the twenty-carbon polyunsaturated acids, but there was some doubt that arachidonic acid was involved, because of the lack of activity of the linoleic acid. Alkali-isomerization confirmed previous evidence that pentaene and hexaene were increased following linolenate feeding, and chromatography of the polyunsaturated acids from the experiment revealed that most of the activity was in fractions other than that containing the arachidonic acid. These data suggest that linoleate and linolenate are the progenitors of two families of polyunsaturated acids formed by the addition of double bonds toward the carboxy end (in the 1:4-relationship) continued to a closest approach

¹⁶⁷ R. Reiser, *Arch. Biochem. Biophys.*, **32**, 113-120 (1951).

¹⁶⁸ J. F. Mead, G. Steinberg, and D. R. Howton, *J. Biol. Chem.*, **205**, 683-689 (1953).

¹⁶⁹ G. Steinberg, W. H. Slaton, Jr., D. R. Howton, and J. F. Mead, *J. Biol. Chem.*, **220**, 257-264 (1956).

¹⁷⁰ J. F. Mead, G. Steinberg, W. H. Slaton, Jr., and D. R. Howton, Univ. California (Los Angeles), *Atomic Energy Project, Report No. 360*, Feb. 15, 1956; *Federation Proc.*, **15**, 313-314 (1956).

of (possibly) two methylenes, at which point acetate is added. An example of this type of reaction is proposed below for linoleate. The two families are not readily interconvertible, and compounds derived from linolenic acid are not highly active as essential fatty acids.

Indirect evidence would indicate a conversion of linoleate to arachidonate by the following scheme:

This scheme would explain Thomasson's¹⁶ observations on the requirement that the first double bond from the methyl group should be at position 4 to ensure essential fatty acid activity.

In a search for intermediates in this scheme, it was found that the trienoic acid which accumulates during fat deficiency is not linolenic acid but 5,8,11-eicosatrienoic acid, which appears to be a hydrogenation product of arachidonic acid.¹⁷¹ Why this reaction should take place during fat deficiency is a mystery, unless this acid is very active as essential fatty acid, which is doubtful.

In a separate study by Mead *et al.*¹⁷² it was shown that the fat-deficiency state is generally characterized by increased fatty acid oxidation, and that there is no sparing of linoleate by fat-deficient mice. Linoleate was converted to carbon dioxide at least as rapidly as were oleate and stearate.

¹⁷¹ J. F. Mead and W. H. Slaton, Jr., *J. Biol. Chem.*, **219**, 705-709 (1956).

¹⁷² J. F. Mead, W. H. Slaton, Jr., and A. B. Decker, *J. Biol. Chem.*, **218**, 401-407 (1956).

When the linoleate is altered by conjugation or elaidinization, it cannot be converted to biologically active tetraenoic acids. Thus Reiser¹⁶⁷ reported that, when conjugated trilinolein was fed to hens, the conjugated dienoic acids appeared in the egg-yolk fat, but that no more highly conjugated acids were observed. Holman¹⁹ noted that, when elaidinized linoleic acid was fed to rats, it was converted to spectrophotometrically detectable tetraenoic acid, as demonstrated by alkali isomerization. The tetraenoic acid, however, was reported to be biologically inert.

b. Conversion to Polyunsaturated Acids Other Than Tetraenoic Acid.

Although the experimental data are exceedingly fragmentary, the present indication is that a species difference obtains in the possible transformations of the dienoic acids. Thus, on the one hand, Reiser and Gibson¹⁶⁴ and Reiser *et al.*⁸⁹ found that the laying hen could convert ingested linoleate into pentaenoic acid as well as into tetraenoic acid. However, neither trienoic nor hexaenoic acid was formed in this species. In contradistinction to these results, Rieckehoff *et al.*¹⁰⁰ and Widner and Holman¹⁰⁵ found that hexaenoic and pentaenoic acids, as well as tetraenoic acid, were derived from dienoic acid in the case of rats; hexaenoic acid was present in the greatest amount. Although these workers failed to observe the formation of pentaenoic or hexaenoic acids, after supplementation, when selected tissues were used, evidence for the synthesis of both types of acids was obtained when the whole animal was analyzed.

(2) Interconversions of Linolenic Acid

a. Factors Altering the Trienoic Acid Content of Tissues. The metabolism of linolenic acid shows certain interesting variations as compared with that of linoleic acid. One of the earliest observations was that this trienoic acid was present in exceedingly small quantities, even when a considerable amount was present in the ration. This led Bloor¹⁷³ to postulate that linolenic acid is preferentially utilized, and is not deposited.

However, it now appears that the deposition of linolenic acid in the tissues is dependent upon the species of animal. On the one hand, Ellis and Isbell^{113,114} reported that only small amounts of trienoic acids were to be found in the case of pigs, even when they were fed large amounts of soybeans. However, Beadle *et al.*¹⁷⁴ did find as much as 11.4% of this acid in the "yellow" fat of swine. Rats which had received a linseed oil diet were found to have as much as 25.6% of linolenate in their fat depots.¹⁷⁴ Brooker

¹⁷³ W. R. Bloor, *Biochemistry of the Fatty Acids*, Reinhold, New York, 1943.

¹⁷⁴ B. W. Beadle, O. H. M. Wilder, and H. R. Kraybill, *J. Biol. Chem.*, **175**, 221-229 (1948).

and Shorland¹⁷⁵ reported that linolenate comprised as much as 17% of the fat of pasture-fed horses. In most species, however, linolenate is absent from the depot fat.

The most striking fact is that the proportion of trienoic acid in the storage fat is *decreased* by feeding polyunsaturated acids and is *increased* by the administration of fat-free diets. Thus, Reiser *et al.*⁸⁹ observed that, in hens on normal rations, the neutral fat and phospholipid of the egg-yolk fat contained only 0.18% and 0.00%, respectively, of trienoic acids. After fifty-two weeks on a fat-free diet, the trienoic acid contents of the above fractions of egg yolk were found to be 0.42% and 2.3%, respectively. Heart fatty acids, which normally had a content of 22% of trienoic acid, contained only about 4% after supplementation with corn oil or cod-liver oil.¹⁰⁰ In 1953 Klein and Johnson⁹ noted that the trienoic acid content of the mitochondria, poorly sedimentable layer, and microsomes of the livers of senescent rats and of rats on a fat-free diet, was increased over the control level in both cases. Smedley-MacLean,¹⁷⁶ who had also reported an increase in trienoic acid in fat deficiency, expressed the opinion that the trienoic acid present may be a partially hydrogenated arachidonic acid, resulting from the attempt on the part of the animal to make the most of its stores of arachidonate. Mead and Slaton¹⁷¹ confirmed this finding.

b. Transformations of Administered Linolenate. Reiser and co-workers⁸⁹ reported that the hen is able to convert ingested linolenic acid into dienoic, tetraenoic, pentaenoic, and hexaenoic acids and to deposit these polyunsaturated acids in egg-yolk fat. On the other hand, Holman¹⁷⁷ is of the opinion that the rat converts trienoic acid mainly to hexaenoic acid, although in another report this author¹⁹ did note the formation of unsaturated acids containing four, five and six double bonds in the rat after the administration of linolenic acid.

It is difficult to harmonize the above data with the information available on the nutritional value of linolenic acid. A number of workers, including Burr⁵ and Greenberg *et al.*,^{82,178} have adduced evidence to show that linoleic and linolenic acids have different functions. Whereas linoleic acid and arachidonic acid can support growth and also alleviate skin symptoms, it is suggested that linolenic acid only supports growth. Holman,^{19,177} believes that the tetraenoic acid produced in the animal as a result

¹⁷⁵ E. G. Brooker and F. B. Shorland, *Biochem. J.*, **46**, 80-85 (1950).

¹⁷⁶ I. Smedley-MacLean, *The Metabolism of Fat*, Methuen, London, 1943.

¹⁷⁷ R. T. Holman, *Proc. Third Conf. on Research, Am. Meat Inst.*, Chicago, 1951, pp. 1-10.

¹⁷⁸ S. M. Greenberg, H. J. Deuel, Jr., and J. B. Brown, *Abst. Twenty-fourth Fall Meeting, Am. Oil Chemists' Soc.*, San Francisco, Sept. 26-29 (1951).

of the administration of linolenate is probably an inactive isomer. The whole subject must be considered to be in a state of flux, in view of the finding of Thomasson¹⁶ that γ -isolinolenic acid (6,9,12-octadecatrienoic acid) is the active essential fatty acid (131 U. per gram), and that ordinary linolenic acid (9,12,15-octadecatrienoic acid) possesses no significant biopotency (9 U. per gram).

(3) *Interconversions of Elaeostearic Acid*

Miller and Burr¹⁷⁹ found that, when the conjugated isomer of linolenic acid, namely, elaeostearic acid (9,11,13-octadecatrienoic acid), is given to rats, it rapidly loses its absorption band at 270 m μ , and that a new maximum appears at 235 m μ . This is interpreted to mean that one double bond has been saturated, with the resultant formation of a dienoic acid. According to Reiser,¹⁶⁷ a similar change of trienoic acid to dienoic acid occurs in the case of the hen when tung oil, which contains principally α -elaeostearic acid, is fed. In another test of Reiser *et al.*,⁸⁹ it was noted that α -elaeostearic acid was transformed to acids having two to six double bonds.

(4) *Interconversions of the More Highly Unsaturated Acids*

Information regarding the changes of the tetraenoic and more highly unsaturated acids is quite limited. Holman and Taylor¹⁸⁰ presented some

Fig. 1. The biologic interconversions of polyunsaturated fatty acids.

¹ Dienoic acid is formed from diene-free fatty acids of cod-liver oil, by the chick.

² Trienoic acid increases upon depletion of other acids, and may be formed from arachidonic acid.

³ The tetraenoic acid formed from trienoic acid appears to be a biologically inactive isomer of arachidonic acid.

⁴ Uncertain, because the tetraene used was impure.

¹⁷⁹ E. S. Miller and G. O. Burr, *Proc. Exptl. Biol. Med.*, **36**, 726-729 (1937).

¹⁸⁰ R. T. Holman and T. S. Taylor, *Arch. Biochem.*, **29**, 295-301 (1950).

evidence that hexaenoic acid is deposited in increased amounts in the heart and brain, and pentaenoic acid in the liver of rats after supplementation with ethyl arachidonate. However, it is questionable whether or not the tetraenoic acid was converted to the more highly unsaturated fatty acids, since there is some indication that impurities in the preparation of arachidonate were sufficient to account for the newly deposited pentaenoic and hexaenoic acids. Reiser and Gibson¹⁶⁴ noted that a significant increase in dienoic acid, but no augmentation in trienoic acid, occurred in growing chicks fed cod-liver oil which was free from dienoic and trienoic acids. According to Clément and May,¹⁸¹ conjugated tetraenoic acid can be hydrogenated by rats to yield conjugated trienoic acid.

Figure 1 presents some known interconversions of the several polyunsaturated acids.

7. Physiologic Functions Related to the Essential Fatty Acids

(1) Growth

The classical demonstration of the necessity of the essential fatty acids in the diet has been their effect on growth when administered to animals on a fat-free diet. When weanling rats are placed on a fat-free diet, they continue to grow normally for two or three weeks, after which the rate of gain-in-weight is decreased below that observed in the case of rats on a diet containing essential fatty acids. Within eight to ten weeks, the body weights usually reach a plateau, and this may be followed by some decrease in body weight.⁵¹ When hydrogenated coconut oil is included in the fat-free regimen during the depletion period, the length of time required for fat depletion is curtailed, and the average body weight at which depletion occurs is reduced.²² Skin symptoms characteristic of the fat-deficiency syndrome invariably develop concomitantly with the retardation in growth. All of these fat-deficiency symptoms can be counteracted when cottonseed oil or other EFA-containing fats are incorporated in the diet; they can also be prevented or cured by the administration of the essential fatty acids, either as the free acids or as their esters.

The above results are best explained on the basis that the EFA provide a necessary factor to permit growth, either by furnishing essential building stones in the form of EFA themselves or by providing required components for certain enzyme systems. Smedley-MacLean and Hume¹⁶³ reported that, when the Walker tumor is implanted in rats receiving a fat-free diet,

¹⁸¹ G. Clément and P. May, *J. physiol. (Paris)*, 48, 79-83 (1953).

there is marked decrease in the ratio of highly unsaturated fatty acids to the fat-free dry weight of the subcutaneous tissues. This effect is interpreted as an indication that the EFA are used up in the formation of new tumor tissue; the subcutaneous fat depots are undoubtedly called upon to furnish the extra EFA required. Thus, growth may well be associated with the metabolism of the essential fatty acids.

Aaes-Jørgensen and Dam¹⁸²⁻¹⁸⁶ studied the effect on growth of several levels of fat in the diet, as related to the essential fatty acid content of the fats. It was found that male rats maintained on diets containing 7% of fat grew much better on lard (presumably containing essential fatty acids) than on hydrogenated peanut oil,¹⁸² whereas rats on a fat-free ration grew at the same rate as did the rats on the hydrogenated peanut oil diet. An increase of the amount of lard to 28% of the diet had little effect upon the average growth rate, whereas an increase of the level of hydrogenated peanut oil to 28% decreased the growth rate considerably.¹⁸³ When the lard or peanut oil was supplemented with linoleic acid, raw skim milk, or both, an increase in the fat level from 7% to 28% increased the growth rate of female rats. When supplemented hydrogenated peanut oil was given, the increase in fat level had considerably less effect upon the weight gain.^{185, 186} Rats given hydrogenated whale oil at a 7% level, and those on a fat-free regimen, had the lowest growth rate.¹⁸⁴

Panos and Finerty¹⁸⁷ reported that female rats kept on a fat-free diet for thirty-four weeks from the time of weaning attained a weight which was 80% of that of the controls. On the basis of organ weight per 100 g. of body weight, the thyroid and adrenal glands were significantly smaller, while the kidneys, liver and heart were larger, in the fat-free group than in the controls. When fat was added to the diet of the deficient animals, a rapid increase in body weight to control values occurred, associated with a progressive relative decrease in the size of the kidneys, liver, and heart.

These workers¹⁸⁷ also demonstrated that the estrus cycle was distinctly irregular in female rats subjected to a fat-free diet. Ovarian weight was not affected, but large numbers of deficient nuclei were present in the interstitial tissues. The follicles and corpora lutea, however, appeared to be normal. Since a significant decrease in the percentage of acidophils, and an increase in basophils, occurred in the pituitary cells, it is suggested

¹⁸² E. Aaes-Jørgensen and H. Dam, *Brit. J. Nutrition*, 8, 281-285 (1954).

¹⁸³ E. Aaes-Jørgensen and H. Dam, *Brit. J. Nutrition*, 8, 285-289 (1954).

¹⁸⁴ E. Aaes-Jørgensen and H. Dam, *Brit. J. Nutrition*, 8, 290-296 (1954).

¹⁸⁵ E. Aaes-Jørgensen and H. Dam, *Brit. J. Nutrition*, 8, 296-301 (1954).

¹⁸⁶ E. Aaes-Jørgensen and H. Dam, *Brit. J. Nutrition*, 8, 302-306 (1954).

¹⁸⁷ T. C. Panos and J. C. Finerty, *J. Nutrition*, 49, 397-424 (1953).

that the fat deficiency reduces the secretion of luteinizing hormone from the acidophil cells, and that the consequent reduction in ovarian stimulation results in a diminished estrogen secretion, with consequent disturbance of the estrus cycle. The increased percentage of basophils can be attributed to the reduction in estrogen secretion.

The activity of the cytochrome oxidase system in the liver is markedly increased in rats deficient in essential fatty acids, according to Kunkel and Williams.¹⁸⁸ These workers noted a slight decrease in choline oxidase activity, but no alteration was observed in that of succinic oxidase, during the fat-deficiency syndrome. Succinic dehydrogenase and butyric dehydrogenase in the liver were found by Tulpule and Patwardhan¹⁸⁹ to be significantly reduced in EFA deficiency. Glutamic dehydrogenase, also, was present in a lower concentration when fats were excluded from the diet.¹⁸⁹

The lipolytic enzymes of the liver were considered by Saka and Sipahioğlu¹⁹⁰ to be related to the content of unsaturated fatty acids in the diet. It was observed that arachidonic acid was more effective in furthering the reestablishment of liver lipases after the ingestion of a fat-free diet than were other unsaturated acids. It was reported¹⁶² that a minimum intake of arachidonate is required before the cell depots are loaded with fat. Subsequently, during the true growth process, comparatively large amounts of arachidonate disappear from the tissues. However, the EFA are not concerned with the synthesis of lecithin. Hevesy and Smedley-MacLean¹⁹¹ reported that the uptake of radioactive phosphorus was surprisingly constant on a fat-free diet, or on one containing arachidonate. In the case of the rats on a fat-free regimen, the phosphorus uptake in the muscle was one-third as great as in rats on a diet containing EFA.

It is suggested that the increased activity of the cytochrome oxidase system may largely account for the augmented level of metabolism in fat deficiency. It will be recalled that Wesson and Burr,¹⁹² as early as 1931, and also Wesson alone,¹⁹³ reported that high metabolic rates and high respiratory quotients regularly accompanied the fat-deficiency syndrome. Engel¹⁹⁴ postulated that, in the absence of the essential fatty acids, choline fails to exert its normal lipotropic action. Smedley-MacLean and Nunn¹⁹⁵

¹⁸⁸ H. O. Kunkel and J. N. Williams, Jr., *J. Biol. Chem.*, **189**, 755-761 (1951).

¹⁸⁹ P. G. Tulpule and V. N. Patwardhan, *Arch. Biochem. Biophys.*, **39**, 450-456 (1952).

¹⁹⁰ M. O. Saka and Ü. Sipahioğlu, *Am. J. Physiol.*, **174**, 49-50 (1953).

¹⁹¹ G. C. Hevesy and I. Smedley-MacLean, *Biochem. J.*, **34**, 903-905 (1940).

¹⁹² L. G. Wesson and G. O. Burr, *J. Biol. Chem.*, **91**, 525-539 (1931).

¹⁹³ L. G. Wesson, *J. Biol. Chem.*, **100**, 365-371 (1933).

¹⁹⁴ R. W. Engel, *J. Nutrition*, **24**, 175-185 (1942).

¹⁹⁵ I. Smedley-MacLean and L. C. A. Nunn, *Biochem. J.*, **35**, 983-989 (1941).

stated that fat cannot be laid down in the tissues on an EFA-deficient diet. This situation would also result in failure of growth.

The problem of the mechanism of action of EFA in the animal body is largely unsolved. Greenberg and Ershoff¹⁹⁶ demonstrated an interesting relationship between EFA and the development of the prostate and seminal vesicle. A marked reduction in the weights of both organs occurred in EFA deficiency. This could be corrected either by gonadotropin or by the administration of methyl linoleate.

(2) Normal Skin Development

The most consistent symptom of EFA deficiency throughout the various species of animals is the condition of the skin and hair. In the case of man, absence of EFA is associated with an eczematous condition. The chief symptoms in rats, mice, dogs and calves are scaliness of the tail, and roughness of the skin. In the rat, mouse, and dog, another common symptom is scaliness of the paws.

The skin symptoms resulting from pyridoxine deficiency and from lack of EFA closely resemble each other. Thus, Birch and György¹⁴⁶ reported that dermatitis, produced by a low-pyridoxine diet, could be cured when lard was added to the diet. In later work, Birch¹⁴⁷ demonstrated that the presence of fat in the diet delayed the onset of skin symptoms on a diet low in vitamin B₆; in fact, it was proved that the skin symptoms resulting from pyridoxine deficiency could be prevented by fat feeding even up to the time the animals succumbed from avitaminosis. Quackenbush *et al.*¹⁹⁷ noted that, although pyridoxine produced a temporary alleviation, it did not effect a cure. Pantothenic acid was completely without effect in counteracting the symptoms produced by fat deficiency.

Linoleic and arachidonic acids are the most effective of the acids in clearing up the skin symptoms resulting from pyridoxine deficiency or from fat deficiency. Quackenbush and co-workers¹⁴⁹ are of the opinion that linoleic acid is more effective than linolenic acid as an antidermatitis factor. In line with this result, Burr *et al.*⁹³ reported that corn oil (which has no linolenic acid) is more potent in preventing the skin disorders of fat-deficient animals than is linseed oil (which is relatively rich in linolenic acid at the expense of linoleic acid). In fact, the ability of natural fats to counteract the skin disorders has been found to be in proportion to the linoleic

¹⁹⁶ S. M. Greenberg and B. H. Ershoff, *Proc. Soc. Exptl. Biol. Med.*, 78, 552-554 (1951).

¹⁹⁷ F. W. Quackenbush, H. Steenbock, F. A. Kummerow, and B. R. Platz, *J. Nutrition*, 24, 225-234 (1942).

acid content of the diet. According to Richardson *et al.*¹⁵² methyl arachidonate and methyl linoleate are equally effective in the treatment of the dermatitis produced by vitamin B₆ deficiency.

In a comprehensive study of skin structure as related to diet, Hansen *et al.*¹²³ proved that the absence of fat in the diet of dogs brings about definite alterations in the epidermis and dermis, including the hair follicles, the sebaceous glands, and the capillaries. Wiese and Hansen¹⁹⁸ observed that the nutritional status of dogs which were maintained on different levels and kinds of fat could be assessed from the level of serum linoleate and serum arachidonate. In a later study, Hansen and associates¹³³ stated that dietary fat supplies a factor necessary for the maturation of epithelial, sebaceous, and sudoriparous cells. When fat is absent from the diet of either puppies or adult dogs, distinct skin abnormalities occur, which can be recognized both microscopically and macroscopically. When EFA are available, the changes in the stratum corneum, the collagen structure, and the cellular infiltration of the dermis, the changes in hair follicles, in sebaceous glands, and in sudoriparous glands are all related to changes in the epidermis. The most rapid restoration of the normal microscopic structure was noted when the dietary fat was high in unsaturated fatty acids. Williamson¹⁹⁹ reported that the epidermis of rats on a fat-free diet became thicker and more differentiated than that of normal rats. The stratum granulosum became especially distinct, and the horny layer thickened.

The specificity of the skin picture in EFA deficiency was demonstrated by Ramalingaswami and Sinclair²⁰⁰ in rats; the histologic picture of the skin in EFA deficiency closely resembled that of phrynoderma, whereas the microscopic skin changes in vitamin A deficiency bore only a slight resemblance to this condition.

(3) *Pregnancy and Lactation*

There is considerable evidence that the level of dietary fat may be of considerable importance in physiologic functions such as pregnancy and lactation. It is now certain that at least a portion of the beneficial effect of fats on pregnancy and lactation is to be ascribed to their content of EFA.

Evans and collaborators^{201,202} were the first to prove that normal reproduction is impossible when the EFA are absent from the diet. The addi-

¹⁹⁸ H. F. Wiese and A. E. Hansen, *Texas Repts. Biol. Med.*, **9**, 516-544, 545-554 (1951).

¹⁹⁹ R. Williamson, *Biochem. J.*, **35**, 1003-1005 (1941).

²⁰⁰ V. Ramalingaswami and H. M. Sinclair, *Brit. J. Dermatol.*, **65**, 1-22 (1953).

²⁰¹ H. M. Evans, S. Lepkovsky, and E. A. Murphy, *J. Biol. Chem.*, **106**, 431-440 (1934).

²⁰² H. M. Evans, S. Lepkovsky, and E. A. Murphy, *J. Biol. Chem.*, **106**, 441-444 (1934).

tion of saturated acids to fat-free diets failed to alleviate the symptoms.²⁰² The mothers were unable to suckle their young in the absence of the required nutrients; male rats were also found by Evans *et al.*²⁰³ to become sterile on the fat-free diets. The addition of small amounts of EFA to the fat-free regimens cured the difficulties in reproduction, and even prevented their development.

More recent studies have contributed some information as to the quantitative aspects of EFA required for pregnancy and lactation. Quackenbush *et al.*¹⁴⁹ noted that the quantity of EFA required for reproduction in rats was approximately twice that necessary to cure the dermal lesions arising on a fat-free diet. Ordinary linolenic acid (9,12,15-octadecatrienoic acid) was found to be relatively ineffective in permitting normal reproduction. There appears to be no information as to whether or not γ -linolenic acid (6,9,12-octadecatrienoic acid), which Thomasson¹⁶ reported to be as active as linoleic acid in promoting growth on a fat-free diet, is effective in allowing normal reproduction. Linoleic and arachidonic acids have about the same biopotency insofar as reproduction is concerned.¹⁴⁹ Deuel and co-workers²⁰⁴ reported data on pregnancy and lactation of female rats which were supplemented with several levels of cottonseed oil or of methyl linoleate during the period of breeding and lactation. Although fat was not required by the female rat for conception, when the diet was otherwise complete, the pups were invariably born dead or died immediately after birth. It was found that, whereas daily doses of cottonseed oil as low as 10 mg. insured the survival of the pups for three days, in some cases as much as 200 mg. of this fat were required per day for an optimal effect. Since comparable results were observed when 80 mg. of methyl linoleate were fed, it was concluded that the essential fatty acids constitute the protective component in fat. The requirement of the female rat for EFA during pregnancy and lactation is as high, if not higher, than the optimum required for growth in this sex.

From the standpoint of lactation performance, fat has long been known to stimulate this function, as demonstrated by Maynard and McCay,²⁰⁵ McCay and Maynard,²⁰⁶ and Maynard and Rasmussen.²⁰⁷ Loosli *et al.*²⁰⁸

²⁰³ H. M. Evans, S. Lepkovsky, and E. A. Murphy, *J. Biol. Chem.*, **106**, 445-450 (1934).

²⁰⁴ H. J. Deuel, Jr., C. R. Martin, and R. B. Alfin-Slater, *J. Nutrition*, **54**, 193-199 (1954).

²⁰⁵ L. A. Maynard and C. M. McCay, *J. Nutrition*, **2**, 67-81 (1929).

²⁰⁶ C. M. McCay and L. A. Maynard, *J. Biol. Chem.*, **92**, 273-280 (1931).

²⁰⁷ L. A. Maynard and E. Rasmussen, *J. Nutrition*, **23**, 385-398 (1942).

²⁰⁸ J. K. Loosli, J. F. Lingensfelder, J. W. Thomas, and L. A. Maynard, *J. Nutrition*, **28**, 81-88 (1944).

did observe improved lactation performance in the case of rats on diets containing corn oil, as compared with animals on a fat-free diet or on one containing hydrogenated coconut oil, which might be interpreted as related to an intake of EFA. However, no improvement in lactation was noted when as much as 125 mg. of linoleate was given per day to the mother or directly to the pups. In the most recent studies of Deuel *et al.*,²⁰⁴ 100% mortality was observed before weaning when the fat-free diet of the mothers was supplemented with 10 mg. of cottonseed oil or with 10 mg. of linoleate. However, the lowest mortality rate and the highest weaning weights of the pups were noted in rats receiving 100 or 200 mg. of cottonseed oil daily, or 80 mg. of linoleate. On this basis, the latter workers ascribe the beneficial effect of fat upon reproduction and also upon lactation to the EFA present in this foodstuff.

(4) Protection against X-Irradiation

Cheng and her co-workers¹⁴⁰ reported that cottonseed oil in amounts as low as 2% in the diet of male rats affords protection against multiple sublethal doses of x-ray, as compared with the situation noted in rats on a fat-free diet. Decker *et al.*¹¹⁰ had observed earlier that a typical EFA deficiency could be provoked in fat-depleted mice when they were exposed to x-irradiation. Although cottonseed oil had a protective effect against x-irradiation in the experiments of Cheng *et al.*,¹⁴⁰ irrespective of whether old rats or young mature male animals were employed in the tests, it was found that fat protected only the old female rats against x-irradiation. It was suggested that, because of the higher linoleate requirement of the male animals, they were depleted of EFA sooner on the fat-free diet than were the female rats. Consequently, the EFA level was not the limiting factor in the protection of young rats. This hypothesis was supported by the longer survival of the control females than of the control males in the group of young mature animals. In another study, it was found that the linoleate protection was in addition to that afforded by liver powder; moreover, Cheng and Deuel²⁰⁹ proved that hydrogenated coconut oil gave no protection.

In subsequent studies by Deuel, Cheng *et al.*,²¹⁰ it was demonstrated that the protective effect of cottonseed oil was a function of the EFA present therein. Thus, when 10 mg. of ethyl linoleate were given daily to fat-depleted rats which were subsequently exposed to weekly doses of x-rays

²⁰⁹ A. L. S. Cheng and H. J. Deuel, Jr., *Federation Proc.*, **12**, 410-411 (1953).

²¹⁰ H. J. Deuel, Jr., A. L. S. Cheng, G. D. Kryder, and M. E. Bingemann, *Science*, **117**, 254-255 (1953).

of 300 r, a significantly greater survival rate was noted in the case of the supplemented rats, despite the fact that the linoleate-treated animals received as much as 45% more x-irradiation than the control rats did. More recently, Cheng *et al.*²¹¹ established the fact that the optimal daily protective dose of linoleate for male rats against x-irradiation injury probably exceeds 100 mg. Thus, it was demonstrated that the average survival period for LD₂₅, LD₅₀, and LD₇₅, were progressively greater when the protective dosages of linoleate were 10, 50, and 100 mg. per day. The beneficial effect of fat in protection from injury due to x-irradiation has been shown²¹² to obtain with widely varying doses of x-ray and with considerable variations in the frequency with which it was applied. Cheng *et al.*²¹³ later found that linolenic acid afforded only slight protection against x-irradiation when given in doses as high as 80 mg. per day to male rats. On the other hand, when linoleate was administered simultaneously with linolenate, a marked synergism was noted,⁸² as was also the case with growth.

The reason for the beneficial effect of the EFA against x-irradiation damage can only be conjectured. Since there is some indication that linoleate is required for the growth of new tissues, as well as for the repair of damaged ones, the restitution of such injured tissues would proceed more rapidly in the cases in which an adequate supply of EFA was available. The primary tissue injured by exposure to x-rays under most conditions is the skin. Since it is known that the EFA are concentrated in the subcutaneous fat, the protective effect of linoleate may be ascribable to the rapid local healing afforded in the subcutaneous tissues; the variation in time required for this process to take place with a high EFA storage, as compared with that in an EFA-deficient animal, may spell the difference in survival in the presence and absence of a fat which contains EFA.

(5) *Relation to Capillary Fragility*

Kramár and Kovács²¹⁴ were the first to call attention to the fact that the capillary resistance of human subjects, especially of those with allergic manifestations, was higher in patients who had received vegetable oils

²¹¹ A. L. S. Cheng, M. Ryan, R. B. Alfin-Slater, and H. J. Deuel, Jr., *J. Nutrition*, **52**, 637-644 (1954).

²¹² A. L. S. Cheng, R. B. Alfin-Slater, and H. J. Deuel, Jr., *J. Nutrition*, **54**, 201-207 (1954).

²¹³ A. L. S. Cheng, T. Graham, R. B. Alfin-Slater, and H. J. Deuel, Jr., *J. Nutrition*, **55**, 647-653 (1955).

²¹⁴ J. Kramár and J. Kovács, Report presented at Annual Meeting of the Hungarian Pediatric Society (June, 1939); cited by J. Kramár and V. E. Levine, *J. Nutrition*, **50**, 149-160 (1953), p. 149.

than in those who had partaken exclusively of a diet containing animal fats. In studies on rats fourteen years later, Kramár and Levine²¹⁵ were able to prove decisively that the resistance of the skin capillaries of immature rats, fed a diet devoid of fats or fatty acids, or one containing 5% of methyl stearate, began to decrease at fifty days, and soon reached pathologically low levels. This change in capillary resistance was shown to be inversely proportional to the increase in capillary permeability. This increased capillary permeability occurred simultaneously with the flattening of the growth curve, but before other symptoms of EFA deficiency were evident. Caudal necrosis did not occur.

The low capillary resistance of the fat-deficient rat could be restored to normal by the administration of small amounts of linseed oil or of linoleic acid itself. It would appear that one must consider decreased capillary resistance (or increased capillary permeability) as an additional manifestation of the fat-deficiency syndrome in rats, and also in man.

(6) Relation to Cholesterol Metabolism

Fats have been widely maligned as a food in recent years, both as a carrier of cholesterol (animal fats) and as a source of endogenous cholesterol (animal and vegetable fats). Since the acetate molecule is known to be incorporated in newly synthesized cholesterol, and because acetate is formed on the oxidation of the fatty acid molecule, it has been suggested that the ingestion of fat stimulates the synthesis of cholesterol. However, the rate of synthesis of cholesterol is independent of the amount of fat available; furthermore, the acetate required in this reaction can originate from dietary carbohydrate, either directly from the ingested carbohydrate, or indirectly from the fat formed from it. In order to prevent an overproduction of cholesterol, because of its supposed relationship to arteriosclerosis, patients have been given diets practically devoid of all fats. It has been recently reported, in the case of the rat, that the administration of a fat-free diet does result in a reduction of blood cholesterol; however, there is a concomitant increase of liver and adrenal cholesterol which may reach twice the normal level, according to Alfin-Slater *et al.*^{92,93} The normal liver cholesterol, 2.06 mg. per g. liver, which was found in the group of rats receiving the diet containing 12.5% of cottonseed oil, compares with averages of 3.60 mg. per g. in one group on the fat-free regimen, and of 4.48 mg. per g. in another group which received no fat. On the other hand, the plasma cholesterol was only 44.4 and 43.0 mg. % in the case of the groups on the fat-

²¹⁵ J. Kramár and V. E. Levine, *J. Nutrition*, 50, 149-160 (1953).

free diets, whereas the average value for the group receiving 12.5% cotton-seed oil was 64.4 mg. %; this compares with the normal value generally obtained on fat-containing diets devoid of cholesterol. In a later study with weanling rats, Deuel and associates²² observed that the increased level of liver cholesterol and the decreased figure for blood cholesterol developed within one week after the animals were placed on a fat-free diet. The divergence continued, and increased in the course of the subsequent thirteen-week period during which the test was continued.

The role of linoleate in cholesterol deposition and transport is not entirely clear. Kelsey and Longenecker²¹⁶ proved that 62% of the plasma cholesterol of cattle occurred in combination with linoleate. It is only natural to postulate that, in the absence of EFA, cholesterol is deposited in the liver, because there is insufficient linoleate available to transport it to other tissues for metabolism and excretion. However, it has been shown that, in such conditions, the increased cholesterol is deposited in the liver as an ester. The cholesterol esters in the liver of rats have been proved by Achaya *et al.*²¹⁷ to consist almost entirely of those of saturated and oleic acids; only approximately 10% of the cholesterol occurs in combination with linoleic acid, irrespective of whether or not the diet contains EFA. It would thus appear that linoleic acid is of prime importance in the control of the distribution and deposition of cholesterol in the rat. Whether or not the same situation exists in the case of man is a moot question.

Mead and his co-workers²¹⁸ have indicated that ingested polyunsaturated fatty acids appear in the blood largely as sterol esters and phospholipids, whereas oleic and stearic acids appear largely as triglycerides, and slowly appear in the phospholipids.

The results of Cochrane *et al.*,²¹⁹ of Kinsell *et al.*,²²⁰ and of Kinsell alone²²¹ do indicate that a profound relationship may exist in man between the intake of EFA and the level of plasma cholesterol. These workers have reported that elevated levels of blood cholesterol in conditions such as diabetes can be gradually decreased to normal when large amounts of vegetable fats are included in the diet. On the other hand, this pattern was immediately reversed when animal fats, for instance egg yolk, were

²¹⁶ F. E. Kelsey and H. E. Longenecker, *J. Biol. Chem.*, **139**, 727-740 (1941).

²¹⁷ K. T. Achaya, R. B. Alfin-Slater, and H. J. Deuel, Jr., Unpublished results, 1954.

²¹⁸ J. F. Mead, Personal communication to R. Reiser, 1955.

²¹⁹ G. C. Cochrane, G. D. Michaels, and L. W. Kinsell, *J. Clin. Nutrition*, **1**, 295-298 (1953).

²²⁰ L. W. Kinsell, G. D. Michaels, J. W. Partridge, L. A. Boling, H. E. Balch, and G. C. Cochrane, *J. Clin. Nutrition*, **1**, 224-231 (1953).

²²¹ L. W. Kinsell, *J. Am. Dietet. Assoc.*, **30**, 685-688 (1953).

taken. The latter effect was not believed to be ascribable to the cholesterol present in the ingested animal fats. Although it is uncertain whether or not the vegetable oil effect is to be attributed to the EFA, it is only natural to assume that the variation in the response of the blood cholesterol to vegetable and animal fats is to be traced to this variation in composition. Sinclair²²² considers that human diets in most civilized countries are becoming increasingly deficient in essential polyethenoid fatty acids (arachidonic acid), due to hydrogenation and hardening of food fats and to other manufacturing processes. As a result, the cholesterol in the diet is esterified with abnormal or unusually saturated fatty acids. These abnormal esters are not readily disposed of, and may cause atheroma. Similarly, abnormal phospholipids containing abnormal or unusually saturated fatty acids are less readily disposed of, and are retained in the plasma; they increase the coagulability of the blood, and contribute to coronary and cerebral thrombosis.

The importance of the essential fatty acids in relation to atherosclerosis in the rabbit has been clearly demonstrated by Kritchevsky and co-workers.²²³ Rabbits were fed complete diets containing the following varied lipid components: I, no fats or cholesterol (control group); II, cholesterol (3%), corn oil (9%); III, cholesterol (3%), shortening (9%); IV, cholesterol (3%); V, corn oil (9%); VI, shortening (9%). The corn oil had an iodine value of 130, and the shortening was a vegetable oil hydrogenated to give a solid fat having an iodine number of 72. After two months, the rabbits were sacrificed and the extent of atherosclerosis was given a mathematical score based upon 0, +, ++, +++, and +++++, considered as 0, 1, 2, 3, and 4. The following averages were obtained on the state of the atherosclerosis in the several groups: I, 0.06; II, 2.71; III, 3.71; IV, 3.80; V, 0.10; and VI, 0.10. It is thus evident that the feeding of corn oil, which contains a high proportion of essential fatty acids, is able to suppress the deposition of cholesterol in the aorta of rabbits to a marked extent, even when the animals are subjected to such a severe strain as is afforded by the presence of 3% cholesterol in the diet. On the other hand, the shortening did not appear to alleviate the condition. These data likewise refute the concept that fat *per se* has an atherogenic effect.

²²² H. M. Sinclair, *Lancet*, 1956, I, 381-383.

²²³ D. Kritchevsky, A. W. Moyer, W. C. Tesar, J. B. Logan, R. A. Brown, M. C. Davies, and H. R. Cox, *Am. J. Physiol.*, 178, 30-32 (1954).

8. Distribution of Essential Fatty Acids

(1) *Distribution in Foods*

a. Introduction. The most concentrated sources of the EFA are the vegetable fats. Linoleic acid is the chief representative of this group,

TABLE 1
THE RELATIVE MAXIMUM AMOUNTS OF ESSENTIAL FATTY ACIDS IN VEGETABLE FATS,
AS DETERMINED BY ANALYSIS AND BY BIOASSAY^a

Vegetable fat or oil	Values by analysis		Total essential fatty acids by bioassay ^b
	Linoleic acid	Linolenic acid	
Almond	19.9	0	—
Avocado	10.3	0	—
Beechnut	38.0 ^c	2.9 ^c	30.1
Castor oil	3.6 ^c	0 ^c	3.8
Cocoa butter	21.1	0	2.2
Coconut	2.6	0	1.1
Corn	39.1	0	—
Cottonseed	50.4	0	48.5, 48.0 ^d
Hempseed	68.8	24.3	—
Kapokseed	31.3 ^c	0 ^c	26.9
Linseed	46.7	60.9	11.9, 25.6
Olive	15.0	0	15.8
Palm	10.9	0	10.6
Palm kernel	0.7 ^c	0 ^c	1.5
Peanut	27.4	0	31.1
Poppyseed	62.2	—	—
Rapeseed	29.0	3.5	17.3
Safflower	78.0	0	78.8
Sesame	40.4	0	28.2
Soybean	58.8	8.1	62.4
Sunflower	68.0	0	55-65 ^e
Walnut	75.5	10.0	—
Wheat germ	—	—	52.9

^a Unless otherwise noted, data are from the summary of H. J. Deuel, Jr., *Nutritional Significance of the Fats, Progress in the Chemistry of Fats and Other Lipids*, 2, 99-192 (1954), and from H. J. Deuel, Jr., *The Role of Fat in Human Nutrition*, in A. E. Bailey, *Cottonseed and Cottonseed Products*, Interscience, New York and London, 1948, 763-811. Dashes indicate that no data are available.

