C I B A LECTURES IN MICROBIAL BIOCHEMISTRY

**BACTERIAL
FERMENTATIONS**

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Received July 26, 1958

Accession No. 73817

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New York City

Place, _____

MBL/WHOI

0 0301 0020771 8

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1956 H. A. Barker, *Bacterial Fermentations*

1957 E. P. Abraham, *Biochemistry of Some Peptide and Steroid Antibiotics*

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BACTERIAL FERMENTATIONS

By H. A. BARKER

1956

NEW YORK · JOHN WILEY & SONS, INC.

London · Chapman & Hall, Ltd.

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BIOLOGICAL FORMATION OF METHANE

Methane is the characteristic product of the microbial decomposition of organic compounds in the absence of oxygen. The escape of bubbles of marsh gas from decaying vegetation in swamps and lake beds is a conspicuous phenomenon which can hardly be overlooked by anyone who observes rivers or lakes, particularly in the late summer when the water is warm and microbial activity is high. The gas consists largely of methane with smaller amounts of carbon dioxide and sometimes a little hydrogen.

Historical Background

Although the escape of combustible gas from various places on the earth's surface was described in Roman times by Pliny and was undoubtedly known much earlier, credit for the discovery of the common formation of such gas in nature must be given to the Italian physicist Alessandro Volta.¹ On November 14, 1776, Volta wrote a letter to a friend describing his unexpected discovery that "combusti-

ble air" was being formed continuously and in substantial quantities in all the lakes, ponds, and streams in the vicinity of Como in northern Italy. The initial observation was made in Lake Verbano. Volta found that to detect the gas it was necessary only to disturb the bottom sediments, whereupon a shower of bubbles would rise to the surface. He further recognized a close relation between the abundance of combustible gas and the amount of plant material in the sediment, and concluded that the former was derived from the latter. Volta collected samples of the gas from many different sites and determined the relative proportion of gas and air that would give the most vigorous explosion.

Chemical knowledge of 1776 did not permit the characterization of Volta's inflammable gas. This was first accomplished in 1806 by William Henry, who showed that Volta's gas was apparently identical with the main constituent of synthetic illuminating gas, which was later called methane.

During the next sixty years several eminent scientists, including Bunsen and Boussingault, became interested in methane formation. Their work, which consisted mainly of collecting and analyzing gas samples from various sources, served to corroborate Volta's conclusion that methane is formed abundantly in nature and is generally associated with the decay of plant materials covered with water, but no new concepts were developed.

The first definite indication that methane is formed by a microbiological process was obtained in 1868 by Béchamp,² a student of Pasteur. Béchamp had previously studied the decomposition of sugar and starch that occurred when these substances were added to a simple inorganic medium containing chalk and incubated in the absence of oxygen. He ascribed the resulting fermentation to a living "ferment" which he thought was originally present in the chalk and

therefore called *Microzyma cretae*. The description of this organism was remarkably vague; it was reported to be so small that even when examined under the highest magnification of the microscope it appeared only as a motile point. For some reason, now not altogether clear, Béchamp decided to find out whether the chalk and its microbe could also cause an anaerobic decomposition of ethyl alcohol. He was rewarded after some weeks by seeing the development of a vigorous fermentation which produced a large amount of methane, a little carbon dioxide, and a mixture of various fatty acids. Béchamp reported that when the gas evolution stopped the only "ferment" to be seen was *M. cretae*! Despite the naive interpretation of the microscopic observations, Béchamp's experiment clearly showed that methane can be formed from ethyl alcohol and calcium carbonate by a process probably caused by microorganisms. This was the first demonstration of biological methane formation from simple carbon compounds. Unfortunately, Béchamp's work was overlooked by most subsequent investigators.

More adequate proof of the microbiological origin of methane was provided by Tappeiner in 1882.³ He set up three identical anaerobic cultures provided with plant materials as substrate and with considerable amounts of the intestinal contents of ruminants as an inoculum and a possible source of soluble catalysts. One of the three cultures was treated with an antiseptic to inhibit bacteria without inactivating soluble "ferments," the second was boiled to destroy both bacteria and "ferments," and the third was left untreated. The microbial nature of the fermentation was deduced from the observation that only the untreated culture produced methane.

During the last quarter of the 19th century, interest in the methane fermentation centered largely on the utilization of cellulose as a substrate. Since cellulose is the most

abundant constituent of plants it was reasonable to suppose that it must be a major source of methane in decomposing plant materials. Furthermore, methane had been shown to be formed in large amounts in the digestive tracts of herbivorous animals; therefore the possible role of methane-producing bacteria in cellulose digestion was of some concern to animal physiologists.