^b Unless otherwise noted, data are from H. J. Thomasson, *Intern. Z. Vitaminforsch.*, 25, 63-82 (1953). Bioassay values of essential fatty acids are expressed in units (1 Unit = activity of 10 mg. linoleic acid), which are practically identical with % composition.

^c Data from H. J. Deuel, Jr., *The Lipids, I. Chemistry*. Interscience, New York and London, 1951.

^d Data are from H. J. Deuel, Jr., S. M. Greenberg, L. Anisfeld, and D. Melnick, *J. Nutrition*, 45, 535-550 (1951). Essential fatty acids are expressed in % as linoleic acid.

^e Employed as a standard based upon analytical values obtained spectrophotometrically.

although, in some cases, such as in linseed oil, appreciable amounts of linolenic acid may occur. Arachidonic acid, on the other hand, is never found in vegetable fats. In contradistinction to the relatively high content of EFA in most vegetable fats, the proportion in animal fats is much lower, and, in many cases, practically none are present. Although arachidonic acid is frequently present in animal fats, linoleic acid may be present in a much larger proportion in these fats. Moreover, after a high intake of linolenate-containing fats, this trienoic acid may also appear in animal fats. For a more complete treatment of this topic, the reader is referred to two recent reviews of Deuel.^{11,138}

b. Presence in Vegetable Fats. As noted above, the vegetable fats have the highest EFA content of any of the natural or processed fats. Data on the essential fatty acid composition of vegetable fats are summarized in Table 1.

In the case of linseed oil and of several other vegetable fats, a poor agreement has been noted between the results recorded spectrophotometrically and by bioassay. Thus, although linoleate values as high as 46.7% and figures for linolenate in some cases as high as 60.9% have been recorded for linseed oil, the bioassay values of two samples reported by Thomasson¹⁶ were only 11.9% and 25.6%. In the case of cocoa butter, the bioassay value is given as 2.2% as compared with a figure of 21.1% determined spectrophotometrically. Coconut (and especially hydrogenated coconut oil) and castor oils have been reported to contain practically no essential fatty acids, as determined by spectrophotometric and bioassay procedures.

c. Presence in Animal Fats. The quantity of EFA in animal fats is largely a matter of the species from which the fat was obtained; also, in cases in which the EFA can be stored, the proportion in the fat varies with the previous diet of the animal. The essential fatty acid contents of some common animal fats are given in Table 2.

Andrews and Richardson²²⁴ made a comprehensive study of the distribution of linoleic, oleic, and saturated acids in various samples of lard, butterfat, margarine fat, hydrogenated shortening and mixed animal and vegetable shortening, while Holman and Greenberg²⁴ studied the distribution of tetraenoic, pentaenoic, and hexaenoic acid in the non-adipose tissue of lambs, hogs, and beef cattle. In lambs and cattle the highest content of arachidonic acid was in the testes. Hog ovaries had the highest content for this animal, followed closely by testes and uterus.²⁴

The animal fats which may contain relatively high amounts of EFA include that from the hog, from the fowl, and especially that in the egg.

²²⁴ J. T. R. Andrews and A. S. Richardson, *Oil & Soap*, 20, 90-94 (1943).

TABLE 2

THE RELATIVE MAXIMUM AMOUNTS OF ESSENTIAL FATTY ACIDS IN ANIMAL AND FISH FATS AS DETERMINED BY ANALYSIS AND BY BIOASSAY^a

Animal and fish fat or oil	Values by analysis			Total essential fatty acids by bioassay ^b
	Linoleic acid	Linolenic acid	Arachidonic acid	
Animal fats				
Egg-yolk (hen)				
Hempseed oil diet	41.9	10.0	0	—
Linseed oil diet	24.9	17.4	0	—
Various diets	21.7	2.9	2.3	—
Phosphatides	8.2	0	0	—
Goose fat (body)	19.3	0	0	—
Hen fat (body)	21.3	0	0.6	—
Human fat	11.0	0	1.0	—
Ox depot fat	5.3	0	0.5	1.5 ^c
Pig depot fat	15.6	0	2.1	6.9 ^d
Soybean oil diet	38.9	0.5	—	—
Peanut diet	19.7	—	—	—
Cottonseed oil diet (12%)	26.8	—	—	—
Cottonseed oil diet (8%)	18.2	—	—	—
Cottonseed oil diet (4%)	13.3	—	—	—
Sheep depot fat	5.0	—	—	—
Fish fats				
Cod-liver oil	9.5 ^e	0 ^e	16.5 ^f	3.9
Herring oil	10.6 ^e	0 ^e	26.0 ^f	7.9
Menhaden oil	13.2 ^e	0 ^e	19.2 ^f	4.4
Whale oil	11.0 ^e	0 ^e	33.4 ^f	6.4

^a Unless otherwise noted, data are from the summary of H. J. Deuel, Jr., *Nutritional Significance of the Fats, Progress in the Chemistry of Fats and Other Lipids*, 2, 99-192 (1954), and from H. J. Deuel, Jr., *The Role of Fat in Human Nutrition*, in A. E. Bailey, *Cottonseed and Cottonseed Products*, Interscience, New York and London, 1948, 763-811. Dashes indicate that no data are available.

^b Unless otherwise noted, data are from H. J. Thomasson, *Intern. Z. Vitaminforsch.*, 25, 63-82 (1953). Bioassay values of essential fatty acids are expressed in units (1 Unit = activity of 10 mg. linoleic acid), which are practically identical with % composition.

^c Value given for "beef suet."

^d Value given for "lard."

^e Based upon spectrophotometric data for diene and triene reported by H. J. Thomasson. See footnote^b.

^f Based upon spectrophotometric data for acids with four or more double bonds, reported by H. J. Thomasson. See footnote^b.

Thus, on a hempseed oil diet, egg-yolk contained as much as 41.9% of linoleate and 10% of linolenate. Likewise, pig fat was reported as having as much as 38.9% of linoleate and 0.5% of linolenate following a soybean oil diet¹¹ (see Table 2). However, these high values have not been con-

firmed by bioassay. Thomasson¹⁶ reported a value of only 6.9% for lard when tested by the latter procedure. Andrews and Richardson²²⁴ reported an average linoleic content varying between 13.7 and 9.5% (average 11.6%) for twenty-seven different samples of eighteen different brands of lard. However, such analyses do not take account of the fact that arachidonic acid is present in lard, and that this has a somewhat higher biopotency than does linoleic acid.

In contradistinction to the relative high content of EFA in chicken and hog fat, the storage fat of beef cattle and of sheep represents only a minimum level of EFA. Although ox depot fat has been reported to have 5.3% of linoleate and 0.5% of arachidonate, Thomasson,¹⁶ employing a bioassay procedure, obtained a figure of only 1.5% for beef suet. Sheep depot fat has been reported to have 5% of linoleate, but this has not been confirmed by bioassay. More precise data, confirmed by bioassay, are needed in the case of animal fats.

d. Presence in Hydrogenated Fats. When fats are completely hydrogenated, as, for example, coconut oil, they obviously lose all their EFA. On the other hand, in the case of the vegetable fats which have been partially hydrogenated for the preparation of margarines and shortenings, appreciable quantities of the EFA may remain. One assay, which is based upon a new chemical "isolation" method of Simmons and Quackenbush,⁵⁰ assigned a value of 20.5% for the linoleate content of a common shortening (*Primex*). On the other hand, Deuel and associates⁷⁶ recorded values of 4.2% to 5.9% for the EFA content of different samples of a hydrogenated vegetable margarine, whereas the value for a shortening prepared by selective hydrogenation was 2.7% and that for one prepared by non-selective hydrogenation was 13.2%.

For a summary of the relative amounts of essential acids in hydrogenated fats and in butters, see Table 3.

It seems probable that the newer types of margarine, recently introduced on the American market, which are composed of one fraction of oil hydrogenated to a higher melting point than that of the former margarines, and a second fraction only lightly hydrogenated, may be expected to have a higher EFA content than those reported in Table 3. According to investigations carried on in the author's laboratory, new types of margarine contained 6.9 to 8.4 % of essential fatty acids. Andrews and Richardson²²⁴ reported variations in the linoleic acid content of various types of margarine. They studied the linoleic acid content of fifty-seven samples of various types of margarine obtained from different localities. The linoleic acid

TABLE 3

THE RELATIVE MAXIMUM AMOUNTS OF ESSENTIAL FATTY ACIDS IN HYDROGENATED FATS AND IN BUTTERS, AS DETERMINED BY ANALYSIS AND BY BIOASSAY

Hydrogenated fats or butters	Values by analysis			Total essential fatty acids by bioassay
	Linoleic acid	Linolenic acid	Arachidonic acid	
Hydrogenated fats ^a				
Coconut oil	0	0	0	0
Margarine oil I	4.8	0	0	4.17
Margarine oil II	5.5	0	0	5.05
Margarine oil	4.4	0	0	5.92
Margarine oil	4.9	0	0	5.06
Shortening (selective)	2.1	0	0	2.74
Shortening (non-selective)	6.8	0	0	13.24
Shortening (Primex) ^b	20.5	0	0	—
Butters				
Butterfat I ^a	3.1	0.9	0.2	2.48
Butterfat II ^a	3.8	1.2	0.2	3.81
Summer butterfat I ^c	1.8	0	0	0.6
Summer butterfat II ^c	2.2	0	0	1.3
Summer butterfat III ^c	1.0	1.3	0.4	1.3
Milk fat (cow) ^d	5.8	0	0.4	—
Milk fat (goat) ^d	1.5	0	0	—
Milk fat (human) ^d	7.9	5.4	0	—

^a Data are from H. J. Deuel, Jr., S. M. Greenberg, L. Anisfeld, and D. Melnick, *J. Nutrition*, 45, 535-550 (1951). Essential acids (bioassay) are expressed as % linoleic acid. Analytical values are determined spectrophotometrically for dienes, trienes, and tetraenes.

^b Data from R. O. Simmons and F. A. Quackenbush, *J. Am. Oil Chemists' Soc.*, 30, 614-616 (1953). A chromatographic separation of saturated acids, oleic, linoleic, and linolenic acids by the use of 2,4-dinitrobenzenesulfonyl chloride derivatives. Total analysis was as follows: saturated acids, 15.8%; oleate, 56.2%; linoleate, 20.5%; and linolenate, 0%.

^c Data are from H. J. Thomasson, *Intern. Z. Vitaminforsch.*, 25, 62-82 (1953). Bioassay values of essential fatty acids are expressed in units (1 Unit = activity of 10 mg. linoleic acid) which are practically identical with % composition. Analytical values, determined spectrophotometrically, except values for arachidonic acid, are for four or more double bonds.

^d Unless otherwise noted, data are from the summary of H. J. Deuel, Jr., *Nutritional Significance of the Fats, Progress in the Chemistry of Fats and Other Lipids*, 2, 99-192 (1954), and from H. J. Deuel, Jr., *The Role of Fat in Human Nutrition*, in A. E. Bailey, *Cottonseed and Cottonseed Products*, Interscience, New York and London, 1948, 763-811. Dashes indicate that no data are available.

content as determined by the thiocyanogen method was found to vary from 23.4% to 1.8%, with an average of 10.9%. In the case of various hydrogenated shortenings, Andrews and Richardson²²⁴ reported a linoleate content ranging from 22.4% to 2.9% for sixty samples, with an overall average of 11.6%. However, no bioassay data are

available for these samples. The method of hydrogenation was shown to have an interesting effect not evident from the spectrophotometric assays.⁷⁶ Thus, a shortening prepared by selective hydrogenation showed a close agreement between the EFA content determined spectrophotometrically and biologically (2.1% *vs.* 2.74%). On the other hand the corresponding figures for shortening prepared by non-selective hydrogenation were 6.8% and 13.24%. This would indicate that the actual EFA content can be established only by actual bioassay, and cannot always be predicted from analytical results.

e. Presence in Butters and Milk Fats. The EFA content of butter is the lowest present in any single group of fats (see Table 3). Thus, in a series of forty-one butters analyzed by the thiocyanogen method by Andrews and Richardson,²²⁴ the range of linoleic acid content varied only from 4.8 to 1.4%, with an average of 3.1%. However, this does not take into consideration the arachidonic acid which is also present in butter. In an examination of the EFA content of two butters from the New York market (one with the lowest and the other with the highest iodine number), values of 2.4% and 3.81% of EFA were reported on bioassay, although the spectrophotometric analysis might lead one to expect somewhat higher values.⁷⁶ According to Thomasson,¹⁶ the value as determined by bioassay for three summer butters averaged only 1.1 (0.6, 1.3, 1.3). Human milk fat appears to have a higher content of essential fatty acids than does that of cow's milk fat. Further experiments are in order to obtain additional data on the EFA content of butters and milks by bioassay, as influenced by various environmental conditions and by feeding of the animal.

(2) *Distribution in Animal Tissues*

The quantity of the several EFA present in the tissues depends to a considerable extent upon the dietary conditions. This is particularly true in the case of the blood. Thus, Wiese *et al.*¹³² showed that the average EFA contents of the blood of sixty healthy children who had partaken of diets containing approximately 3% of the total calories as linoleate were as follows, in percentages of the total fatty acids: dienoic acids, 30.3%; trienoic acids, 1.5%; and tetraenoic acids, 10.2%. The value for hexaenoic acid in the serum of healthy children was variable. These workers state that the role of the pentaenoic and hexaenoic acids, if any, is not known. On the other hand, Hansen and Wiese²²⁵ were unable to find any significant differences in the total fatty acids in the blood of poorly nourished children

²²⁵ A. E. Hansen and H. F. Wiese, *J. Nutrition*, 52, 367-374 (1954).

as compared with well-nourished individuals. However, the dienoic, tetraenoic, and hexaenoic acid levels were distinctly lower, and the proportion of trienoic acid was higher, in the poorly nourished group as compared with the well-nourished children. The following figures were reported for sixty well-nourished and thirty-four malnourished children, respectively: total fatty acids, 301 and 278 mg. %; dienoic acids, 30.3% and 12.0%; trienoic acids, 1.5% and 2.4%; tetraenoic acids, 10.2% and 7.0%; and hexaenoic acids, 4.2% and 1.8%.

The polyunsaturated fatty acids have a wide distribution in tissues; however, because of the relatively difficult procedures for isolation, and because of the low concentration, there is little information in this field. Arachidonic acid, either as its polybromide or as the pure acid, has been isolated from a number of the tissues. These include the following: liver lecithin,²²⁶ brain phospholipids,²²⁷ corpus luteum,²²⁸ egg yolk,²²⁹ pig liver,²³⁰ beef adrenal phosphatides obtained by debromination of methyl octabromoarachidate by Ault and Brown,²³¹ and by the use of chromatography by White and Brown²³² and by Herb *et al.*^{48,49}

Holman and Greenberg²⁴ investigated the proportion of tetraenoic, pentaenoic, and hexaenoic acids in several non-adipose tissues from lambs, hogs, and beef cattle. The highest proportion of arachidonic acid was found in the testes and ovaries. Of the fatty acids of lamb testes, for example, 15.6% is hexaenoic acid and 10.3% is arachidonic acid. From the standpoint of isolation, the most practical sources of hexaenoic acid were beef testes lipid and hog brain lipid, while arachidonic acid could best be prepared from hog liver lipid.

9. Conclusions

The essential fatty acids, linoleic, linolenic, and arachidonic acids, have been found to mediate a number of reactions in the animal body. Thus, not only are they important in growth and in the nutrition of the skin, as has been recognized since the discovery of their indispensability, but they are now recognized as specific agents in protecting the animal against x-irradiation injury. They also maintain capillary pressure in the subcutane-

²²⁶ P. A. Levene and H. S. Simms, *J. Biol. Chem.*, **51**, 285-294 (1922).

²²⁷ P. A. Levene and I. P. Rolf, *J. Biol. Chem.*, **54**, 91-98, 99-100 (1922).

²²⁸ G. F. Cartland and M. C. Hart, *J. Biol. Chem.*, **66**, 619-637 (1925).

²²⁹ P. A. Levene and I. P. Rolf, *J. Biol. Chem.*, **51**, 507-513 (1922).

²³⁰ J. B. Brown, *J. Biol. Chem.*, **80**, 455-460 (1928).

²³¹ W. A. Ault and J. B. Brown, *J. Biol. Chem.*, **107**, 607-614 (1934).

²³² M. F. White and J. B. Brown, *J. Am. Chem. Soc.*, **70**, 4269-4270 (1948).

ous blood vessels. The EFA are required for successful pregnancy and lactation. It has recently been shown that the essential fatty acids are necessary for normal cholesterol transport and metabolism in the rat. In the absence of fats from the diet, the cholesterol content in the liver and adrenal tissue reaches abnormally high values; it promptly returns to normal when fats containing the EFA, or the EFA themselves, are added to the diet. The requirement for EFA in the rat has been shown to be a sex function; apparently much larger amounts are required for optimum growth by the male than by the female rat. One interesting recent report, which has not as yet been confirmed, is the finding that γ -linolenic acid, 6,9,12-octadecatrienoic acid, is the active isomer of linolenic acid in causing growth, rather than ordinary linolenic acid, 9,12,15-octadecatrienoic acid, as was formerly believed.

CHAPTER XIV

THE NUTRITIONAL VALUE OF FATS

1. Introduction

Fat fulfills a number of functions in the animal organism. Until recently, it was generally considered that these functions were non-essential, since they could be carried out by other foodstuffs. It has now become evident that fats play a unique role in many respects. Fats must therefore be considered as essential components of the diet rather than as only optional factors.

One of the roles which fat plays is as a carrier of fat-soluble vitamins. Fats also assist in the absorption of these compounds from the gastrointestinal tract, even though they may not themselves contain the vitamins. A number of years ago Evans and Lepkovsky¹ demonstrated that fats exert a marked sparing action on the antineuritic vitamin B (thiamine). Furthermore, fats are important in the diet in that they improve palatability. Low-fat diets prove extremely unappetizing, and their use is often associated with a voluntary reduction in food consumption. According to Starling,² the civilian population in Great Britain lost weight during World War I partly as a result of the failure to consume as great a number of Calories on the high-carbohydrate, low-fat diet as were needed to maintain a caloric equilibrium. Fats also serve as important adjuncts in the preparation of many baked foods. Finally, it is well known that fats have the highest caloric density of any of the common foodstuffs. Each gram of fat yields 9.3 calories (1 Kilo calorie equals 1,000 Calories), whereas proteins and carbohydrates yield only 4.1 Calories per gram under similar conditions.² Since fats are hydrophobic, they are usually laid down in the tissues without additional water. On the other hand, proteins and carbohydrates are usually associated with considerable amounts of water. For this reason the ratio of the caloric content of fatty foods and of non-fatty foods is usually greater than 2.3:1, which is the ratio of the caloric density of dry fats to that of carbohydrates and proteins.

¹ H. M. Evans and S. Lepkovsky, *J. Biol. Chem.*, **83**, 269-287 (1929).

² E. H. Starling, *Brit. Med. J.*, *II*, Part I, 105-107 (1918).

Fat is therefore the foodstuff to be employed in greatest proportion when it is desirable to reduce the bulk of the food to the minimum.

Since the classical experiments of Burr and Burr,³ first published in 1929, there has been positive evidence of the specific nutritional value of fats themselves. As a result of this discovery it is now known that certain polyunsaturated fatty acids are required in the diet, since they cannot be manufactured in the body. The primary polyunsaturated fatty acids undoubtedly are linoleic and arachidonic acids, although linolenic acid and certain other related compounds can also serve in the same capacity, probably because of the ability of the animal body to convert them to one of the primary types. These "essential" acids are known to be required for growth and for the maintenance of normal skin conditions by a wide variety of animals, and by man. More recently it has been shown that the essential fatty acids mediate not only growth but also certain diverse functions such as protection from x-irradiation injury, maintenance of capillary resistance in the skin capillaries, and the normal transport and metabolism of cholesterol. Essential fatty acids are also required for pregnancy and lactation. Thus, the extent to which the essential fatty acids contribute to the metabolic processes of the body must be considered as indicating the indispensability of fats in nutrition.

In addition to the importance of fat in serving as a source of the essential fatty acids there is also some evidence that fat *per se* may likewise be a required foodstuff, since certain physiologic functions are carried out better when it is present in the diet. Thus fats exert a protein-sparing action, increase work capacity, extend the length of survival during fasting, and protect the subject from stress factors such as occur in hypothyroidism. It is not believed that all of these beneficial effects of fat are to be attributed solely to the essential fatty acids.

In addition to the several functions already listed for fats and lipids, they are essential for the normal functioning of the tissues and organs, as well as of the body as a whole. In this capacity, they are secondary only to protein. Thus, in the master tissue of the body, namely the brain, as well as in the spinal cord and associated nervous system, phospholipids, cholesterol, and galactolipids form a large part of the essential portion of the functioning tissue. The lipids are part of the structural elements, and these cells could not function without them. Moreover, all cell membranes throughout the body apparently contain lecithin, a phospholipid which is derived from fat. One of the effects of prolonged fat deficiency in rats is the failure of normal kidney function; this is in all probability to be ascribed to the failure of the body to build up normal cell

³ G. O. Burr and M. M. Burr, *J. Biol. Chem.*, **82**, 345-367 (1929).

membranes. Fats also serve as a mechanical protection for nerves and for bony projections. The layer of subcutaneous fat is undoubtedly of considerable importance in maintaining body temperature, particularly because of its function as an insulator. Adipose tissue, in addition to connective tissue, acts as a protective covering for organs such as kidneys and ovaries, which are frequently imbedded in large amounts of storage fat. Finally, the capability of fat deposits to furnish energy over long periods of time of fasting is another most important function which should not be overlooked.

Fats and oils are generally considered to have the same type of composition. Both are fatty acid esters of the trihydric alcohol, glycerol. The term "fats" is usually accepted as referring to substances solid at ordinary temperatures, while "oils" are products which are liquid under certain conditions. The terms are not necessarily absolute; one must recognize that a compound which is a fat under one environmental condition may be an oil under different temperature conditions. However, one should distinguish between "fats" and "fatty foods." Most of the fats of commerce contain not only the triglycerides but also other substances which are dissolved in the fats. These comprise chiefly the non-saponifiable fraction, and contain sterols, tocopherols (vitamins E), carotenoids and, in some cases, vitamin A. In the case of crude fats, phospholipids frequently also comprise an appreciable proportion of the material, although these are usually almost completely removed in the processes of refining. One should also include in the category of "fatty foods" substances such as butter and margarine in which triglyceride fats comprise only about 80% of the total material, the balance being made up of non-fat milk solids, water, salt, fat-soluble vitamins, and other materials. It is obvious that some of the materials frequently considered as nutritional components of the fats are actually present in solution in the fats and are not really part of them. The only substances having an absolute nutritional value which is part of the triglyceride molecule are the essential fatty acids. The nutritional significance of the fats has been discussed in a number of other publications by the present author.⁴⁻⁸ The physi-

⁴ H. J. Deuel, Jr., *Nutrition Aspects of Cottonseed Oil Utilization*, in A. E. Bailey, *Cottonseed and Cottonseed Products*, Interscience, New York, 1948, pp. 763-811.

⁵ H. J. Deuel, Jr., *Nutritional Value of Soybeans and Soybean Products*, in K. S. Markley, *Soybeans and Soybean Products*, Vol. II, Chap. 18, Interscience, New York, 1951, pp. 727-786.

⁶ H. J. Deuel, Jr., and S. M. Greenberg, *Some Biochemical and Nutritional Aspects in Fat Chemistry*, *Fortschr. Chem. org. Naturstoffe*, 6, 1-86 (1950).

⁷ H. J. Deuel, Jr., in R. T. Holman, W. O. Lundberg, and T. Malkin, *Progress in the Chemistry of Fats and Other Lipids*, Vol. 2, Pergamon Press, London, 1954, pp. 99-192.

⁸ H. J. Deuel, Jr., *Federation Proc.*, 14, 639-649 (1955).

ology and biochemistry of the essential fatty acids are reviewed in Chapter XIII and also by Deuel and Reiser.⁹

2. Fats as Essential Dietary Components

(1) *The Effect of Dietary Fat upon Growth*

a. Experiments in Which *ad libitum* Feeding Was Employed. There is considerable evidence in the literature to prove that the growth-promoting properties of diets are progressively improved as the proportion of fat is increased. This greater increase in body weight on diets containing large amounts of fat may possibly be attributed merely to the fact that the animals are able to consume a larger number of calories because they are ingesting diets of a higher caloric density when large amounts of fat are included, or because the presence of increased amounts of fat *per se* may have some special beneficial effect on growth. Hoagland and Snider¹⁰ proved that, when rats were fed diets containing 5, 30, or 55% of four types of lard the best growth was obtained on those regimens which had 30 or 55% of fat. In another series of tests carried on by these same workers at a later date,¹¹ it was found that, when steam-rendered lard and hydrogenated cottonseed oil were fed at levels of 5, 15, 30, or 54% in the diet, optimum growth occurred in the animals which received the 30% fat diet, and the minimal growth was noted in the group receiving the lowest fat level, namely 5%. Deuel and co-workers¹² also reported that weanling rats which were fed on several levels of cottonseed oil or margarine fat grew best when the fat comprised 20 to 40% of the total weight of the diet. These authors suggest that the optimum level of fat estimated from these tests would be approximately 30% by weight, or 50% on the basis of calories.¹³ Figure 1 represents the mean body weights, at the several periods, of weanling rats fed over an eighteen-week period on diets containing several levels of fat. According to these data the best growth was obtained on diet 63, which contained 20% by weight of cottonseed oil or margarine fat.

(a) *Experiments on Rats Maintained on Restricted Calories.* The improvement in nutrition and growth which results from the inclusion of

⁹ H. J. Deuel, Jr., and R. Reiser, *Vitamins and Hormones*, 13, 29-70 (1955).

¹⁰ R. Hoagland and G. G. Snider, *U. S. Dept. Agr. Tech. Bull.*, No. 725, 1-12 (1940).

¹¹ R. Hoagland and G. G. Snider, *J. Nutrition*, 22, 65-76 (1941).

¹² H. J. Deuel, Jr., E. R. Meserve, E. Straub, C. Hendrick, and B. T. Scheer, *J. Nutrition*, 33, 569-582 (1947).

¹³ H. J. Deuel, Jr., *J. Am. Dietet. Assoc.*, 26, 255-259 (1950).

fat in the diet has been demonstrated by Scheer and collaborators¹⁴ in weanling rats which had previously been stunted by a sufficiently drastic restriction of the food intake so that practically no increase in body weight occurred for a period of twelve weeks after weaning. The rats were then allowed to eat diets containing varying quantities of fats *ad libitum*.

Fig. 1. The average body weight of weanling male rats at the start (to top of lower blank space), after three weeks (to top of solid black), after six weeks (to top of stippled area), after twelve weeks (to top of cross-lined area), and after eighteen weeks (to top of upper blank area). The figures in the lower blank area are the diet numbers, which are as follows: 60a, 0% fat; 60b, 0% fat with essential fatty acids; 61, 5% fat; 62, 10% fat; 63, 20% fat; 64, 40% fat; 65, 30% fat; 66, 50% fat; 67, margarine fat (7.2%) and cottonseed oil (2.8%) and S, stock diet.¹² (From original of Fig. 1, H. J. Deuel, Jr., *et al. J. Nutrition*, 33, 573 (1947).)

Markedly superior growth was noted in the animals receiving 10, 20, and 40% fat in the diet, as compared with those which had only 0 or even 5% fat in the diet, as compared with those which had only 0 or even 5% of cottonseed oil. Although this superiority in growth was evident during the first period after *ad libitum* feeding was initiated, it continued to be

¹⁴ B. T. Scheer, D. F. Soule, M. Fields, and H. J. Deuel, Jr., *J. Nutrition*, 33, 583-592 (1947).

the dominant factor up to the last period investigated, namely, nine to twelve weeks, at which time the greatest potentiality for growth was still exhibited by the animals whose diet contained generous quantities of fat. In another series of tests which were carried out by Scheer *et al.*¹⁵ on young adult rats, it was found that the loss of weight by the rats, when subjected to severe caloric restriction, was less in the groups receiving the high-fat diets than in those receiving the isocaloric low-fat diets. Moreover, the mortality rate observed in rats receiving the high-fat diet during the period of caloric restriction was much less than the figure obtained for those on the low-fat regimen. Finally, when the depleted animals were again allowed to replete on the same diets, but given *ad libitum*, those animals which were receiving the high-fat rations made a much better recovery during the subsequent period than did those which were on the low-fat diets. This gain was due to fat rather than to the essential fatty acids contained, as is shown by the experiments of Pearson and Panzer,¹⁶ which demonstrated that rats fed a diet containing 8% of corn oil or lard *ad libitum* gained 29% more in body weight than did animals which were maintained on a similar diet minus the fat but with the addition of ethyl linoleate. In general the fat content of the body is not closely related to the fat content of the diet. Thus it was shown by Scheer and colleagues¹⁷ that rats receiving 20% of fat in the diet, rather than those receiving 40%, had the highest content of body fat. It was calculated in these experiments that the greater body size of animals on diets containing liberal amounts of fat, as compared with fat-free diets, is not solely the result of deposition of fat. It would thus appear from the experiments carried out employing *ad libitum* feeding that animals do better on high-fat diets than when a low-fat regimen is employed. However, these data do not answer the question whether or not improved body weights are to be ascribed to the fact that the animal is able to consume a larger proportion of calories on the higher fat diets than on the lower fat diets, or whether some other condition is responsible for the better growth.

Lundbaek and Stevenson^{17a} observed greater weight gains in rats on a high-fat, non-carbohydrate diet than on diets high in carbohydrate. A greater efficiency of utilization of the calories was noted on high-fat diets. These workers advanced the hypothesis that this greater efficiency was

¹⁵ B. T. Scheer, J. F. Codie, and H. J. Deuel, Jr., *J. Nutrition*, **33**, 641-648 (1947).

¹⁶ P. B. Pearson and F. Panzer, *J. Nutrition*, **38**, 257-265 (1949).

¹⁷ B. T. Scheer, E. Straub, M. Fields, E. R. Meserve, C. Hendrick, and H. J. Deuel, Jr., *J. Nutrition*, **34**, 581-586 (1947).

^{17a} K. Lundbaek and J. A. F. Stevenson, *Am. J. Physiol.*, **151**, 530-537 (1947).

due to the fact that the animals receiving the fat did not experience a loss of energy entailed by the transformation of carbohydrate to fat.

(b) *Experiments in Which Paired Feeding Tests Were Employed.* In most of the tests in which variabilities in caloric intake and palatability of the food were eliminated by the use of isocaloric diets, growth tests in general still favored the high-fat regimen. Thus, in the experiments of Forbes *et al.*,¹⁸ which were carried out with pair-fed rats on isocaloric diets containing 2, 5, 10, or 30% of fat, which was mostly lard, the gains in weight, the digestibility of the fat, and the retention of nitrogen increased proportionally as the amount of fat in the diet was augmented. In another series of tests carried on by Forbes and co-workers¹⁹ with 2, 10, and 30% of fat, and with increasing proportions of vitamins as compared with the amount given in the earlier series, it was proved that the greater efficiency of the diets having the highest fat content was manifested by significant gains of fat energy and by a decrease in the total heat production. In a later report from this laboratory²⁰ it was found that the fat content of the diets had little effect upon nitrogen utilization in the case of adult rats. With a supermaintenance food intake, there was a slight increase in urinary nitrogen and a decrease in nitrogen retention in proportion to the increasing fat content of the diets. However, as the fat in the diets increased there were slight decreases in the metabolizable energy, and larger decreases in heat production, in the order of the increasing fat content. The heat increments therefore decreased with increasing fat. The decreasing energy expenditure entailed by utilization of the isocaloric diets, in the order of their increasing fat content, was due mainly to decreasing heat derived from the catabolism of carbohydrates.²¹ No fat was catabolized from a 2% fat diet, while considerable heat was produced from fat in the 30% diet (for a further discussion of this point see the following section on associative dynamic action). French, Black and Swift²² did report that, when the protein intake of growing rats was decreased from 22% to 7% of the diet, the superior energy utilization of high-fat diets was still maintained. The only discordant result in this

¹⁸ E. B. Forbes, R. W. Swift, R. F. Elliott, and W. H. James, *J. Nutrition*, **31**, 203-212 (1946).

¹⁹ E. B. Forbes, R. W. Swift, W. H. James, J. W. Bratzler, and A. Black, *J. Nutrition*, **32**, 387-396 (1946).

²⁰ E. B. Forbes, R. W. Swift, E. J. Thacker, V. F. Smith, and C. E. French, *J. Nutrition*, **32**, 397-403 (1946).

²¹ E. B. Forbes, R. W. Swift, R. F. Elliott, and W. H. James, *J. Nutrition*, **31**, 213-227 (1946).

²² C. E. French, A. Black, and R. W. Swift, *J. Nutrition*, **35**, 83-88 (1948).

category has been that of Hoagland, Snider, and Swift,²³ who reported that they were unable to demonstrate any statistical difference in the gains of weight, storage of fat or protein, or the efficiency of utilization of feeding energy in rats on diets containing 4, 9, or 14% of fat (5.0, 10.98, or 18.27% of the calories, respectively). The most recent results of French and his collaborators²⁴ have confirmed the fact that identical growth can be brought about in male and female rats on a high-fat diet (22.7%), and on a low-fat regimen (3.4%). The difference in food consumption amounted to 4.8% and 5.7% less total calories consumed on the high-fat diet for males and for females, respectively. The preponderance of evidence, therefore, would seem to favor the hypothesis that greater increases in weight, and improved efficiencies of the diet, are associated with fat consumption in the rat. Siedler and Schweigert²⁵ reported that the rates of gain of cocker spaniel pups when 4, 6, or 8% of fat was added to the basal diet, or when 6% of fat was added to commercial meal, were equal or superior to those obtained when no fat was added. No significant differences in the food or caloric efficiency were noted between groups fed different levels of fat, indicating that the calories from fat were well utilized.

b. Associative Dynamic Action. The greater gain in weight observed by many different investigators for rats on isocaloric diets containing generous proportions of fat, as compared with weight gains in animals on low-fat regimens, is best accounted for by differences in the specific dynamic action exerted by the fat under these conditions. This variation in the effect of the fats, which was first discovered by Forbes and Swift,²⁶ and which has been termed "Associative Dynamic Effect," may be explained by the fact that the increased growth of animals on a high-fat diet represents an accumulated saving of calories due to the fact that smaller amounts of waste energy are lost, as a result of specific dynamic action. This saving can be reflected in the calories utilized for growth instead of for heat production.

It has long been recognized that respiratory metabolism is increased above the basal level during the period of absorption, transport and inter-conversion of the foodstuffs in the animal body. This increase in metabolism over and above the normal basal rate is termed specific dynamic action. On the basis of caloric intake, protein exerts the greatest stimula-

²³ R. Hoagland, G. G. Snider, and C. E. Swift, *J. Nutrition*, 47, 399-409 (1952).

²⁴ C. E. French, R. H. Ingram, J. A. Uram, G. P. Barron, and R. W. Swift, *J. Nutrition*, 51, 329-339 (1953).

²⁵ A. J. Siedler and B. S. Schweigert, *J. Nutrition*, 48, 81-90 (1952).

²⁶ E. B. Forbes and R. W. Swift, *J. Nutrition*, 27, 453-468 (1944).

tory effect upon heat production, and this is followed in turn by carbohydrate and fat. Other foodstuffs such as vitamins and water apparently exert no similar calorigenic action. Although it was shown by Rubner²⁷ that the extra heat produced due to specific dynamic action may be used by the body *in lieu* of a stimulation of metabolism by shivering, the extra heat produced by the metabolism of the foodstuffs usually represents waste heat. Any condition which will reduce the output of this waste heat will obviously result in a greater efficiency of food utilization.

The earlier experimental work indicated that the specific dynamic action of the several foodstuffs is a result of different types of stimulation. It has been generally believed that these different types of specific dynamic action are completely additive. Thus Murlin and Lusk²⁸ demonstrated by experiments on dogs that the total heat produced when 20 g. glycerol was fed together with 70 g. glucose and 75 g. fat over a two-hour period was identical with the sum of extra heats produced during the same interval when the foodstuffs were fed separately (20 g. glycerol and 50 g. glucose four hours after the administration of 75 g. fat). However, in these data the calculations were based only upon the short period following the ingestion of the several foodstuffs, at which time the specific dynamic action was at the maximum. It is possible, however, that variations may have been caused by the action of one foodstuff or another on the specific dynamic action produced at a time remote from the period at which the materials were fed.

In the studies by Forbes and Swift²⁶ on the rat, heat production as effected by the various combinations of foodstuffs was followed during the entire twenty-four-hour period after the ingestion of the several diets. When the several foodstuffs were fed to the rats the amounts of extra heat produced were 323 calories per 1,000 calories of beef protein, 202 calories per 1,000 calories of carbohydrate (cerelose), and 160 calories for 1,000 calories of fat (lard). When glucose and protein were given simultaneously, the response calculated from the proportion of each of the foodstuffs fed agreed closely with the value actually found. However, when lard was included in the diet, either with beef protein or with cerelose, the calculated values exceeded those actually observed by a considerable amount. Thus, when the fat was administered with beef protein, only 113 extra calories were produced, as compared with a theoretical value more than twice as

²⁷ M. Rubner, *Die Gesetze des Energieverbrauchs bei der Ernährung*, Leipzig & Vienna, 1902, p. 109; cited by G. Lusk, *The Elements of the Science of Nutrition*, 4th ed., Saunders, Philadelphia & London, 1928, p. 278.

²⁸ J. R. Murlin and G. Lusk, *J. Biol. Chem.*, 22, 15-29 (1915).

great, namely, 244 calories. If fat can normally reduce the loss of heat due to specific dynamic action to such an extent in animals other than the rat it should result in a greater efficiency of utilization of fat diets in other species also. As far as the author can see, there is no reason why this associated dynamic action should be confined to the rat. Figure 2 gives a graphic representation of the results on associative dynamic action obtained by Forbes and Swift.²⁶

Fig. 2. The dynamic effects per 1000 calories of gross energy of nutrients as affected by nutrient combinations.²⁶ (From original of Fig. 2, E. B. Forbes, R. W. Swift, *J. Nutrition*, 27, 464 (1944).)

Table 1 indicates the breakdown as to the source of the energy, the metabolizable calories and the output of energy in the groups of rats receiving 2%, 5%, 10%, or 30% of the diet in the form of fat.²⁹

It is evident from an inspection of Table 1 that the decreased expenditure of total calories which occurs when fat replaces carbohydrate in the isocaloric diets is sufficient to account for the greater efficiency of growth observed when high-fat diets are utilized, as compared with that when

²⁹ R. W. Swift and A. Black, *J. Am. Oil Chemists' Assoc.*, 26, 171-176 (1949).

TABLE 1
THE PARTITION OF THE AVERAGE DAILY INTAKE
OF ENERGY PER RAT DURING 70 DAYS^a

Category	Diets containing fat at levels of			
	2%	5%	10%	30%
Energy intake, calories				
Gross	2854	2854	2854	2854
Protein	799	799	799	799
Carbohydrate	1912	1713	1456	657
Fat	143	342	599	1398
Metabolizable calories	2601	2605	2610	2615
Energy output, calories				
In feces	128	126	121	123
In urine	125	123	123	116
As heat	2195	2165	2154	2155
Energy retained, calories	406	440	456	460

^a R. W. Swift and A. Black, *J. Am. Oil Chemists' Assoc.*, 26, 171-176 (1949).

low-fat diets are fed. Although the authors do not propose an explanation of this divergent effect on specific dynamic action, it is possible that the prolongation of the absorption time resulting from the increased fat content in the diet might reduce the period during which an excess of amino acids and of carbohydrate occurs in the animal body, inasmuch as the specific dynamic action of protein and that of carbohydrate are both regarded as plethora phenomena. It is possible that extending the period of absorption over a longer time interval would effectively decrease the concentration present in the body at a given time, and thus lessen the total specific dynamic action produced. According to Forbes and Swift,²⁶ these data suggest that it is unnecessary to diminish the protein content of the diet during hot weather in order to insure a low heat increment resulting from specific dynamic action; one need only substitute an additional proportion of fat for some of the carbohydrate in the diet. There is no evidence as yet that the so-called associative dynamic action observed in the rat also occurs in man or in other animals.²⁶

(2) *The Effect of Dietary Fat upon the Time of Sexual Maturity*

Sexual maturity is not so obvious an indication of nutritional value as is growth, nevertheless it is a factor which can be used to assess the nutritional value of a foodstuff. The age at which sexual maturity develops in the female rat can be determined with considerable accuracy from the opening of the vagina. Diets which induce the completion of this change

at an earlier period may be considered as having a higher nutritional value than do regimens following which maturation is delayed. In tests made by Deuel *et al.*¹² it was found that the female rats receiving a fat-free diet *ad libitum* matured at 81 days of age, whereas those receiving diets containing different levels of fat experienced sexual maturity, on an average, at periods as early as 65.1 days. The average occurrence of maturity for the animals on the several levels of fat were as follows: 0% fat, 81 days; 5% fat, 70 days; 10% fat, 66.5 days; 20% fat, 65.8 days; 40% fat, 65.1 days. In rats which had been fed on diets so low in calories, after weaning, as to prevent an appreciable growth, sexual maturity did not occur.¹⁴ However, as soon as the animals were allowed to consume various diets *ad libitum* after the prolonged restriction period, sexual maturity took place within a short time. The number of days required before sexual maturity was noted, after the initiation of feeding the diets on an *ad libitum* basis, were as follows: 0% fat, 14.7 days; 5% fat, 11.9 days; 10% fat, 10.5 days; 20% fat, 7.7 days; 40%, 9.8 days.¹⁴ It would thus appear that, in the first instance, the sexual maturity occurred about as well on the diets containing 10, 20, or 40% of fat, whereas in the animals on the previously restricted diet the shortest time requirements for maturity were observed when the diets contained 20% of fat. It is uncertain in the latter case whether the augmented effect, on the high-fat diets, in promoting sexual maturity is a direct action exerted upon this function, or whether it is an indirect result of the increased rate of growth. In either case one would appear to be justified in interpreting the data as indicative of the beneficial effect of fat in this change.

(3) *The Effect of Dietary Fat upon Pregnancy and Lactation*

Growth, pregnancy, and lactation entail successively increasing nutritional stresses upon the animal which must be counteracted by means of diets having progressively greater nutritional values. A dietary regimen which may be satisfactory for providing optimal growth may prove unsatisfactory for allowing a normal pregnancy, particularly if it is continued over more than one pregnancy. Moreover, a regimen which proves entirely satisfactory both for growth and for sustaining pregnancy may be found to be inadequate in supplying the necessary dietary components to insure a successful lactation. On this diet the mother may be unable to nurse her litter successfully or to ensure the survival of healthy pups until weaning. A number of authors have recorded the fact that reproductive failure and unsuccessful lactation occur in rats raised to maturity on fat-

free diets.³⁰⁻³³ In experiments carried out by Kummerow and co-workers,³⁴ animals on a diet containing no essential fatty acids but including 5% hydrogenated coconut oil did give birth to living young, but these did not survive more than seventy-two hours. The mothers and young were deficient in arachidonic acid, and not in fat *per se*. However, when the diet contained 5% corn oil, 85% of the young survived to weaning. After the feeding of corn oil to the mothers the phospholipids of the young contained five to ten times more arachidonic acid than did the phospholipids of pups from rats on fat-free regimens. Although it is obvious that marked differences would be expected to exist in the performance of rats during pregnancy and lactation when fat-free diets were used, as contrasted with the results when fat-containing diets were employed, it is somewhat more difficult to demonstrate variations in these physiologic functions when different levels of dietary fat were fed. Thus no differences in reproductive and lactation performance have been noted in normal rats which received 5 to 40% of fat in the diet, but in all cases the results were superior to those in rats which were fed a fat-free diet.¹² However, Scheer and co-workers¹⁴ reported that, when animals were first subjected to a twelve-weeks period of undernutrition followed by an *ad libitum* feeding period, the best performance as far as both the number in the litter and the total weaning weights were concerned, was obtained in the group receiving 40% of cottonseed oil in the diet. Thus the average litter weights of the rats, at weaning, from mothers on diets containing different levels of cottonseed oil were as follows: 0% fat but containing 1% linoleate, 152 g.; 5% fat, 219 g.; 10% fat, 167 g.; 20% fat, 230 g.; and 40% fat, 283 g. The average weight of the individual weanling rats approximated 30 g. in the four groups receiving fats. The increased litter weights are a reflection of the larger litters which the rats on the high-fat diets were able to bring to weaning. In another group of tests of Scheer and co-workers,¹⁵ a study was made of the fertility of animals which had been subjected to severe caloric restriction to such a degree that they had lost approximately half of their body weight over a twelve-weeks period; this period of undernutrition was followed by an

³⁰ H. M. Evans, S. Lepkovsky, and E. A. Murphy, *J. Biol. Chem.*, **106**, 431-440 (1934).

³¹ E. C. Maeder, *Anal. Record*, **70**, 73-88 (1937).

³² F. W. Quackenbush, F. A. Kummerow, and H. Steenbock, *J. Nutrition*, **24**, 213-224 (1942).

³³ H. J. Deuel, Jr., C. R. Martin, and R. B. Alfin-Slater, *J. Nutrition*, **54**, 193-199 (1954).

³⁴ F. A. Kummerow, H. P. Pan, and H. Hickman, *J. Nutrition*, **46**, 489-498 (1952).

interval during which diets containing several fat levels were given *ad libitum*. As soon as recovery occurred, breeding tests were carried out. The following percentage of fertility was noted in the groups on various diets: 0% fat, 20%; 5% fat, 0%; 10% fat, 20%; 20% fat, 80%; and 40% fat, 80%. In no case were the mothers on a fat-free diet able to raise their progeny through the age of twenty-one days.

French, Ingram, Knoebel, and Swift³⁵ concluded, as a result of tests on reproduction over three generations of albino rats fed diets *ad libitum* composed of natural foods which varied in fat and carbohydrate content, that there was a significant decrease in the reproductive performance of the rats when 23% of fat was fed, as compared with that noted when the animals received a diet containing 4.4% fat, or one high in carbohydrate (3.4% fat). However, a larger number of litters was produced by mothers on the high-fat diet, and the total number of young was also considerably greater, although the number per litter was less. Thus, on the control diet the average number of surviving young was 10.0 ± 0.44 , while on the carbohydrate diet the average was 10.2 ± 0.37 , as compared with a value of 9.3 ± 0.32 for litters produced by mothers on the high-fat regimen. The average birth weight of the rats was slightly lower in the high-fat group, i.e. 6.1 ± 0.08 for the males, as contrasted with 6.5 ± 0.08 and 6.4 ± 0.07 for the males receiving the low-fat and carbohydrate diets, respectively, and 5.7 ± 0.07 for the females in the high-fat group as contrasted with 6.0 ± 0.07 for those whose mothers were on the other two diets. The fact which was particularly striking was the increased number of successful litters obtained on the high-fat diet in the third generation as contrasted with the first. Thus, the number of litters was increased from twenty-one to forty-three for the animals in the high-fat group, while the values for the high-carbohydrate group increased only from twenty-one to thirty, and for the control group from fifteen to twenty-two successful litters.

On the basis of these results it appears to the author that one might still ascribe certain nutritional benefits to high-fat diets as contrasted with low-fat diets, if one considers the number of successful pregnancies as the dominant factor, rather than the weight of the newborn rats. Most of the experimental evidence indicates the great importance of fat in rendering normal pregnancy and lactation possible. To what extent this favorable

³⁵ C. E. French, R. H. Ingram, L. K. Knoebel, and R. W. Swift, *J. Nutrition*, 48, 91-102 (1952).

effect is to be traced to the essential fatty acid content of the fat is not entirely clear. In this connection, it was demonstrated by Deuel, Martin, and Alfin-Slater³³ that female rats are able to cast normal litters and to raise their pups to weaning quite satisfactorily when they are fed a fat-free diet supplemented with 40 to 80 mg. of methyl linoleate daily. It would appear that the most important factor contributed by fats to pregnancy and lactation is the presence of the essential fatty acids.

(4) *The Effect of Dietary Fat upon Work Capacity*

Although carbohydrate has long been regarded as the main source of energy for muscular activity, a number of recent observations point to the fact that fats may play a very important role in this function. Krogh and Lindhard³⁶ reported, many years ago, as a result of respiration experiments on human subjects, that carbohydrate was utilized about 10% more efficiently for muscular work than was fat. These results are diametrically opposed to the very exhaustive studies made by Anderson and Lusk³⁷ on dogs. The latter investigators reported that the animals ran with equal efficiency, from the caloric standpoint, irrespective of whether they had a non-protein respiratory quotient of 1.0 (indicating oxidation mainly of carbohydrate) or one of 0.71 (indicating fat oxidation), which was obtained after a thirteen-day fast. The present author is inclined to accept the latter experimental findings, since it is far easier to control the experimental conditions in tests on dogs than it is in the case of experiments on man. It is of course possible that the variation in results obtained by these two groups of investigators may have been due to the fact that the first group employed human subjects while the second group used dogs. However, there is no evidence that variations of such a nature occur in the utilization of carbohydrates and fats in man and in dogs. More recently the total working capacity has provided a closely related index for comparing the nutritional values of foodstuffs. Thus Deuel and collaborators¹² employed a swimming test which has been devised by Scheer and co-workers³⁸ as a method for testing the maximum work capacity possible from different animals. It was demonstrated that the maximum period of swimming of the rats was considerably prolonged as the proportion of fat in the diet was increased. This was not to be traced to variations in buoyancy of the animals, since all test animals were re-

³⁶ A. Krogh and J. Lindhard, *Biochem. J.*, **14**, 290-363 (1920).

³⁷ R. J. Anderson and G. Lusk, *J. Biol. Chem.*, **32**, 421-436 (1917).

³⁸ B. T. Scheer, S. Dorst, J. F. Codie, and D. Soule, *Am. J. Physiol.*, **149**, 194-203 (1947).

duced to the same specific gravity at the start of tests by adding a sufficient number of weights to their bodies. The average swimming period in three series of tests on rats receiving diets containing several proportions of cottonseed oil were as follows: 0%, 654 seconds; 0% but containing 1% of linoleate, 636 seconds; 5%, 863 seconds; 10%, 849 seconds; 14% (stock diet), 1021 seconds; 20%, 1043 seconds; and 40%, 1044 seconds. The results were similar, irrespective of whether cottonseed oil or a vegetable margarine fat served as a source of fat in their diets.¹² Confirmatory evidence of the increased physical capacity of rats as related to the diets can be deduced from the work of Samuels and collaborators,³⁹ who used an entirely different method of approach. Tests were made on the amount of spontaneous activity of rats as well as on their forced activity during a period of starvation following a period of twenty-eight days throughout which the rats had received diets with either high-fat (80% of total calories), high-carbohydrate (0.5% of total calories), or high-protein (19.5% of total calories) contents. Not only was the spontaneous activity in general greater in the case of rats which had previously received the fat diet than in those which had received the other foodstuffs, but also the total forced activity in this group greatly exceeded that in the other groups. The latter phenomenon is partly to be ascribed to the longer survival time of the fat-fed group. These data are summarized in Table 2.

TABLE 2
THE EFFECT OF PREVIOUS DIET ON THE SPONTANEOUS AND FORCED
ACTIVITY AND ON THE PERIOD OF SURVIVAL OF RATS
DURING FASTING^a

Category	Previous diet ^b		
	Fat	Carbohydrate	Protein
Spontaneous activity, cage revolutions			
2-4 days	6730 ± 915 (12)	4040 ± 411 (16)	3600 ± 262 (7)
9-11 days	8800 ± 1020 (13)	8660 ± 1017 (14)	7220 ± 500 (4)
16-18 days	7960 ± 738 (7)	8650 ± 0000 (3)	— (0)
Forced activity, cage revolutions	19700 ± 1780 (12)	13800 ± 1630 (14)	6465 ± 1650 (9)
Survival time, days	18.3 ± 0.44 (12)	15.5 ± 0.46 (13)	10.2 ± 1.68 (9)

^a Adapted from L. T. Samuels, R. C. Gilmore, and R. M. Reinecke, *J. Nutrition*, **36**, 639-651 (1948), p. 644.

^b Including Standard Error of the Mean. The figures in parentheses designate the number of rats in each group.

³⁹ L. T. Samuels, R. C. Gilmore, and R. M. Reinecke, *J. Nutrition*, **36**, 639-651 (1948).

All of the data which have been advanced in comparing work capacity would appear to support the view that rats which receive a high-fat diet or which, previous to fasting, have received high-fat diets, use fat more economically insofar as the source of calories is concerned than they do the other foodstuffs. In the case of the fasting rats this would allow the animals to survive for a longer period of time and to carry out a larger proportion of work than did animals which received a non-fat diet. However, Ershoff,^{39a} using different experimental conditions, observed that the swimming performance of rats and mice at a water temperature of 20°C. was inversely proportional to the fat content of the diet. In this later experiment, the stressor agent was not work *per se* but, at least in part, the stress of immersion in cold water, and the attendant loss of body temperature.

(5) *The Effect of Dietary Fat upon Survival Time during Subsequent Fasting*

The length of life and of survival during fasting are two nutritional indices which are of importance in the evaluation of the nutritive value of foodstuffs. In the case of the first index, namely longevity, the data are somewhat controversial. Carlson and Hoelzel⁴⁰ reported that rats fed on a mixed diet containing 28% of fat and 35% of protein (chiefly meat) lived somewhat longer than did animals which were fed on a vegetarian diet containing 8% of fat and 30% of protein (whole wheat, beans, and other vegetable proteins) but supplying more roughage on an isocaloric basis. Because of variations in the type of proteins employed and in that of the other foodstuffs, it is difficult to attribute the variations in longevity in the aforementioned experiments solely to the variation in the fat content. French and co-workers²⁴ observed, in 1953, that the life span of male rats ingesting a high-fat diet (22.7%) was somewhat shorter than that of the animals on a high-carbohydrate diet containing only 3.4% of fat. In the case of females, less pronounced differences in the span of life were noted. The average ages of the group on the several diets at death were as follows: Males: high-fat diet, 676 ± 19 days; low-fat (high-carbohydrate) diet, 765 ± 22 days; and stock diets 747 days. In the case of the females the figures were as follows: high-fat diets, 752 ± 25 days; low-fat diets, 793 ± 24 ; and stock diets 794 ± 31 days. The growth rate was found to be identical on the high-fat and low-fat diets, although the male and

^{39a} B. H. Ershoff, *J. Nutrition*, **53**, 439-449 (1954).

⁴⁰ A. J. Carlson and F. Hoelzel, *J. Nutrition*, **34**, 81-96 (1947).

female rats received 4.8% and 5.7% less total calories in the former case. The increased efficiency of utilization of the high-fat diet was correlated with the decreased life span, but there was no histopathologic evidence that fat induced premature death. On the other hand, when one considers the survival time of animals which are fasted following the feeding of single foodstuffs, the results are quite different. Under such conditions fat exhibits a superior response, as compared with that of protein and carbohydrate,⁴¹ when the procedures of Samuels and collaborators³⁹ mentioned in the last section were used as the basis of assessment. Roberts and Samuels⁴² have likewise demonstrated that the period of survival of rats previously subjected to evisceration (removal of a section of the intestine and of the liver) was considerably prolonged when the animals had previously received an exclusive fat diet beyond that when their previous food intake had consisted either of protein or of carbohydrate. Thus, on an average, the eviscerated animals which had received the high-fat diets survived approximately seventeen hours as compared with the figure of under ten hours for those on the high-carbohydrate diet.⁴³ As a probable explanation for the superior behavior of fat in prolonging survival under these several conditions, it is possible that the metabolic pattern changes in the animals during the time when they have received the high-fat diet. The original study of the relationship of diet to insulin tolerance was carried out by Abderhalden and Wertheimer.⁴⁴ Roberts and Samuels⁴⁵ reported that rats which had been accustomed to a high-fat diet had a markedly lower susceptibility to insulin injection than did animals which were on a high-carbohydrate ration. This resistance to insulin on the part of the fat-fed rats was ascribed to a prolonged retention of liver glycogen in these animals. Roberts *et al.*⁴³ suggested that the rats in whose case 85% of the calories had been derived from fat developed a sparing action for liver glycogen. Thus it was found that rats which had previously received a fat regimen were free from hypoglycemic symptoms and survived for a longer period during subsequent fasting than did rats which had received the high carbohydrate diet.⁴¹ This phenomenon has been termed "preferential utilization."^{42,46} The characteristic metab-

⁴¹ S. Roberts and L. T. Samuels, *Am. J. Physiol.*, 158, 57-62 (1949).

⁴² S. Roberts and L. T. Samuels, *Bull. Univ. Minnesota Med. Foundation*, 4, 55-59, 67 (1944); *Am. J. Physiol.*, 146, 358-365 (1946).

⁴³ S. Roberts, L. T. Samuels, and R. M. Reinecke, *Am. J. Physiol.*, 140, 639-644 (1944).

⁴⁴ E. Abderhalden and E. Wertheimer, *Pflüger's Arch. ges. Physiol.*, 205, 547-558 559-570 (1924).

⁴⁵ S. Roberts and L. T. Samuels, *Proc. Soc. Exptl. Biol. Med.*, 53, 207-208 (1943).

⁴⁶ S. Roberts and L. T. Samuels, *J. Biol. Chem.*, 151, 267-271 (1943).

olism of the whole animal which has previously received fat is also reflected in the respiration of isolated tissue from these animals. Thus Gilmore and Samuels⁴⁷ observed that the rate of glucose utilization in diaphragm tissue removed from animals previously on a fat diet differed from that of the tissue from rats which had been fed carbohydrate prior to fasting. These data also support the earlier results of Lundbaek and Stevenson,⁴⁸ who reported that the diaphragm of a carbohydrate-fed rat utilized glucose *in vitro* at approximately twice the rate that was noted in the muscle of the fat-fed rat when both tissues were immersed in the glucose solution.

Making use of a somewhat different procedure, Templeton and Ershoff⁴⁹ studied the effect of the foodstuff ingested on the length of survival. When the stress agent employed was thyroxine, it was found that rats fed margarine fat or carbohydrate (sucrose) lived appreciably longer than did those which received only the protein, casein. However, no significant differences were noted in control tests when saline was injected instead of the thyroxine solution. Moreover, when the stress of low temperature (2°C.) was employed, the rats fed fat and carbohydrate exhibited a greater resistance than did those which consumed only protein. However, this variation in survival time was obliterated when the rats were exposed to a temperature of 23°C.

Some time is required before the body can adapt itself to high-fat diets when the previous regimen has been rich in carbohydrates and low in fat. According to Geiger, El Rawi, and Catz,⁵⁰ thyroidectomy does not interfere with this adaptation, nor are the adrenal glands required to effect it. It was found that adrenalectomized rats utilized dietary fat with the same efficiency as did normal animals. Fat-adapted animals have been found to survive longer following adrenalectomy than did animals on a high-carbohydrate regimen. Finally, it was demonstrated by these authors⁵⁰ that hypophysectomized animals utilize fat normally, and that adaptation can occur after this operation.

(6) *The Effect of Dietary Fat upon Longevity*

Although fasting animals undoubtedly survive longer when the prefast diet has been composed of fat rather than of carbohydrate or protein,

⁴⁷ R. C. Gilmore, Jr., and L. T. Samuels, *J. Biol. Chem.*, **181**, 813-820 (1949).

⁴⁸ K. Lundbaek and J. A. F. Stevenson, *Federation Proc.*, **7**, 75 (1948).

⁴⁹ H. A. Templeton and B. H. Ershoff, *Am. J. Physiol.*, **159**, 33-39 (1949).

⁵⁰ E. Geiger, I. El Rawi, and B. Catz, *XIXth Intern. Physiol. Congress, Abst.*, 384-385 (1953).

some evidence indicates that the longevity of animals on high-fat diets may be somewhat less than that of animals receiving lower levels of fat in the diet. Although Carlson and Hoelzel⁴⁰ reported that rats fed a mixed diet with a high fat and protein content (see page 851) lived somewhat longer, on the average, than did those receiving a vegetarian diet containing less fat and protein, French and co-workers²⁴ reached opposite conclusions. They point out that, in the experiments of Carlson and Hoelzel, only one rat lived longer than 1000 days on the mixed diet, while four of the vegetarian group exceeded this age at the time of death. When diets containing 22.7% of fat were fed to rats, they grew as rapidly as did those on carbohydrate diets containing only 3.4% of fat and a lower proportion of calories. However, the life span of the male rats on this relatively high-fat diet decreased markedly from that of the animals on the low-fat diet, and there was also a less pronounced decrease in the longevity of the females.^{24,51} Thus, the increased efficiency of utilization of the diet, but not the caloric intake *per se*, could be correlated with a decrease in life span. A higher fat content was noted in the livers of the male rats fed the high-fat diet, as contrasted with those on the carbohydrate diet.^{24,51} The adverse effects of high-fat diets upon the life span, like those of underfeeding, appear to depend upon the age, strain, and sex of the test animal, and upon the duration of the fat-feeding period. The optimal regimens to ensure longevity lie between the extremes of undernourishment and of high dietary enrichment. Retardation of growth *per se* does not appear to contribute materially to longevity, and mere acceleration of growth is not directly related to the life span. Evidently growth and longevity are coördinated phenomena governed by the same metabolic principles, but are not mutually interdependent.⁵²

In longevity studies conducted in the author's laboratory,⁵³ in which rats were fed diets containing 3, 9, 18, and 30% cottonseed oil, the best survival was obtained in the two middle ranges. When cellulose was added to the higher fat diets to render them isocaloric with the 3% fat diet, animals receiving the diet containing cellulose and cottonseed oil at the 18% level exhibited the lowest mortality rate during the observation period.

⁵¹ C. E. French, R. H. Ingram, G. P. Barron, J. A. Uram, and R. W. Swift, *Federation Proc.*, **12**, 414 (1953).

⁵² M. Silberberg and R. Silberberg, *Physiol. Revs.*, **35**, 347-362 (1955).

⁵³ H. J. Deuel, Jr., L. Aftergood, and R. B. Alfin-Slater, Unpublished data, 1956

(7) *The Effect of Dietary Fat upon Nitrogen Retention*

The ability of a foodstuff to spare protein has always been considered to be an important index of nutritional value. For a number of years it has been generally assumed and accepted that nitrogen equilibrium was more readily established when carbohydrate was given simultaneously with protein foods than when fat served as the auxiliary foodstuff. In the classical experiments of Graham Lusk made over sixty years ago⁵⁴ it was reported that men on a constant protein intake exhibited a reduced urinary nitrogen when carbohydrate was ingested concomitantly, but that no such decrease in nitrogen elimination was observed when fat was the additional foodstuff consumed.

However, it has recently been demonstrated by a different type of experimental approach that fat actually does play a commanding role in protein metabolism when one is concerned solely with the endogenous protein metabolism. Thus Willman and collaborators⁵⁵ were able to demonstrate that the low level of "wear and tear quota" or the so-called nitrogen minimum (which is the lowest level of protein catabolism obtained after a prolonged period on a protein-free diet) could be maintained only when generous amounts of fat were present in the diet. In experiments on rats no differences were noted in the level of urinary nitrogen when the protein-free diets were fed at 100% or at 75% of the twenty-four-hour caloric requirement, irrespective of whether the diet contained 20% of fat or was fat-free. On the other hand, when only 50% of the required calories were given, the increase in nitrogen excretion over the basal level, namely the level when 100% of required calories were fed, was only 24 mg. when the animals received the protein-free diet containing 20% of fat, as contrasted with an increase of 311 mg. for the group of rats which received the protein-free diet which was also deficient in fat. When the caloric intake was reduced to 25% of the required level, the variations between the fat-containing and fat-free diets were exaggerated to an even greater extent. Thus the increase in nitrogen excretion over the basal was 254 mg. for the animals receiving the fat diet as contrasted with the mean of 569 mg. for those on the fat-free regimen. Similar though less striking results were noted when methionine was added to the protein-free diet.⁵⁶ Apparently the same situation exists in the case of

⁵⁴ G. Lusk, *Z. Biol.*, 27 (n.s.), 459-481 (1890).

⁵⁵ W. Willman, M. Brush, H. Clark, and P. Swanson, *Federation Proc.*, 6, 423-424 (1947); also personal communication from P. Swanson.

⁵⁶ D. Schwimmer and T. H. McGavack, *New York State J. Med.*, 48, 1797-1799 (1948).

man as occurs with rats, insofar as the effectiveness of fat in protecting the endogenous protein level is concerned. Thus Schwimmer and McGavack⁵⁶ demonstrated that the ingestion of fat results in the maintenance of a low level of urinary nitrogen in human subjects who partook of protein-free diets in amounts considerably below that necessary for caloric equilibrium, while fat-free diets did not exhibit this behavior under similar experimental conditions. The sparing effect of fat on protein is exhibited not only in relation to nitrogen minimum but also under several other conditions. Thus, when rats were previously fed on diets containing 80% of one foodstuff for a period of twenty-eight days and then were fasted according to the procedure of Samuels *et al.*,³⁹ those which had previously received the high-fat regimen subsequently excreted the smallest proportion of nitrogen in the urine during the fast period, and apparently had the most economical utilization of the protein during this time. The data for these experiments on the several types of foodstuff are included in Table 3.

TABLE 3
THE AVERAGE NITROGEN EXCRETION OF RATS ON SPECIAL DIETS
AND DURING A SUBSEQUENT PERIOD OF FASTING WITH
VOLUNTARY ACTIVITY, AND OF RATS UNDERGOING
VIOLENT EXERCISE^a

Category	Period during fast	Nitrogen excretion, grams/day/rat ^b		
		Protein diet	Carbohydrate diet	Fat diet
Total nitrogen excretion	(days)			
	Before (on diet)	1.369 ± 0.38	0.3375 ± 0.0053	0.3165 ± 0.0080
	0-4	0.2547 ± 0.0314	0.1770 ± 0.0068	0.1104 ± 0.0057
	6-11	0.1427 ± 0.0392	0.1318 ± 0.0111	0.1004 ± 0.0094
	13-death	—	0.2286 ± 0.0131	0.1407 ± 0.0272
Nitrogen excretion during work, mg./100 revolutions	—	0.0521 ± 0.0095	0.0465 ± 0.0058	0.0342 ± 0.0044

^a Adapted from L. T. Samuels, R. C. Gilmore, and R. M. Reinecke, *J. Nutrition*, 36, 639-651 (1948).

^b Including Standard Error of the Mean.

An inspection of Table 3 will indicate that the amount of nitrogen excreted in the group which had previously received the fat diet was invariably considerably less than that of those which had received carbohydrate; this in turn was considerably lower than that for the group which had been fed protein. Moreover the nitrogen excretion in mg. per hun-

dred revolutions of work was found to be lowest in the fat group; thus the figures are 0.0521 for the protein group, 0.0465 for the carbohydrate group and, finally, 0.0342 for the group which had previously received the high-fat diet.

It was demonstrated by French, Black and Swift⁵² that a more efficient energy utilization with an increase in weight, and decreased heat production, obtained in rats on a low-protein diet when 30% of fat was present than when only 2% of this foodstuff was available. Obviously the high-fat level enabled the protein to be better utilized. Geiger⁵⁷ and Salmon⁵⁸ likewise observed that the sparing effect of cystine on the utilization of casein is exerted only when fat is present in the diet. Rogers and co-workers⁵⁹ demonstrated another situation in which fat has an especially beneficial effect upon the retention of proteins as compared with other foodstuffs. Thus, the rate of liver regeneration, after a 70% partial hepatectomy was carried out in the case of rats previously depleted of protein, was found to be better when the animals were given a 30% fat diet than when they received a 3% fat regimen *ad libitum*. It would therefore appear that the inclusion of fats in the diet has a beneficial effect upon protein metabolism. Further data on the mechanism of action of fat in sparing protein are given in the review by Munro.⁶⁰

(8) *The Effect of Dietary Fat in Stress Conditions*

The foregoing discussion has been concerned largely with the role of fats in general in helping the animal meet physiologic forms of stress, such as growth, pregnancy and lactation, work, maintenance of protein stores, and similar demands. It is also of considerable importance whether or not fat may function in a specific manner when the animal is subjected to exogenous forms of stress, such as thyrotoxicosis, induced by the administration of thyroid, x-irradiation, cold, and hepatectomy.

a. Thyrotoxicosis. The thyroid gland is associated with so many varied functions in the animal body that any increased activity of this gland may cause wide-spread changes in certain physiologic indices. The stress which can be brought about by the administration of the thyroid hormone is similar to that produced by the feeding of desiccated thyroid powder, and also parallels the effect of hyperactivity of the gland. Among

⁵⁷ E. Geiger, Personal communication, 1952.

⁵⁸ W. D. Salmon, *J. Nutrition*, 33, 155-168 (1947).

⁵⁹ C. S. Rogers, C. C. Ferguson, C. E. Friedgood, and H. M. Vars, *Am. J. Physiol.*, 163, 347-353 (1950).

⁶⁰ H. N. Munro, *Physiol. Revs.*, 31, 449-488 (1951).

the effects of hyperthyroidism, the most important are suppression of growth and an increased basal metabolism, together with an augmented heart rate, which is proportional to the metabolic effect.

Fat has been shown to be the food which has the greatest beneficial effect in cases of hyperthyroidism. Abelin and his co-workers⁶¹⁻⁶⁴ were the first to note this effect. These workers reported that the high-fat diet counteracted the increased metabolic rate and likewise prevented the rapid reduction of liver glycogen which normally follows the administration of desiccated thyroid. The beneficial effect of fat in hyperthyroidism has been confirmed by a number of workers.⁶⁵⁻⁷⁰ Although most of the experimental evidence was obtained on rats, Berg⁷¹ corroborated the fact that dietary fat causes a reduction in the basal metabolic rate following hyperthyroidism in dogs, as well.

There is some question as to whether the beneficial effects of fat in hyperthyroidism are due to the fat *per se* or to the essential fatty acids which it contains. Guerra⁷² correlated the protective effect of fat with its unsaturation; Keeser⁷³ reported that the unessential sodium oleate, which is also unsaturated, is likewise able to prevent the increase in metabolism. On the other hand, Zain^{74,75} observed that linoleic acid, but not stearic acid, prevented the loss of liver glycogen which occurred after the injection of massive doses of the thyroid hormone. However, oleic acid had an intermediate effect, while it should respond in an entirely negative manner if the action of fat were dependent exclusively upon the essential fatty acid content. It is also possible that the negative effect

⁶¹ I. Abelin, *Klin. Wochschr.*, 5, 367 (1926).

⁶² I. Abelin, M. Knuchel, and W. Spiechtin, *Biochem. Z.*, 228, 189-210 (1930).

⁶³ I. Abelin, *Biochem. Z.*, 228, 211-232 (1930).

⁶⁴ I. Abelin and P. Kursteiner, *Biochem. Z.*, 198, 19-46 (1928).

⁶⁵ F. Hoffman, E. J. Höffman, and J. Talesnik, *Rev. méd. chile*, 73, 393-394 (1945).

⁶⁶ E. Muñoz. Ortiz, *Acción de la tiroxina sobre la cuantía de la metabolismo en ratas sometidas a dietas ricas en grasa y relativamente pobres en colina*. Tesis Cirjuano-Dentista Univ. de Chile, Inst. Fisiol. (1944).

⁶⁷ W. Spalloni Cialdea, *Influencia de dietas grasas sobre la elevación del consumo de oxígeno en ratas tratadas con tiroxina*. Tesis. Químico Farmaceutico Univ. de Chile, Inst. Fisiol. (1945).

⁶⁸ S. M. Greenberg and H. J. Deuel, Jr., *J. Nutrition*, 42, 279-284 (1950).

⁶⁹ B. H. Ershoff, *Metabolism*, 2, 175-181 (1953).

⁷⁰ B. Kennelly and L. A. Maynard, *J. Nutrition*, 49, 599-608 (1953).

⁷¹ J. Berg, *Z. ges. expil. Med.*, 93, 143-154 (1934).

⁷² E. Z. Guerra, *Acción inhibitoria de diversas grasas dietéticas (saturadas y no saturadas) sobre elevación del consumo de oxígeno producida por el tiroxina*. Tesis Medico-Cirujano, Univ. de Chile. Inst. Fisiol. (1947).

⁷³ E. Keeser, *Klin. Wochschr.*, 17, 1100-1103 (1938).

⁷⁴ H. Zain, *Klin. Wochschr.*, 15, 1722 (1936).

⁷⁵ H. Zain, *Arch. expil. Pathol. Pharmacol.*, 187, 302-323 (1937).

of stearic acid can be attributed to the failure of the latter acid to be satisfactorily absorbed.

Two recent American reports have indicated that the beneficial effect of fat in thyrotoxicosis is not necessarily proportional to the essential fatty acid content of the fats fed. Thus, Ershoff⁷⁶ demonstrated that the growth retardation of rats treated with thyroid was counteracted equally well by olive oil, cottonseed oil, peanut oil, corn oil, soybean oil, hydrogenated cottonseed oil, wheat germ oil, and lard, fats which are known to have widely varying contents of the essential fatty acids. Although growth inhibition was prevented, according to these tests, the fats failed to counteract the inhibition of ovarian development or the increase in renal, adrenal, and ventricular weight. In tests carried out by Greenberg and Deuel⁸⁸ the presence of cottonseed oil in the diet not only permitted normal growth of the hyperthyroid rats but also decreased premature mortality. Thus, in the case of the male rats, the following gains-in-weight and mortality rates were noted: low-fat diet alone, 162.2 g., 0%; low-fat with thyroid powder, 98.0 g., 50%; low-fat with thyroid powder plus a high vitamin B₁₂ level, 98.0 g., 83%; high-fat diet alone, 229.9 g., 0%; high-fat diet with thyroid powder, 236.4 g., 0%. Practically identical results were obtained in the case of female rats. Greenberg⁷⁷ later reported that methyl linoleate and cottonseed oil were both able to protect immature rats against growth retardation when the rat was fed a fat-free diet to which 0.05% desiccated thyroid had been added. Although 50 mg. of linoleate per day, or 500 mg. of cottonseed oil daily, were largely able to counteract the growth retardation in the rat on the fat-free diet to which thyroid powder had been added, these substances were not able to protect the hyperthyroid rat against enlargement of the heart, and protected it only partially against enlargement of the kidney. Although Ershoff⁶⁹ was able to demonstrate direct correlation between the fat content of the diet and the gain in body weight and length of survival of immature hyperthyroid rats, he also found that a defatted liver residue was as effective as cottonseed oil, when fed at a 10% level in the diet, in prolonging survival of the immature hyperthyroid rats on an otherwise fat-free ration. This defatted liver residue was found to be more effective than cottonseed oil in promoting growth.

Thus, although the beneficial effect of fat in hyperthyroidism may be partly attributable to the essential fatty acid content, the requirements for which might be increased in hyperthyroidism, and partly to the higher

⁷⁶ B. H. Ershoff, *J. Nutrition*, 39, 259-281 (1949).

⁷⁷ S. M. Greenberg, *J. Nutrition*, 47, 31-39 (1952).

caloric density of the high-fat diets, which enable the animal with the high metabolic level to obtain sufficient caloric supply to meet the energy needs, the beneficial effect of fat in this abnormal condition cannot be questioned, and is, in fact, one of the most striking examples of the value of dietary fat. On the other hand, fat may prove a detriment in hypothyroidism. It was shown by Kennelly and Maynard⁷⁰ that, when young rats are given sufficient thiouracil to retard growth, to decrease body temperature and oxygen consumption, the effects are more pronounced when the substance is given to animals on a high-fat diet than when it is fed to animals receiving a low-fat regimen. Thus, a high-fat diet tends to alleviate the effects of increased thyroid activity, but it augments the disturbances due to depressed thyroid action. Templeton and Ershoff⁴⁹ reported that both carbohydrate and fat are superior to protein (casein) in permitting the survival of hyperthyroid rats (see page 853).

b. X-Irradiation. Fats have a protective effect against x-irradiation injury in rats which seems to be primarily a function of the essential fatty acid content. For a discussion of this relationship the reader is referred to Chapter XIII.

c. Cold Stress. Cold offers an excellent example of stress for studies of nutrition, since it produces a marked increase in the level of metabolism. More calories are required under such conditions. Mitchell and co-workers⁷⁸ reported that, when human subjects were exposed to severe cold, a high-fat diet was superior to a high carbohydrate regimen in maintaining general psychomotor performance and visual efficiency, as well as in permitting a maximum speed in tapping tests. It was likewise observed that high-fat rations were superior to high-carbohydrate regimens in maintaining normal internal tissue temperatures in the cold environment, provided the interval between meals did not exceed two hours. It is believed that this is related to a decrease in heat emission, rather than to an increased heat production in the subjects ingesting the high-fat regimen. The results of the effect of high-fat and low-fat diets upon the resistance and acclimatization of animals to cold are somewhat equivocal, and are therefore not discussed here.^{78a}

d. Hepatectomy. Since the liver is responsible for performing a wide variety of functions, any interference with its activity will result in a critical form of stress. Although Mann⁷⁹ was able to keep dogs alive for

⁷⁸ H. H. Mitchell, N. Glickman, E. H. Lambert, R. W. Keeton, and M. K. Fahnestock, *Am. J. Physiol.*, **146**, 84-96 (1946).

^{78a} A. C. Burton and O. G. Edholm, *Man in a Cold Environment*, Monogr. Physiol. Soc. (London), Arnold, London, 1955, p. 148.

⁷⁹ F. C. Mann, *Am. J. Physiol.*, **55**, 285 (1921).

five to eight hours after the removal of the liver, and Markowitz and Soskin⁸⁰ extended this survival time somewhat beyond this period by a modification in the operative procedure, it is impossible to circumvent an early fatal outcome following complete removal of the liver. On the other hand, because of the rapid regeneration of liver tissue, it is possible to remove as much as 70% of the organ in rabbits and still permit survival for several weeks.⁸¹ This offers an opportunity to test the nutritive efficiency of various diets in stimulating the regeneration of such tissue. Although Brues and collaborators⁸² reported that the rate of regeneration of the liver cells of rats was slower, when fat was the chief dietary component, than when protein or carbohydrate served in this capacity, Rogers and his associates⁸³ reported that a moderately high-fat diet does serve as efficaciously in permitting a new growth of liver tissue as do rations low in fat. Actually when rats on a 30% diet fat were limited in caloric intake to that of animals on the 3% fat regimen, a significantly higher nitrogen balance, and a higher increment of protein in the liver, as well as a greater increase in body weight, obtained in the high-fat group. When *ad libitum* feeding was employed, the rats did considerably better when they received the high-fat diet than when they were offered the low-fat regimen.

e. Alloxan Diabetes. High-fat diets have frequently been recommended in diabetes mellitus in human patients. The diabetic organism can extensively utilize the calories from fat, which is impossible in the case of carbohydrate. However, there is always the difficulty that the increased fat intake may exaggerate the existing ketonuria.