The experiments of Popoff,⁴ Tappeiner, and Hoppe-Seyler⁵ on the utilization of cellulose by crude enrichment cultures of bacteria obtained from soil or the digestive tracts of herbivorous animals demonstrated that cellulose is in fact decomposed under anaerobic conditions, frequently with the formation of methane and other products including carbon dioxide, hydrogen, and acetic and butyric acids. At first this was taken to mean that the bacteria which attacked cellulose also form methane. However it was soon realized that another interpretation is possible, namely, that the methane is formed not by the cellulose-decomposing bacteria but by the action of other associated microorganisms on one or more of the products of the cellulose fermentation. This interpretation was supported by two lines of evidence. First, certain cellulose-fermenting cultures were found to produce carbon dioxide and hydrogen but no methane. This proved that cellulose fermentation is not necessarily associated with methane formation. Second, Hoppe-Seyler, Omelianski, and particularly Söhnngen¹ demonstrated that the products of cellulose fermentation, such as formate, acetate, butyrate, ethanol, and even hydrogen and carbon dioxide, can be readily used as substrates by methane-producing bacteria.

Although these results were consistent with the conversion of cellulose to methane by a two-stage process, they did not exclude the existence of methane-producing bacteria that attack cellulose. Omelianski (1895-1904)⁶ tried to resolve

this problem by isolating pure cultures of cellulose-fermenting bacteria derived from soil. This approach proved to be unexpectedly difficult and was finally abandoned. Omelianski then tried more indirect methods. By analysis of the gas formed by various cellulose-fermenting enrichment cultures, he found that the ratio of hydrogen to methane varied greatly from culture to culture. Ultimately, by a combination of heating and transferring procedures, he was able to obtain cellulose-fermenting cultures producing either methane without hydrogen or hydrogen without methane. Both cultures appeared to contain only spore-forming bacteria which were similar in appearance. The organism presumed to be responsible for the formation of methane from cellulose was given the name of *Bacillus methanigenes*.

Omelianski's conclusion that he had demonstrated the existence of a cellulose-fermenting, methane-forming bacterium may be correct but is open to doubt. The description of Omelianski's methods shows that he was not dealing with pure cultures. Consequently the possibility cannot be excluded that the observed methane formation was the result of the action of two types of bacteria, similar in morphology but differing in physiology. Subsequent research has provided no confirmation of Omelianski's conclusion; none of the cellulose-fermenting bacteria since isolated in pure culture has been found to produce methane.

The Methane Bacteria

The formation of methane in decomposing organic materials is the result of the action of a specialized physiological group of bacteria, often referred to as the "methane-producing bacteria" or, more simply, as the "methane bacteria." These bacteria are entirely different from the aerobic methane-oxidizing bacteria. The former produce methane

from various organic and inorganic compounds; the latter oxidize methane to carbon dioxide and water.

Enrichment and Isolation. The methane bacteria have not been studied as extensively as most other groups of bacteria of comparable scientific and practical importance. The reason for this is readily apparent. In order to study the biology and biochemistry of bacteria most effectively, it is necessary to use pure cultures, that is, to study one species at a time. Unfortunately, with the methane bacteria, this elementary but basic requirement has been difficult and in many instances impossible to achieve.

Until 1936 all attempts to isolate pure cultures, or even to obtain growth of colonies in solid media, were unsuccessful.⁷ Consequently all the early studies and many of the more recent studies of methane bacteria were of necessity carried out with enrichment cultures, i.e., cultures in which the substrate and environmental conditions were chosen in such a way as to favor the development of certain species of methane bacteria, without, however, excluding a substantial and frequently significant number of other bacterial species, both methane-producing and non-producing types. By the use of enrichment cultures it was possible to obtain considerable information about the existing morphological types of methane bacteria, the general environmental conditions that favor their development, the kinds of substrates attacked and the over-all chemical reactions involved, but many basic points of biology, nutrition, and biochemistry could not be studied effectively with these cultures.

Since 1936 four species of methane bacteria (*Methanobacillus omelianskii*,⁸ *Methanobacterium formicicum*,⁹ *Methanosarcina barkerii*,⁹ *Methanococcus vannieli*¹⁰) belonging in three different genera have been isolated in pure culture. An additional four species (*Mbact. suboxy-*

dans,¹¹ *Mbact. söhngeniei*,⁷ *Ms. methanica*, *Mc. mazéi*) have been purified by isolation and reisolation of colonies from agar media until they were free of other species of methane bacteria, but they may still have been contaminated by other anaerobic bacteria. The complete or partial purification of these eight species indicates that the isolation of pure cultures of other species should also be possible. It must be emphasized, however, that the isolation of methane bacteria is generally a difficult and time-consuming task requiring great patience and considerable experience with anaerobic culture techniques. So far, pure cultures have been obtained in only three laboratories. Even after a pure culture has been isolated its preservation is no easy problem. Most of the pure cultures have been lost after a few years. Only one species, *Mbac. omelianskii*, has been kept in pure culture for a long period of time.