Burn and associates⁸³ pointed out that glycosuria disappears in rats which have been rendered diabetic with alloxan when a high-fat diet (80 to 90%) is substituted for a normal diet containing 10% of fat. Janes and Prosser⁸⁴ confirmed these findings in the case of twenty adult male diabetic rats. It was found that, when the amount of dietary fat was increased progressively at weekly intervals for as long as the rats could tolerate the diet, or until the 60% level of fat in the diet was reached, the sugar levels in the blood and urine remained low, and most of the animals gained weight. In fact, when ten of these animals were again given the basic diet for three weeks and the severity of the diabetes was compared with that of the original basic period, it was found that in 50% of the cases there was some improvement in the diabetic symptoms. The weight gain

⁸⁰ J. Markowitz and S. Soskin, *Proc. Soc. Exptl. Biol. Med.*, 25, 7-8 (1927).

⁸¹ P. D. McMaster and D. R. Drury, *J. Exptl. Med.*, 49, 745-758 (1929).

⁸² A. M. Brues, D. R. Drury, and M. C. Brues, *Arch. Pathol.*, 22, 658-673 (1936).

⁸³ J. H. Burn, T. H. C. Lewis, and F. D. Kelsey, *Brit. Med. J.*, 1944, II, 752-754.

⁸⁴ R. G. Janes and M. Prosser, *Am. J. Physiol.*, 151, 581-587 (1947).

is believed by these investigators to be caused by an increase in the deposition of fat. Similar effects were reported by Bornstein and Nelson⁸⁵ for rats suffering from alloxan diabetes. These workers observed that diabetic rats were able to utilize fat and to convert it to glycogen, regardless of the severity of the diabetes produced by alloxan.

Reports in the literature indicate that the nature of the fat has some effect upon its ability to combat the diabetogenic action of alloxan. Angelico and Galamini Ligori⁸⁶ found that rabbits on diets supplemented with olive oil were most sensitive toward alloxan. These animals developed disturbances of the nervous system within eight days after the initiation of the oil supplementation. In some cases, complete paralysis of the hind quarters ensued. It was found that alloxan was more toxic for rabbits fed on a high-fat diet than for those on normal regimens. In fact, alloxan administered to rabbits on a standard diet proved toxic and even lethal when the animals had previously received a high-fat diet (olive oil).⁸⁷

Houssay and Martínez⁸⁸ pointed out the variation in sensitivity to alloxan and to the development of pancreatic diabetes in rats as influenced by different diets. They found that the toxic and diabetogenic action increased in rats fed a low-protein diet, and to an even greater degree when large amounts of lard or ox fat were given.⁸⁸⁻⁹⁰ Methionine, thiamine or thiouracil⁹¹ added to the diets decreased the toxicity of the alloxan, and complete protection was afforded when a diet high in coconut oil was employed.⁸⁹ In pancreatic diabetes, Martínez⁹² found that, in general, a high carbohydrate diet afforded better protection than did a diet rich in fat. The susceptibility of partially pancreatectomized rats on a high-lard diet was especially marked in the over-fed group, and in the case of rats which received one large feeding per day; it was less notable when the animals were fed smaller amounts in three installments.⁸⁸ Rodríguez and Krehl⁹³ confirmed this finding in alloxan diabetes, and also found that coconut oil decreased the incidence of the condition.

Pancreatectomized rats, receiving minimal doses of insulin, were forced a diet containing large amounts of corn oil and smaller proportions of

⁸⁵ J. Bornstein and J. F. Nelson, *Med. J. Australia*, 1949, I, 121-126.

⁸⁶ R. Angelico and M. Galamini Ligori, *Bull. soc. chim. biol.*, 31, 536-539 (1949).

⁸⁷ R. Angelico and M. Galamini Ligori, *Bull. soc. chim. biol.*, 31, 540-543 (1949).

⁸⁸ B. A. Houssay and C. Martínez, *Science*, 105, 548-549 (1947).

⁸⁹ B. A. Houssay and C. Martínez, *Rev. soc. argentina biol.*, 24, 242-248 (1948).

⁹⁰ C. Martínez, *Rev. soc. argentina biol.*, 21, 332-337 (1945).

⁹¹ C. Martínez, *Acta Physiol. Latinoamer.*, 1, Part II, 135-162 (1951).

⁹² C. Martínez, *Rev. soc. argentina biol.*, 22, 414-425 (1946).

⁹³ R. R. Rodríguez and W. A. Krehl, *Federation Proc.*, 10, 391-392 (1951).

wheat germ oil and cod liver oil, in conjunction with propyl thiouracil. A definite remission of the glycosuria was noted, without the loss of body weight which might otherwise be expected.⁹⁴ However, the protective effect of thiouracil, of propyl thiouracil, of thyroidectomy, and also of estradiol benzoate, against pancreatic and alloxan diabetes was noted even when the animals were fed *ad libitum* on diets containing only small amounts of these fats.⁹⁵⁻⁹⁷

In the case of the lard-fed animals, the addition of choline or of sulfonamide to the diet, or an increase in the protein ration, did not reduce the toxicity. When the rats were fed diets high in olive oil or butter, the effect of alloxan was not modified, but there was a slight decrease in mortality when oleomargarine or corn oil was given, and the mortality was zero when coconut oil (34%) was fed.⁸⁸ Houssay⁹⁸ found that, in human subjects, as well as in animals, "certain fats" (which he did not identify) favored the development of alloxan and pancreatic diabetes, while others inhibited it. In general, a previous diet poor in carbohydrates and rich in fats decreased the utilization of the carbohydrates, and the response to insulin.⁹⁸

It would appear that, when short-chain fatty acids are fed, such as those which occur in coconut oil, an improvement in alloxan diabetes is to be expected, while no change is noted, or the symptoms are more severe, when the usual fats containing long-chain fatty acids are included in the diet. Later reports from the South American laboratory indicated that the higher the degree of unsaturation of the fatty acids in the various fats and oils, the higher was the incidence of diabetes.⁹⁹⁻¹⁰² Rodríguez *et al.*¹⁰² noted, for example, that diabetes occurred in from 0 to 12% of rats fed alloxan (40 mg./kg. body weight) in conjunction with caprylic + capric acid esters (0%), palmitic + stearic acid (12%), and palmitic + stearic acid esters (5%), while the incidence increased up to 100% in the case of animals receiving alloxan (160 mg./kg.) with fats containing fatty acids

⁹⁴ C. Martínez, *Rev. soc. argentina biol.*, 27, 14-18 (1951).

⁹⁵ R. R. Rodríguez and C. Martínez, *Rev. soc. argentina biol.*, 26, 127-134 (1950).

⁹⁶ C. Martínez, *Rev. soc. argentina biol.*, 22, 135-146 (1946).

⁹⁷ B. A. Houssay, V. G. Foglia, H. P. Diaz, and J. G. Sara, *Rev. soc. argentina biol.*, 21, 232-242 (1945).

⁹⁸ B. A. Houssay, *Rev. asoc. méd. argentina*, 64, 1-19 (1950).

⁹⁹ R. R. Rodríguez, P. Cattaneo, B. A. Houssay, and B. Uno, *Rev. soc. argentina biol.*, 28, 224-230 (1952).

¹⁰⁰ R. R. Rodríguez and W. A. Krehl, *Am. J. Physiol.*, 169, 295-300 (1952).

¹⁰¹ R. R. Rodríguez, personal communication cited by L. F. Leloir and C. E. Cardini, *Ann. Rev. Biochem.*, 22, 179-210 (1953), p. 202.

¹⁰² R. R. Rodríguez, P. Cattaneo, B. A. Houssay, and B. Uno, *J. Nutrition*, 51, 441-448 (1953).

with increasing iodine numbers such as coconut oil (29%), lard (75%), oleic acid (80%), and corn oil (100%). When sunflower seed oil was partially hydrogenated, the pronounced toxic and diabetogenic action was reduced.¹⁰² It was concluded that a marked and direct correlation exists between the iodine number of the fat fed and the diabetogenic effect of the alloxan. In general, high-fat diets containing primarily saturated fatty acids had a marked protective action against the toxic and diabetogenic effects of alloxan, as compared with high carbohydrate diets.¹⁰² György and Rose¹⁰³ found that, in rats injected with alloxan, a high early mortality occurred when the rations contained large amounts of lard or coconut oil. The toxicity of the alloxan in these cases could be reduced by supplementing the diet with tocopherol or with yeast. The condition was not alleviated by supplements of methionine or choline, or by a higher proportion of protein in the diet.

However, the beneficial effect of certain fats against alloxan diabetes is not specific, as many other compounds have been shown to fall in the same category.^{104,105}

In the case of rats on a high-lard diet (38%) the survival rate at seven days after injection was raised from 10% for those receiving alloxan alone to 88% for those given boric acid as well; in the group receiving a low-fat diet, containing 3 drops of corn oil and 3 drops percomorph oil per day, the survival rate at seven days was 80% for those receiving alloxan alone and 100% for those given alloxan + boric acid.¹⁰⁶

f. Thiamine Deficiency. It was discovered as early as 1929 by Evans and Lepkovsky¹ that fat exhibits a sparing action on thiamine (at that time called the "antineuritic vitamin B"). It was later found by Evans, Lepkovsky, and Murphy¹⁰⁷ that fats did not behave similarly in sparing riboflavin. In a further study of this phenomenon, Banerji¹⁰⁸ demonstrated that rats excreted a smaller amount of bisulfite-binding substances in their urine on a vitamin B₁-deficient diet, and that the presence of high fat alleviated the severity of the brachycardia. It is therefore evident that the thiamine-sparing action of fat is not limited to its effect in protecting such animals against loss of weight or the development of the

¹⁰³ P. György and C. S. Rose, *Science*, **108**, 716-718 (1948).

¹⁰⁴ J. S. Dunn, J. Kirkpatrick, N. G. B. McLetchie, and S. V. Telfer, *J. Pathol. Bacteriol.*, **55**, 245-257 (1943).

¹⁰⁵ S. Banerjee, *Science*, **106**, 128-130 (1947).

¹⁰⁶ C. S. Rose and P. György, *Proc. Soc. Exptl. Biol. Med.*, **70**, 476-749 (1949).

¹⁰⁷ H. M. Evans, S. Lepkovsky, and E. A. Murphy, *J. Biol. Chem.*, **107**, 443-447 (1934).

¹⁰⁸ G. G. Banerji, *Biochem. J.*, **34**, 1329-1333 (1940).

prominent symptoms of deficiency, but that it is also demonstrated in the absence of these two characteristic signs of the avitaminosis. On the other hand, although rats can thrive on a diet free from vitamin B₁ if it contains a large proportion of fat or of protein, the oxidation of pyruvate is nevertheless defective, as demonstrated by Banerji and Yudkin¹⁰⁹ with *in vitro* tests of kidney slices. The defective utilization of pyruvate could be corrected by the addition of vitamin B₁ to the respiring tissue. The defect was present in all animals deprived of the vitamin, irrespective of the presence or absence of other signs of deficiency. According to De Caro and Rindi,¹¹⁰ the hyperpyruvemia of animals suffering from avitaminosis B₁ drops to the normal amount six hours after the feeding of fat (olive oil). It is believed that this occurs because, under these conditions, the organism metabolizes fats in preference to carbohydrates and proteins; the latter foodstuffs give rise to pyruvic acid. In a further investigation of the interrelations of thiamine and fat, Krider and Guerrant¹¹¹ found that the increase in body weight of rats varied directly with the fat content of the diet, and was not affected by the amount of thiamine fed when the intake of the latter vitamin was 2 mg. or more per day. Krider and Guerrant¹¹¹ reported that the increase in weight was due primarily to deposited fat. According to Waisman and colleagues,¹¹² the resistance of thiamine-deficient mice to infection with Lansing poliomyelitis or Theiler's encephalomyelitis virus can be explained only in part by the accumulation of pyruvate or of other metabolites from incomplete carbohydrate breakdown. The addition of 4% of sodium pyruvate to a complete diet containing thiamine decreased the incidence of paralysis, but not to such an extent as occurred in thiamine-deficient rats. It was found that the substitution of high-fat diets to prevent the formation of pyruvic acid was not wholly effective, because fat apparently has little sparing action on thiamine in the case of mice.

g. Bacterial Action. Since insufficient data are available concerning the effects of lipids on microorganisms, the author preferred to discuss the papers on the subject in more or less chronological order, rather than to construct what could only be an incomplete system.¹¹³ Fats, and es-

¹⁰⁹ G. G. Banerji and J. Yudkin, *Biochem. J.*, **36**, 530-541 (1942).

¹¹⁰ L. De Caro and G. Rindi, *Nature*, **167**, 114-115 (1951).

¹¹¹ M. M. Krider and N. B. Guerrant, *J. Nutrition*, **41**, 115-126 (1950).

¹¹² H. A. Waisman, H. C. Lichstein, and P. F. Clark, *Arch. Biochem.*, **8**, 203-210 (1945).

¹¹³ Nomenclature in bacteriology is rather confused, since there are so many synonyms for one organism. Insofar as possible the author has followed the system of nomenclature used by D. H. Bergey, *Manual of Determinative Bacteriology*, 6th ed., Williams & Wilkins, Baltimore, 1948.

pecially fatty acids, inhibit the growth of certain bacteria, especially that of the tubercle bacillus. Thus, Hedgecock¹¹⁴ showed that the administration of a non-lipid, casein-supplemented ration to resistant albino mice retarded the progress of experimental mouse tuberculosis. The condition was also retarded when 20% of the total fatty acids of coconut oil were added to the dietary mixture. On the other hand, olive oil, linseed oil, and oleic acid appeared to aggravate the experimental tuberculosis when these compounds were added to the ration to the extent of 20%. Hänel and Piller¹¹⁵ reported that dietary fats in general tend to alleviate tuberculosis in man. However, cod liver oil apparently has a superior therapeutic action. It is suggested that the unsaturated fatty acids in cod liver oil account for this favorable action upon tuberculosis, as it was demonstrated that these acids, diluted 1 to 50,000, will retard the growth of tubercle bacilli *in vitro*. On the other hand, Miura¹¹⁶ reported, on the basis of examination of the action of sixty-four fatty acid derivatives against *Mycobacterium avium* that the most effective derivative was α -ethylmyristic acid; α -phenylmyristic acid was effective only *in vitro*, and had no therapeutic value in mice. Among the alkyl fatty acids having eight to eighteen carbon atoms, the C₁₄ acid (myristic), had the greatest antibacterial action. In the C₁₈ series, the unsaturated acids were found to be more effective than the saturated forms. Of the unsaturated acids, the *cis* forms are more active than are the *trans* isomers. Free acids have greater activity than do the esters. Of the branched-chain acids, those with the ethyl group are the most potent.¹¹⁶ Dubos¹¹⁷ reported that the short-chain aliphatic acids such as acetic, propionic, butyric, caproic, caprylic, and capric acids exerted a marked bacteriostatic effect upon mammalian tubercle bacilli *in vitro*. Lactic acid in low concentrations also inhibited growth. However, keto and dicarboxylic acids exhibited no inhibitory action. More recently (1952) Weitzel¹¹⁸ reported that there was no relation between the tuberculobacteriostatic action of the total fatty acids and their iodine values. The most pronounced antibacterial action was found in the case of the pure C₁₈ acids with two double bonds, especially when the bonds were conjugated. Acids with three double bonds, however, did not exert any stronger antibacterial action than did those with two double bonds.

¹¹⁴ L. W. Hedgecock, *Proc. Soc. Exptl. Biol. Med.*, **68**, 106-110 (1948).

¹¹⁵ F. Hänel and S. Piller, *Beitr. Klin. Tuberk.*, **103**, 239-244 (1950).

¹¹⁶ Y. Miura, *J. Biochem. (Japan)*, **37**, 387-395 (1950).

¹¹⁷ R. J. Dubos, *J. Exptl. Med.*, **92**, 319-332 (1950).

¹¹⁸ G. Weitzel, *Z. physiol. Chem.*, **290**, 252-265 (1952).

McKee and co-workers¹¹⁹ demonstrated that a mixture of fatty acids could be obtained from the bacteria-free cultures of the ciliated protozoon *Tetrahymena geleii*. The fatty acids in these lysates consisted of an approximately fifty-fifty mixture of saturated and unsaturated fatty acids, with a fairly high proportion of myristic acid. Partially purified preparations were active *in vitro* against pneumococci, streptococci, mycobacteria, and staphylococci, but not against Gram-negative bacilli. Their activity was greatly reduced in the presence of blood or of serum, and could not be demonstrated in mice infected with mycobacteria or pneumococci. Costilow and Speck¹²⁰ demonstrated that caprylic (C₈), capric (C₁₀) and lauric (C₁₂) acids inhibit the growth of *Streptococcus lactis*, usually present in rancid milk; the degree of inhibition varies directly with the concentration of the acid. It would thus appear that several components of the fatty acid structure are of importance in inhibiting the growth of certain types of bacteria. Properties such as chain length, degree of unsaturation, and the presence of side chains all may affect the ability to inhibit bacterial growth.

Hardwick *et al.*¹²¹ observed that volatile and non-volatile fatty acids from commercial peptone inhibited sporulation of aerobic bacilli in a synthetic medium, in concentrations not inhibitory to growth. The non-volatile fraction was the more active. Saturated fatty acids such as capric, lauric, tridecylic, and myristic acids were especially active in antispore formation.

By means of a Warburg respirometer, Franke and Schillinger¹²² tested a number of acid-fast bacteria, including *Mycobacterium rubrum*, *M. phlei* ("timothy bacillus"), *M. lacticola*, *M. avium*, and also several cocci and bacilli, and parasitic fungi, including *Actinomyces chromogenes*. They observed inhibition of the growth of these organisms by both saturated and unsaturated fatty acids and dicarboxylic acids. The respiratory activity was found to decrease with the degree of resistance to acid of these acid-fast organisms. It was lowest for tubercle bacilli. The respiratory inhibition by saturated C₁₀ to C₁₄ acids and by higher unsaturated acids was related to the number of double bonds.

According to Drea,¹²³ the growth of human tubercle bacilli was inhibited to a notable extent by C₁₂, C₁₄, and C₁₆ straight-chain acids. The anti-

¹¹⁹ C. M. McKee, J. D. Dutcher, V. Groupé, and M. Moore, *Proc. Soc. Exptl. Biol. Med.*, **65**, 326-332 (1947).

¹²⁰ R. N. Costilow and M. L. Speck, *J. Dairy Sci.*, **34**, 1104-1110 (1951).

¹²¹ W. A. Hardwick, B. Guirard, and J. W. Foster, *J. Bacteriol.*, **61**, 145-151 (1951).

¹²² W. Franke and A. Schillinger, *Biochem. Z.*, **316**, 313-334 (1944).

¹²³ W. F. Drea, *J. Bacteriol.*, **48**, 547-553 (1944).

bacterial effect of saturated fatty acids was most pronounced in the case of myristic (C_{14}) and palmitic (C_{16}), while stearic acid (C_{18}) had a somewhat less notable action, and lauric acid (C_{12}) was less inhibitory. The acids with one to five carbon atoms had little or no growth-inhibitory effects. Chaulmoogric acid, which is an unsaturated, straight-chain C_{13} acid, had the same effect as did myristic acid. In a later report, Drea¹²⁴ stated that octyl, decyl, undecyl, dodecyl, and tetradecyl-chain resorcinols inhibited the growth of human tubercle bacilli (strain H 37) to a greater extent than did the corresponding fatty acids or resorcinol itself. Tetradecyl resorcinol had the same effect as did myristic acid in inhibiting growth.

Dubos¹²⁵ confirmed the fact that long-chain fatty acids exert a toxic effect upon a number of microorganisms, especially the Gram-positive and acid-fast species, and that their toxicity for the bacteria is directly related to the number of unsaturated bonds in the molecule. All the fatty acids tested (capric, lauric, myristic, palmitic, stearic, oleic, ricinoleic, linoleic, linolenic, arachidonic) exerted a bacteriostatic and bactericidal effect on micrococci and on tubercle bacilli in protein-free media. Stearic acid was the least toxic, whereas the unsaturated oleic, ricinoleic, linoleic, linolenic, and arachidonic acids had the greatest bacteriostatic and bactericidal action. Human tubercle bacilli were the most sensitive of the organisms tested. Inhibition of growth of small inocula of these organisms in synthetic media could be detected in concentrations of 0.00001 to 0.0001% of the fatty acids. Avian tubercle bacilli were ten times less sensitive, and the *Micrococcus* spp. culture was much more resistant. None of the esters was significantly toxic for *Micrococcus* cultures. The water-soluble esters of lauric acid and of palmitic acid (Tween 20 (polyoxyethylene sorbitan monolaurate), G 2144, and Tween 40 (polyoxyethylene sorbitan monopalmitate)) exhibited appreciable bacteriostatic and bactericidal activity against tubercle bacilli in concentrations of 0.01 to 0.001%, but esters of stearic and oleic acid (Tween 60 (polyoxyethylene sorbitan monostearate) and Tween 80 (PSM, polyoxyethylene sorbitan monooleate)) were inhibitory only in higher concentrations. In all cases the toxicity was decreased or abolished by the addition of crystalline serum albumin (0.1 to 0.5%), blood plasma, or glucose. Oleic acid acted as a growth factor for *Corynebacterium diphtheriae* and for *Clostridium tetani* in concentrations of 1 to 50 μ g./ml. of medium. Metabolic studies showed

¹²⁴ W. F. Drea, *J. Bacteriol.*, **51**, 507-511 (1946).

¹²⁵ R. J. Dubos, *J. Exptl. Med.*, **85**, 9-22 (1947).

that unsaturated acids in general, especially linolenic acid, inhibited the oxygen uptake by tubercle bacilli.

Although Tween 80 promotes the growth of tubercle bacilli, it inhibits small inocula, because of its content of unesterified oleic acid. Bovine serum albumin neutralizes the inhibition by tightly binding the fatty acid; it also contains a trace of lipase, which releases enough fatty acid to equal the binding capacity of the albumin. Growth from very small inocula (two to three cells) is possible only when hydrolysis of the Tween by this lipase is prevented.^{126,127}

Davis and Dubos¹²⁷ suggested that the lipolytic activity accounts for the bacteriostatic effect, in a medium containing horse serum and a culture filtrate of *Mycobacterium phlei*.

Stanley and Adams¹²⁸ found that, of a large number of aliphatic acids which were bactericidal *in vitro* for *Mycobacterium leprae*, all the bactericidally effective compounds markedly depressed surface tension, and had a molecular weight of approximately 256 (C₁₆ acids). However, this is not the sole criterion, inasmuch as α -bromopalmitic acid, the total acids of cod liver oil, and some C₁₉ and higher acids were good surface depressors, but were relatively non-effective as bactericides. However, with these exceptions, surface tension paralleled bactericidal effectiveness.

Spector¹²⁹ observed that the fatty acids of butterfat and of corn oil exerted varying degrees of inhibitory action up on the metabolism of Escherich's intestinal bacillus (*Escherichia coli*), *Lactobacillus casei*, and *L. arabinosus*, depending upon the concentrations employed, and upon the level of the vitamin or amino acid also affecting growth.

On the other hand, Williams *et al.*^{130,131} found that oleic acid, detoxified with Tween 40, was required for the growth of *Lactobacillus bulgaricus*. Williams and Fieger¹³² were able to stimulate the growth of *Lactobacillus casei* and *L. arabinosus* by the addition of oleic and linoleic acid to the medium, provided biotin-free bovine serum albumin was also added. Oleic acid is so toxic for the lactic acid bacilli that its growth-promoting action can be observed only within a narrow range of concentrations and pH, and in the absence of biotin.¹³¹ The addition of Tween 40 greatly

¹²⁶ B. D. Davis and R. J. Dubos, *Arch. Biochem.*, **11**, 201-203 (1946).

¹²⁷ B. D. Davis and R. J. Dubos, *J. Bacteriol.*, **55**, 11-23 (1948).

¹²⁸ W. M. Stanley and R. Adams, *J. Am. Chem. Soc.*, **54**, 1548-1557 (1932).

¹²⁹ H. Spector, *Arch. Biochem.*, **11**, 167-177 (1946).

¹³⁰ W. L. Williams, E. Hoff-Jørgensen, and E. E. Snell, *J. Biol. Chem.*, **177**, 933-940 (1949).

¹³¹ W. L. Williams, H. P. Broquist, and E. E. Snell, *J. Biol. Chem.*, **170**, 619-630 (1947).

¹³² V. R. Williams and E. A. Fieger, *J. Biol. Chem.*, **177**, 739-744 (1949).

extends the pH range over which the activity of oleic acid is observed. Tween 80, in adequate amounts, has the same effect as does Tween 40 with the addition of oleic acid.¹³¹

Kodicek and Worden¹³³ observed that the inhibitory effects of oleic, linoleic, and linolenic acids for *Lactobacillus helveticus* could be prevented by the addition of several compounds, such as lecithin, cholesterol, calciferol, lumisterol, α -tocopherol, α -tocopherol acetate, and sometimes even calcium. The toxicity of oleic acid for *Erysipelothrix rhusiopathiae* was counteracted by saponin and by bile salts.¹³⁴ The bacteriostatic action of linoleic acid was also noted in the case of other Gram-positive bacteria, such as *Streptococcus agalactiae*, *Micrococcus pyogenes*, var. *albus*, *Bacillus anthracis*, and *Listeria monocytogenes*. It was not observed in the case of Gram-negative organisms such as *Proteus vulgaris* or *Escherichia coli*. Dubos and Davis^{135,136} reported that growth of the tubercle bacillus (*Mycobacterium tuberculosis*), was stimulated by long-chain fatty acids only if these acids were first esterified or bound in some manner to serum albumin. This was especially true of oleic acid.

According to Hedgecock,¹³⁷ the dietary administration of a mixture of fatty acid esters in proportions simulating those found in coconut oil, and consisting of methyl laurate, methyl myristate, methyl palmitate, methyl stearate, methyl oleate, and methyl linoleate, increased the resistance of mice to infection with *Mycobacterium tuberculosis*. Methyl linoleate alone, and lard alone, did not have this antibacterial effect. The resistance was greatest if the animals had also received rations with 20% protein. The increase in resistance may be due to: (1) humoral factors in the immunity reaction of the host, (2) alterations in the biochemical environment in the tissues of the host which are unfavorable to proliferation of the organism, (3) direct inhibition of *Mycobacterium tuberculosis* by the fatty acids. Hedgecock¹¹⁴ had previously found that mice on a synthetic diet containing coconut oil were more resistant to tuberculosis than were those which had received olive or linseed oil. Dubos¹³⁸ found that dietary peanut oil increased the resistance of mice to the tubercle bacillus, and he attributed this bacteriostatic action to the effect of the oil on the intermediary metabolism.

The concentration of fatty acids in *in vitro* studies of microorganisms

¹³³ E. Kodicek and A. W. Worden, *Biochem. J.*, **39**, 78-85 (1945).

¹³⁴ S. H. Hutner, *J. Bacteriol.*, **43**, 629-640 (1942).

¹³⁵ R. J. Dubos and B. D. Davis, *J. Exptl. Med.*, **83**, 409-423 (1946).

¹³⁶ B. D. Davis and R. J. Dubos, *J. Exptl. Med.*, **86**, 215-228 (1947).

¹³⁷ L. W. Hedgecock, *J. Bacteriol.*, **70**, 415-419 (1955).

¹³⁸ R. J. Dubos, *J. Exptl. Med.*, **101**, 59-84 (1955).

affects the reaction. For example, Kidder and Dewey¹³⁹ found that propionate had a slight stimulatory effect upon the growth of the ciliate *Tetrahymena geleii* in low concentration, but was inhibitory at higher concentrations, while butyrate was never stimulatory at any concentration, and rapidly became inhibitory. Lauric acid also inhibited growth, as did all the higher fatty acids tested (oleic, linoleic, linolenic, arachidonic, vaccenic), and a C₂₀ unsaturated fatty acid. Concentrations as low as 30 to 70 µg./ml. were sufficient to suppress growth completely.

Trager¹⁴⁰ studied chickens and ducks experimentally infected with avian malaria (*Plasmodium lophurae* and *P. cathemerium*). When *P. lophurae* was cultured *in vitro* in suspensions of duck erythrocytes, plasma protein fractions rich in a fat-soluble factor resembling biotin inhibited multiplication, while comparable concentrations of plasma fractions poor in the fat-soluble factor did not. The lipid substance in the plasma which yields this unidentified fat-soluble factor is considered to be directly concerned in resistance to avian malaria. Biotin deficiency produced by synthetic, biotin-deficient diets decreased the resistance of chickens to *P. lophurae* and *P. cathemerium*.

Eggerth¹⁴¹ tested the action of sulfur-containing soaps (α -mercapto and α -disulfo soaps prepared from caprylic, capric, lauric, myristic, palmitic, and stearic acids) against *Diplococcus pneumoniae*, *Streptococcus pyogenes*, *Corynebacterium diphtheriae*, *Micrococcus pyogenes* var. *aureus*, *Pasteurella multocida*, and *Brucella melitensis* (Malta fever) and other organisms. He found that the germicidal titers of the acids increased rapidly with the length of the chain until a maximum was reached, and then decreased. The most active soap in the series was not always the same for the various organisms. At a pH of 7.5, in the α -mercapto series, mercaptolaurate was most active against *Micrococcus ovalis* and *Pseudomonas aeruginosa*. Mercaptolaurate and mercaptomyristate were equally active against *Corynebacterium diphtheriae*, *Pasteurella multocida*, *Brucella melitensis*, *Vibrio comma*, and *Salmonella typhosa* (typhoid fever organism). Mercaptomyristate was most potent against *Diplococcus pneumoniae*, *Streptococcus pyogenes*, and *Micrococcus pyogenes* var. *aureus*. In the disulfo series, dicaprinate was the most germicidal for *Pasteurella multocida*, *Vibrio comma*, and *Salmonella typhosa*. Dilaurate was most effective against *Corynebacterium diphtheriae*, *Micrococcus pyogenes* var. *aureus*, *Micrococcus ovalis*,

¹³⁹ G. W. Kidder and V. C. Dewey, *The Biochemistry of Ciliates in Pure Culture*, in A. Lwoff, *The Biochemistry and Physiology of Protozoa*, Vol. I, Academic Press, New York, 1951, pp. 323-400 (p. 337).

¹⁴⁰ W. Trager, *J. Exptl. Med.*, 85, 663-683 (1947).

¹⁴¹ A. H. Eggerth, *J. Exptl. Med.*, 53, 27-42 (1931).

and *Brucella melitensis*. Dimyristate had the greatest bactericidal effect against *Diplococcus pneumoniae* and *Streptococcus pyogenes*.

According to Friedewald,¹⁴² the immunizing effect of a PRS strain of influenza vaccine was enhanced by the addition of killed acid-fast *Mycobacterium butyricum* (from milk and butter) suspended in sesame oil. The influenza virus induced four times as much antibody formation when the *M. butyricum* was given in sesame oil as when it was suspended in saline alone or in sesame oil in combination with killed typhoid bacilli.

In 1908 Landsteiner and Ehrlich¹⁴³ noted the strong bactericidal action of aqueous organic extracts of animal fats on *Bacillus anthracis* and on other bacteria. They found that serum and bone-marrow fat acted as bacteriolysin, and suggested that the bactericidal combination of protein + lipid acts in a similar manner as amboceptor and complement. The complements in the blood serum are probably lipoids. The importance of this possibility in immunity is suggested. In this connection, Eagle,¹⁴⁴ in his studies of the serology of syphilis, discusses the complement-fixing properties of the lipid-reagin precipitate. A film of normal serum protein does not interfere with the specific reaction between antigen particles and subsequently added syphilitic serum (reagin). The protein is only loosely bound, and does not obscure the specific reacting groups of the lipid. However, the lipid-reagin precipitate incubated with very strongly positive syphilitic serum does not give any further reaction; the first incubation has covered the reacting groups of the lipid with closely adherent reagin-globulin, so firmly bound as not to allow combination with more reagin, until the protein film has been destroyed by heating.

M. Lwoff¹⁴⁵ found that certain bacteria, for example *Hemophilus pertussis* (found in whooping cough), produce fatty acids which inhibit their own growth and that of other bacteria. Cholesterol may neutralize these acids, as it is essential to the growth of the organism. Very low concentrations of oleic acid (0.01 to 0.03 mg./ml.) stimulate *Entameba terrapinae* of reptiles, but the acid has a toxic effect at high concentrations. A mixture of oleic acid and cholesterol does not permit multiplication of the organism. Von Brand¹⁴⁶ found that, although the halogenated acetic acids

¹⁴² W. F. Friedewald, *J. Exptl. Med.*, 80, 477-490 (1944).

¹⁴³ K. Landsteiner and H. Ehrlich, *Centr. Parasitenk.*, 45, Abt. I, 247-257 (1908).

¹⁴⁴ H. Eagle, *J. Exptl. Med.*, 52, 717-738 (1930).

¹⁴⁵ M. Lwoff, *Nutrition of Parasitic Amebae*, in A. Lwoff, *Biochemistry and Physiology of Protozoa*, Vol. I, Academic Press, New York, 1951, pp. 235-250.

¹⁴⁶ T. von Brand, *Metabolism of Trypanosomidae and Bodonidae*, in A. Lwoff, *Biochemistry and Physiology of Protozoa*, Vol. I, Academic Press, New York, 1951, pp. 177-234.

proved too toxic to the host for practical use, they were highly effective in reducing or eliminating African pathogenic trypanosomes from the blood stream of experimental animals.

The interrelation of fats and microorganisms has several aspects. In contradistinction to the bactericidal effect of fats and fatty acids, a number of workers have observed synthesis of fatty acids by bacteria and fungi, and also stimulation of bacterial growth by certain fatty esters. The degree of concentration is sometimes the determining factor, and growth conditions also have a bearing. For example, while Rittenberg *et al.*¹⁴⁷ observed the anaerobic toxicity of certain fatty acids for *Escherichia coli*, these workers¹⁴⁸ also found that aerobic bacteria have the ability to oxidize C₉ and C₁₀ fatty acids. Glucose-grown cells of *Serratia marcescens* (also called *Erythrobacillus prodigiosus*), a non-pathogenic bacterial organism which produces a red pigment, is able to oxidize all straight-chain saturated fatty acids from acetic through myristic, with the exception of propionic and valeric acids.

Hesse¹⁴⁹ reviewed the findings of a number of workers who observed the synthesis of fat by various yeasts and penicillia (fungi), including among many others the ascosporogenous yeast (*Endomyces vernalis*), the milk mold (*Oidium lactis*), a mold from decaying vegetable matter (*Aspergillus fischeri* (*fumigatus*)), the non-pathogenic fungus from feces (*Mucor mucedo*), the yeast-like fungus (*Torula utilis*), which forms fat rapidly from alcohol, the ascomycetous fungus from brewer's yeast (*Saccharomyces cerevisiae*), the pigment-producing fungus (*Rhodotorula glutinis*), which forms a reddish pigment resembling carotene, the plant pathogen mold causing flax blight (*Fusarium lini*), etc. The fat produced by these microorganisms contains both saturated and unsaturated fatty acids (palmitic, stearic, oleic, linoleic, linolenic); oleic acid is the main constituent.

Ramakrishnan and Banerjee¹⁵⁰ found that the molds grown on sesame (*Sesamum indicum*) and on coconut developed considerable lipolytic activity. The blue-green mold (*Penicillium chrysogenum* S₁) and the yellow-green variety (*Aspergillus flavus* S₆) grown on sesame oil, showed the greatest activity.

Goldman and Rayman¹⁵¹ studied the hydrolysis of fats by a saprophytic bacterium in baker's yeast (*Pseudomonas fluorescens*), named for the

¹⁴⁷ S. C. Rittenberg, J. H. Silliker, and M. E. Ward, *Bacteriol. Proc.*, 1950 *Abst.*, 145.

¹⁴⁸ J. H. Silliker and S. C. Rittenberg, *J. Bacteriol.*, 61, 653-659, 661-673 (1951).

¹⁴⁹ A. Hesse, *Advances in Enzymol.*, 9, 653-704 (1949).

¹⁵⁰ C. V. Ramakrishnan and B. N. Banerjee, *Arch. Biochem. Biophys.*, 37, 131-135 (1952).

¹⁵¹ W. L. Goldman and M. M. Rayman, *Food Research*, 17, 326-337 (1952).

fluorescent pigment which it produces, *P. oleovorans* (which forms fluorescent colonies on oil-water emulsions), and *P. fragi* (from milk and dairy products, characterized by its strawberry odor) which is strongly lipoclastic. *P. fluorescens* was found to be especially active in the hydrolysis of butter oil (90.7%); coconut oil and tallow were also strongly hydrolyzed (80.8%), and lard, soybean oil, and peanut oil were split to a lesser degree (74.3 to 79%). *P. fragi* also hydrolyzed butter oil to the extent of 75.2%. On the other hand, the bacterial growth rate in butter oil emulsion increased sharply up to the fifth day of incubation, from an initial count of 350 to 7000 million bacteria/ml. of culture, and then fell precipitously after the twenty-fifth day from 1300 to 35, and to 0.001 on the forty-second day. As the acidity of the culture increased, the number of viable cells declined.

Trypanosoma evansi (an animal microparasite, found in India, which causes "surra" disease of horses and mules), suspended in a buffered Ringer solution, is able to oxidize the sodium salts or methyl esters of the lower fatty acids, formic, acetic, propionic, butyric; the acids are split by both β - and ω -oxidation.¹⁵²

Fat is found in surface cultures of *Aspergillus nidulans* (a fungus causing white mycetozoa). The amounts of both saturated and unsaturated acids (oleic, linoleic, linolenic) increase up to the ninth day of incubation, and then gradually decrease.¹⁵³ Singh *et al.*¹⁵⁴ found that the proportion of saturated fatty acids produced by *Penicillium lilacinum* (soil mold forming rosy and violet colonies) was markedly greater than in most fats of microbiologic origin. Palmitic comprised 32.3% of the total fatty acids (75% of the total saturated acids), myristic, 0.1%; stearic, 9.4%; arachidic, 1.4%; hexadecenoic, 3.4%; oleic, 38.6%; linoleic, 13.4%; C₂₀ unsaturated acids, 1.4%.

h. Miscellaneous Stress Factors. Clayton and Baumann¹⁵⁵ reported that, when rats and mice were given 2,4-dinitrotoluene, growth was better and mortality was lower on a 30% fat diet than when the diet provided only 5% of fat. Beneficial effects of fat in minimizing the toxicity of DNT were noted with cottonseed oil, corn oil, Crisco, hydrogenated coconut oil, lard, and butterfat. It was suggested that the harmful effects of dinitrotoluene on the low-fat diets could be ascribed to the low caloric intake of the mice. Rancid cottonseed oil, on the other hand, was found to accentu-

¹⁵² M. Manozzi-Torini, *Arch. sci. biol. (Italy)*, **26**, 565-580 (1940).

¹⁵³ J. Singh and T. K. Walker, *Biochem. J.*, **62**, 286-289 (1956).

¹⁵⁴ J. Singh, S. Shah, and T. K. Walker, *Biochem. J.*, **62**, 222-224 (1956).

¹⁵⁵ C. C. Clayton, and C. A. Baumann, *Arch. Biochem.*, **16**, 415-422 (1948).

ate the toxicity of this chemical. In the case of rats suffering hypothalamic injury, Lundbaek and Stevenson^{17a} observed that the weight gains were greater on the high-fat, non-carbohydrate diet than on high-carbohydrate diets.

Although normal growth was observed when a low-fat diet (0.5% corn oil) was fed to growing mice, a retardation of growth was noted when metabolic stresses such as certain intestinal bacteriostatic agents were added to the diet. The substances which produced this retardation in growth included succinylsulfathiazole, streptomycin and atabrine, as well as thyroid and triacetin.¹⁵⁶ The retardation in growth could be prevented if fatty acids or partially defatted cottonseed meal or water-extracted liver residue were included in the diet. The deleterious effect of the bacteriostatic agents is believed to be due to the fact that they interfere with the synthesis of long-chain fatty acids by the organism. It was suggested that cottonseed meal and liver contain a factor essential for the synthesis of fat by the animal.