Because of the difficulties encountered in the isolation and maintenance of pure cultures of methane bacteria only a rather limited amount of information has been obtained concerning the biological and physiological characteristics of the group as a whole. Nevertheless some tentative generalizations can be made that are useful in defining the group and indicating the probable behavior of its members under a variety of circumstances.

Relation to Oxygen. All of the species are strictly anaerobic; they develop only in the absence of oxygen and in the presence of a suitable reducing agent.⁷ The methane bacteria are much more sensitive to oxygen or certain other oxidizing agents, such as nitrate, than are most other anaerobic bacteria. For this reason they can be grown more easily in liquid or semisolid media than on the surface of an agar medium. Even with liquid media not fully protected from air, sufficient oxygen may leak in to inhibit cultures that have passed the peak of their activity. The

beneficial effect on the growth of methane bacteria caused by the addition of solid sediments, such as diatomaceous earth and shredded asbestos, to a liquid medium may be attributed at least partially to a mechanical shielding of the bacteria from dissolved oxygen, although other explanations are possible.

The most satisfactory reducing agents for pure cultures are generally sodium sulfide and hydrosulfite. Care must be used in adjusting the concentration of these substances since above the optimal level they become toxic to some species. Mylroie and Hungate¹² have recently described the successful use of hydrogen and palladium chloride as a reducing system in media for *Mbact. formicicum*. The effectiveness of this reducing system supports the conclusion that a low oxidation-reduction potential in the medium is more important than the presence of a specific concentration of a reduced sulfur compound per se.

Energy Metabolism. A second characteristic of methane bacteria is their energy metabolism which is specialized for a process that produces methane as a major product. A little later we shall take a closer look at the nature of the energy-yielding reactions. Now it is sufficient to emphasize that the ability to form methane is not a common and widely distributed property of anaerobic bacteria but is restricted to a specialized group. A few possible exceptions to this generalization are recorded in the literature, particularly in the work of Laigret.¹³ This investigator reported that *Clostridium perfringens*, which normally does not produce methane, can be induced to do so in a peptone-formate medium by the addition of a small amount of iodine. However, because of the absence of experimental details, the validity of Laigret's observations is difficult to assess. A thorough reinvestigation of this problem is necessary before a definite conclusion can be reached concern-

ing the ability of *Cl. perfringens* and other species to form methane.

Compounds Fermented. Methane bacteria specialize not only in the chemical mechanisms of energy metabolism but also in respect to the types of substrates which they utilize. For some obscure reason, all of the methane bacteria whose substrate requirements have been studied effectively by the use of pure or nearly pure cultures are unable to decompose the more usual substrates for bacteria such as carbohydrates and amino acids. Early reports of cellulose- or glucose-fermenting methane bacteria have not been confirmed; probably they were based on observations with mixed cultures containing both carbohydrate-fermenting and methane-producing species. Of course the possibility that still unknown species of carbohydrate- or amino acid-fermenting, methane-producing bacteria do exist cannot be excluded, but convincing evidence for such organisms is still lacking.

As a group the methane bacteria appear to be restricted to the utilization of relatively simple organic and inorganic compounds, many of which are products of the better-known types of bacterial fermentations. Table 1 gives a list of the oxidizable substrates that have been shown by studies with pure or nearly pure cultures to be decomposed by methane bacteria. These substrates fall into three groups, the lower normal fatty acids containing from one to six carbon atoms, the normal and iso alcohols containing from one to five carbon atoms, and three inorganic gases, hydrogen, carbon monoxide,¹⁴ and carbon dioxide. In Table 2 are listed some additional compounds that are probably decomposed by methane bacteria, judging from observations made with enrichment cultures. These compounds include several long-chain fatty acids, dicarboxylic acids, aromatic compounds, acetone, and butylene glycol.

They have all been shown, mainly by Buswell and his collaborators,^{15,16} to be converted more or less quantitatively into methane and carbon dioxide by crude cultures

TABLE 1

Compounds Known to Be Decomposed by Methane Bacteria
(Based on experiments with pure or purified cultures)

Fatty Acids	Alcohols	Gases
Formic	Methanol	Hydrogen
Acetic	Ethanol	Carbon monoxide
Propionic	<i>n</i> -Propanol	Carbon dioxide
	Isopropanol	
<i>n</i> -Butyric	<i>n</i> -Butanol	
	Isobutanol	
<i>n</i> -Valeric	<i>n</i> -Pentanol	
<i>n</i> -Caproic		

derived from sewage sludge. Since these compounds are not known to be attacked by other anaerobic bacteria under the test conditions used, they are probably decomposed by methane bacteria.