A relationship has also been shown to exist between the duration of thiopental anesthesia and the amount of fat available. Thus, according to Hermann and Wood¹⁵⁷ a 5 g.% decrease in the body fat of a rat resulted in a 100% increase in the duration of thiopental anesthesia at a fixed thiopental dosage, or a 40% decrease in the thiopental required for a given period of anesthesia. In contrast to this, the anesthetic effect of pentobarbitol was not affected by the amount of body fat. It was also demonstrated by Anderson and Magee¹⁵⁸ that the ingestion of oil by rats preliminary to thiopental anesthesia decreased the period of anesthesia by about 50%. It is believed that this is to be explained by the production of hyperlipemia by the oil, with the resultant uptake of the circulating thiopental by the chylomicrons.

(9) *The Effect of Dietary Fat upon Galactose Absorption*

Schantz, Elvehjem, and Hart¹⁵⁹ first called attention to the fact that galactose appeared in the urine of rats and of other animals which were fed on a mineralized skim-milk diet. On the other hand, it was shown that the excretion of galactose did not occur when a diet consisting of whole milk was employed. Likewise, when 3 or 4% of fat (butter, lard, corn oil, coconut oil, linseed oil, palmitic and oleic acids) was added to skim

¹⁵⁶ D. K. Bosshardt and J. W. Huff, *J. Nutrition*, **50**, 117-127 (1953).

¹⁵⁷ G. Hermann and H. A. Wood, *Proc. Soc. Exptl. Biol. Med.*, **80**, 318-319 (1952).

¹⁵⁸ E. G. Anderson and D. F. Magee, *Proc. Soc. Exptl. Biol. Med.*, **83**, 110-111 (1953).

¹⁵⁹ E. J. Schantz, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.*, **122**, 381-390 (1938).

milk, the galactosuria could be completely prevented. These workers suggested that fat may play a role in the metabolism of lactose and of galactose other than by altering the rate of absorption. It was later shown that several different synthetic even-chain fatty acids containing twelve or more carbon atoms were equally effective in preventing the excretion of galactose in the urine, when fed with skim milk.¹⁶⁰ In contradistinction to this earlier work, Zialcita and Mitchell¹⁶¹ were unable to confirm the effect of fat as such on the metabolism of galactose. These workers conclude that there is no reason which would lead one to expect such an interrelationship. It was found by these latter investigators that corn oil, but not butterfat, was able to decrease galactosuria by about 27% on a diet containing 48% of lactose. It was believed by Zialcita and Mitchell that this effect may be due to some non-glyceride component present in the corn oil. In a later communication of the Wisconsin group, Geyer and co-workers¹⁶² extended the earlier work by employing skim milk and synthetic diets containing lactose or galactose. It was concluded that fat decreased the resultant galactosuria, and increased the utilization of galactose. These conflicting viewpoints of the several observers leave the question open as to whether the effect of fat on galactosuria originates primarily in the absorptive or in the metabolic phase of the utilization of galactose. Niefert and Deuel¹⁶³ attempted to throw some further light on this problem. Their data strongly support the hypothesis that fat affects primarily the absorption of galactose. It was suggested that the delayed evacuation of the stomach due to the presence of fat might slow down the rate at which lactose (or galactose) reaches the intestine; this would result in a lower galactosemia, with a resultant increase in the proportion of the sugar which is retained. When fat was fed at a level of 20% the relative effectiveness of butterfat, cottonseed oil and corn oil, respectively, in reducing the excretion of sugar in the urine was found to be identical. Although favoring the theory that fat acts upon the absorptive phase of galactose metabolism, Niefert and Deuel¹⁶³ leave the question open as to whether or not there is also some effect upon the metabolic phase. It was reported earlier from the laboratory of these investigators that galactose exerts a greater ketolytic effect than does glucose, in the case of both rats¹⁶⁴ and man.¹⁶⁵ Banerjee¹⁶⁶ found that, although the glycogen and fat

¹⁶⁰ E. J. Schantz and C. F. Krewson, *Proc. Soc. Exptl. Biol. Med.*, **42**, 577-579 (1939).

¹⁶¹ L. P. Zialcita, Jr., and H. H. Mitchell, *J. Nutrition*, **30**, 147-150 (1945).

¹⁶² R. P. Geyer, R. K. Boutwell, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.*, **162**, 251-259 (1946).

¹⁶³ M. L. Niefert and H. J. Deuel, Jr., *J. Biol. Chem.*, **167**, 521-525 (1947).

¹⁶⁴ J. S. Butts, *J. Biol. Chem.*, **105**, 87-96 (1934).

¹⁶⁵ H. J. Deuel, Jr., M. Gulick, and J. S. Butts, *J. Biol. Chem.*, **98**, 333-351 (1932).

¹⁶⁶ S. Banerjee, *Proc. Soc. Exptl. Biol. Med.*, **77**, 585-587 (1951).

content of the liver are significantly diminished in rats fed skim milk, the cerebroside content of the brain of rats fed skim milk or whole milk, respectively, did not differ significantly. Moreover, the urinary excretion of galactose by the skim-milk-fed rats might be due to the non-conversion of galactose into glycogen in the liver under such conditions. In contradistinction to rats, rabbits fed skim milk for two months did not excrete galactose in the urine.

(10) *The Effect of Dietary Fat upon Calcium Utilization*

It has become increasingly evident in recent years that an interrelationship exists between the utilization of calcium and that of fat. Thus, certain high-fat diets may decrease the utilization of calcium, whereas in an analogous manner high-calcium diets may reduce the utilization of fats. Givens¹⁶⁷ reported that, when the absorption of fats or of fatty acids was poor, the excretion of fecal calcium was increased. In fact, they found that the calcium storage was decreased to a great extent under such conditions, even when the calcium intake was kept at a comparatively high level. In investigating this condition Bosworth and co-workers¹⁶⁸ concluded that the presence and composition of calcium compounds in the intestine are the determining factors in soap formation, and that the amount of calcium ultimately excreted in the feces also depends upon the solubility of the soap formed. These workers suggested that calcium oleate is more readily soluble in bile than is calcium palmitate or calcium stearate. Therefore, when the fats consist largely of oleic acid, calcium would have a less depressing effect upon the absorption of the fatty acids than when palmitic and stearic acids were the predominant fatty acids. Boyd, Crumm, and Lyman¹⁶⁹ arrived at essentially similar conclusions on the basis of experiments on rats, although they found that palmitic acid calcium soaps were also well utilized. In a further investigation of this problem, Cheng, Morehouse, and Deuel¹⁷⁰ reported that, although the presence of calcium and magnesium in the diet did not influence the digestibility of low-melting fats, it markedly decreased that of the higher-melting simple triglycerides and hydrogenated fats. In the absence of calcium in the diet, the proportion of soap in the feces was markedly di-

¹⁶⁷ M. H. Givens, *J. Biol. Chem.*, **31**, 441-444 (1917).

¹⁶⁸ A. W. Bosworth, H. I. Bowditch, and L. A. Giblin, *Am. J. Diseases Children*, **15**, 397-407 (1918).

¹⁶⁹ O. F. Boyd, C. L. Crumm, and J. F. Lyman, *J. Biol. Chem.*, **95**, 29-41 (1932).

¹⁷⁰ A. L. S. Cheng, M. G. Morehouse, and H. J. Deuel, Jr., *J. Nutrition*, **37**, 237-250 (1949).

minished. Calcium and magnesium were shown to have a progressive effect. That is, the larger the amount present in the diet the greater the depression in digestibility of the fat and the larger the proportion of soap eliminated in the feces.

The proportion of calcium lost in the stool or, conversely, the amount of fatty acid which fails to be absorbed because it is excreted as a calcium soap, has also been shown to vary with the type of fat. Thus Steggerda and Mitchell¹⁷¹ found that, in the case of milk fat, the calcium metabolism was not affected by the proportion of butterfat present in the diet. On the other hand, when cocoa butter was employed,¹⁷² it was found that the average calcium utilization by rats was decreased from a level of 73.9% for a low-fat diet, or 72.1% for a 20% lard diet, to a value of only 57.6% for a 20% cocoa butter diet. It is believed that the inhibiting effect of cocoa butter on calcium utilization is in this case largely or entirely accounted for by the greater formation of insoluble calcium soaps.

Rao and De¹⁷³ reported that the inclusion of 10% of coconut oil containing both the saturated and the unsaturated fatty acid fraction in a normal diet increased the utilization of calcium in young growing rats. However, the excretion of calcium in the urine was only indirectly affected by the presence of fat in the diet. Thus, when the fecal excretion of calcium was considerably increased, there was a decrease in the urinary elimination of the metabolite.

(11) *The Effect of Dietary Fat upon Vitamin Requirements*

a. Thiamine and Fat. It was recognized by Evans and Lepkovsky¹⁷⁴ that, on diets adequate except for their content of vitamin B₁, the onset of polyneuritic symptoms was retarded on high-fat diets. On the other hand, when carbohydrate diets were employed, polyneuritis developed at an accelerated rate. It was found that many vegetable and animal fats were equally efficacious in retarding the onset of avitaminosis. Thus, lard, coconut oil, synthetic coconut oil, hydrogenated coconut oil, partially hydrogenated cottonseed oil (Crisco), cottonseed oil, and perilla oil were all effective.^{175, 176}

The only fat which did not possess a thiamine-sparing action was hydrogenated perilla oil.¹⁷⁶ This substance was poorly absorbed¹⁷⁶ and,

¹⁷¹ F. R. Steggerda and H. H. Mitchell, *J. Nutrition*, **45**, 201-211 (1951).

¹⁷² J. R. Beadles, H. H. Mitchell, and T. S. Hamilton, *J. Nutrition*, **45**, 399-405 (1951).

¹⁷³ M. N. Rao and S. S. De, *Ind. J. Med. Research*, **40**, 235-242 (1952).

¹⁷⁴ H. M. Evans and S. Lepkovsky, *Science*, **68**, 298 (1928).

¹⁷⁵ H. M. Evans and S. Lepkovsky, *J. Biol. Chem.*, **92**, 615-622 (1931).

¹⁷⁶ H. M. Evans and S. Lepkovsky, *J. Biol. Chem.*, **96**, 165-177 (1932).

presumably, was inactive, inasmuch as it did not contribute to the utilizable calories in the diet. In addition to digestibility, several other factors are important in relation to the sparing action of fat on thiamine. Unsaturation of the fat *per se* apparently does not play a role, except when a complete saturation of the fat is associated with such a high melting point as to render the fat unabsorbable, as was the case with hydrogenated perilla oil. Thus Evans and Lepkovsky¹⁷⁶ found that fats having an iodine number of only 8 spared thiamine as effectively as did an unsaturated oil with an iodine number of 187. Furthermore, saturated triglycerides such as tricaprylin and trimyristin were found to be very effective in sparing vitamin B₁, although Evans and Lepkovsky¹⁷⁷ found that tristearin was inactive. We would expect this to be the case, since it is now known that this triglyceride is practically unabsorbed by the rat.^{170,178}

Salmon and Goodman¹⁷⁹ demonstrated that the thiamine-sparing action of the synthetic esters varied according to the chain length of the fatty acids. The maximum vitamin-sparing action was found in the case of the C₈-fatty acid ester. The most obvious manner in which fat spares vitamin B₁ is by decreasing the oxidation of carbohydrate, whereby the production of pyruvic acid is markedly reduced.

Another method whereby fat may serve as a sparing agent for thiamine is by influencing the bacterial synthesis of thiamine in the gastrointestinal tract. Whipple and Church¹⁸⁰ demonstrated that the feces of rats contained some thiamine if lard was incorporated in the diet, even though a thiamine-deficient diet was fed. However, when the lard was omitted from the ration, the thiamine content of the stools dropped to 0. It has also been shown that, in the case of human subjects, the ingestion of carbohydrate decreases the excretion of thiamine in the urine. This would seem to indicate that the utilization of thiamine is greater under these conditions. A high-fat diet reduces the thiamine requirement in experimental animals,^{1,181,182} but the actual amount of thiamine ingested by human subjects under various dietary conditions is directly correlated with the urinary excretion of vitamin B₁, regardless of the caloric intake.¹⁸³ All the vitamin ingested is apparently absorbed from the gastrointestinal

¹⁷⁷ H. M. Evans and S. Lepkovsky, *J. Biol. Chem.*, **96**, 179-188 (1932).

¹⁷⁸ R. Hoagland and G. G. Snider, *J. Nutrition*, **26**, 219-225 (1943).

¹⁷⁹ W. D. Salmon and J. G. Goodman, *J. Nutrition*, **13**, 477-500 (1937).

¹⁸⁰ D. V. Whipple and C. F. Church, *J. Biol. Chem.*, **109**, xcvi-xcix (1935).

¹⁸¹ A. Arnold and C. A. Elvehjem, *Am. J. Physiol.*, **126**, 289-298 (1939).

¹⁸² F. E. Stirn, A. Arnold, and C. A. Elvehjem, *J. Nutrition*, **17**, 485-495 (1939).

¹⁸³ K. O'Shea Elsom, J. G. Reinhold, J. T. Nicholson, and C. Chernock, *Am. J. Med. Sci.*, **203**, 569-577 (1942).

tract, and the thiamine in the feces may be accounted for by the synthetic action of intestinal microorganisms.¹⁸⁴ Reinhold *et al.*¹⁸⁴ found no evidence of a thiamine-sparing action by fat. However, another possibility to account for a thiamine-sparing action of fat is suggested by Whipple and Church,¹⁸⁵ who offered experimental evidence to support the hypothesis that animals on fat-free diets receiving thiamine gained more weight than did their mates on the same diet but without the vitamin. They suggest that thiamine functions in the synthesis of fat within the animal body. The decreased excretion of thiamine during a high-carbohydrate intake, observed by Reinhold and co-workers¹⁸⁴ was interpreted as indicating an increased need for the vitamin in the metabolism of the extra carbohydrate, and consequent retention in the tissues. A continuous conversion of carbohydrates into fatty acids is assumed to occur under normal conditions.¹⁸⁶ If, when fat is fed, the extent of a carbohydrate-fat conversion is reduced, one might expect thiamine to be spared.

b. Riboflavin and Fat. The nutritional interrelation between fat and riboflavin is even less clear than is that of fat and thiamine. Mannering and his co-workers^{187,188} are of the opinion that more riboflavin is required by rats when they are metabolizing a high-fat diet than when they are receiving a low-fat regimen. One explanation for this augmented requirement of riboflavin when high-fat diets are employed was suggested by Elvehjem,¹⁸⁹ according to this hypothesis the normal intestinal synthesis of riboflavin is inhibited when fat is substituted for dextrin in the diet, and a greater amount must be received from outside sources. According to Czaczkes and Guggenheim,¹⁹⁰ at least twice as much riboflavin must be given for the maintenance of the normal riboflavin level in the organs and in the urine when rats are on high-protein and high-fat diets as is required when they are kept on a "normal" diet. According to Potter *et al.*¹⁹¹ no similar increase in riboflavin requirements on high-fat diets was observed in experiments with puppies. Kaunitz and co-workers¹⁹² noted

¹⁸⁴ J. G. Reinhold, J. T. L. Nicholson, and K. O'Shea Elsom, *J. Nutrition*, **28**, 51-62 (1944).

¹⁸⁵ D. V. Whipple and C. F. Church, *J. Biol. Chem.*, **114**, cvii-cviii (1936).

¹⁸⁶ R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **114**, 381-396 (1936).

¹⁸⁷ G. J. Mannering, M. A. Lipton, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **46**, 100-104 (1941).

¹⁸⁸ G. J. Mannering, D. Dasini, and C. A. Elvehjem, *J. Nutrition*, **28**, 141-156 (1944).

¹⁸⁹ C. A. Elvehjem, *J. Am. Dietet. Assoc.*, **22**, 959-963 (1946).

¹⁹⁰ J. W. Czaczkes and K. Guggenheim, *J. Biol. Chem.*, **162**, 267-274 (1946).

¹⁹¹ R. L. Potter, A. E. Axelrod, and C. A. Elvehjem, *J. Nutrition*, **24**, 449-460 (1942).

¹⁹² H. Kaunitz, H. Wiesinger, F. C. Blodi, R. E. Johnson, and C. A. Slanetz, *J. Nutrition*, **52**, 467-482 (1954).

that high dietary fat levels actually accentuated the riboflavin deficiency produced by the administration of galactoflavin. Protein utilization is probably better in riboflavin deficiency when no fat is present. According to the best available evidence, it would appear that high-fat diets require supplementation with increased doses of riboflavin in order to maintain a constant amount of the vitamin in the tissues, since the synthesis of riboflavin in the intestine is reduced by the high-fat regimen. Reiser and Pearson¹⁹³ reported that chicks fed moderately high levels of refined cottonseed oil together with diets low in riboflavin ceased growing earlier than did those on a low-fat diet. This cessation of growth was corrected by the addition of riboflavin. Lard did not retard growth, nor did a commercial hydrogenated vegetable fat. Since there is no reason to believe that unsaturated fatty acids such as those present in cottonseed oil require more riboflavin for their metabolism than do saturated fatty acids, it seems probable that the unsaturated acids interfere primarily with the intestinal synthesis of riboflavin.

c. Pantothenic Acid and Fat. A similar type of dermatitis appears to be produced by a deficiency in pantothenic acid, pyridoxine, or linoleic acid. Salmon¹⁹⁴ is of the opinion that these essential components have similar functions, and that all three are required simultaneously to cure the acrodynia of rats caused by a pantothenic acid deficiency. According to Stotz,¹⁹⁵ pantothenic acid serves as a component of coenzyme A, and is therefore involved in the many biologic processes which are mediated through this important coenzyme. Thus it takes part in the synthesis and the degradation of the fatty acid molecule. The requirement for this vitamin appears to depend upon the amount and type of fat present in the diet.

d. Pyridoxine (Vitamin B₆) and Fat. Some years ago Birch and György¹⁹⁶ and Hogan and Richardson¹⁹⁷ pointed out the close relationship between the requirement for fat and that for pyridoxine. It has already been mentioned that the skin symptoms which develop when the essential fatty acids are absent from the diet are similar to those which have been noted in pyridoxine deficiency. Thus Birch and György¹⁹⁶ showed that the dermatitis which occurs on a diet low in pyridoxine but containing 10% of fat can be cured when small amounts of lard are added. In fact,

¹⁹³ R. Reiser and P. B. Pearson, *J. Nutrition*, **38**, 247-256 (1949).

¹⁹⁴ W. D. Salmon, *J. Biol. Chem.*, **140**, cix-cx (1941).

¹⁹⁵ E. Stotz, *J. Am. Oil Chemists' Soc.*, **26**, 341-345 (1949).

¹⁹⁶ T. W. Birch and P. György, *Biochem. J.*, **30**, 304-315 (1936).

¹⁹⁷ A. G. Hogan and L. R. Richardson, *Nature*, **136**, 186 (1935).

somewhat later Birch¹⁹⁸ proved that the addition of fat to the diet delayed the onset of dermatitis produced by pyridoxine deficiency. In some cases it completely prevented the development of this abnormal skin condition. Salmon¹⁹⁹ also demonstrated that pyridoxine and the fat supplement each other in curing dermatitis.

The bulk of the evidence indicates that the effectiveness of the fat in curing dermatitis is directly proportional to the degree of its unsaturation and thus presumably to the essential fatty acid content. The latter explanation might readily account for the fact that lard, which ordinarily has a very high essential fatty acid content, will be effective in alleviating pyridoxine deficiency, whereas butter, which has a relatively low essential fatty acid content, is somewhat less efficacious in this respect. According to Schneider²⁰⁰ the low antidermatitis potency of rancid butter is attributable to the fact that the antidermatitis factor has been destroyed by the oxidation.

Kummerow and collaborators²⁰¹ reported that small quantities of oxidized fats cure acrodynia in rats; rancid methyl linoleate was less effective than was the freshly-distilled ester. Tocopherol did not improve the effectiveness of rancid ethyl linoleate, while ethanolamine aggravated the symptoms. Drastically oxidized fats, and 10,12-ethyl linoleate, were ineffective as curative agents. It is indicated that the various supplements of oxidized linoleate esters were converted to oleic acid and to saturated fatty acids.

All of the commonly occurring essential fatty acids are effective in alleviating pyridoxine deficiency. Quackenbush *et al.*²⁰² reported that, of these several essential fatty acids, ethyl linoleate is the most effective, especially when given in conjunction with pyridoxine. Linolenic and arachidonic acids were somewhat less satisfactory in alleviating the dermatitis. Schneider and co-workers²⁰³ agree that linoleic acid possesses the greatest potency. They call attention to the fact that the effectiveness of several natural fats in clearing up acrodynia is in proportion to their linoleate content. Thus corn oil was shown to have a greater activity than did linseed oil. While other fats exerted a curative effect proportional to their linoleate content, cod liver oil was completely ineffective.¹⁹⁹ Ac-

¹⁹⁸ T. W. Birch, *J. Biol. Chem.*, **124**, 775-793 (1938).

¹⁹⁹ W. D. Salmon, *J. Biol. Chem.*, **123**, civ-cv (1938).

²⁰⁰ H. A. Schneider, *Proc. Soc. Exptl. Biol. Med.*, **44**, 266-267 (1940).

²⁰¹ F. A. Kummerow, T. K. Chu, and P. Randolph, *J. Nutrition*, **36**, 523-536 (1948).

²⁰² F. W. Quackenbush, H. Steenbock, F. A. Kummerow, and B. R. Platz, *J. Nutrition*, **24**, 225-234 (1942).

²⁰³ H. A. Schneider, H. Steenbock, and B. R. Platz, *J. Biol. Chem.*, **132**, 539-551 (1940).

cording to Richardson *et al.*,²⁰⁴ the methyl esters of arachidonic and linoleic acids were useful in counteracting the dermatitis caused by pyridoxine deficiency, but did not afford permanent protection. These workers suggest that the unsaturated acids do not replace pyridoxine but simply delay the onset of the skin symptoms. They found that pyridoxine and pantothenic acid are both required.

Growth inhibition in rats brought about by feeding diets containing suboptimal amounts of pyridoxal or pyridoxine is accentuated, according to Sarma and associates,²⁰⁵ when oleic acid is added to the ration. However, this inhibitory effect of oleate can be counteracted by giving additional quantities of vitamin B₆.

There has been considerable speculation as to whether or not the deficiencies in essential fatty acid and pyridoxine are entirely similar insofar as the skin symptoms are concerned. Although Medes *et al.*²⁰⁶ emphasized the interrelation between the two types of deficiency, these results might well be interpreted to mean that two different factors are involved. Thus, when the diet was lacking in both of these components, a relief from the deficiency was obtained by the administration of either ethyl linoleate or of pyridoxine. However, the resulting growth response was less with maximum amounts of ethyl linoleate than when optimum doses of pyridoxine were given. The best results were obtained when both of these components were included in the diets simultaneously. According to more recent work of Beaton *et al.*²⁰⁷ the inclusion of corn oil in a fat-free, pyridoxine-free diet had no effect upon the rate of appearance of acrodynia, but it did prevent the development of severe external deficiency symptoms. Acrodynia appeared more frequently and was more severe in male rats than in the females.

In a comprehensive study of the nature of the pyridoxine deficiency in acrodynia, and the deficiency resulting from a diet lacking the essential fatty acids, Sinclair²⁰⁸ observed that the lesions in the skin of rats differ in these two abnormal conditions. In EFA deficiency, the concentration of pyridoxine in the body was found to be normal. Sinclair²⁰⁸ postulates that, in EFA deficiency, the failure in growth occurs because phosphatides (and therefore cell membranes) cannot be formed. In pyridoxine deficiency, however, the retardation in growth is due to interference with

²⁰⁴ L. R. Richardson, A. G. Hogan, and K. F. Itschner, *Univ. Missouri Agr. Expt. Sta., Research Bull.* 333, 3-12 (1941); *Chem. Abst.*, 36, 2591 (1942).

²⁰⁵ P. S. Sarma, E. E. Snell, and C. A. Elvehjem, *J. Nutrition*, 33, 121-128 (1947).

²⁰⁶ G. Medes, D. C. Keller, and A. Kurkjian, *Arch. Biochem.*, 15, 19-29 (1947).

²⁰⁷ J. R. Beaton, J. L. Beare, and E. W. McHenry, *J. Nutrition*, 48, 325-334 (1952).

²⁰⁸ H. M. Sinclair, *Biochem. J.*, 51, xvi (1952).

protein metabolism because of deficient transamination and decarboxylation. Moreover, it is believed that the skin lesions in EFA deficiency allow the water to pass through the skin more readily than normally, whereas in pyridoxine deficiency an inadequate amount of histaminase is present, with a resultant incomplete destruction of histamine.

Another indication that EFA deficiency and acrodynia result from two different types of deficiency is the fact that the liver dehydrogenases vary according to the nature of the deficiency. Thus, Tulpule and Patwardhan²⁰⁹ reported that, whereas the level of succinic dehydrogenase was not affected in pyridoxine deficiency, its activity was reduced in the liver in EFA deficiency, or in combined EFA and pyridoxine deficiencies. Furthermore, the amount of butyric dehydrogenase in the liver was decreased in EFA deficiency, while it was unchanged when pyridoxine was lacking in the diet. Combined EFA and pyridoxine deficiency did not reduce the activity of butyric dehydrogenase any further. On the other hand, either EFA or pyridoxine deficiency significantly decreased the glutamic dehydrogenase in the liver. It is postulated that the higher saturation of the fatty acids in the liver which occurs in EFA or pyridoxine deficiency is to be ascribed to a decrease in the desaturating capacity of the liver or to interference with the metabolism of other compounds from which unsaturated fatty acids are derived.

When a low content of dienoic acid was produced in the tissues of rats by the withdrawal of EFA from the diet or by the exclusion of pyridoxine or thiamine from the diet, coupled with a severe caloric restriction, characteristic EFA deficiency symptoms were observed only in the first case.²¹⁰ This led Medes and co-workers²¹⁰ to conclude that pyridoxine exerts no special supplementary action on the production or conservation of EFA.

According to McHenry and Gavin,^{211 212} pyridoxine is a factor required by the mammal for the synthesis of fat. Therefore, larger amounts are apparently required in the diet when preformed fat is not fed, and it must be produced in the body itself.

Sherman²¹³ reviewed the interrelationship of pyridoxine and fat metabolism.

e. Niacin, Folic Acid, Biotin, Vitamin B₁₂, and Fat. Since both niacin and folic acid are synthesized in the intestine of the rat, it is logical to

²⁰⁹ P. G. Tulpule and V. N. Patwardhan, *Arch. Biochem. Biophys.*, **39**, 450-456 (1952).

²¹⁰ G. Medes, M. V. Mann, and J. B. Hunter, *Arch. Biochem. Biophys.*, **32**, 70-79 (1951).

²¹¹ E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **138**, 471-475 (1941).

²¹² E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **125**, 653-660 (1938).

²¹³ H. Sherman, *Vitamins and Hormones*, **8**, 55-68 (1950).

assume that any conditions which change the nutrient material available to the intestinal flora will influence the amount of these vitamins which can be utilized by the animal. Elvehjem¹⁸⁹ reported that the rate of synthesis is accelerated on a diet containing fat. Butterfat is believed to have a greater effect than does corn oil in this synthesis. In addition to its sparing action on thiamine and pyridoxine, fat exerts a similar action on niacin. Salmon⁵⁸ reported that the requirement for nicotinic acid in the rat is lower when fat is the chief source of calories than when the diet is predominantly carbohydrate.

Biotin is also believed to exert a pronounced effect upon fat metabolism. As early as 1927, Boas²¹⁴ observed that practically no body fat was present in the tissues of rats which were subjected to severe biotin deficiency. When this vitamin was administered to the rats, an increase in fat synthesis occurred.²¹⁵ According to Pavcek and Shull,²¹⁶ biotin is readily destroyed by rancid fats, but tocopherol serves as an excellent agent in preventing this destruction.

Biotin also plays a major role in the growth of microorganisms,²¹⁷ whose requirements vary according to the amount of oleic acid or of other unsaturated acids present in the medium.

Spivey and co-workers²¹⁸ reported that the addition of 20% of fat to a vitamin B₁₂-deficient diet in chickens increased the weight difference between the chicks receiving the vitamin B₁₂ and the unsupplemented birds. Ling and Chow²¹⁹ concluded that vitamin B₁₂ plays an important role in both carbohydrate and lipid metabolism.

f. Fat-Soluble Vitamins. In some cases, the nature of dietary fat markedly affects the requirement for the several fat-soluble vitamins. Since some fats are excellent media for the solution and stabilization of the fat-soluble vitamins, it is obvious that the quality and quantity of fat in the diet are of prime importance in the absorption of these vitamins from the gastrointestinal tract, and in their ultimate utilization. Muelder and Kelly²²⁰ reported that the absorption of vitamin A by depleted rats was aided by dietary fat, although its subsequent utilization, as evidenced by growth in body length, was independent of the level of this foodstuff

²¹⁴ M. A. Boas, *Biochem. J.*, **21**, 712-724 (1927).

²¹⁵ G. Gavin and E. W. McHenry, *J. Biol. Chem.*, **141**, 619-625 (1941).

²¹⁶ P. L. Pavcek and G. M. Shull, *J. Biol. Chem.*, **146**, 351-355 (1942).

²¹⁷ D. B. Melville, *Vitamins and Hormones*, **2**, 29-69 (1944).

²¹⁸ M. R. Spivey, G. M. Briggs, and L. O. Ortiz, *Proc. Soc. Exptl. Biol. Med.*, **85**, 451-453 (1954).

²¹⁹ C. T. Ling and B. F. Chow, *J. Biol. Chem.*, **206**, 797-805 (1954).

²²⁰ K. D. Muelder and E. Kelly, *J. Nutrition*, **23**, 335-344 (1942).

in the diet. On the other hand, Russell and co-workers²²¹ reported that the utilization of vitamin A in the chicken was not related to the presence of fat in the diet, although the absorption of carotene by fowl was improved by fat. The retention of vitamin A in the liver was greater in hens which had received a ration with a normal fat content.

The presence of fat may also influence the relative effectiveness of the various forms of vitamin A. In general, the natural ester of vitamin A is less efficiently absorbed from oil solution than is either the acetate or the free alcohol. In some cases, the acetate ester, also, is poorly absorbed.^{7,222} For a further discussion of the effect of fats on the absorption of vitamin A, see Chapter IX of this volume.

A number of different investigators have demonstrated that the utilization of the provitamin A, β -carotene, by the rat depends upon the solvent in which it is fed.^{221,223-227} Thus Fraps and Meinke,²²⁵ using a liver storage test as a criterion, reported that carotene was less readily utilized when present in vegetables than when fed as a component of cottonseed oil. Kreula and Virtanen²²⁶ observed that, in human subjects, the absorption of carotene from carrots was less efficient than when it was fed dissolved in butterfat. It has already been reported that Russell and co-workers²²¹ noted that β -carotene is efficiently utilized by the chick only if fat is present. According to Burns *et al.*,²²⁷ both carotene and vitamin A are more effective in the rat when fed with the diet containing 5% of lard than when given with a fat-free diet which was supplemented with 0.1 g. of corn oil daily. Deuel and collaborators²²⁸ found that carotene was more efficiently utilized when it was incorporated in margarine than when fed with cottonseed oil. These workers suggest that the greater physiologic availability of carotene is potentiated by the emulsifying agents present in the margarine.

²²¹ W. C. Russell, M. W. Taylor, H. A. Walker, and L. J. Polskin, *J. Nutrition*, **24**, 199-211 (1942).

²²² E. F. Week and F. J. Sevigne, *J. Nutrition*, **39**, 233-250 (1949).

²²³ E. J. Lease, J. G. Lease, H. Steenbock, and C. A. Baumann, *J. Nutrition*, **17**, 91-102 (1939).

²²⁴ W. C. Sherman, *J. Nutrition*, **22**, 153-165 (1941).

²²⁵ G. S. Fraps and W. W. Meinke, *Food Research*, **10**, 187-196 (1945).

²²⁶ M. Kreula and A. I. Virtanen, *Uppsala Läkarefören. Förh.*, **45**, 355-362 (1939); *Chem. Abst.* **34**, 5897 (1940); cited by D. Melnick and B. L. Oser, *Vitamins and Hormones*, **5**, 39-92 (1947), 59.

²²⁷ M. J. Burns, S. M. Hauge, and F. W. Quackenbush, *Arch. Biochem.*, **30**, 341-346 (1951).

²²⁸ H. J. Deuel, Jr., S. M. Greenberg, E. E. Savage, and D. Melnick, *J. Nutrition*, **43**, 371-387 (1951).

Nightingale *et al.*²²⁹ observed an avitaminosis K in rats when dihydroxystearic acid triglyceride was incorporated in the diet to the extent of 25%. It is possible that this hydroxy acid may interfere with the synthesis of vitamin K in the intestine.

There is no explanation as yet for the observation by Booth, Henry, and Kon,²³⁰ that fats exert an antirachitic effect when they are fed to rats receiving high-calcium, low-phosphorus, rachitogenic diets. Thus it was found that vegetable fats devoid of vitamin D and of triglycerides resynthesized from fatty acids of butter and peanut oil exerted an antirachitic effect differing from that of vitamin D. It is possible that the differential effect of several fats on calcium excretion *via* the intestine, as demonstrated by Cheng and her co-workers,¹⁷⁰ and by Givens¹⁶⁷ might offer an explanation for this phenomenon.

(12) Fatty Acids as Required Growth Factors for Bacteria

a. Oleic Acid as a Substitute for Biotin. Although oleic acid can be readily synthesized by the higher animals and cannot be considered as an essential fatty acid, it fails to be synthesized by certain bacteria in the absence of the B vitamin, biotin. Hence, in the absence of biotin, oleic acid can serve as a stimulant to growth for a number of bacteria. Cohen *et al.*²³¹ were the first to report that oleic acid is required by bacteria. They stated that oleic acid was essential for the growth of *Corynebacterium diphtheriae*. Subsequently it was also found to be essential for *Clostridium tetani*, *Clostridium perfringens*, and for certain other bacteria. Kodicek and Worden¹³³ noted that oleic, linoleic, and linolenic acids inhibited the growth and lactic acid production of *Lactobacillus helveticus*. Williams, Broquist, and Snell¹³¹ reported that several cultures of lactic acid bacteria require oleic acid, linoleic acid, or a combined source of these materials to effect growth. The even-numbered saturated acids from C₂ to C₁₈ were found to be completely inactive. In the case of *Lactobacillus bulgaricus*, oleic acid, although essential for growth, is so toxic that its growth-promoting action can be observed only within a narrow range of concentrations and pH. The addition of detergents such as Tween 40, which are inactive by themselves, renders oleic acid non-toxic, and extends the pH range over which activity can be observed. Tween 80, a water-soluble ester of oleic acid, is an excellent non-toxic source of oleic acid for use in

²²⁹ G. Nightingale, E. E. Lockhart, and R. S. Harris, *Arch. Biochem.*, **12**, 381-387 (1947).

²³⁰ R. G. Booth, K. M. Henry, and S. K. Kon, *Biochem. J.*, **36**, 445-455 (1942).

²³¹ S. Cohen, J. C. Snyder, and J. H. Mueller, *J. Bacteriol.*, **41**, 581-590 (1941).

culture media for these experiments. However, most of the lactic acid bacteria commonly used as assay organisms do not require oleic acid for growth on complete media. This acid becomes essential when biotin is omitted, and its effect is enhanced by Tween 40.¹³¹ Whitehill, Oleson, and SubbaRow²³² reported that an essential growth factor present in fish solubles and necessary for the growth of a *Lactobacillus* isolated from the cecum of a rat was oleic acid. This particular *Lactobacillus* could be transferred repeatedly on synthetic media, but only in the presence of sodium oleate. The requirement for oleic acid could not be modified by high levels of sodium acetate, biotin, riboflavin, pantothenic acid or by any of the other known factors tested. In the case of a number of lactic acid bacilli, Broquist and Snell²³³ reported that the growth-promoting action could not be nullified by an excess of avidin when biotin was absent. *Lactobacillus arabinosus* did not respond to oleic acid in the presence of egg white (which contains avidin, a substance which combines with biotin). Broquist and Snell²³³ believe that the inhibitory action of avidin on the growth of *L. arabinosus* in the presence of oleic acid is due to the ability of avidin to combine with traces of biotin in the cell, which are essential for growth.

Williams and Fieger²³⁴ were the first to call attention to the similarity in the stimulating action of oleic acid, of whole rice oil and of biotin on *Lactobacillus casei*. Although maximum growth (as determined from acid production) could be demonstrated in the presence of lipids,²³⁴ no synthesis of biotin occurred.²³⁵ Of a number of fatty acids tested, lauric, myristic, and linoleic acids strongly inhibited the growth of *L. casei*, whereas elaidic acid was even more stimulatory than oleic acid.²³⁵ It would seem, therefore, that the bacteria adapt themselves to either the biotin-free or the biotin-low environment.

Broquist and Snell²³⁶ found that, in the absence of biotin, not only are the unsaturated fatty acids required to permit growth of *Lactobacillus arabinosus*, *L. casei*, and *Streptococcus faecalis*, but the dicarboxylic acid, aspartic acid, is also required. On the other hand, *Lactobacillus fermenti* and *Clostridium butyricum* require only the unsaturated fatty acids.

For some time it has been known that there is a fat-soluble and ether-soluble biotin-like component in acid-hydrolyzed plasma capable of

²³² A. R. Whitehill, J. J. Oleson, and Y. SubbaRow, *Arch. Biochem.*, **15**, 31-37 (1947).

²³³ H. P. Broquist and E. E. Snell, *Arch. Biochem. Biophys.*, **46**, 432-442 (1953).

²³⁴ V. R. Williams and E. A. Fieger, *Ind. Eng. Chem., Anal. Ed.*, **17**, 127-130 (1945).

²³⁵ V. R. Williams and E. R. Fieger, *J. Biol. Chem.*, **166**, 335-343 (1946).

²³⁶ H. P. Broquist and E. E. Snell, *J. Biol. Chem.*, **188**, 431-444 (1951).

stimulating growth of *L. casei* and of other lactic acid bacilli. Trager²³⁷ considered that this growth factor was related to oleic acid, and that both were related to the utilization and function of biotin. However, he found that the properties of the material did not correspond to those of oleic acid or of any previously described vitamer of biotin. Although Trager²³⁷ was of the opinion that this ether-soluble substance was a component of the non-saponifiable residue, Hofmann and Axelrod²³⁸ were unable to confirm this hypothesis. The latter workers reported that the substance with biotin-like activity in beef and human plasma occurred exclusively in the saponifiable portion of the lipid. When the fraction was esterified with diazomethane, the resulting methyl esters obtained by distillation possessed high biologic activity. On a weight basis, oleic acid was shown to have about the same potency as the plasma distillate. It was also found²³⁹ that the liquid acid fraction of the plasma which contains oleic, linoleic, and arachidonic acids had a greater growth activity for the *Lactobacillus* than did the original fraction, although the unsaturated acids were also found to have some synergistic action.²³⁹ A number of suggestions have been offered as to the reason why biotin and the unsaturated fatty acids can replace each other in promoting bacterial growth. Trager²⁴⁰ studied the growth of yellow fever mosquito larvae (*Aedes aegypti*) in biotin-free media, and found that larval growth was very slow, and that metamorphosis did not occur. However, relatively low concentrations of oleic acid, lecithin, and related compounds supported growth commensurate with that obtained with low concentrations of biotin. He concluded that biotin functions in the synthesis of fatty compounds or of substances containing fatty acids, such as lecithin. Rubin and Scheiner²⁴¹ demonstrated that biotin is primarily concerned with the synthesis of oleic acid. Thus, biotin antagonists such as homobiotin, trishomobiotin, homobiotin sulfone and trishomobiotin sulfone have no depressing effect upon the growth of *Lactobacillus casei* on a biotin-free medium, provided oleic acid is used as a biotin substitute. Moreover, Williams and Fieger²⁴² reported that avidin sufficient to combine with 1,000 m μ of biotin did not prevent the lipid stimulation of *L. casei* in the absence of biotin. These facts would seem to indicate that oleic acid is not active by virtue of effecting a syn-

²³⁷ W. Trager, *Proc. Soc. Exptl. Biol. Med.*, **64**, 129-134 (1947).

²³⁸ K. Hofmann and A. E. Axelrod, *Arch. Biochem.*, **14**, 482-483 (1947).

²³⁹ A. E. Axelrod, M. Mitz, and K. Hofmann, *J. Biol. Chem.*, **175**, 265-274 (1948).

²⁴⁰ W. Trager, *J. Biol. Chem.*, **176**, 1211-1223 (1948).

²⁴¹ S. H. Rubin and J. Scheiner, *Arch. Biochem.*, **23**, 400-410 (1949).

²⁴² V. R. Williams and E. A. Fieger, *J. Biol. Chem.*, **170** 399-411 (1947).

thesis of biotin. It is therefore apparent that biotin normally functions in bacterial metabolism by bringing about the synthesis of oleic acid.

Although oleic acid functions as a substitute for biotin with many bacteria in *in vitro* culture tests, apparently it does not behave in the same manner when injected into animals. For example, Trager²⁴³ reported that oleic acid injected intramuscularly into chicks did not have the same effect in reducing biotin deficiency produced by the ingestion of avidin from raw egg white as had biotin or the fat-soluble fraction from hydrolyzed plasma. Moreover, Williams and Fieger¹³² demonstrated that the lipids from rice polishings, which have a biotin-like activity in the case of *L. casei* and *L. arabinosus*, will not reduce the biotin-deficiency symptoms in white leghorn chicks. Hutchings and Boggiano²⁴⁴ reported that the addition of sodium oleate to a medium containing a yeast extract allowed for maximum growth with several lactobacilli. The amount of sodium oleate producing maximum growth varied with each organism. Increasing amounts (500 to 4000 $\mu\text{g.}/10\text{ ml.}$) proved toxic for *Lactobacillus leichmannii* and *L. plantarum*.

b. Comparative Potencies of Octadecenoic Acids. In addition to oleic acid, a large number of isomers, both geometric and positional, can serve as adjuncts in the growth of bacteria. In the first place the *trans*-isomer of oleic acid, namely elaidic acid, has been found by a number of workers²⁴⁵⁻²⁴⁹ to be approximately as active as oleic acid. On the other hand, Axelrod and co-workers²³⁹ assigned a much lower biopotency to elaidic acid, and Williams and Fieger²³⁵ observed a somewhat greater effect than was the case with oleic acid. The activity of *cis*-octadecenoic acids is not markedly influenced by the location of the double bond. Thus, petroselinic acid (6-octadecenoic acid),^{246,247,249} palmitoleic acid (*cis*-9-hexadecenoic acid),²⁴⁷ vaccenic acid (*trans*-11-octadecenoic acid),^{245,249} *cis*-12-octadecenoic acid,²⁴⁹ and *cis*-8-octadecenoic acid²⁴⁹ all exert about the same growth-stimulating effect as does oleic acid, although the *cis*-8 acid appeared to have a somewhat greater effect. On the other hand, myristoleic acid (*cis*-4-octadecenoic acid) has been reported to be inactive,

²⁴³ W. Trager, *J. Biol. Chem.*, **176**, 133-146 (1948).

²⁴⁴ B. L. Hutchings and E. Boggiano, *J. Biol. Chem.*, **169**, 229-230 (1947).

²⁴⁵ B. W. Boughton and M. R. Pollock, *Biochem. J.*, **50**, xxxi (1952).

²⁴⁶ M. R. Pollock, G. H. Howard, and B. W. Boughton, *Biochem. J.*, **44**, lii (1949).

²⁴⁷ M. R. Pollock, G. H. Howard, and B. W. Boughton, *Biochem. J.*, **45**, 417-422 (1949).

²⁴⁸ A. E. Axelrod, K. Hofmann, and B. F. Daubert, *J. Biol. Chem.*, **169**, 761-762 (1947).

²⁴⁹ A. L. S. Cheng, S. M. Greenberg, H. J. Deuel, Jr., and D. Melnick, *J. Biol. Chem.*, **192**, 611-622 (1951).

as has also the *trans*-isomer of this compound.²⁵⁰ Shull *et al.*²⁵⁰ found vacenic acid about as effective as oleic acid in replacing biotin, both in the basal medium and in the arginine-tyrosine medium. The *trans* forms of these acids are usually equally potent, although marked variations do occur in some cases. Cheng and her associates²⁴⁹ found that elaidic acid possesses the highest potency of any of the octadecenoic acids tested. As the double bond shifts in either direction from the 9 position, a reduction in potency occurs, until at the 6 position the acids are completely inactive. Similar results are noted in the case of octadecenoic acid, which forms no geometric isomers. These results are summarized in Table 4.

TABLE 4
BIOTIN-LIKE EFFECT OF OCTADECENOIC ACIDS AND LINOLEIC ACID,
ALONE AND WITH BIOTIN, USING *Lactobacillus Arabinosus*^a

Compound	<i>cis</i> -Acid			<i>trans</i> -Acid		
	Melting point, °C.	Biotin equivalent (mμ g.) per mg. fatty acid		Melting point, °C.	Biotin equivalent (mμ g.) per mg. fatty acid	
		Alone	With biotin		Alone	With biotin
6-Octadecenoic acid	29.8	11.6	12.4	53.0	0.4	3.7
7-Octadecenoic acid	—	—	—	43.5–44.5	2.7	15.2
8-Octadecenoic acid	22.7–23.8	14.7	13.4	51.5–52.3	2.8	9.1
9-Octadecenoic acid	13.0	10.3	10.2	44.5–45.5	11.5	10.4
	—	—	—	41.5–42.5	9.6	9.8
10-Octadecenoic acid	—	—	—	52.0–52.6	6.1	11.0
11-Octadecenoic acid	13.0–14.0	12.0	11.4	43.5–44.5	2.6	7.3
	—	—	—	39.0	1.5	7.2
12-Octadecenoic acid	26.8–27.6	10.8	13.4	52.0–53.0	2.7	15.2
17-Octadecenoic acid ^b	55.5–56.1	0.0	1.8	—	—	—
<i>cis</i> -9,10, <i>cis</i> -12,13-Octadecadienoic acid (Linoleic)	—5	5.3	6.9	—	—	—

^a A. L. S. Cheng, S. M. Greenberg, H. J. Deuel, Jr., and D. Melnick, *J. Biol. Chem.*, 192, 611–622, 1951.

^b No geometric isomers of this compound. Included in *cis* column for convenience.

c. Comparative Potency of Other Unsaturated Acids and of Related Compounds. Axelrod and co-workers²³⁹ reported that a number of compounds related to oleic acid are effective stimulants for the growth of *Lactobacillus arabinosus* in the absence of biotin. The following activities

²⁵⁰ G. M. Shull, R. W. Thoma, and W. H. Peterson, *Arch. Biochem.*, 20, 227–241 (1949).

(expressed in $\mu\text{g.}$ of biotin per mg. of fatty acid) were obtained: oleic acid, 6.0; methyl oleate, 3.2; oleic acid amide, 6.3; oleyl alcohol, less than 0.05; elaidic acid, 1.0; vaccenic acid, 1.2; dihydroxystearic acid (94°C.), less than 0.05; and dihydroxystearic acid (130.7°C.), less than 0.05. Linoleic acid (12-octadecadienoic acid) also possesses a high biotin-like activity in promoting the growth of *Clostridium butyricum* and of *Streptococcus faecalis*,²³⁷ and *Cl. sporogenes*.²⁵⁰ However, some workers found that its activity was lower than that of oleic acid, and that it was inhibitory at high concentrations.^{239, 246, 247, 250, 251} Linolenic acid (9,12,15-octadecatrienoic acid) is likewise effective in stimulating bacterial growth,^{237, 239, 246, 247} but its potency was found to be only a fraction of that of oleic acid.²³⁹

The ability of unsaturated fatty acids to substitute for biotin is not limited to the octadecenoic acid. Thus, palmitoleic acid (7-hexadecenoic acid) was shown by Hassinen, Durbin, and Bernhart,²⁵² as well as by Boughton and Pollock,^{245, 253} and by Pollock, Howard, and Boughton,²⁴⁷ to have a stimulatory capacity approximating that of oleic acid. In the case of *L. arabinosus*, hexadecenoic acid was able to stimulate the growth of bacteria in a manner similar to that of oleic acid, while the growth stimulation of *Lactobacillus bifidus* occurred only at a low concentration.²⁵² However, Boughton and Pollock alone²⁴⁵ and with Howard^{246, 247} were able to demonstrate positive stimulation of an oleic acid-requiring diphtheroid species of *Corynebacterium* ("Q") by *cis*- and *trans*-palmitoleic acid, as well as by the odd-carbon acids *cis*- and *trans*-heptadec-9-enoic acids.²⁴⁶

Not all unsaturated acids are active. Thus Boughton and Pollock²⁴⁵ reported negative results with the following acids: *cis*-undec-9-enoic acid; *cis*-undec-10-enoic acid; *cis*- and *trans*-myristoleic acid (9-tetradecenoic acid); *cis*- and *trans*-gadoleic acid (9-eicosenoic acid).²⁵³ Pollock, Howard, and Boughton²⁴⁶ reported that erucic acid (*cis*-13-docosenoic acid), brassidic acid (*trans*-12-docosenoic acid), α,β -oleic acid and ricinoleic acid (12-hydroxy-9-octadecenoic acid) were all quite inactive, although Shull *et al.*²⁵⁰ noted that ricinoleic acid exhibited activity in replacing biotin.

d. The Effect of Saturated Acids upon Bacterial Growth. In contradistinction to the activity of a number of unsaturated fatty acids which substitute for biotin as growth factors in bacterial cultures, the saturated fatty acids have been found to be completely inactive when supplied alone. This is true not only of the various even-chain carbon acids from C_8 to

²⁵¹ F. W. Norris and K. J. Lynes, *Biochem. J.*, **43**, xlv-xlvi (1948).

²⁵² J. B. Hassinen, G. T. Durbin, and F. W. Bernhart, *Arch. Biochem.*, **25**, 91-96 (1950).

²⁵³ B. W. Boughton and M. R. Pollock, *Biochem. J.*, **53**, 261-265 (1953).

C₁₆, but also of stearic acid (octadecanoic acid).²³⁹ Williams and Fieger²³⁵ reported that lauric (C₁₂) and myristic (C₁₄) also inhibit the growth of *Lactobacillus casei*. Hassinen and colleagues²⁵⁴ observed that the C₈ and C₁₀ saturated fatty acids inhibited the growth of both Gram-positive and Gram-negative organisms, but that the C₄, C₆, and C₈ saturated acids exhibited no significant depressing action against the growth of such organisms. The C₁₂ saturated acid inhibited only the Gram-positive organisms, while the C₁₄ acid exerted some inhibiting action upon *Lactobacillus bifidus* and *L. casei*. On the other hand, Cheng *et al.*²⁴⁹ reported that the saturated acids exhibited a synergistic action upon bacterial growth when present along with oleic acid. This effect is discussed in the next section.

e. Growth Stimulation of Bacteria Produced by Mixed Fatty Acids from Various Natural and Artificial Fats. A study of the biotin-like activities of mixed fatty acids prepared from a number of fats showed that their activity could not be correlated with the content of oleic and linoleic acids only; it was necessary to consider the proportion of the several saturated acids present. The following formula, which takes into account the variation in activity of oleic and linoleic acids, and corrects for the synergistic action for the saturated acids, was found to give excellent results in predicting the activity of the fatty acid mixtures. Thus, the biotin equivalent, expressed as millimicrograms of biotin per milligram of fatty acids, equals:

$$\frac{[(\% \text{ oleic times } 10.3) + (\% \text{ linoleic times } 5.3)]}{100} \times \frac{[(1.00 + 2.3 \times \% \text{ saturated fatty acids})]^{249}}{100}.$$

When this formula was employed, the calculated biotin-like effect of the mixed fatty acids was almost identical with that found by bioassay. A comparison of the estimated and found values of the fatty acids from the several fats is given in Table 5.

Spector¹²⁹ reported that the fatty acids of butterfat and of corn oil produce similar results in stimulating the growth of five strains of *Escherichia coli*. He found no consistent distinction between their effects upon bacterial growth, and presumably upon bacterial synthesis of the vitamins.

f. Effects Other Than the Biotin-Like Effect of Unsaturated Fatty Acids on Growth of Bacteria. Kodicek^{255, 256} demonstrated that most fatty acids

²⁵⁴ J. B. Hassinen, G. T. Durbin, and F. W. Bernhart, *Arch. Biochem. Biophys.*, 31 183-189 (1951).

²⁵⁵ E. Kodicek, *Symposia Soc. Exptl. Biol.*, No. III, 217-232 (1949).

²⁵⁶ E. Kodicek, *Bull. soc. chim. biol.*, 30, 946-960 (1948).

TABLE 5
BIOTIN-LIKE ACTIVITY OF FATTY ACIDS FROM SEVERAL FATS AND OILS AS FOUND BY
MICROBIOLOGICAL ASSAY AND BY CALCULATION FROM FATTY ACID COMPOSITION^a

Fatty acids from	Composition of total mixed fatty acids			Biotin equivalents of mixed fatty acids		Synergistic effect of saturated acids		Biotin equivalents of mixed fatty acids	
	Saturated (1) %	Oleic (2) %	Linoleic (3) %	Found (1) mg./mg. fatty acid	Antici- pated (2) + (3) mg./mg. fatty acid	Increase ^b (6), %	Ratio (6):(1)	Calculated ^c (7) mg./mg. fatty acid	Deviation, ^d %
Coconut oil	92	6	2	2.0	0.7	186	2.0	2.2	-9
Cottonseed oil	26	22	52	9.2	5.0	84	3.2	8.0	+15
Olive oil	12	81	7	12.0	8.7	38	3.2	11.2	+7
Soybean oil	13	25	62 ^e	7.7	5.5	40	3.1	7.2	+7
Butterfat	59	37	3	9.1	4.0	127	2.2	9.4	-3
Margarine fat	21	74	5	11.8	7.8	51	2.4	11.7	+1
Shortening C	38	55	7	8.2	6.1	34	0.9	11.3	-27
Shortening D	32	66	2	10.6	6.9	54	1.7	12.0	-12

^a A. L. S. Cheng, S. M. Greenberg, H. J. Denel, Jr., and D. Melnick, *J. Biol. Chem.*, 192, 611-622 (1951), 618.

^b $((4) - (5)/(5)) \times 100$.

^c Based upon the composition of the fatty acid mixture, using the formula given above

^d $((4) - (7)/(7)) \times 100$.

^e Including 7% linolenic acid.

possess an inhibitory effect upon the growth of various Gram-positive organisms. This behavior applies both to the long-chain saturated and to the unsaturated acids alike. Lauric and myristic acids inhibit bacterial growth in concentrations as small as 1:100,000. In the case of unsaturated acids, the *cis* forms exhibit a marked retarding effect on growth, and an increased effect is noted in the case of the polyunsaturated acids. Thus, the toxicity occurs in the following order: oleic acid < linoleic acid < linolenic acid. The bacteriostatic action of the unsaturated acids can be reversed by surface-active agents such as lecithin, sterols, tocopherols, proteins, and similar compounds. On the other hand, esters of the fatty acids are not bacteriostatic. In contradistinction to the reversal of the inhibitory effect of the surface-active agents upon the unsaturated acids, these agents were ineffective in the case of the long-chain saturated acids.

Laser^{257,258} found that *cis*-vaccenic acid inhibited respiration and growth of *Bacillus subtilis*. The respiratory inhibition decreased with time, and was followed by an increase in oxidation rate. This phenomenon is explained on the basis of a penetration of the acid into the cell which results in an unspecific inhibition of the succinic oxidase system within the cell. The organism is gradually able to metabolize the penetrated acid, and the metabolism returns to normal. *Trans*-vaccenic acid was found to have only about half of the activity of the *cis* acid.²⁵⁷ In later studies by Laser,^{259,260} it was found that the respiration of *B. subtilis* was inhibited qualitatively in a similar manner but quantitatively to different degrees by stearic, oleic, vaccenic, and oleic acid. However, after a certain length of time, resistance develops in the growing cells; this is believed to be the result of the production of an adaptive enzyme. Tomarelli *et al.*²⁶¹ found that long-chain fatty acids have a bacteriostatic effect upon *Lactobacillus bifidus*. Lauric and myristic acids were found to be the most toxic, but sodium acetate, oleic, and linoleic acid were also markedly inhibitory to the bifid strain. The presence of factors which alleviate the toxicity of the fatty acids was demonstrated in both human and cow's milk. These factors were found in bovine serum albumin, in dialyzed human whey, and in acacia suspension. A similar reversal of fatty acid toxicity was afforded by surface-active compounds such as Tween 80, Tween 60, and lipositol. Broquist and Snell²⁶² demonstrated that the oleic acid "in-

²⁵⁷ H. Laser, *Biochem. J.*, **47**, xxxii-xxxiii (1950).

²⁵⁸ H. Laser, *Biochem. J.*, **48**, 164-170 (1951).

²⁵⁹ H. Laser, *Biochem. J.*, **49**, lxvi-lxvii (1951).

²⁶⁰ H. Laser, *Biochem. J.*, **51**, 57-62 (1952).

²⁶¹ R. M. Tomarelli, R. F. Norris, C. S. Rose, and P. György, *J. Biol. Chem.*, **187**, 197-204 (1950).

²⁶² H. P. Broquist, and E. E. Snell, *J. Biol. Chem.*, **173**, 435-436 (1948).

activating" potencies of raw egg white and of avidin were directly proportional to the biotin-binding activities of the two preparations. Thus 0.1 μ g. of the purified avidin was found to be sufficient to combine with only 0.2 m μ g. of biotin to nullify the growth-promoting action of 50 μ g. of oleic acid.

(13) Fatty Acids as Growth Factors for Lower Organisms

In a study of pathogenic fungi of the "dermatophyte" genera, *Trichophyton*, *Epidermophyton*, *Microsporum*, and *Candida*, Keeney²⁶³ reported that long-chain acids exhibited a fungicidal activity. Sodium propionate, sodium valerate, sodium caproate, sodium caprylate, sodium caprate, and sodium undecylenate were all approximately equal in effectiveness. Much less or no fungicidal action resulted when the fungi were exposed to the short-chain members of the fatty acid series.²⁶⁴ Wyss and co-workers²⁶⁴ also found that the long-chain saturated and unsaturated fatty acids caused the greatest inhibition in growth of fungi, and killed more spores than did those with a shorter chain length.

Trager²⁴⁰ reported that oleic acid can replace biotin in the growth of the larvae of the yellow fever mosquito, *Aedes aegypti*. In the absence of biotin, the larval growth was very slow, and metamorphosis did not occur. However, when biotin was present to the extent of only 50 m μ g./ml. of medium, excellent growth was obtained. Relatively low concentrations of oleic acid or of an oil from hydrolyzed horse plasma, lecithin, or related compounds, when used in place of biotin, supported larval growth comparable with that obtained with the lower effective doses of biotin. On the other hand, oleic acid and Tween 80, either alone or with aspartic acid, produced some growth response on the part of red bread mold (*cholineless Neurospora crassa*), although Hodson²⁶⁵ was unable to demonstrate that biotin could be replaced completely.

(14) Miscellaneous, Beneficial, and Harmful Effects of Fats

Schweigert *et al.*²⁶⁶ pointed out that the isocaloric substitution of 10 or 20 parts of lard for sucrose in a purified ration reduced the incidence and extent of carious lesions in the cotton rat (*Sigmodon hispidus*) in proportion

²⁶³ E. L. Keeney, *Bull. Johns Hopkins Hosp.*, 73, 379-390 (1943); *J. Clin. Invest.*, 23, 929-930 (1944).

²⁶⁴ O. Wyss, B. J. Ludwig, and R. R. Joiner, *Arch. Biochem.*, 7, 415-425 (1945).

²⁶⁵ A. Z. Hodson, *J. Biol. Chem.*, 179, 49-52 (1949).

²⁶⁶ B. S. Schweigert, J. H. Shaw, M. Zepplin, and C. A. Elvehjem, *J. Nutrition*, 31, 439-447 (1946).

to the amount of lard added. When 50 parts of casein and 10 parts of lard were fed, the protective effect was additive. The number of cavities observed was comparable to that observed when the ration contained 20 parts of lard. Aaes-Jorgensen²⁶⁷ pointed out that kidneys from rats reared on a fat-free ration revealed necrosis, apical disintegration, and calcification in the papilla, and a rather high number of calculi on the corticomedullary border. Although similar changes were observed in the kidneys of rats fed diets containing various hydrogenated fats, the calculi were less numerous.

Among the harmful effects of fat is the hemolytic anemia caused by fat and choline. Thus, when fats are fed to dogs, an increase in the fragility of the red blood cells is noted. The hemolytic agents in fat are presumably the fatty acids and soaps which have escaped resynthesis during absorption.²⁶⁸ When choline is administered to dogs, in addition to a high-fat diet, the rapid development of an acute hemolytic anemia is noted.²⁶⁹ Davis^{270,271} concludes that the choline aids in the production of hemolytic and hyperchromic anemia, not by virtue of a lipotropic action, but rather because of its vasodilator or pharmacologic action. However, Clarkson and Best²⁷² were unable to confirm the general conclusions of Davis in regard to the production of various anemias;^{270,271} they did not observe any trace of macrocytic anemia in dogs following the addition of choline or choline plus fat to the stock diet.

Silberberg and Silberberg²⁷³ called attention to the fact that male mice fed a diet containing 29% of fat exhibited an increased incidence of osteoarthritis, and an accelerated onset as compared with controls fed 5% of fat. Apparently overweight was not a significant factor in the occurrence of this degenerative joint disease. According to Perdue and Phillips,²⁷⁴ a diet containing 0.03% potassium and 20% of corn oil increased the incidence and extent of mild myocardial necrosis, and of the fibrosis and dilation of the renal tubules typical of potassium deficiency to a greater extent than did an isocaloric 5% corn oil diet containing the same amount of potassium, when the two diets were pair-fed. No statistically signifi-

²⁶⁷ E. Aaes-Jorgensen, *XIXth Intern. Physiol. Congress*, Montreal, Aug.-Sept., 1953, *Abst.*, p. 146.

²⁶⁸ J. Longini and V. Johnson, *Am. J. Physiol.*, **140**, 349-353 (1943).

²⁶⁹ J. E. Davis and J. B. Gross, *Am. J. Physiol.*, **144**, 444-446 (1945).

²⁷⁰ J. E. Davis, *Science*, **105**, 43-44 (1947).

²⁷¹ J. E. Davis, *Am. J. Physiol.*, **147**, 404-411 (1946).

²⁷² M. F. Clarkson and C. H. Best, *Science*, **105**, 622-623 (1947).

²⁷³ M. Silberberg and R. Silberberg, *Arch. Pathol.*, **50**, 828-846 (1950).

²⁷⁴ H. S. Perdue and P. H. Phillips, *Proc. Soc. Exptl. Biol. Med.*, **81**, 405-407 (1952).

cant differences, however, were observed in other criteria of potassium deficiency.

Davis and Dubos¹³⁶ demonstrated that serum albumin has the ability to bind oleic acid in the ratio of 9:1. A fat embolism developed in dogs when the amounts injected exceeded this protective range.²⁷⁵ Thus, Jefferson and Necheles²⁷⁵ reported that oleic acid does not produce a massive edema of the lungs by embolism but by toxic effects on the lung capillaries. Both fatty acids and soaps are considered to be equally deleterious. Li and Freeman²⁷⁶ reported that rats exposed to benzene and maintained on a high-fat diet manifested a greater incidence of leucopenia than did control groups, irrespective of the protein content of the diet. Horlick, Katz, and Stamler²⁷⁷ stated that, in the case of white Leghorn cockerels fed a diet of chick starter mash containing 5% of crude fat, the incidence of arteriosclerotic lesions was greater than in control tests in which the fat content was reduced to 0.3% by alcohol-ether extraction, but the ration was supplemented with vitamins A, B, D, and E. However, variations other than the level of fat may be responsible for these differences.

Certain natural fats may in some cases be toxic to animals if consumed in sufficient amounts. Horse fat may be in this category when it contains large amounts of linolenic acid. Lalor and co-workers²⁷⁸ are of the opinion that the disease "yellow fat" in the mink may be the result of the relatively high trienoic acid content in the horse fat which these animals were fed; the trienoic acid was found to be present to the extent of 16%. A condition resembling "yellow fat" could be produced in the mink by feeding 3% linolenic acid in the form of raw linseed oil to these animals. It was noted that α -tocopherol exerted a protective action against this condition.

3. Factors Altering the Nutritional Value of Fats and Oils

(1) *Comparison of the Nutritional Value of Fats and Oils Based upon Composition*

For a discussion of the role of the essential fatty acids, see Chapter XIII of this volume.

a. Fat-Soluble Vitamin Content. (a) *Vitamins A and Carotene.* Vita-

²⁷⁵ N. C. Jefferson and H. Necheles, *Proc. Soc. Exptl. Biol. Med.*, **68**, 248-250 (1948).

²⁷⁶ T. W. Li and S. Freeman, *Am. J. Physiol.*, **145**, 158-165 (1945).

²⁷⁷ L. Horlick, L. N. Katz, and J. Stamler, *Am. Heart J.*, **36**, 472 (1948).

²⁷⁸ R. J. Lalor, W. L. Leaschke, and C. A. Elvehjem, *J. Nutrition*, **45**, 183-188 (1951).

min A occurs in some animal fats, but it is never present as such in vegetable fats. Although the vitamin A content of butters varies with the season, with the breed and diet of the cows from which the milk has been obtained, the average vitamin A content of butter in the United States has been estimated, on the basis of a cooperative study, as 15,000 International Units per pound.²⁷⁹ The most concentrated sources of vitamin A are the fish-liver oils. Cod-liver oil (*Gadus morrhua*), which has generally been considered to be an excellent source of this vitamin, and which has served as a biologic standard for vitamin A, has a relatively low concentration of this vitamin, namely, 600 I.U./g., when compared with liver oils of the soupfin shark (*Galeorhinus zyopterus*), which has been reported to have as much as 640,000 I.U./g. of oil,²⁸⁰ and even, in one case, as high as 800,000 I.U./g.²⁸¹ Moderate amounts of vitamin A are found in animal fats other than the fish-liver oils, in sources such as egg yolk fat and in the body fat of chickens. On the basis of growth experiments, Nieman²⁸² estimated the amount of vitamin A in fresh lard as 0.7 I.U./g. of the lard.

On the other hand, the plants are the principal sources of vitamin A in the diet. In this case the provitamin A, β -carotene, and related carotenoids, serve as sources of vitamin A. Under ordinary conditions, these carotenoids may occur with the minimum of fat. This is the case with various green leafy vegetables, such as turnip greens, spinach, kale, and broccoli, as well as with yellow, orange, or red-colored foods such as squash, carrots, apricots, persimmons, and tomatoes. Although β -carotene is not widely distributed in vegetable oils, red palm oil does contain the carotenoid in average amounts of 1900 I.U./g. of fresh food,²⁸³ and in some cases in quantities as high as 3,000 I.U./g.²⁸⁴ In fatty foods such as butter, vitamin A occurs both in the form of the vitamin itself and as the provitamin A, namely β -carotene. It has been the practice in the United States and in some foreign countries for a number of years to add β -carotene to margarines. To parallel the average level of total vitamin A in butter, established as 15,000 I.U./pound,²⁷⁹ practically all margarine manufactured in the United States contains vitamin A at this same level.

²⁷⁹ Anonymous, *U. S. Dept. Agr., Miscell. Pub., No. 571*, 1-14 (1945).

²⁸⁰ W. E. Ripley and R. A. Bolomey, *Calif. Dept. Nat. Resources, Div, Fish and Game, Bur. Marine Fisheries, Fish. Bull. No. 64*, 39-72 (1946).

²⁸¹ L. A. Swain and B. H. McKercher, *Fisheries Research Bd. Canada, Progr. Repts. Pacific Coast Sta. No. 65*, 67-69 (1945); *Chem. Abst.*, 40, 1681 (1946).

²⁸² C. Nieman, *Voeding*, 15, 232-240 (1954); *Chem. Abst.*, 48, 12263 (1954).

²⁸³ T. A. Buckley, *Malayan Agr. J.*, 24, 485-488 (1936).

²⁸⁴ H. P. Kaufmann, *Fette u. Seifen*, 48, 53-59 (1941).

Since legislation removing the restrictions on colored margarine has been passed, a number of brands have employed carotene both as a coloring matter and as a source of part of the vitamin A. This provitamin A may now be included as a portion of the vitamin A declared on the label, according to the recent ruling of the U. S. Food and Drug Administration,²⁸⁵ which stipulates that the oleomargarine must contain not less than 15,000 I.U./lb. Margarine has been reported to be an especially effective vehicle for facilitating the utilization of β -carotene in the rat.²²⁸

(b) *Vitamins D.* Vitamin D is found only rarely in animal fats, and never in vegetable fats. Small amounts of this vitamin may occur in butter, depending to a considerable extent upon the diet of the cows from which the butter is obtained. Some vegetable margarines are also fortified with vitamin D. The most concentrated natural sources of vitamin D (chiefly vitamin D₃) are the fish-liver oils. The potencies of these various liver oils vary from a comparatively low figure of 100 I.U./g. for cod-liver oil (*Gadus morrhua*),²⁸⁶ which was formerly considered to be an important source of vitamin D, to as high values as 40,000 I.U./g. for the liver oil of the bluefin tuna (*Thunnus thynnus*), while the oil of many fishes, such as the broadbill swordfish (*Xiphias gladius*) and the yellowfin tuna (*Neothunnus macropterus*) contains approximately 10,000 I.U./g. Actually cod-liver oil, which was long regarded as the standard treatment for rickets, holds twenty-third place, as regards the content of vitamin D, in the values listed by Bills²⁸⁶ for various fish-liver oils. In addition to the food sources of vitamin D, it can also be formed by the irradiation of the provitamins D with ultraviolet light. This may either occur in the skin of the animal or by irradiation of the foodstuffs in the course of their preparation. As a result of this the irradiation of foods for the production of vitamin D has become wide-spread, and the distribution of the D vitamins has been greatly extended. In cases in which vitamin D is required in increased amounts, such as in infancy, one does not ordinarily depend entirely upon dietary sources for the supply of this vitamin, but rather resorts to the fish-liver oils or to preparations of irradiated ergosterol, or even to direct irradiation of the body with sunlight or with ultraviolet rays. Consequently, although the distribution of vitamin D in food fats is of academic interest, it is not of too great importance in practical nutrition, because of the uncertainty of supply and because of the availability of much more concentrated sources of the material.

²⁸⁵ J. L. Thurston, *Federal Register*, 17, No. 100, 4613-4615 (May 21, 1952). Food and Drug Admin., Federal Security Agency, Part 45.

²⁸⁶ C. E. Bills, *Physiol. Revs.*, 15, 1-97 (1935).

TABLE 6
THE TOCOPHEROL CONTENT OF REPRESENTATIVE VEGETABLE
FATS AND OILS AND OF HYDROGENATED VEGETABLE FATS^d

Vegetable fat or oil or hydrogenated fats	Tocopherol in mg./100 g. oil					Refer- ence
	Total	α	β	γ	δ	
Vegetable oils						
Babassu, crude	3	—	—	—	—	<i>b</i>
Castor bean	50	—	—	—	—	<i>b</i>
Coconut, refined	3	—	—	—	—	<i>b</i>
Coconut	8.3	3.6	—	—	4.7 ^c	<i>d</i>
Corn, "Mazola"	119	—	—	—	—	<i>e</i>
Corn, European brand	250	—	—	—	—	<i>f</i>
Corn, refined	87	7	—	—	80 ^c	<i>d</i>
Cottonseed, Wesson	87	493	—	37.7	—	<i>g</i>
Cottonseed, refined	90 ^b	56	—	—	34 ^c	<i>d</i>
Linseed	113	—	—	—	—	<i>h</i>
Olive	14	—	—	—	—	<i>h</i>
Palm	56	30	—	—	26 ^c	<i>d</i>
Peanut, crude	52 ^b	30	—	22	—	<i>i</i>
Peanut, refined	22	11	—	—	11 ^c	<i>d</i>
Poppyseed, crude	44	—	—	—	—	<i>j</i>
Rapeseed, crude	55	—	—	—	—	<i>j</i>
Safflower, crude	80	—	—	—	—	<i>b</i>
Sesame, refined	50	—	—	—	—	<i>b</i>
Soybean	168 ^k	20 ^a	—	98 ^a	50 ^a	<i>k</i>
Soybean, refined	140	10	—	—	130 ^c	<i>d</i>
Sunflower seed, crude	70	—	—	—	—	<i>j</i>
Wheat germ	520	—	—	—	—	<i>l</i>
Wheat germ, Merit	268	161	107	—	—	<i>g</i>
Hydrogenated vegetable shortenings						
Crisco	104.2	—	—	—	—	<i>h</i>
Margarine Base Stock L (P & G)	100.5	—	—	—	—	<i>h</i>
Primex	98.4	—	—	—	—	<i>h</i>
Sweetex	101.0	—	—	—	—	<i>h</i>

^a Most of the data are from W. Lange, *J. Am. Oil Chemists' Soc.*, 27, 414-422 (1950).

^b A. E. Bailey, *Industrial Oil and Fat Products*, Interscience, New York, 1945, p. 32; cf. also H. J. Deuel, Jr., in A. E. Bailey, *Cottonseed and Cottonseed Products*, Interscience, New York and London, 1948, pp. 763-811.

^c Sum of γ - and δ -tocopherol.

^d P. L. Harris, M. L. Quaife, and W. J. Swanson, *J. Nutrition*, 40, 367-381 (1950).

^e F. W. Quackenbush, H. L. Gottlieb, and H. Steenbock, *Ind. Eng. Chem.*, 33, 1276-1278 (1941).

^f A. Emmerie and C. Engel, *Z. Vitaminforsch.*, 13, 259-266 (1943).

^g M. L. Quaife, *J. Biol. Chem.*, 175, 605-617 (1948).

^h Procter and Gamble unpublished data cited by W. Lange. (see footnote ^a).

ⁱ G. S. Fisher, *Ind. Eng. Chem., Anal. Ed.*, 17, 224-227 (1945).

^j M. Kofler, *Helv. Chim. Acta*, 26, 2166-2176 (1943).

^k L. Weisler, C. D. Robeson, and J. G. Baxter, *Anal. Chem.*, 19, 906-909 (1947).

^l P. Karrer and H. Keller, *Helv. Chim. Acta*, 21, 1161-1169 (1938).

TABLE 7
THE TOCOPHEROL CONTENT OF SOME ANIMAL FATS,
FISH LIVER OILS, AND DAIRY PRODUCTS^a

Fat sample	Tocopherol in mg./100 g. oil		Reference
	Total	α	
Animal fats			
Beef, fat	1.0	—	<i>b</i>
Hog, fat	0.22	—	<i>c</i>
Hog, ruffle fat	0.78, 2.87 (γ)	—	<i>d</i>
Hog, leaf fat	0.67, 2.13 (γ)	—	<i>d</i>
Hog, back fat	0.64 (γ)	—	<i>d</i>
Hog, lard	1.2	—	<i>b</i>
Hog, lard	2.7	2.3	<i>e</i>
Oleo oil	2.0	—	<i>f</i>
Rabbit fat	0.6	—	<i>g</i>
Fish-liver oils			
Cod-liver oil	26	—	<i>h</i>
Mangona shark liver oil	10	ca. 0.10	<i>i</i>
Soupin shark liver oil	4	—	<i>i</i>
Dairy products:			
Butter	2.1-3.3	—	<i>j</i>
Butter	2.1-2.5	—	<i>k</i>
Butter	2.40	—	<i>e</i>
Butter	—	2.4	<i>l</i>
Butter, summer cow's butterfat	4.2	—	<i>m</i>
Butter, winter cow's butterfat	1.7-3.0	—	<i>n</i>
Egg, lipid	13.0	(4.4 γ)	<i>o</i>
Egg, whole	2.0	1.16	<i>e</i>

^a Most of the data are from W. Lange, *J. Am. Oil Chemists' Soc.*, **27**, 414-422 (1950).

^b M. Kofler, *Helv. Chim. Acta*, **26**, 2166-2176 (1943).

^c P. Karrer, W. Jaeger, and H. Keller, *Helv. Chim. Acta*, **23**, 464-465 (1940).

^d J. R. Chipault, W. O. Lundberg, and G. O. Burr, *Arch. Biochem.*, **8**, 321-335 (1945).

^e P. L. Harris, M. L. Quaife, and W. J. Swanson, *J. Nutrition*, **40**, 367-381 (1950).

^f A. E. Bailey, *Industrial Oil and Fat Products*, Interscience, New York and London, 1945.

^g M. Kofler, *Helv. Chim. Acta*, **28**, 26-31 (1945).

^h F. W. Quackenbush, H. L. Gottlieb, and H. Steenbock, *Ind. Eng. Chem.*, **33**, 1276-1278 (1941).

ⁱ C. D. Robeson and J. G. Baxter, *J. Am. Chem. Soc.*, **65**, 940-943 (1943).

^j A. Emmerie and C. Engel, *Z. Vitaminforschung*, **13**, 259-266 (1943).

^k H. Lieck and H. Willstaedt, *Svensk. Kem. Tid.*, **57**, 134-139 (1945); *Chem. Abst.*, **40**, 4759 (1946).

^l M. L. Quaife and P. L. Harris, unpublished data cited by K. C. D. Hickman, *Record Chem. Progress* (Kresge-Hooker Sci. Lib.) **9**, 104-121 (1948), p. 108.

^m P. L. Harris, W. J. Swanson, and K. C. D. Hickman, *J. Nutrition*, **33**, 411-427 (1947).

ⁿ M. L. Quaife, *J. Biol. Chem.*, **169**, 513-514 (1947).

^o M. L. Quaife and P. L. Harris, *Ind. Eng. Chem., Anal. Ed.*, **18**, 707-708 (1946).

(c) *Tocopherols (Vitamins E)*. The tocopherols occur in varying amounts in most animal and vegetable fats. Actually there are four types of tocopherols which may occur in fats, namely, α -tocopherol (5,7,8-trimethyltolcol), β -tocopherol (5,8-dimethyltolcol), α -tocopherol (7,8-dimethyltolcol), β -tocopherol (4,8-dimethyltolcol), γ -tocopherol (7,8-dimethyltolcol), and δ -tocopherol (8-methyltolcol). Tocopherol has a number of different functions. Probably the most important one is its function as vitamin E, as originally discovered by Mattill and Conklin²⁸⁷ and by Evans and Bishop.^{288,289} It was shown by these workers to be an antisterility vitamin, although it was not yet identified by name (1923) but was called "substance X." A second important function which can be assigned to the tocopherols is their antioxidant effect. Of the four tocopherols now known, the α -type has the greatest effect upon fertility, and the lowest activity as an antioxidant. Conversely, δ -tocopherol is the most active form as an antioxidant, and it has practically no "vitamin E" activity. Table 6 summarizes the tocopherol values for the common vegetable oils insofar as the data are available, while Table 7 furnishes corresponding information on animal fats, fish-liver oil, and dairy fats.

An examination of Tables 6 and 7 reveals the fact that the tocopherol content of most of the common vegetable oils is relatively high, and many times exceeds that present in animal fats or in dairy fats. The highest reported value was recorded for wheat germ oil, namely 550 mg. %, while corn, cottonseed, and soybean oils are excellent sources, with tocopherol contents varying between 80 and 168 mg. %. Olive oil is a relatively poor source (14 mg. %), while refined coconut oil is practically devoid of this vitamin (only approximately 3 mg. %). δ -Tocopherol is especially abundant in corn and soybean oils. The level of tocopherols in the fats is not adversely affected by hydrogenation, as evidenced by the fact that the figures approximated 100 mg. % in all four samples of commercial hydrogenated products tested.

In the case of animal fats, most values for the tocopherols are under 5 mg. %. Lards and butters have been reported to contain between 1 and 3 mg. %, although a value of 4.2 mg. % was assigned to one sample of cow's butterfat obtained in the summer. Values as high as 13 mg. % have been reported for egg lipid, while cod liver oil contains 26 mg. % of this vitamin. In the case of animal fat, the larger proportion of the tocopherols is in the form of α -tocopherol.