TABLE 2

Some Compounds Probably Decomposed by Methane Bacteria
(Based on experiments with enrichment cultures)

<i>n</i> -Caprylic acid	Benzoic acid	Oxalic acid
<i>n</i> -Capric acid	Phenylacetic acid	Succinic acid
Isobutyric acid	Hydrocinnamic acid	Acetone
Stearic acid	Cinnamic acid	2,3-Butylene glycol
Oleic acid		

In the older literature concerned with anaerobic decomposition processes in nature it is often stated that many other compounds such as sugars, cellulose, proteins, purines, glycerol, lactate, and citrate are also suitable substrates for

a methane fermentation. This is true in the sense that these substrates are decomposed by crude cultures with the formation of methane and carbon dioxide. However, since these compounds are rapidly decomposed by many common bacteria that do not produce methane, it is virtually certain that the conversion of these substrates to methane is a two- or multistage biological process in which various bacteria that cannot form methane convert the substrates to volatile fatty acids, alcohols, and other common fermentation products which are then transformed by methane bacteria to their characteristic products.

Species Substrate Specificity. In addition to the apparently severe limitations of the methane bacteria as a group with respect to utilizable substrates, each species characteristically is restricted to the use of a few compounds. A few examples of the substrate specificity of different species are shown in Table 3. *Methanobacterium*

TABLE 3

Substrate Specificity of Some Methane Bacteria

Species	Oxidizable Substrates
<i>Methanobacterium formicicum</i>	H ₂ , CO, formate
<i>Methanobacillus omelianskii</i>	H ₂ , ethanol, primary and secondary alcohols
<i>Methanobacterium suboxydans</i>	Butyrate, valerate, caproate
<i>Methanosarcina barkerii</i>	H ₂ , CO, methanol, acetate

*formicicum*⁹ oxidizes only hydrogen, carbon monoxide and formate. *Methanobacillus omelianskii* cannot oxidize carbon monoxide or formate but specializes in the decomposition of primary and secondary short-chain aliphatic alcohols¹⁷ and hydrogen.¹⁸ *Methanobacterium suboxydans* cannot attack any of the previously mentioned compounds except possibly hydrogen which was not tested, but spe-

cializes in the oxidation of C_4 to C_6 fatty acids to acetate and propionate.¹¹ Finally, *Ms. barkerii* is restricted to the decomposition of hydrogen, carbon monoxide, methanol, and acetate.⁹ This species does not attack longer chain alcohols or fatty acids.

Since all the available evidence indicates that these species are representative of the group, it is apparent that methane bacteria show a rather extreme substrate specificity. This implies that several species of methane bacteria must be required for the complete methane fermentation of the variety of compounds that are present in sewage and decomposing plant materials. Even for the complete fermentation of so simple a compound as valeric acid, as many as three species of methane bacteria may be required. Valerate is oxidized by *Mbact. suboxydans* to acetate and propionate, which are not further attacked by this organism.¹¹ Methane is formed in a coupled reduction reaction. A second species, *Mbact. propionicum*, converts the propionate to acetate, carbon dioxide, and methane. Since this organism cannot attack acetate, a third species such as *Mc. mazéi* is required to ferment the acetate. The need to establish a balanced population of bacteria to participate in this type of fermentation sequence undoubtedly explains why considerable time is always required to develop a culture capable of causing a rapid and complete fermentation of complex mixtures of organic compounds. When such a culture is obtained, it is capable of maintaining itself more or less indefinitely when a fresh supply of organic materials is added continually, because the major products of the fermentation are gases which escape from the medium leaving behind very little in the way of potentially toxic byproducts.

Nutrition. The general nutritional requirements of methane bacteria appear to be very simple, although this

can be said with certainty only for those species which have been studied in pure culture. These species all grow satisfactorily in media containing the usual nutritive salts, carbon dioxide, a reducing agent, a single oxidizable compound suitable for the organism, and an ammonium salt as a source of nitrogen.⁸ One species, *Mbac. omelianskii*, has been shown to utilize nitrogen gas.¹⁹ The general physiological properties of the methane bacteria suggest, by comparison with other nitrogen-fixing bacteria, that the ability to fix nitrogen may be a common characteristic of the group. The addition of extracts containing amino acids, growth factors, and other nutritional supplements to synthetic media does not have a beneficial effect on the rate or magnitude of growth of the few species that have been studied in this respect.

Special attention should be drawn to the carbon dioxide requirement of methane bacteria. Qualitatively, there is nothing unusual in this since all microorganisms require small amounts of carbon dioxide to initiate and maintain growth. However, several species of methane bacteria need a large amount of carbon dioxide which they use as a major substrate.²⁰ The specific role of carbon dioxide in the energy metabolism of methane bacteria is considered in a later section.

pH Range. Another environmental factor that is important for the methane bacteria is the hydrogen ion concentration. As a general rule these bacteria are most active in the pH range from 6.4 to 7.2. Below pH 6 and above pH 8 the growth rate of methane bacteria falls off rapidly. At the high pH this may be the result of the greatly diminished concentration of free carbon dioxide. There is one exception to the generalization that an alkaline medium is unfavorable. The formate-fermenting species *Mc. vanniellii* grows best between pH 8 and 9.¹⁰

So far no methane bacteria preferring acid media have been reported. Nevertheless the slow formation of methane in peat bogs having a *pH* as low as 4.0 suggests that acid tolerant species probably exist.

Taxonomy. The system of classifying methane bacteria depends upon the relative importance given to morphology and physiology. The editors of *Bergey's Manual of Determinative Bacteriology*²¹ have placed primary emphasis upon

TABLE 4

Classification of Methane Bacteria

Family: Methanobacteriaceae

- A. Rod-shaped cells
 - I. Non-sporulating: *Methanobacterium*
 1. *Mbact. formicicum*: formate, carbon monoxide, hydrogen
 2. *Mbact. propionicum*: propionate
 3. *Mbact. söhngenii*: acetate, butyrate
 - II. Sporulating: *Methanobacillus*
 1. *Mbac. omelianskii*: primary and secondary alcohols, hydrogen
- B. Spherical cells
 - I. Cells not in sarcina arrangement: *Methanococcus*
 1. *Mc. mazéi*: acetate, butyrate
 2. *Mc. vanniellii*: formate, hydrogen
 - II. Cells in sarcina arrangement: *Methanosarcina*
 1. *Ms. barkerii*: methanol, acetate, carbon monoxide, hydrogen
 2. *Ms. methanica*: acetate, butyrate (?)

morphology, and consequently have dispersed the methane bacteria among the families and genera of better-known bacteria of similar forms. In contrast, those investigators who have had personal experience with the methane bacteria have been impressed by the striking physiological characteristics of all members of the group, and have preferred to think of them as belonging to a physiological

family, which might well be called Methanobacteriaceae. Within the physiological family, four genera can be recognized on the basis of morphology (Table 4). The known methane bacteria are either rods or cocci. The rod-shaped organisms may be further subdivided into two groups on the basis of sporulation. The non-sporulating types are placed in the genus *Methanobacterium*, and the sporulating types in the genus *Methanobacillus*.* The spherical organisms whose cells occur singly, in pairs, or in irregular masses are put in the genus *Methanococcus*, whereas the organisms with more or less spherical cells arranged in cubical sarcina packets are included in the genus *Methanosarcina*. The species in these four genera differ with respect to size and arrangement of cells, presence or absence of flagella, or the ability to utilize various substrates. Substrate specificity has been of particular value in species identification; therefore the substrates known to be attacked by different species are included in Table 4 following the species name. So far, a total of eight species of methane bacteria have been described in these four genera. These species have been obtained entirely from sewage sludge and black mud. Undoubtedly many more species will be discovered as more adequate isolation methods are developed, and as the organisms associated with other habitats, such as the digestive tracts of ruminants, are systematically investigated.

Chemistry of Methane Fermentations

The early studies on the chemistry of methane fermentations were done entirely with enrichment cultures containing a single substrate and a mixture of organisms. The

* The one species of this genus that has been studied extensively, *Mbac. omelianskii*, has previously been included in the genus *Methanobacterium*.

main result of such studies was the demonstration that a variety of compounds can be more or less quantitatively converted to methane and carbon dioxide.²² Equations summarizing some of the observed reactions are given in Table 5. The equations show that fermentations of acetate,

TABLE 5

Fermentation of Organic Compounds by Mixed Cultures

propionate, butyrate, ethanol, or acetone give the same products, the ratio of methane to carbon dioxide depending on the oxidation state of the substrate. The remarkable aspect of this result is that the nature of the products is independent of the structure of the substrate. A plausible explanation for the carbon dioxide was easily found: carbon dioxide might be formed by the complete oxidation of part of the substrate. But it was difficult to understand why methane was always the other product. For one substrate, acetate, a simple and reasonable explanation was suggested, namely, that the acid is decarboxylated to form carbon dioxide from the carboxyl group and methane from the methyl group.²² Unfortunately this explanation is of no help with propionate which, according to the hypothesis, should yield ethane on decarboxylation. Actually no ethane could be detected by ordinary analytical methods.