²⁸⁷ H. A. Mattill and R. E. Conklin, *J. Biol. Chem.*, **44**, 137-158 (1920).

²⁸⁸ H. M. Evans and K. S. Bishop, *Science*, **56**, 650-651 (1922).

²⁸⁹ H. M. Evans and K. S. Bishop, *J. Metabolic Research*, **3**, 233-283 (1923).

b. Résumé of the Effect of Composition on the Nutritional Value of Fats and Oils. There are wide variations in the proportion of EFA, tocopherol, vitamin A and carotene, and vitamin D, in the various animal and vegetable fats and oils. Since all of these factors are important from a nutritional standpoint, it is evident that variations in the proportion of these several nutrients will result in variations in the nutritional value of the several fats considered.

No single fat is high in all of these nutritional components. However, the vegetable fats and oils in general excel in having a high proportion of essential fatty acids, as well as a high content of the tocopherols. On the other hand, animal fats usually have an extremely low EFA content, and the proportion of tocopherol is usually so small that these animal fats become rancid much more readily than do the vegetable fats. Although vitamin A *per se* is found under normal conditions in animal fats, its distribution is quite limited in these fats. The provitamin A, on the other hand, does occur in a number of vegetable fats, as well as in such animal fats as butter and egg yolk. The vitamins D have an extremely limited distribution but, when they do occur, they are normally found exclusively in animal fats. However, since certain types of vitamins D can be produced by direct irradiation of the foods, vitamin D is found in a number of non-animal fat foods. Its presence in these irradiated foods does not, therefore, imply the presence of animal fat. Even in animal fats the distribution of vitamin D is extremely limited, and one must rely upon other sources when the requirement for vitamin D is high. The beneficial features in the composition of vegetable fats are to a considerable extent retained in the fats after hydrogenation. Thus, hydrogenation does not destroy any large proportion of tocopherol, and considerable amounts of essential fatty acids are still retained in margarines and in most hydrogenated fats. Fatty foods such as margarines are further improved by the addition of vitamin A alone or with carotene and vitamin D, so that they contain all of the nutritional adjuncts ordinarily dissolved in fat.

(2) *Comparison of the Nutritional Value of Fats and Oils, Based upon Physiologic Response*

a. Introduction. In addition to the presence of the above-mentioned compounds in fats and oils, further factors which affect the nutritional values are associated with the ability of the animal to utilize these fats. Information including the digestibility of the fats, and the rate of absorp-

tion is obviously important in assessing to what extent the fat can be utilized by the animal.

b. The Digestibility of Fats and Oils. The extent of digestibility provides one of the most exacting indices of the nutritional value. The so-called *coefficient of digestibility* is a constant which serves as a criterion of the extent of utilization of a foodstuff. It is the ratio of the absorbed material to the total amount ingested; in the case of fat, corrections are made for the "metabolic fat" in the feces. This latter fraction represents the lipid-like material which would be excreted irrespective of the diet. For a complete discussion on the digestibility of fats, the reader is referred to *The Lipids*, Vol. II, pages 195-246.

(a) *The Digestibility of Vegetable and of Animal Fats in Man.* With very few exceptions, all natural animal and vegetable fats melting below 50°C. (122°F.) are practically completely digested by the normal individual. In a comprehensive study made by the author and by the late Dr. C. F. Langworthy and Dr. A. D. Holmes and others of the Office of Home Economics of the U. S. Dept. of Agriculture,²⁹⁰⁻²⁹² it was found that the coefficients of digestibility of thirty-four vegetable fats ingested with a fat-free diet in amounts of 50 to 100 g. daily, varied between 94 and 99; the only exceptions were avocado fat and teaseed oil, in which the coefficients of digestibility were 88 and 91, respectively.²⁹² The figures for digestibility of some common vegetable fats are as follows:²⁹² cocoa butter, 95; coconut oil, 98; corn oil, 97; cottonseed oil, 98; English walnut oil, 98; hempseed oil, 96; olive oil, 98; peanut oil, 98; poppyseed oil, 96; rapeseed oil, 99; sesame oil, 98; soybean oil, 98; and sunflowerseed oil, 96. In the studies which were made with eighteen different animal fats, the digestibility figures varied between 93 and 99. Some of these values for animal fats are as follows:²⁹² beef fat, 93; butter, 97; chicken fat, 97; cod-liver oil, 98; egg yolk fat, 94; goose fat, 95; lard, 97; oleo oil, 97; ox-marrow fat, 94. One criticism of the earlier work is that no account was taken of the fat excreted in the form of soap in the feces. It is possible that, had this factor been taken into consideration, the calculated coefficients of digestibility might have been somewhat lower. However, Deuel *et al.*²⁹³ have reinvestigated the digestibility of cottonseed and rapeseed oil

²⁹⁰ H. J. Deuel, Jr., and A. D. Holmes, *U. S. Dept. Agr. Bull. No. 1033*, 1-15 (July 27, 1922).

²⁹¹ C. F. Langworthy and A. D. Holmes, *U. S. Dept. Agr. Bull. No. 505*, 1-20 (Feb. 13, 1917).

²⁹² C. F. Langworthy, *Ind. Eng. Chem.*, 15, 276-278 (1923).

²⁹³ H. J. Deuel, Jr., R. M. Johnson, C. E. Calbert, J. Gardner, and B. Thomas, *J. Nutrition*, 38, 369-379 (1949).

in a number of human subjects, allowing for the excretion of fecal soaps. The coefficients of digestibility obtained in these latter experiments are identical with the ones reported by the U. S. Dept. of Agriculture. This result was obtained in spite of the increased fat accounted for as soaps, because of a corresponding increase in the quantity of "metabolic fat" when soaps were included in the latter correction. It is our opinion that the failure to consider the fecal soaps in the case of experiments on vegetable fats or on low-melting animal fats probably does not alter their digestibility figures. Moreover, the diet was extremely constant in the large number of experiments carried on at the U. S. Dept. of Agriculture, which would probably not involve much variation in the proportion of calcium contained in the diet. However, in the case of the fats containing largely stearic acid, the figures recorded for digestibility in these earlier studies are probably too high. Obviously, further studies in this field are necessary. For a further discussion of the effect of calcium and magnesium on fat digestibility see page 877.

(b) *Résumé of the Comparative Digestibility of Fats and Oils.* In the case of man there are no marked variations in the high utilization of most common natural fats. Thus, the same high coefficients of digestibility have been noted in vegetable fats and in animal fats melting below 50°C. Moreover, hydrogenated fats, such as shortenings and margarines, present the same high values for utilization as is the case with natural fats, provided that the melting points of these fats are below 50°C. Both butter and a margarine prepared from a hydrogenated vegetable fat had coefficients of digestibility of 97%.

Animal fats melting above the critical level of 50°C. (122°F.) are progressively less efficiently utilized by man and by animals as the melting points are further increased. However, this situation is not confined to the animal fats, as it likewise occurs in the case of vegetable fats which have been hydrogenated to melting points exceeding this critical temperature. Thus, from the standpoint of digestibility, animal fats, butters, vegetable fats, and hydrogenated oils are almost equal, provided that the samples considered do not have excessively high melting points. The various factors which alter the digestibility of fat may also affect the several types of fat in a similar manner.

c. The Effect of Absorption on the Nutritional Value of Fats. Nutritionists do not always differentiate between absorption and digestibility of foodstuffs. However, these biologic determinations actually constitute two separate indices for evaluating the nutritional value of a fat, and variations in absorption rate may, under some conditions, be of considerable

importance in relation to the utilization of the fat studied. Absorption denotes the rate at which fat is removed from the intestine into the body, while digestibility is an index of the completeness of utilization as the food-stuff passes through the gastrointestinal tract. Ordinarily fats which have a high absorption rate also have a high digestibility. However, the converse may not necessarily be true.

Although no differences in nutritional value may be observed between two fats which have the same coefficients of digestibility when the fats are ingested in moderate quantities, variations in nutritional value may well be noted if fats are taken in large amounts. Thus, a fat having a high rate of absorption would still be completely utilized at a high level of intake; on the other hand, a fat having a low rate of absorption may be only incompletely absorbed, and may cause digestive disturbances, including diarrhea, when taken in large amounts. The slowly-absorbed fat is definitely inferior to the rapidly-absorbed one under such circumstances. If slowly-absorbed and rapidly-absorbed fats are taken in small quantities, then the coefficient of digestibility of the two fats would be the same, and they would provide an equal number of calories. Absorption therefore provides an important index as to the possible changes in nutritional value which are dependent upon the size of the dosage. The subject of absorption of fats is discussed *in extenso* in *The Lipids*, Vol. II, pages 115-134, while the rate of absorption is discussed on pages 172-194.

(a) *Absorption Rates of Different Fats.* The rate of absorption of various fats and oils is generally highest when the concentration of the fats is at a maximum. Thus, the figures in the test carried out at 0 to three hours were generally found to be somewhat higher than those for the second three-hour period after feeding, although considerable amounts of fats still remained in the gut at the end of a six-hour period. The variations in absorption rates of the different fats investigated are shown in *The Lipids*, Vol. II, pages 176, 177.

Although the number and variety of fats on which the rate of absorption has been tested are exceedingly limited, certain variations appear to be fairly regular. In the first place, the variations between natural animal and vegetable fats are not great. The highest rate of absorption, in the case of both males and females, seems to occur with butter. However, this rapid absorption is noted only during the first three-hour period, and it has largely disappeared by the second three-hour period. It is possible that the more rapid absorption rate may be a reflection of the absorption of the short-chain acids present in the butter triglycerides. The rate of absorption of simple short-chain triglycerides is very rapid. Thus Deuel

and Hallman²⁹⁴ reported that the most rapid absorption occurred with triacetin and tributyrin, while it was somewhat slower in the case of tri-caproin and tricaprylin. As soon as the short-chain triglycerides in the butterfat are absorbed, the rest of the butter triglycerides are utilized at a rate comparable with that of other fats.

Among the fats liquid at ordinary temperatures, rapeseed oil offers an interesting result. Although the figures for absorption during the first three-hour period are only slightly less than those of other fats, the figure for the second three-hour period are definitely low.²⁹⁵ In this case the low absorption can be correlated with a low reported digestibility in rats,²⁹⁶ but not in man.²⁹³ High-melting fats, also, are slowly absorbed. Hydrogenated cottonseed oil melting at 46°C. and 54°C., and hydrogenated lard melting at 55°C., are absorbed more slowly than are other natural fats. This effect can likewise be correlated with the low digestibilities of these products.^{297,298}

(b) *Résumé of the Comparative Absorption Rates of Fats and Oils.* There is no valid evidence as to whether fat should preferably be absorbed rapidly or slowly. When fatty foodstuffs are absorbed quickly, they can be tolerated in larger amounts than otherwise, and they furnish body energy more promptly than in the case of samples which are absorbed more slowly. Conversely, when the materials are absorbed slowly they prevent hunger over a longer period, and cause the feeling of satiety for a prolonged interval of time, thus reducing the caloric intake. In the latter case, the increase of blood lipids associated with absorption is less marked when absorption proceeds over a long period, and this might be expected to tax the body to a lesser extent. However, if absorption is so slow that it is not completed before the fat leaves the small intestine, the slow absorption rate may be considered to be a factor contributing to a lower nutritional value, inasmuch as the coefficient of digestibility of the fat would therefore be reduced.

In summarizing the limited data which are available, one is impressed with the fact that most of the natural fats and oils are absorbed at quite similar rates. During the early period of absorption, butter seems to be more rapidly absorbed; on the other hand, during the later periods the rate of absorption of butter agrees quite closely with that of other natural fats. Rapeseed oil is absorbed very rapidly after the first preliminary period of absorption. Hydrogenated fats such as margarines, lard, and

²⁹⁴ H. J. Deuel, Jr., and L. Hallman, *J. Nutrition*, **20**, 227-232 (1940).

²⁹⁵ H. J. Deuel, Jr., L. Hallman, and A. Leonard, *J. Nutrition*, **20**, 215-226 (1940).

²⁹⁶ H. J. Deuel, Jr., A. L. S. Cheng, and M. G. Morehouse, *J. Nutrition*, **35**, 295-300 (1948).

²⁹⁷ M. E. Crockett and H. J. Deuel, Jr., *J. Nutrition*, **33**, 187-194 (1947).

²⁹⁸ V. Augur, H. S. Rollman, and H. J. Deuel, Jr., *J. Nutrition*, **33**, 177-186 (1947).

Crisco are absorbed at rates comparable to those of the natural fats. It is only in the case of hydrogenated fats having melting points above 50°C that a retardation in absorption rate becomes evident.

d. The Nutritional Value of Animal vs. Vegetable Fats. There has been considerable discussion in the literature as to whether or not animal fats are superior to vegetable fats as shown by the several physiologic indices which are used for establishing nutritional value. Most of the work has centered around a comparison of the nutritional value of butter as contrasted with that of vegetable fats, including vegetable fats modified by hydrogenation. A number of different criteria have been employed for making this comparison, but growth-promoting effect, pregnancy, and lactation, and the effect in multigeneration experiments, have been the principal methods for attacking this problem.

(a) *Relative Nutritional Value Based upon Growth Tests.* a'. Experiments on Normal Rats: There has been a long-continued discussion on the question of whether or not butterfat *per se* possesses a specific nutritional value not shared by other animal fats or by vegetable fats. On the positive side of this discussion the more recent work has centered around the report of Schantz, Elvehjem, and Hart²⁹⁹ in 1940, which stated that rats grew better over the period of six weeks following weaning if they received a liquid diet of skim milk homogenized with butterfat as compared with similar diets in which corn oil, cottonseed oil, coconut oil, or soybean oil was employed in place of butterfat. This difference in growth-promoting capacity disappeared after a period of six weeks. It was later reported from the same laboratory^{300, 301} that the saturated fatty acids of the butterfat compose the effective fraction, and afford more growth than does a comparable amount of whole butter. The volatile and unsaturated fatty acid fractions were found to be ineffective in producing a greater augmentation of growth. However, the unsaturated fatty acids were found to become active in promoting growth, after they had been hydrogenated. The greater rate of growth reported in the earlier experiments of Schantz and co-workers²⁹⁹ was ascribed to the presence of certain undefined long-chain fatty acids which were believed to be in butterfat³⁰⁰ but not in other fats.³⁰¹ However, two different groups of workers^{302, 303} have failed to confirm the

²⁹⁹ E. J. Schantz, C. A. Elvehjem, and E. B. Hart, *J. Dairy Sci.*, **23**, 181-189 (1940).

³⁰⁰ E. J. Schantz, R. K. Boutwell, C. A. Elvehjem, and E. B. Hart, *J. Dairy Sci.*, **23**, 1205-1210 (1940).

³⁰¹ R. K. Boutwell, R. P. Geyer, C. A. Elvehjem, and E. B. Hart, *J. Dairy Sci.*, **24**, 1027-1034 (1941).

³⁰² K. M. Henry, S. K. Kon, T. P. Hilditch, and M. L. Meara, *J. Dairy Research*, **14**, 45-58 (1945).

³⁰³ E. L. Jack, J. L. Henderson, D. F. Reid, and S. Lepkovsky, *J. Nutrition*, **30**, 175-181 (1945).

finding that the saturated fatty acid moiety of butterfat possesses specific nutritive properties. Jack and co-workers³⁰³ suggested that either the oleic acid content or the total unsaturation might be a factor contributing to differences in growth of weanling rats fed glyceride fractions separated from milk fat. Heftmann³⁰⁴ could find no significant difference in weight gain or efficiency of food conversion between two groups of rats, regardless of whether the fat component of a Sherman B diet was the entire fatty acid fraction of butter or the same fatty acids stripped of their low molecular weight fraction. In contradistinction to earlier evidence, Heftmann³⁰⁴ reported that, on partial hydrogenation of the butterfat, the efficiency of food utilization was somewhat decreased in male rats when the vitamin A and vitamin D were low in the diet. In contradistinction to earlier reports,²⁹⁹⁻³⁰¹ Geyer *et al.*³⁰⁵ observed that a specific growth-promoting action was exerted by a liquid portion of butter fractionated from an acetone solution by cold crystallization at -4°C . to remove the solid glycerides. These workers reported a positive result in only a single case; on repetition of the test, they failed to confirm their earlier findings. Jack and Hinshaw,³⁰⁶ as well as Jack *et al.*,³⁰³ reported that the liquid fraction of butterfat had higher nutritive properties, provided care was taken to prevent oxidation of the unsaturated fraction. Thus, on the one hand, Schantz and co-workers³⁰⁰ ascribed the growth-promoting action of butterfat to the saturated portion of the fatty acids, while Geyer *et al.*³⁰⁵ from the same laboratory, and Jack and co-workers,³⁰³ attributed this effect to the unsaturated fraction. This difference in interpretation leaves open to question the validity of both experimental results.

In addition to the experiments of the Wisconsin group, Boer and his associates reported that butterfat produces better growth in young rats than do certain vegetable oils. Thus Boer³⁰⁷ reported better growth on butterfat than on olive oil. However, the choice of olive oil as a representative vegetable fat is a rather unfortunate one, since this oil readily becomes rancid, probably due to its relatively low tocopherol content. One must question the results of experiments in which olive oil was employed unless special precautions were taken to prevent it from becoming rancid. Clausen *et al.*³⁰⁸ called attention to the toxicity caused by the feeding of rancid fat.

³⁰⁴ E. Heftmann, *J. Nutrition*, **34**, 455-467 (1947).

³⁰⁵ R. P. Geyer, B. R. Geyer, P. H. Derse, H. Nath, V. H. Barki, C. A. Elvehjem, and E. B. Hart, *J. Dairy Sci.*, **30**, 299-305 (1947).

³⁰⁶ E. L. Jack and E. B. Hinshaw, *J. Nutrition*, **34**, 715-724 (1947).

³⁰⁷ J. Boer, *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.*, **11**, 180-182 (1941).

³⁰⁸ D. F. Clausen, R. H. Barnes, and G. O. Burr, *Proc. Soc. Exptl. Biol. Med.*, **53**, 176-178 (1943).

In a later study Boer and co-workers³⁰⁹ reported that rapeseed oil diets were decidedly inferior to regimens containing butterfat in stimulating growth. This finding has been confirmed by Deuel and associates,³¹⁰ as well as by Euler, Euler, and Lindeman.³¹¹ The latter workers also found margarine superior to rapeseed oil in stimulating growth. Undoubtedly the low growth-promoting activity of rapeseed oil in the rat is associated with its poor digestibility in this species.²⁹⁶

The preponderance of experimental evidence would seem to indicate that satisfactory growth is obtained with a variety of vegetable oils, equal to that observed with butter. Thus Deuel and co-workers³¹² observed that the extent of growth in rats during the first twelve weeks after weaning was the same, irrespective of whether butterfat, corn, cottonseed, olive, peanut, or soybean oil, or a vegetable margarine fat, was chosen as the source of fat. In these latter experiments rancidity was prevented by refrigeration of the food and by the addition of tocopherol to the diet. The fact that the similar gains-in-weight obtained after the administration of the diets containing the several vegetable fats and butter represent true growth was confirmed by measurement of the bone length,³¹² as well as by the demonstration that the body composition of the animals was similar in these several groups.³¹³ Euler, Euler, and Säberg³¹⁴ likewise reported that butter exhibits no superiority over margarine insofar as growth-promoting properties in the case of rats is concerned. In fact, these workers observed better growth when rats were fed diets containing margarine than when butter served as the source of dietary fat. In later reports,³¹⁵ the comparison of the growth on butter and on margarine was extended to twenty-six months; growth was found to be equally satisfactory with the two fats over the entire period, although the individual weights of the offspring were higher on the butter diet. Deuel and co-workers,³¹⁶ Zialeita and Mitchell,³¹⁷

³⁰⁹ J. Boer, B. C. P. Jansen, and A. Kentie, *J. Nutrition*, **33**, 339-358 (1947).

³¹⁰ H. J. Deuel, Jr., S. M. Greenberg, E. E. Straub, D. Jue, C. M. Gooding, and C. F. Brown, *J. Nutrition*, **35**, 301-314 (1948).

³¹¹ B. v. Euler, H. v. Euler, and G. Lindeman, *Arkiv. Kem. Mineral. Geol.*, **B 26**, No. 3, 1-5 (1948).

³¹² H. J. Deuel, Jr., E. Movitt, L. F. Hallman, and F. Mattson, *J. Nutrition*, **27**, 107-121 (1944).

³¹³ H. J. Deuel, Jr., L. F. Hallman, E. Movitt, F. H. Mattson, and E. Wu, *J. Nutrition*, **27**, 335-338 (1944).

³¹⁴ B. v. Euler, H. v. Euler, and I. Säberg, *Ernährung*, **7**, No. 3, 65-74 (1942).

³¹⁵ B. v. Euler, H. v. Euler, and I. Rönneham-Säberg, *Arkiv. Kemi, Mineral., Geol.*, **A 22**, No. 8, 1-12 (1946).

³¹⁶ H. J. Deuel, Jr., S. M. Greenberg, E. E. Straub, T. Fukui, C. M. Gooding, and C. F. Brown, *J. Nutrition*, **38**, 361-368 (1949).

³¹⁷ L. P. Zialeita, Jr., and H. H. Mitchell, *Science*, **100**, 60-62 (1944).

Henry *et al.*,³⁰² and Lassen and Bacon³¹⁸ also reported that the growth-promoting properties of vegetable oils and of butter were similar. One explanation for the greater growth of the rats on the butterfat diets as compared with those receiving diets containing other fats, as observed in the experiments of Schantz *et al.*,²⁹⁹ might be that the rats ate more of the butter-containing diet. Thus it was shown that rats preferred a diet with artificial butter flavor (diacetyl) to a bland unflavored one.³¹⁹ Parrish *et al.*³²⁰ observed that, although the average efficiency of conversion of the food to body tissue over a period of six weeks was similar for rats receiving a butterfat ration and for those fed corn oil, the average consumption of the ration was greater on the butterfat diet. The gain in weight on either ration was related to the amount of food consumed. Although Boutwell and co-workers³²¹ failed to demonstrate a sufficiently marked improvement in the consumption of corn oil diets with the addition of diacetyl to produce growth equal to that observed when butterfat was fed, there was an appreciable increase in food consumption as a result of adding this flavoring. According to Scott and Verney,³²² however, young rats preferred diets containing hydrogenated vegetable oil rather than food including butterfat, or corn or cottonseed oil.

One criticism raised against the technic used in the original experiments of Deuel and co-workers³¹² was that the beneficial effects of the vegetable fat diets may have resulted from butterfat present in the skim milk powder,³²³ since this contained 1.1% of residual fat. In order to answer this criticism, tests were repeated in which the residual fat was previously removed as completely as possible from the skim milk powder by the use of a combined ethyl alcohol and diethyl ether extraction. Even under these conditions, no difference could be observed in the rate of growth of weanling rats during a twelve-week period on diets containing the extracted skim milk powder, irrespective of whether a butterfat, a vegetable margarine, or one of a number of vegetable oils was used in the diet.³²⁴

b'. Experiments on Prematurely Weaned Rats: According to Boutwell *et al.*³²⁵ the need of the young rats for the specific fatty acids reputed to be

³¹⁸ S. Lassen and E. K. Bacon, *J. Nutrition*, **39**, 83-91 (1949).

³¹⁹ H. J. Deuel, Jr., and E. Movitt, *J. Nutrition*, **27**, 339-346 (1944).

³²⁰ D. B. Parrish, E. R. Shiner, and J. S. Hughes, *J. Nutrition*, **31**, 321-331 (1946).

³²¹ R. K. Boutwell, R. P. Geyer, C. A. Elvehjem, and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, **55**, 153-155 (1944).

³²² E. M. Scott and E. L. Verney, *J. Nutrition*, **36**, 91-98 (1948).

³²³ Anonymous, *Nutrition Revs.*, **2**, 267-270 (1944).

³²⁴ H. J. Deuel, Jr., C. Hendrick, E. Movitt, M. E. Crockett, I. M. Smyth, and R. J. Winzler, *J. Nutrition*, **31**, 747-753 (1946).

³²⁵ R. K. Boutwell, R. P. Geyer, C. A. Elvehjem, and E. B. Hart, *J. Dairy Sci.*, **26**, 429-437 (1943).

in butter is most marked during the early days of growth following weaning. The specific growth-promoting properties of butter as compared with corn oil could no longer be demonstrated when the rats were maintained on a stock diet until they were thirty days of age, instead of being weaned and placed on the test diets at the usual period of twenty-one days. It was also indicated that the need for butter was accentuated when the animals were placed on test diets at fourteen days of age rather than at the usual period of twenty-one days. However, these results have not been supported by tests carried out in other laboratories. For example, Deuel and Movitt³²⁶ reported that rats weaned at fourteen days of age grew as rapidly on a diet containing corn, cottonseed, olive, peanut, or soybean oil, or a vegetable margarine fat, as they did on one containing butterfat. Moreover, Zialcita and Mitchell³¹⁷ demonstrated that rats weaned at the early age of seven days could be raised as satisfactorily on a special liquid diet containing corn oil as was possible on one in which butterfat was employed. These negative findings would seem to discount the age factor as related to a specific requirement for butterfat.

c'. Experiments on Rats Subjected to Undernutrition: Another procedure which has been employed to compare the nutritional value of fats is to study their effect upon growth when added to diets which yield insufficient calories to meet the normal need. Under such conditions, if a fat possesses a special merit in times of stress, its superiority should be evident. When this procedure was tested and the caloric levels were such that growth progressed at a subnormal rate, no differences were noted by the Deuel group, irrespective of whether butterfat, corn, cottonseed, olive, peanut, or soybean oil, or a vegetable margarine fat, was the fat employed.³²⁷ When the animals were given the same diets *ad libitum*, the growth response to the vegetable fat diets and to the butterfat regimen remained similar.³²⁷

d'. Experiments on Rats Receiving Growth Hormone: A somewhat different method for comparing nutritional value which has been employed in the study of the comparative nutritional value of fats consists in using the growth hormone as a stress factor. By means of this method one can gain information as to whether or not deficiencies may develop in association with the greater growth rate which occurs. If fats have different nutritional values one fat should presumably be able to supply this need to a greater extent than another fat is capable of doing. It has been reported that, when growth hormone is given to rats on a vitamin A-deficient diet,

³²⁶ H. J. Deuel, Jr., and E. Movitt, *J. Nutrition*, 29, 237-244 (1945).

³²⁷ H. J. Deuel, Jr., C. Hendrick, and M. Crockett, *J. Nutrition*, 31, 737-746 (1946).

the symptoms of avitaminosis A are accentuated.³²⁸ However, when the relative efficiencies of the several vegetable fats and of margarine fat and butterfat were tested in rats injected with growth hormone, no differences were found in the ability of the various groups to sustain the increased rate of tissue formation attributable to the hormone.³²⁷

e'. Experiments on Calves: Gullickson, Fountaine, and Fitch³²⁹ reported that calves fed on a skim milk diet into which lard, tallow, coconut oil, peanut oil, corn oil, cottonseed oil, or soybean oil was homogenized, in place of butterfat, did poorly. Some of the calves which received corn oil, cottonseed oil, or soybean oil died, while others were saved only by changing their diet to whole milk. In a later report by the Gullickson group,³³⁰ the authors stated that calves showed no ill effects from consuming homogenized milk containing 3.5% of hydrogenated vegetable oil for a period of over forty-five days, although the feeding of the non-hydrogenated vegetable oil and milk diet resulted in rapid depletion of the vitamin A and tocopherol in the blood plasma of the calves, and in tissue changes and lesions of the heart and striated muscles. These symptoms were relieved by the substitution of butterfat for the vegetable oil in the ration. Certain recent reports tend to minimize the importance of the early Gullickson work.³²⁹ Cunningham and Loosli³³¹ reported that newborn calves did not thrive on a fat-free diet, but developed leg weakness and muscular twitches within one to five weeks, and died unless a source of fat was supplied. This condition could be cured by feeding an artificial milk containing 4% of lard, and could be prevented if the synthetic diet contained 2% of lard. When hydrogenated coconut oil in an amount of 2% was added, the development of these symptoms was prevented. Arrington and Reaves³³² also noted that fats are essential food components in the calf diet. More recently Lambert and co-workers³³³ observed that growing calves require the EFA. Thus, when calves were placed on a fat-free diet, typical fat-deficiency symptoms developed which could be cured not only with butter oil but also with hydrogenated soybean oil plus lecithin. Thus, the gain-in-weight of calves on the fat-free regimen for the first fifty-six days of life averaged

³²⁸ B. H. Ershoff and H. J. Deuel, Jr., *Endocrinology*, **36**, 280-282 (1945).

³²⁹ T. W. Gullickson, F. C. Fountaine, and J. B. Fitch, *J. Dairy Sci.*, **25**, 117-128 (1942).

³³⁰ T. W. Gullickson, R. S. Adams, J. Gander, and J. H. Sautter, *J. Dairy Sci.*, **36**, 599-600 (1953).

³³¹ H. M. Cunningham and J. K. Loosli, *J. Dairy Sci.*, **37**, 453-461 (1954).

³³² L. R. Arrington and P. M. Reaves, *J. Dairy Sci.*, **31**, 1-9 (1948).

³³³ M. R. Lambert, N. L. Jacobson, R. S. Allen, and J. H. Zaletel, *J. Nutrition*, **52**, 259-274 (1954).

10.9 lb., while the corresponding increase was 30.3 lb. when an isocaloric diet containing hydrogenated soybean oil and lecithin was fed.

f'. Experiments on Children: Leichenger, Eisenberg, and Carlson³³⁴ made a comparative study of the growth of orphanage children who received either a vegetable margarine only or butter only as a table spread. This experiment lasted over a two-year period. Although this is a relatively short interval in the life-span of the human, the experiments were sufficiently decisive to leave no question as to their interpretation. It was demonstrated that the well-being of the children, as well as their growth (as determined by measurements of weight and height), were similar in the two groups during the test. These data would seem to indicate that the results obtained on rats are applicable to the human.

g'. The Relationship of the Vaccenic Acid Content to the Alleged Superior Growth-Promoting Activity of Butter: The Boer group suggested that the greater growth rate of rats on butter-containing diets, as compared with those receiving rapeseed oil or olive oil in their diets, could be traced to the presence of a particular fatty acid in butterfat, namely vaccenic acid, or 11-octadecenoic acid. Boer, Jansen, and Kentie³⁰⁹ were the first to observe that summer butter contains appreciable amounts of an unsaturated fatty acid which is absent from winter butter, as well as from vegetable fats. This acid was identified as vaccenic acid, and its presence in butterfat and in other fats has since been established by several investigators.³³⁵⁻³³⁷ Although vaccenic acid was not found as a component of vegetable fats, it has been reported in hydrogenated vegetable fats such as margarine.^{335,336} Considerable experimental evidence now refutes the hypothesis that vaccenic acid has a specific effect upon growth.

Vaccenic acid was identified as the specific nutritional adjunct in butter, not only on the basis of its presence in this fat,³⁰⁹ but also due to the demonstration of an increased gain-in-weight of rats receiving a rapeseed oil diet when vaccenic acid obtained from China wood oil was added to the rapeseed oil.^{338,339} It was further suggested by Boer *et al.*³⁰⁹ that the failure of Euler and co-workers^{314,340} to confirm the early results of Boer³⁰⁷ and of

³³⁴ H. Leichenger, G. Eisenberg, and A. J. Carlson, *J. Am. Med. Assoc.*, 136, 388-391 (1948).

³³⁵ S. H. Bertram, *Biochem. Z.*, 197, 433-441 (1928).

³³⁶ J. Grossfeld and A. Simmer, *Z. Untersuch. Lebensm.*, 59, 237-258 (1930).

³³⁷ R. P. Geyer, H. Nath, V. H. Barki, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.*, 169, 227-228 (1947).

³³⁸ J. Boer, B. C. P. Jansen, A. Kentie, and H. W. Knol, *J. Nutrition*, 33, 359-360 (1947).

³³⁹ J. Boer, B. C. P. Jansen, and A. Kentie, *Nature*, 158, (1946).

³⁴⁰ B. v. Euler, H. v. Euler, and I. Säberg, *Ernahrung*, 8, 257-264 (1943); *Chem. Abst.*, 39, 4921-4922 (1945).

Boer and Jansen,³⁴¹ which demonstrated the superiority of butterfat over margarine or over olive oil, might well be ascribed to the fact that the Swedish investigators used winter butter, from which vaccenic acid, in all probability, was lacking.

However, a number of reports fail to confirm the fact that vaccenic acid has a stimulatory effect on growth. Although Deuel *et al.*³¹⁰ did confirm the fact that rapeseed oil provides a poorer growth than does butterfat or cottonseed oil, no added growth could be demonstrated when vaccenic acid was added to the diet. Similar results were recorded by Euler and co-workers,³¹¹ and by Nath, Barki, Elvehjem, and Hart.³⁴² Sankar and Sarma³⁴³ also concluded that neither butter nor paddy grass contains unidentified nutrients necessary for the rat, and that vaccenic acid has no growth-promoting properties for this species. In fact, Boer and collaborators³⁴⁴ have retracted their earlier statement that vaccenic acid has specific nutritional properties.

12-Octadecenoic acid, which has physical properties similar to those of vaccenic acid, and which might well have been mistaken for it, was tested by the Deuel group for specific growth-promoting activity, and was found to be completely inactive.³¹⁶ Even if butterfat could be shown to have a growth-promoting activity superior to that of other animal fats or vegetable fats, it appears definite that neither vaccenic acid nor 12-octadecenoic acid could account for it.

(b) *Relative Nutritional Value as Based upon Pregnancy and Lactation Tests.* As stated earlier, pregnancy and lactation provide successively more critical indices of nutritional value than does growth. It has already been established that adequate lactation and survival of the young rat require the presence of definite amounts of essential fatty acids.³³ It would be expected therefore that if the importance of fat in pregnancy and lactation were largely or entirely to be ascribed to the presence of EFA, vegetable oils and margarine fat would function equally well or even better than butterfat as a nutritional requirement under these conditions. It has been reported from the laboratory of the author³⁴⁵ that a number of different vegetable oils, a vegetable margarine fat, and butterfat are equally

³⁴¹ J. Boer and B. C. P. Jansen, *Arch. néerland. physiol.*, 26, 1-177 (1942); *Chem. Zentr.*, 1942, I, 2012-2013.

³⁴² H. Nath, V. H. Barki, C. A. Elvehjem, and E. B. Hart, *J. Nutrition*, 36, 761-772 (1948).

³⁴³ D. V. S. Sankar and P. S. Sarma, *J. Sci. Ind. Research (India)*, 9, B, No. 7, 170-173 (1950).

³⁴⁴ J. Boer, E. H. Groot, and B. C. P. Jansen, *Voeeding*, 9, 60-62 (1948); *Chem. Abst.*, 42, 7847 (1948).

³⁴⁵ H. J. Deuel, Jr., E. Movitt, and L. F. Hallman, *J. Nutrition*, 27, 509-513 (1944).

effective in ensuring normal pregnancy and lactation. Data illustrating this are given in Table 8.

TABLE 8
THE COMPARATIVE NUTRITIONAL VALUE, AS DETERMINED BY
PERFORMANCE IN PREGNANCY AND LACTATION, IN RATS
RECEIVING DIETS CONTAINING BUTTERFAT
OR VARIOUS VEGETABLE OILS^a

Dietary fat	Pregnancy tests			Lactation tests	
	Animals bred	Litters cast	Av. 3-day weight per rat, g.	Rats dead before weaning at 21 days	Av. 21-day weight per rat, ^b g.
Butterfat	29	29	7.8	23	33.4 (84)
Corn oil	31	28	7.7	1	32.3 (112)
Cottonseed oil	31	30	8.0	2	32.7 (98)
Vegetable margarine fat	35	33	7.8	8	31.0 (112)
Olive oil	21	18	7.7	1	35.7 (70)
Peanut oil	32	31	7.9	4	31.5 (84)
Soybean oil	28	26	8.0	1	34.3 (98)

^a Adapted from H. J. Deuel, Jr., E. Movitt, and L. F. Hallman, *J. Nutrition*, 27, 509-513 (1944).

^b Figures in parentheses indicate the number of rats weaned. Only seven-rat litters are included in the average.

Euler, Euler, and Rönnestam-Säberg³⁴⁵ carried out a very extensive two-year study of the reproductive performance of rats on diets containing butter or margarine without and with added vitamin E. The total weight of the offspring at the age of thirty days, which gives an index of the number of young and the effectiveness of the lactation, over a period of one and a half years, was 8,508 g. for the group on the butter-containing diet, as compared with the figure of 15,955 g. for those on the margarine fat regimen. Although supplementation with vitamin E improved the performance of the butterfat group, the total weight of such litters was still less satisfactory than was that of the unsupplemented margarine group. Euler and Euler³⁴⁶ reported later that, for a period of eighteen months, covering four generations, butter and margarine, when fed in a diet consisting of 24% skim milk powder, 10% fat, 65% wheat powder, 1% sodium chloride, 5 g. of spinach, and 5 g. of meat per animal per week, together with 100 I.U. of vitamin A and 5.8 I.U. of vitamin D, resulted in no significant differences in longevity and fertility or in growth rate. In view of these results, and especially

³⁴⁶ B. v. Euler and H. v. Euler, *Arkiv. Kemi*, 3, 31-39 (1951).

because of the relatively high vitamin E content of vegetable oils as contrasted with that of butterfat, it is difficult to explain the recent findings of Viswanatha *et al*,³⁴⁷ who reported that butterfat provided better growth for rats on a lactose or sucrose diet than did corn oil, even when the latter diet was supplemented with vitamin E.

(c) *Multigeneration Tests as a Method for Comparing the Nutritional Value of Animal and of Vegetable Fats.* The so-called "multigeneration" method was employed by several investigators as a criterion of the nutritional value of foodstuffs. In this procedure, the rats are raised on a diet containing the test substances until mature; they are then bred, and the progenies are continued on the same diet. After the young are weaned, the parents are discarded and the new generation is bred at maturity.³⁴⁸ Sherman and Campbell^{349,350} devised a simple diet which has been employed successfully in carrying out multigeneration experiments with diets containing butterfat as the sole source of fat. This experiment has progressed further than any other similar type of experiment on record. The diet was successful in enabling rats to continue growth, reproduction, and lactation for at least sixty-seven generations; when last reported (1949) it was still in progress.³⁵¹ In the 1949 report the authors stated that the adequate vitamin A intake for a laboratory rat family of the sixty-seventh generation was 3 I.U./g. dry food. The so-called Sherman B diet, which has been used from the fortieth generation on in their tests, contains one-third whole milk powder, and two-thirds ground wheat berries to which 2% of sodium chloride is added. A test similar to that of Sherman was instituted in the author's laboratory in 1940. In the 1945 test, skim milk powder was employed in place of whole milk powder, and an amount of vitamin A-fortified vegetable margarine fat comparable to that of the butterfat present in the whole milk powder was added to the skim milk powder. At the end of ten generations of this series of tests, the rate of growth of both males and females had reached a plateau considerably higher than at the start of the test.³⁵² Fertility and lactation, also, had been maintained at a high level throughout the first ten generations. The results after twenty-five generations mirrored the early tests in all respects.³⁵³ In the twenty-fifth genera-

³⁴⁷ T. Viswanatha, J. E. Gander, and I. E. Liener, *J. Nutrition*, 52, 613-626 (1954).

³⁴⁸ H. J. Deuel, Jr., S. M. Greenberg, C. E. Calbert, R. Baker, and H. R. Fisher, *Food Research*, 16, 258-280 (1951).

³⁴⁹ H. C. Sherman and H. L. Campbell, *J. Biol. Chem.*, 60, 5-15 (1924).

³⁵⁰ H. C. Sherman and H. L. Campbell, *J. Nutrition*, 2, 415-417 (1929-1930).

³⁵¹ H. C. Sherman and H. Y. Trupp, *Proc. Nat. Acad. Sci.*, 35, 90-92 (1949).

³⁵² H. J. Deuel, Jr., L. F. Hallman, and E. Movitt, *J. Nutrition*, 29, 309-318 (1945)

³⁵³ H. J. Deuel, Jr., S. M. Greenberg, E. E. Savage, and L. Bavetta, *J. Nutrition*, 42, 239-255 (1950).

tion group, the calcium content of the rats twenty-one days old, and the calcium balance of the growing animals maintained on this modified Sherman diet over a period of ninety days, were more satisfactory than in the case of animals receiving the regular Sherman diet, which contains butter-fat in place of a vegetable margarine fat. When last reported (July, 1955) the forty-second generation was being raised. There was no indication of nutritional failure. Growth, fertility, and lactation were continuing at high levels. The results of these experiments indicate that vegetable fat can supply all the needs of the rat for fat over an indefinite period.

(d) *Other Nutritional Indices for Comparing the Nutritional Value of Animal and of Vegetable Fats.* Several other indices of nutritional value have indicated that the comparative nutritive values of butter and of vegetable fats are essentially the same. Thus the so-called "survival factor," as suggested by Keane and Johnson³⁵⁴ occurs to the greatest extent in wheat germ oil, although smaller amounts are present in butter. This is the factor which is necessary to enable the female rat to rear her young to weaning age successfully. Butter improves the survival rate considerably, but is less potent than wheat germ oil. This particular factor does not appear to be identical with any of the known vitamins or essential fatty acids. Lard does not contain this factor. The primary effect of the survival factor is exerted in the *in utero* nutrition of the pup, but the factor is secreted to some extent in the milk. It does not, however, appear to be necessary for the lactation process.

Stress as produced by cold environmental conditions is another nutritional index which has been employed for comparing butter with vegetable oils. Thus Ershoff, Pagones, and Deuel³⁵⁵ reported that the weight gains of immature female rats raised under cold room temperature conditions on purified rations which differed only in the source of fat were significantly less than the gains of rats maintained at normal room temperature. However, the gain in weight was approximately the same, irrespective of whether butter, corn oil, cottonseed oil, or a vegetable margarine fat was employed as a source of fat.

Another condition which has been employed for the evaluation of butter-fat and of vegetable oils has to do with differences in the carbohydrate content of the foods. Thus, it was reported by Boutwell *et al.*³⁵⁶ that, when rats were placed on a diet containing sucrose, a fructose-glucose mixture,

³⁵⁴ K. W. Keane and B. C. Johnson, *Federation Proc.*, 12, 418-419 (1953).

³⁵⁵ B. H. Ershoff, J. N. Pagones, and H. J. Deuel, Jr., *Proc. Soc. Exptl. Biol. Med.*, 70, 287-290 (1949).

³⁵⁶ R. K. Boutwell, R. P. Geyer, C. A. Elvehjem, and E. B. Hart, *Arch. Biochem.*, 7, 143-157 (1945).

starch, dextrin, dextrimaltose, or lactose, and moderate amounts of thiamine, riboflavin, pyridoxine, pantothenic acid, and choline, the rats which received butterfat grew at a faster rate than did those fed corn oil. This inferiority of corn oil could be minimized in the group on lactose rations, and was eliminated in those on all other rations, by raising the level of these vitamins, and by adding high levels of inositol, *p*-aminobenzoic acid and nicotinic acid, plus 1% of whole liver powder. No differences in growth were noted at any vitamin level when glucose or galactose-glucose mixtures were the rations employed in the tests. On the basis of these experiments it has been assumed that butterfat stimulates the synthesis of the vitamins B in the intestinal tract more than is the case with corn oil. However, in view of the rather small differences in body weight noted when butterfat or the corn oil, respectively, was employed, these experimental data do not seem to be of much practical importance in assessing the comparative nutritional value of the two types of fat.

One criterion employed in evaluating the nutritional efficiency of butterfat as compared with corn oil is the level at which it is fed in the diet. Thus, when high levels of the fat were given (35%) the rats receiving the butterfat diets gained the most weight while, in the groups maintained on lower levels of fat (10%), those receiving the corn oil did better, insofar as gain-in-weight was concerned.³⁵⁷ On the other hand, changes in the level of coconut oil or of soybean oil in the diet did not result in significant changes in growth. It is thus suggested that the different fats produce optimum growth at different levels of intake, so that this fact must be taken into consideration in the comparative evaluation of their nutritive values.³⁵⁷

(3) *The Effect of Heating upon the Nutritional Value of Fats and Oils*

The effect of polymerization was discussed in Vol II of *The Lipids*, pp. 232 ff.

It has been suggested that the heated fats may develop some carcinogenic activity. Dunning *et al.*³⁵⁸ reported that a diet high in fat shortened the latent period and increased the number of malignant mammary tumors induced by diethylstilbestrol in rats. The effect of the high-fat diet could be inhibited by caloric restriction. Lane, Blickenstaff, and Ivy³⁵⁹ reported a greater incidence of cancerous stomach lesions when "browned" lard which had been heated at 350°C. for thirty minutes was fed to rats, than when

³⁵⁷ V. H. Barki, R. A. Collins, C. A. Elvehjem, and E. B. Hart, *J. Nutrition*, **40**, 383-392 (1950).

³⁵⁸ W. F. Dunning, M. R. Curtis, and M. E. Maun, *Cancer Research*, **9**, 354-361 (1949).

³⁵⁹ A. Lane, D. Blickenstaff, and A. C. Ivy, *Cancer*, **3**, 1044-1051 (1950).

unheated lard was employed. Approximately 37% of the animals developed gastric lesions after twelve months, while only 5.7% of the rats in the control group presented lesions. Three of the thirty-one rats which had been given subcutaneous injections of heated lard or of partially hydrogenated vegetable oil developed sarcoma within twelve to eighteen months. No tumors developed in the 150 control rats of the same age and strain.³⁵⁹ Chalmers³⁶⁰ reported that tumors of the forestomach could be induced in mice fed a diet containing heated cottonseed oil. Several reports from Peacock *et al.*³⁶¹ and from Beck and co-workers³⁶² indicate a relationship between the oral administration or subcutaneous injection of heated fats and the presence of cancer.

Further work is necessary before one can decide with certainty whether or not the heating of fats under controlled conditions will induce these deleterious effects. The findings of Farmer, Crampton, and Siddall³⁶³ should be mentioned. They reported that reproduction and lactation were impaired in female rats by diets containing 10% of linseed oil heated at 275°C. This deleterious effect could not be counteracted by α -tocopherol.

(4) *The Effect of Rancidity upon the Nutritional Value of Fats and Oils*

It has long been recognized that rancidity influences the interpretation and outcome of nutritional experiments. According to Greenberg and Frazer,³⁶⁴ there are a number of ways in which rancid fats may have an adverse effect upon growth. Thus they may cause a condition of stress brought about by an irritating effect or by the presence of toxic agents. Secondly, they may interfere with the nutritional properties of fat. Thirdly, when rancid fats are present in the diet they may destroy other nutrients. They may possibly alter the intestinal flora. Finally, the principal respect in which rancidity may affect nutritional experiments is by decreasing the food intake of the test animals. Clausen, Barnes, and Burr,³⁶⁸ in 1943, called attention to the fact that growth on diets containing various fats was markedly influenced by rancidity. They indicated that "The inadvertent destruction of dietary essentials and the possible independent toxicity of rancid fat are factors that might confuse the interpretation of many diet experiments." Greenberg and Fraser³⁶⁴ reported that the presence of

³⁶⁰ J. G. Chalmers, *Biochem. J.*, 52, xxxl (1952).

³⁶¹ P. R. Peacock, *Brit. Med. Bull.*, 4, 364-367 (1946-1947); P. R. Peacock and S. Beck, *Brit. J. Exptl. Pathol.*, 24, 143-146 (1943); *Nature*, 162, 252-253 (1948).

³⁶² S. Beck, *Brit. J. Exptl. Pathol.*, 22, 299-302 (1941); S. Beck, A. H. M. Kirby, and P. R. Peacock, *Cancer Research* 5, 135-139 (1945).

³⁶³ F. A. Farmer, E. W. Crampton, and M. Siddall, *Science* 113, 408-410 (1951).

³⁶⁴ S. M. Greenberg and A. C. Frazer, *J. Nutrition*, 50, 421-440 (1953).

rancid fat in the diet increases the protein requirement of the rat for optimal growth. However, a diet containing 10% of rancid soybean oil supported almost normal growth in rats when all essential nutrients were supplied in large amounts and protein constituted 30% of the diet. Under these conditions the only organ weight seriously affected by the presence of rancid fat was that of the intestinal tract. Greenberg and Frazer³⁶⁴ also showed that whole desiccated liver benefited the rats in proportion to the protein content, but that cortisone exerted no beneficial effect. Drugs given to change the intestinal flora did not modify the growth of rats fed rancid fat.

Kaunitz³⁶⁵ found that the feeding of highly oxidized fats was not as toxic as is generally believed, if additional protein was added to the diet. This worker considers that the protein reduces the active compounds of oxidized fat and thus inactivates them. The effect may possibly be due to relatively easily reduced products which are formed during oxidation.

Burr and Barnes³⁶⁶ reviewed the subject up to 1943. According to these authors, the following substances might be destroyed by the prooxidant effect of the fats: vitamin A, carotene, vitamin D, vitamin E, pantothenic acid, pyridoxine, biotin, ascorbic acid, and linoleic acid. Although the information is not complete on the effect of oxidized fat on all of these substances, considerable experimental data have been amassed. It is suggested that the dermatitis which sometimes occurs in older animals, and the caudal necrosis in the newborn which have been subjected to diets deficient in vitamin E, are relieved by the administration of wheat germ oil.³⁶⁶ Although the effect of rancid fats on pantothenic acid or on pyridoxine is not known, it has been shown that biotin, at least, is destroyed by organic peroxides,^{367,368} and by products formed in rancid fats.²¹⁶ Fritz and co-workers³⁶⁹ demonstrated that rancid fats are also of importance in the destruction of vitamin D, due to the oxidation which occurs. Ascorbic acid, which is known to act as an antioxidant, is not appreciably oxidized during the induction period of rancidity.³⁷⁰

When peroxidation of unsaturated fats occurs, linoleic acid is destroyed. Feeding of these fats has been shown to result in fat-deficiency symptoms in rats³⁷¹ and in skin lesions in dogs.³⁷² These skin lesions suggest linoleic

³⁶⁵ H. Kaunitz, *Arch. exptl. Pathol. Pharmacol.*, **220**, 16-25 (1953).

³⁶⁶ G. O. Burr and R. H. Barnes, *Physiol. Revs.*, **23**, 256-278 (1943).

³⁶⁷ H. A. Mattill and C. Golumbic, *J. Nutrition*, **23**, 625-631 (1942).

³⁶⁸ G. B. Brown and V. du Vigneaud, *J. Biol. Chem.*, **141**, 85-89 (1941).

³⁶⁹ J. C. Fritz, J. L. Halpin, and E. H. Kramke, *Ind. Eng. Chem.*, **34**, 979-982 (1942).

³⁷⁰ C. Golumbic and H. A. Mattill, *J. Am. Chem. Soc.*, **63**, 1279-1280 (1941).

³⁷¹ D. V. Whipple, *Oil & Soap*, **10**, 228-229 (1933); *Am. J. Med. Sci.*, **187**, 864-865 (1934).

³⁷² D. V. Whipple, *Proc. Soc. Exptl. Biol. Med.*, **30**, 319-321 (1932).

acid deficiency, but in a much more severe form than that ordinarily produced by the exclusion of essential fatty acids from the diet. Holman and Greenberg³⁷³ reported that ethyl linoleate, either fresh, slightly oxidized, or fed with benzoyl peroxide, cured fat deficiency symptoms and decreased water consumption in fat-deficient rats. Ethyl linoleate hydroperoxide, thermally decomposed ethyl linoleate hydroperoxide, reduced ethyl linoleate hydroperoxide, and conjugated ethyl linoleate, were all found to be ineffective in curing the fat deficiency symptoms or in decreasing water consumption. However, when all of the essential nutrients were fed separately from the rancid fat, no toxic symptoms developed in rats.³⁷⁴ Thus Kaunitz, Johnson, and Slanetz³⁷⁴ reported that the inclusion of 10% of rancid lard in the purified rat diet did not result in toxic symptoms under these conditions, although the rats were 5% lighter than the control animals. However, when riboflavin was withdrawn, a severe reduction of growth and food intake occurred. A less pronounced effect was observed when mildly rancid lard was fed, or when fresh fat was given. It is therefore apparent that rancidity accentuates the signs of riboflavin deficiency, which may well be due to a reduction of the riboflavin made available by the intestinal flora. It is also believed by these workers that rancid lard increases the requirements for various other essential components.³⁷⁴ Others who have reported on the destruction of vitamins and of other essential dietary components by rancid fats are Quackenbush,³⁷⁵ Barnes and co-workers,³⁷⁶ and Grench³⁷⁷ and Barnes *et al.*³⁷⁶ found that the ingestion of highly rancid fat by rats resulted in oxidative destruction of the biotin synthesized in the intestine.

György and co-workers³⁷⁸ observed that, when diets containing linoleic acid and cod-liver oil became rancid, they were very toxic for rats. The animals lost weight, and developed secondary anemia and leucopenia, and were subject to pediculosis. However, these symptoms could be prevented by the daily addition of yeast to the diet. The toxicity was apparently due to oxidation of the unsaturated acid. It was also found that linoleic acid present in the diet was almost completely destroyed by oxidation on standing at room temperature for three to four weeks.

³⁷³ R. T. Holman and S. I. Greenberg, *Federation Proc.*, **12**, 219 (1953).

³⁷⁴ H. Kaunitz, R. E. Johnson, and C. A. Slanetz, *J. Nutrition*, **46**, 151-159 (1952).

³⁷⁵ F. W. Quackenbush, *Oil & Soap*, **22**, 336-338 (1945).

³⁷⁶ R. H. Barnes, M. Clausen, I. I. Rusoff, H. T. Hanson, M. E. Swendseid, and G. O. Burr, *Arch. sci. physiol.*, **2**, 313-328 (1948).

³⁷⁷ J. M. Grench, Ph.D. Thesis, Univ. Birmingham Med. School (England), 1949; cited by S. M. Greenberg and A. C. Frazer, *J. Nutrition*, **50**, 421-440 (1953), p. 421.

³⁷⁸ P. György, R. Tomarelli, R. P. Ostergard, and J. B. Brown, *J. Exptl. Med.*, **76**, 413-420 (1942).

4. The Nutritional Value of Modified Fats and Oils

(1) *Monoglycerides and Diglycerides*

Although it is generally agreed that mono- and diglycerides are intermediates in fat digestion and in fat absorption, as well as in its intermediary metabolism, experimental evidence on the subject has only recently become available. These compounds have been known since they were synthesized by Berthelot in 1860.³⁷⁹ Moreover, mixtures of mono- and diglycerides have been produced industrially for many years by reacting good fats, chiefly triglycerides, with glycerol according to patents of Edeler and Richardson issued in 1934 and 1940.³⁸⁰ These materials had been widely used as food additives during the period between 1928 and 1932. It was found that the addition of these commercial products in amounts of one to two parts per hundred brought about a modification of the so-called "functional properties" of both margarine and shortening. According to the report of the Food Protection Committee of the National Research Council,³⁸¹ today, in the United States, almost every major brand of retail shortening contains about 2% of added monoglycerides and about 2% of added diglycerides. Shortenings used by bakers for cake making contain over 3% of each of these components, and some shortening products designed for use in baking bread contain as much as 7 to 10% of added monoglycerides and a like amount of added diglycerides. Mono- and diglycerides are used to a somewhat lesser extent in margarine; a maximum of 0.5% is allowed by the Federal Standard of Identity for Oleomargarine.^{381,382} Some experimental evidence indicates that mono- and diglycerides are effectively utilized by the animal body. Thus, Braun and Shrewsbury³⁸³ noted that monostearin and monolinolein were nutritionally approximately equivalent to lard in producing growth in rats when fed at a level of 8%. In 1951 Ames and his co-workers³⁸⁴ reported no difference in growth, repro-

³⁷⁹ M. Berthelot, *Chimie organique fondée sur la synthèse*, 2, 68-100 (1860); cited by B. F. Daubert and C. G. King, *Chem. Revs.*, 29, 269-285 (1941) p. 271.

³⁸⁰ A. Edeler and A. S. Richardson (to Procter and Gamble), *U. S. Patents Nos. 2,206,167* and *2,206,168* (July 2, 1940); *Canadian Patents, Nos. 340,803-340,805* (April 10, 1934).

³⁸¹ Anonymous, *The Safety of Mono- and Diglycerides for Use as Intentional Additives in Foods*, Report of Food Protection Committee, Nat. Research Council, *Pub. No. 251*, 1-14 (Dec., 1952).

³⁸² Anonymous, Oleomargarine. *Definition and Standard of Identity*, Fed. Security Agency, Food and Drug Admin., Service and Regulatory Announcements, Food, Drug and Cosmetic No. 2 Rev., 1, Part 45, 58-60 (Jan., 1949).

³⁸³ W. Q. Braun and C. L. Shrewsbury, *Oil & Soap*, 18, 249-250 (1941).

³⁸⁴ S. R. Ames, M. P. O'Grady, N. D. Embree, and P. L. Harris, *J. Am. Oil Chemists' Soc.*, 28, 31-33 (1951).

duction, or lactation performance between rats fed mono- or triglycerides prepared from cottonseed oil and those fed the refined cottonseed oil. The monoglycerides used in these tests were separated by molecular distillation. Mattson and co-workers³⁸⁵ investigated the mono-, di-, and triglycerides isolated from soybean oil and from coconut oil, as well as purified mono- and triglycerides of oleic, stearic, and lauric acids. It was concluded that the mono-, di-, and triglycerides of corresponding fatty acid composition are calorically equivalent. The caloric efficiencies of the mono- and triglycerides of both stearic and lauric acids were found to be low, but this was attributed entirely or partially to poor absorption. In later investigations, Mattson and associates³⁸⁶ isolated both monoglycerides and diglycerides from the lumen of the intestine of rats, following the feeding of triglycerides; these constituted 16% and 36%, respectively, of the total lipids separated. This fact supports the work of Frazer and Sammons.³⁸⁷ The Food Protection Committee of the National Research Council³⁸¹ concluded that purified monoglycerides, as well as mixtures of mono- and diglycerides ("superglycerinated edible fats"), present no significant differences in nutritive value from that of the triglycerides. Such criteria are based upon measurements of the rate of increase of body weight, food consumption data, reproduction through several generations, lactation, digestibility and absorption, deposition of fatty acids in animal tissues, possible influence upon absorption or metabolism of other substances, and gross and microscopic examination of tissues for any evidence of deviation from the normal.³⁸⁸⁻³⁹³ On the basis of this study the Food Protection Committee of the National Research Council³⁸¹ concluded that the mono- and diglycerides are usual components of foods, that they are normally formed in the gastrointestinal tract, that their metabolism does not differ from that of the triglycerides, and that their efficiency as emulsifiers indicates that "there is no evidence on which to question the safety of mono- and diglycerides as food additives."

³⁸⁵ F. H. Mattson, F. J. Baur, and L. W. Beck, *J. Am. Oil Chemists' Soc.*, **28**, 386-390 (1951).

³⁸⁶ F. H. Mattson, J. H. Benedict, J. B. Martin, and L. W. Beck, *J. Nutrition*, **48**, 335-344 (1952).

³⁸⁷ A. C. Frazer and H. G. Sammons, *Biochem. J.*, **39**, 122-128 (1945).

³⁸⁸ A. D. Holmes, *U. S. Dept. Agr. Bull. No. 613*, 1-25 (April 25, 1919).

³⁸⁹ G. Peretti, *Boll. soc. ital. biol. sper.*, **10**, 873-874 (1935).

³⁹⁰ J. S. Huff, R. K. Waugh, and G. H. Wise, *J. Dairy Sci.*, **34**, 1056-1063 (1951).

³⁹¹ A. R. Bourke, J. P. Frawley, and O. G. Fitzhugh, *Federation Proc.*, **11**, 325 (1952).

³⁹² H. C. Tidwell and M. E. Nagler, *Federation Proc.*, **11**, 298-299 (1952).

³⁹³ A. Argvyrus and O. Frank, *Z. Biol.* [2], **41** (59) 143-164 (1912).

(2) Synthetic Fats

A number of types of synthetic fat were manufactured in Germany during the war, from petroleum products or from brown coal. The fatty acids and neutral fats obtained were not entirely satisfactory, and posed a number of problems as to palatability and the ability to metabolize the material in a normal manner. For a further discussion of the earlier work the reader is referred to *The Lipids*, Vol. I, pages 175-178, as well as to the FAO Report on Synthetic Fats.³⁹⁴ In 1949 Thomas and Weitzel³⁹⁵ reported that the use of synthetic fats can be defended on the following grounds: Oxidation products are now largely removed with superheated steam; isoparaffins, and iso-fatty acids, can be extracted with solvents; the quantity of diacidogenic fatty acids (C_9 - C_{11}) ingested with the synthetic fats is not sufficient to be harmful, since methylated fatty acids are metabolized to a large extent; succinuria is not necessarily caused by the synthetic fats. These synthetic fats have been greatly improved since the war, and further improvement can be expected. Kraut *et al.*³⁹⁶ reported a number of experiments on synthetic fats in which the chain length of the fatty acids was 6 to 12 carbons. When synthetic fat was fed to rats they grew less rapidly than did those fed soya bean oil, partly because of their repugnance toward the high-fat diet containing the synthetic fat. In the case of dogs, however, there was no growth difference between animals given the synthetic or the natural fat. Moreover, young pigs which had been pair-fed on synthetic and soya fat grew to the same extent. No disturbance in health was observed in any case. A fat of a higher iodine number was obtained in the abdominal and back fat of pigs after the feeding of soybean oil. However, none of the short-chain acids could be noted in any case following the administration of a synthetic C_{6-12} fat. The oxyacids present as impurities in the synthetic fats could be identified by taste in the pig fat. This is presumably due to the fact that the oxycarboxylic acids are not completely metabolized, and therefore are deposited in the body fat. It is apparently necessary that all keto- and oxyacids be removed from any such synthetic fat before it can be used for human nutrition. Schaltenbrand and Schorn³⁹⁷ observed toxicity when various fats synthesized from coal were fed to rats at levels of 57 or 64% of the total dietary calories. These toxic symptoms

³⁹⁴ F. A. O. Report, *Synthetic Fats, Their Potential Contribution to World Food Requirements*, Washington, D. C., Nov. 3, 1948.

³⁹⁵ K. Thomas and G. Weitzel, *Klin. Wochschr.*, 27, 784-786 (1949).

³⁹⁶ H. Kraut, A. Weischer, R. Hügel, and G. Stumpff, *Biochem. Z.*, 318, 472-494 (1948).

³⁹⁷ G. Schaltenbrand and J. Schorn, *Deut. Z. Nervenheilk.*, 159, 408-416 (1948).

ensued most frequently following administration of a synthetic fat containing largely C₉-, C₁₀-, and C₁₁-carbon fatty acids. The same diet in which 60% of the calories was present as coconut or as peanut oil produced no similar symptoms. When rats were fed synthetic diets with triglycerides obtained from fatty acids with 10 and 11 carbons, they developed a shaggy coat and severe spastic paresis of the legs within four to six days. Areas of softening in the spinal cord and medulla, and meningeal bleeding, were observed on autopsy. When synthetic fat was removed from the diet, the symptoms gradually regressed. These authors demonstrated similar toxic effects in monkeys. Harris *et al.*³⁹⁸ reported that, when a synthetic triglyceride containing all of the acids as dihydroxystearic acid was fed to weanling rats at levels of about 2% as replacement for equal amounts of hydrogenated fat, the rats gained more weight and showed superior gain-in-weight per g. of food intake. The weight increase was due to better growth and development, and not to deposition of adipose tissue. On the other hand, tri-trihydroxystearoglyceride exerted little if any influence upon growth and development. The difference in effectiveness of these compounds may be due entirely to differences in melting point.

(3) *Acetoglycerides*

When acetyl groups are introduced into the glyceride molecules in place of one or more of the long-chain fatty acids, fats are formed with interesting and unusual properties. These fats were described almost simultaneously by Feuge *et al.*^{399,400} and by Baur, Jackson, and Lange.⁴⁰¹⁻⁴⁰³

Feuge *et al.*³⁹⁹ prepared acetostearins by reacting acetic anhydride with monostearins. The melting points of the products depended upon the purity of the monostearin and the degree of acetylation, and varied between 37°C. and 60°C. Inasmuch as these acetostearins may be useful as coating fats for foods, their melting range is important.³⁹⁹ As far as chemical properties such as resistance to oxidation, and stability at high tempera-

³⁹⁸ R. S. Harris, H. Sherman, and E. E. Lockhart, *Arch. Biochem.*, **5**, 63-70 (1944).

³⁹⁹ R. D. Feuge, E. J. Vicknair, and N. V. Lovegren, *J. Am. Oil Chemists' Soc.*, **29**, 11-14 (1952).

⁴⁰⁰ R. O. Feuge, E. J. Vicknair, and N. V. Lovegren, *J. Am. Oil Chemists' Soc.*, **30**, 283-287 (1953).

⁴⁰¹ F. J. Baur and W. Lange (to the Procter and Gamble Co.), *U.S. Patent No. 2,614,937* (Oct. 21, 1952).

⁴⁰² F. L. Jackson (to the Procter and Gamble Co.), *U. S. Patent No. 2,615,159* (Oct. 21, 1952).

⁴⁰³ F. J. Baur (to the Procter and Gamble Co.), *U. S. Patent No. 2,615,160* (Oct. 21, 1952).

tures, are concerned, the acetylglycerides were found to be as satisfactory as ordinary fats of comparable degrees of unsaturation.

Several types of acetylated glycerides are possible, namely monoacetyl and diacetyl glycerides. The terms "monoacetins" and "diacetins" are widely used to indicate the composition of these glycerides. Baur⁴⁰⁴ proposed the term "acetin fats" to cover this group of compounds.

The preparation and use of acetoglycerides containing predominantly oleic acid as the fatty acid with a high molecular weight were described by Feuge and co-workers.⁴⁰⁵ Baur also reported on the preparation and properties of diacetin fats from common vegetable oils (soybean, cottonseed, rapeseed, and coconut oils).^{403,406} The most useful property of the fats formed by the introduction of the acetyl group into the glyceride molecules is the pronounced lowering of the melting point which occurs.⁴⁰⁴ Up to the time of the discovery of the acetoglycerides, a low-melting fat consisted of either an unhydrogenated oil or of one only slightly hydrogenated. These fats contain such large amounts of highly unsaturated acids that they do not have stability toward oxygen or the keeping quality necessary for their extensive use. This fact has limited their employment in the production of plasticizing coatings. Thus acetoglycerides with a high degree of saturation and a significantly increased resistance to oxidation may be prepared which can replace the more perishable unsaturated fats having similar melting points.⁴⁰⁶

One possible use for the acetin fats is as a coating for processed meats, and for cheese, to retard loss of moisture and deterioration.⁴⁰⁷ They may also be used to coat hard candies, raisins, and nuts. The primary requirement here is for an oil which will not become rancid when a thin film is exposed to air for long periods of time. Acetostearin products are stable to oxidation, especially if they are derived from completely hydrogenated vegetable oils which contain natural antioxidants.⁴⁰⁸ It is possible that mixed acetin fats may be substituted for normal fats as salad oils and spreads when improved keeping quality is essential.⁴⁰⁴ After complete hydrogenation, which renders them immune to oxidation and rancidity, they still retain the waxlike softness necessary for their use as spreads or salad dressings.⁴⁰³

In tests of the solubility of hard fats in liquid oils, which is also of im-

⁴⁰⁴ F. J. Baur, *J. Am. Oil Chemists' Soc.*, **31**, 147-151 (1954).

⁴⁰⁵ R. O. Feuge, A. T. Gros, and E. J. Vicknair, *J. Am. Oil Chemists' Soc.*, **30**, 320-325 (1953).

⁴⁰⁶ F. J. Baur, *J. Am. Oil Chemists' Soc.*, **31**, 196-199 (1954).

⁴⁰⁷ R. O. Feuge, *Food Eng.*, **25**, N. 6, 100-103 (June, 1953).

⁴⁰⁸ R. O. Feuge, *Food Technol.*, **9**, 314-318 (1955).

portance in the utilization of these products in foods, it was found by Ward *et al.*⁴⁰⁹ that tristearin was equally soluble, on a weight basis, in 1,2-diaceto-3-olein, 1,2-dibutyro-3-olein, and cottonseed oil. On a mole fraction basis the tristearin was most soluble in triolein and least soluble in the diaceto-olein. Hydrogenated cottonseed oil was equally soluble, on a weight basis, in the acetoölein product and in cottonseed oil.

Mattson and co-workers⁴¹⁰ reported feeding experiments with diacetin fats containing stearic acid. It was found that the long-chain fatty acids with an iodine value of one were best utilized in the form of diacetin fat, and hence the group consuming this material exhibited greater food efficiency than did the corresponding control group fed triglycerides consisting entirely of long-chain fatty acids with an iodine number of one.

On the other hand, triacetin has been said to have a deleterious effect on growth, in the absence of fat. Bosshardt and Huff¹⁵⁶ found that mice fed a low-fat diet complete in other dietary essentials grew normally, but that the addition to the diet of metabolic stress factors, including succinyl-sulfathiazole (2%) and triacetin (15%), separately or in combination, resulted in retardation of growth. This retardation could be prevented if the diet was supplemented with fat, fatty acids, defatted cottonseed meal, or water-extracted liver residue. These workers concluded tentatively that triacetin interferes with the synthesis of long-chain acids by the animal, and that cottonseed oil and liver contain some factor essential for this synthesis. In these tests the triacetin was used as a fat precursor. Acetate can also be utilized in this manner. However, unutilized acetate, if present in large concentrations, may produce toxic effects in the animal, by upsetting the acid-base balance.¹⁵⁶

Feuge⁴⁰⁸ stated, in 1955, that "the acetoglycerides cannot be recommended for food use until absolute proof of edibility has been established." However, since they are readily hydrolyzed in the intestinal tract, to yield glycerol, acetic acid, fat-forming acids, and mono- and diglycerides,^{408,411} the acetoglycerides would be expected to be nutritionally acceptable. In fact, Cox⁴¹² reported in 1933 that, in feeding tests with rats, the administration of triacetin at a level of 55% of the diet resulted in no deleterious effects, and in fact permitted better growth than did other fatty acid esters tested. Deuel and Hallman²⁹⁴ found that triacetin was absorbed more rapidly from the gastrointestinal tract in three hours than was any other

⁴⁰⁹ T. L. Ward, A. T. Gros, and R. O. Feuge, *J. Am. Oil Chemists' Soc.*, **32**, 316-318 (1955).

⁴¹⁰ F. H. Mattson, F. J. Baur, and L. W. Beck, *Federation Proc.*, **14**, 443 (1955).

⁴¹¹ A. M. Ambrose and D. J. Robbins, *J. Nutrition*, **58**, 113-124 (1956).

⁴¹² W. M. Cox, Jr., *J. Biol. Chem.*, **103**, 777-790 (1933).

fat tested. According to Deuel *et al.*,⁴¹³ triacetin is a source of liver glycogen. McManus, Bender, and Garrett⁴¹⁴ fed triacetin equal in caloric value to 15% glucose, and found that the triacetin was utilized as efficiently as was the glucose. Ambrose and Robbins^{411,415} tested the digestibility and absorption of acetostearin and of acetoölein in rats, over a four-hour period. The acetoöleins appeared to be better absorbed than the acetostearins; both were absorbed at about the same rate as a commercial shortening, under the experimental conditions. The various acetoglycerides were utilized by the rats in much the same manner as were natural fats. The digestibility figures were approximately 99% for the acetoöleins, 80 and 80.8% for the acetostearins melting at 30.7°C. and 57°3., respectively, and 85% for the acetostearin melting at 32.5°C. For the mixtures of 15% of acetostearin and 10% of acetoölein, and of 10% of acetostearin and 10% acetoölein, the digestibilities were 85.1 and 93.7%, respectively. Thus, the acetostearins are not so readily digestible as the acetoöleins. This may be due to the large amount of stearic acid formed during digestion, which combines with calcium to form calcium stearate; the latter is not readily utilized. The digestibility of acetostearin is markedly improved by mixture with acetoölein, as indicated by a decrease in the amount of fatty acid excreted as soap. This would suggest that acetostearins in a suitable mixture with other edible fats and oils, or a mixture of 80% acetoölein and 20% acetostearin, would be completely edible.⁴¹¹ Ambrose and Robbins⁴¹⁶ further stated that the feeding of acetostearin up to 4% or of pure acetoölein up to 1% of the diet of rats, for over a year, did not interfere with growth or food utilization. Commercial acetostearins from hydrogenated lard, or acetoölein from cottonseed oil or lard, when included in concentrations of 5% or 10% of the diet for eighty-six weeks, also failed to interfere with growth or food utilization. The feeding of 20% of acetostearins, however, had a slight inhibitor effect upon growth, while acetoölein, fed as 20% of the diet, did not inhibit growth. In concentrations of 10% of the diet, two of three samples of acetostearin affected the progeny, while two samples of acetoölein had no effect over several generations.

Herting *et al.*⁴¹⁷ found that the digestion by rats of distilled, acetylated saturated monoglycerides prepared from hydrogenated lard corresponded

⁴¹³ H. J. Deuel, Jr., J. S. Butts, H. Blunden, C. H. Cutler, and L. Knott, *J. Biol. Chem.*, **117**, 119-129 (1937).

⁴¹⁴ T. B. McManus, C. B. Bender, and O. F. Garrett, *J. Dairy Sci.*, **26**, 13-23 (1943).

⁴¹⁵ D. J. Robbins and A. M. Ambrose, *Federation Proc.*, **14**, 382-383 (1955).

⁴¹⁶ A. M. Ambrose and D. J. Robbins, *Federation Proc.*, **14**, 314-315 (1955).

⁴¹⁷ D. C. Herting, S. R. Ames, M. Koukides, and P. L. Harris, *J. Nutrition*, **57**, 369-387 (1955).

to that of the hydrogenated lard itself. The absorption of the digestive products of the distilled acetylated monoglycerides depended largely upon their fatty acid composition.^{417,418}

Alexander and Mattson⁴¹⁹ fed a vitamin A supplement to a control group of vitamin A-depleted weanling male rats maintained on a diet containing 14% fat in the form of a mixture of hydrogenated soybean and cottonseed oil, and to a similar group given a mixture of monoacetin and diacetin derived from the control fat. The acetin fat facilitated the absorption of the vitamin A, and promoted its storage in the liver, at least as efficiently as did the control fat. A normal rate of utilization of vitamin A from the liver was noted in the case of the rats fed acetin fats.

Herting⁴²⁰ found that feeding acetylated monoglycerides to rats at dietary levels up to 50% increased the lipid-bound, steam-distillable acids in the blood, but not in the carcass fat. The low level of these acids found in the tissue fat of a dog fed standard dog-meal was not increased by feeding a diet containing 25% of acetylated monoglycerides. Samples of human blood lipids contained relatively large amounts of the acids.

5. Optimum Levels of Fat in the Diet

(1) *Conclusions Based upon Experiments on Rats*

The beneficial effects of fat in the diet are so varied in character as to raise the question whether fats are only optional components of the diet or whether they should be regarded as necessary foodstuffs. The figure at which the optimum level is established depends to some extent upon the method of evaluation. Results of the methods which have been employed on rats, as described in the earlier portion of this chapter, indicated that the best performance was obtained when the diet contained 20 to 40% of fat by weight. There are, however, some instances in which quite satisfactory results were obtained when only 10% of the diet consisted of fat. One factor which certainly plays a role in determining the amount of fat needed in the diet is the type of fat employed. Thus, fats which are completely saturated or which contain none of the EFA probably would not result in optimum performance even if fed in large quantities. The amount of fat needed to supply the EFA varies according to the particular situation

⁴¹⁸ S. R. Ames, D. C. Herting, M. Koukides, and P. L. Harris, *Federation Proc.*, **14**, 173 (1955).

⁴¹⁹ J. C. Alexander and F. H. Mattson, *Federation Proc.*, **15**, 541-542 (1956).

⁴²⁰ D. C. Herting, S. R. Ames, N. D. Embree, and P. L. Harris, *Federation Proc.*, **15**, 556 (1956).

involved. For example, the requirement for these components varies with sex, being higher in males than in females.

Essential fatty acids also serve as protective agents against x-irradiation injury.^{421,422} Most recently it has been shown that the LD₅₀ from a single exposure of x-irradiation reached the maximum level when 80 mg. of linoleate was administered to rats.⁴²¹ The survival rate was considerably higher when the animals had received 15% cottonseed oil than when a fat-free diet had been given.^{423,424}

It is not known whether or not the beneficial effects of fat are to be traced exclusively to their EFA content. There is some indication that the capacity for work, and the sparing action of fats on the "wear and tear quota" of protein metabolism, may not be directly related to the EFA content of the diet.

(2) *Conclusions Based upon Experiments on Man*

There is some question as to how far one can apply the results obtained with rats in interpreting the requirements for human nutrition. According to Brandt⁴²⁵ the daily intake of fat by the British increased from 99 g. per day before World War I to a figure of 124 g. by 1934. A similar fat consumption was noted for Americans and Germans during the latter period; fats accounted for 30% of calories in their dietaries at that time. In sharp contrast to this relatively high intake by the Occidentals, the intake by Orientals was shown to be quite low. Shen⁴²⁶ estimated that the soldiers of South China during World War II ate fat in the amount of only 3% of their total caloric intake while Howe⁴²⁷ calculated the average consumption of the American soldiers in World War II as 40%.

The Food and Nutrition Board of the National Research Council (United States)⁴²⁸ made the following suggestions as to the human requirement for fat:

⁴²¹ A. L. S. Cheng, T. Graham, R. B. Alfin-Slater, and H. J. Deuel, Jr., *J. Nutrition* 55, 647-653 (1955).

⁴²² A. L. S. Cheng, M. Ryan, R. B. Alfin-Slater, and H. J. Deuel, Jr., *J. Nutrition*, 52, 637-644 (1954).

⁴²³ A. L. S. Cheng, Personal communication, 1955.

⁴²⁴ A. L. S. Cheng, T. Graham, R. B. Alfin-Slater, and H. J. Deuel, Jr., *Federation Proc.*, 14, 429-430 (1955).

⁴²⁵ K. Brandt, *Ann. Am. Acad. Polit. Soc. Sci.*, 225, 210-215 (1943).

⁴²⁶ T. Shen, *Science*, 98, 302-303 (1943).

⁴²⁷ P. E. Howe, *Ann. Am. Acad. Polit. Soc. Sci.*, 225, 72-79 (1943).

⁴²⁸ Anonymous, *Recommended Dietary Allowances*, Food and Nutrition Board, Nat. Research Council, *Circ. No. 129*, 3-31 (Oct., 1948), p. 17.

There is available little information concerning the human requirement for fat. Fat allowances must be based at present more on food habits than on physiological requirements. While a requirement for certain unsaturated fatty acids (the linoleic and arachidonic acids of natural fats) has been amply demonstrated with experimental animals, the human need for these fatty acids is not known. In spite of the paucity of information on this subject there are several factors which make it desirable (1) that fat be included in the diet to the extent of at least 20 to 25% of the total Calories, and (2) that the fat intake include essential unsaturated fatty acids to the extent of at least 1% of the total Calories. At higher levels of energy expenditure, *e.g.* for a very active person consuming 4500 Calories and for children and for adolescent persons, it is desirable that 30 to 35% of the total Calories be derived from fat. Since foodstuffs such as meat, cheese, nuts, etc., contribute fat to the diet, it is necessary to use separated or "visible" fats such as butter, oleomargarine, lard, or shortenings to supply only one-third to one-half of the amounts indicated.

Considerable experimental work is still required to determine what quantities of fat are to be considered as optimal for man. It is evident that the capacity to utilize dietary fat is limited and that, when large quantities are consumed, gastrointestinal distress may follow. It is also of considerable importance to determine to what extent tolerance for fat varies with the type of fat, as well as with the method of preparation of the food into which fat is incorporated.

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PLANT AND ANIMAL SOURCES OF LIPIDS

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