No generally applicable chemical explanation for the origin of methane was developed until 1934, when van Niel

suggested the so-called "carbon dioxide reduction theory."²⁰ This theory postulates that the organic compounds fermented by methane bacteria are oxidized completely to carbon dioxide, and that this oxidation is coupled with a reduction of some or all of the carbon dioxide to methane. The application of this idea to the fermentation of acetate is shown in the following equations.

The oxidation of acetate, according to the theory, gives 2 moles of carbon dioxide and 8 equivalents of hydrogen. One mole of carbon dioxide is reduced to methane and water. The net result, the conversion of 1 mole of acetate to 1 mole each of methane and carbon dioxide, is in accordance with the experimental facts.

The main evidence for the general theory at the time it was developed was provided by the early observations of Söhngen²² on the fermentation of a mixture of hydrogen and carbon dioxide. Söhngen found that enrichment cultures could couple the oxidation of hydrogen with the reduction of carbon dioxide to methane according to equation 4. Söhngen's experimental observations were later

confirmed in several laboratories by the use of pure cultures.^{14,18}

Much of the subsequent work on the chemistry of the fermentation has been concerned with determining whether the carbon dioxide reduction theory also applies to fer-

mentations of organic compounds. By now a considerable amount of information has accumulated which shows that the theory is correct for fermentations of many, but not all, substrates.

Examples of the reduction of carbon dioxide to methane are provided by the fermentations of ethanol by *Mbac. omelianskii*, of butyrate by *Mbact. suboxydans*, and of propionate by *Mbact. propionicum*. With all three of these species, the interpretation of the experimental data is greatly simplified by the fact that they oxidize their substrates to acetate which is not further decomposed.

Methanobacillus omelianskii oxidizes ethyl alcohol almost quantitatively to acetate according to equation 5.¹⁷ The

oxidation of alcohol is completely dependent upon the supply of carbon dioxide; when the carbon dioxide is all consumed the oxidation of alcohol stops. Other oxidants, such as sulfate and nitrate, cannot replace carbon dioxide. Now the above equation strongly indicates that the acetate is derived from the alcohol, and that the methane is derived from carbon dioxide. To confirm this interpretation by an independent method, an experiment was done in which unlabeled ethyl alcohol was incubated with C¹⁴-labeled carbon dioxide.²³ At the end of the fermentation, the C¹⁴ content per mole of the methane was found to be essentially equal to that of the carbon dioxide, thus proving that the methane produced in this fermentation is derived entirely from carbon dioxide.

The fermentation of butyrate by *Mbact. suboxydans* is very similar in principle.¹¹ The oxidation of 2 moles of butyrate to 4 moles of acetate is coupled with the reduction of 1 mole of carbon dioxide to methane according to

equation 6. In this fermentation also tracer experiments have shown that at least 98% of the methane is derived from carbon dioxide.

In the preceding examples of methane fermentations involving carbon dioxide reduction, essentially no carbon dioxide is formed by the oxidation of the organic substrate. The fermentation of propionate by *Mbact. propionicum*, shown in the following equations, is somewhat more complicated because it involves both carbon dioxide formation and utilization.¹¹

The observed reaction, shown in equation 9, results in the production of 1 mole of carbon dioxide from 4 moles of propionate. However, although 1 mole of carbon dioxide accumulates, it is reasonable to assume, on the basis of what is known about the oxidation of propionate in other biological systems, that the oxidation of 4 moles of propionate initially gives 4 moles of acetate, 4 moles of carbon dioxide, and 24 "hydrogens" as is shown in equation 7. If these 24 "hydrogens" serve to reduce carbon dioxide, 3 moles would be converted to methane as is shown in equation 8. This accounts for the observed yields of methane and carbon dioxide (equation 9).

The postulated intermediate role of carbon dioxide was tested by experiments with C^{14} -carbon dioxide or C^{14} -propionate. Without discussing the experimental data in detail, it may be stated that the results could be interpreted to indicate that approximately 1 mole of carbon dioxide is formed per mole of propionate, in accordance with equation 7, and that carbon dioxide is a precursor of most, if not all of the methane, as indicated in equation 8.

Buswell and his associates²⁴ have also done tracer experiments on the fermentation of propionate using enrichment cultures, presumably containing a mixture of species, capable of converting propionate completely to carbon dioxide and methane without the accumulation of acetate. These cultures were shown to use carbon dioxide and to convert all three carbon atoms of propionate to both methane and carbon dioxide in varying degrees. Part of these results can be interpreted in terms of the reactions already discussed in combination with a secondary decomposition of acetate. However, some additional reaction would be required to account for the preferential conversion of the α carbon of propionate to methane and a substantial conversion of the β carbon to carbon dioxide. These results could be caused by the formation of a symmetrical intermediate like succinate from propionate. This would allow a randomization of the α and β carbons of propionate and therefore would account for the similarity in their behavior. Other explanations are also possible. This type of problem probably cannot be resolved until pure cultures of all of the organisms involved are available.

The results which I have mentioned, as well as others, show conclusively that, with many substrates and at least several species of methane bacteria, methane is formed mainly or wholly by reduction of carbon dioxide. However, at least two compounds, methyl alcohol and acetate,

are converted to methane by chemical pathways not involving carbon dioxide.

Schnellen,⁹ working in Kluyver's laboratory, showed that methyl alcohol is readily fermented by a species of *Methanosarcina* according to equation 10. At first this reaction was

thought to be the result of a complete oxidation of the alcohol coupled with the reduction of three-fourths of the resulting carbon dioxide. However, this possibility was excluded by tracer experiments which showed that less than 1% of the methane was derived from carbon dioxide.²⁵ Therefore the methane must be formed more or less directly by reduction of methanol.

With acetate, which is decomposed by several species of methane bacteria, the situation is similar. The fermentation of acetate is equivalent to a decarboxylation. As we have seen, according to van Niel's theory, acetate should be completely oxidized to carbon dioxide, half of which is simultaneously reduced to methane. This mechanism was first critically tested by Buswell and Sollo²⁶ in 1948 by the use of C¹⁴-labeled carbon dioxide. They demonstrated that essentially none of the methane is derived from carbon dioxide. Subsequently Thressa Stadtman and I^{23,25} showed by the tracer method that the methane is formed entirely from the methyl carbon, and the carbon dioxide exclusively from the carboxyl carbon, of acetate (equation 11). These

results are inconsistent with the carbon dioxide reduction theory since the methyl carbon of acetate is not oxidized to carbon dioxide and carbon dioxide is not a precursor of methane.

After the fate of the carbon atoms of acetate was established, it became of interest to find out what happens to the hydrogen atoms attached to the methyl group. In particular, it was important to determine whether some or all of these hydrogen atoms are removed by an oxidative reaction during the course of the fermentation of acetate or whether the methyl group is incorporated intact into methane. This question was investigated by Pine.²⁷ In the experiment illustrated by equation 12 acetate labeled

in the methyl group with deuterium was fermented and the amount of deuterium per mole in the evolved methane was compared with that in the substrate. Mass analysis showed that the deuterium contents of the acetate and methane were essentially equal. In another experiment illustrated by equation 13 unlabeled acetate was fermented in the

presence of D_2O . The data indicate that approximately one atom of deuterium per molecule of methane was derived from the solvent. These results demonstrate that the methyl group is transferred from acetate to methane as a unit, without the loss of attached hydrogen or deuterium. In other words, one or more transmethylation reactions occur during the fermentation.

Pine²⁸ later investigated the same problem with methanol by allowing unlabeled methanol to ferment in the presence of a mixture of D_2O and H_2O (equation 14). The methane formed in this experiment contained a large percentage of CH_3D , although somewhat less than the percentage of deuterium in the water. This was interpreted to mean

that the methyl group of methanol, like that of acetate, is transferred intact into methane. The lower percentage of deuterium in the methane could be attributed to a preferential utilization of the mass 1 hydrogen from the water.

The specific reactions involved in methane formation from carbon dioxide, acetate, or methanol are not known. The simplest mechanism proposed for the conversion of carbon dioxide to methane is a stepwise reduction involving formate or carbon monoxide, formaldehyde, and methanol as successive intermediates. A faint suggestion of such a sequence was provided by Stephenson and Stickland,²⁹ who claim to have isolated a bacterium capable of reducing carbon dioxide, carbon monoxide, formate, formaldehyde, and methyl alcohol to methane using hydrogen as a reductant. However, their experiments do not provide any substantial evidence for a sequential reduction of these compounds. Carbon monoxide, formaldehyde, and methanol were reduced far too slowly to be intermediates. Formate was shown to be converted to hydrogen and carbon dioxide more rapidly than to methane; consequently carbon dioxide actually appeared to be an intermediate in formate reduction rather than the reverse.

Kluyver and Schnellen¹⁴ later reinvestigated the fermentation and reduction of one-carbon compounds in the presence of hydrogen by pure cultures of three species of methane bacteria. Cell suspensions of *Mbac. omelianskii* were unable to reduce any one-carbon compound at a significant rate, except carbon dioxide, which reacted rapidly. *Methanosarcina barkerii*, which ferments methanol, acetate, and carbon monoxide, was unable to reduce formate or formaldehyde. A mixture of carbon monoxide and

hydrogen was converted to methane according to equation 15.

However, convincing evidence was obtained that this reaction involves two steps with carbon dioxide being an intermediate (equations 16 and 17). *Methanobacterium*

formicicum ferments both carbon monoxide and formate, and reduces carbon dioxide with hydrogen, but is unable to reduce formaldehyde or methanol. These observations of Kluyver and Schnellen clearly exclude formate, carbon monoxide, formaldehyde, and methanol as intermediates in carbon dioxide reduction despite the fact that all of these compounds, except possibly formaldehyde, can be metabolized by some species. Therefore it is virtually certain that the intermediates in methane formation are not one-carbon compounds.

The only available information which seems to provide evidence concerning the path of carbon dioxide reduction to methane is derived from the previously mentioned studies on the acetate and methanol fermentations, which curiously enough do not involve carbon dioxide. The argument might be made that these fermentations are entirely unrelated processes and have no bearing on the path of carbon dioxide reduction. However, there is a good reason for rejecting this view, namely, the fact that the *Methanosarcina* species which ferments acetate and methanol can also reduce carbon dioxide to methane with hydrogen;⁹ it seems highly improbable that one organism

would possess several types of chemical machinery for making methane from different substrates.

A schematic representation of the possible pathways of carbon in methane formation, which combines the information obtained from studies of the acetate and methanol fermentations with the carbon dioxide reduction theory of van Niel, is presented in Fig. 1. Carbon dioxide is postu-

Fig. 1. Possible Pathways of Methane Formation.

lated to combine with an unidentified organic compound XH to form a carboxylated derivative of X which is then reduced by three successive steps to a methyl derivative of X which on further reduction yields methane and regenerates the carbon dioxide acceptor XH. Methanol and acetate are postulated to react also with XH to give the intermediate XCH_3 of the carbon dioxide reduction pathway. The direct formation of this intermediate from the organic methyl donors might well inhibit carbon dioxide utilization by lowering the concentration of XH or in other ways and thus account for the absence of carbon dioxide reduction with these substrates.

This scheme is to be regarded only as a rough working hypothesis. Undoubtedly the actual reaction mechanism is considerably more complicated. For example, a source of energy, such as adenosine triphosphate, would be required for the reduction of XCOOH to XCHO , and for the methylation of XH by methanol. But these are only matters of biochemical detail that could be worked out once the main pathway is established. At present the most important point to determine is the nature of the hypothetical compound XH , which could be a metabolite, as in photosynthesis, or a coenzyme as in fatty acid oxidation. The possibility must not be overlooked that the one-carbon unit is passed from one carrier to another as its oxidation level changes, so that XH may be not one but several compounds.

The material presented in this chapter shows that knowledge of the biology, physiology, and biochemistry of methane bacteria has developed slowly over a long period of time. Unfortunately this knowledge is still incomplete and superficial in many respects. Much time and effort will be required to bring our understanding of this group to a level comparable with that of many other groups of microorganisms.

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2

THE CHEMISTRY OF

BUTYRIC ACID–BUTANOL FERMENTATIONS

The butyric acid fermentation of sugar and lactate was discovered by Pasteur in 1861. During the next half century Fritz, Beijerinck, Duclaux, Winogradsky, Weizmann, and others studied various butyric acid fermentations and showed that they are caused mainly by several species of clostridia that are widely distributed in nature and as a group are able to decompose a great variety of substrates, including sugars, polysaccharides, and hydroxy acids. The main products of these fermentations were identified as acetic and butyric acids, *n*-butanol, ethanol, acetone, sometimes isopropanol, carbon dioxide, and hydrogen. Also techniques were devised for the commercial exploitation of the fermentation of starch by *Clostridium acetobutylicum* for the production of acetone and butanol.

Early studies in the chemistry of the butyric acid–butanol fermentations were hampered by the variety of the products and the difficulties associated with their quantitative estimation. Precise and extensive quantitative information con-

cerning the fermentation products did not become available until the period between 1925 and 1932, when several fundamental studies such as those of Donker,¹ van der Lek,² and Peterson and Fred³ were published. This information provided a basis for reasonable deductions and speculations concerning the chemical pathways involved in the conversion of glucose and other substrates to fermentation products.

The state of knowledge of the chemistry of butyric acid-butanol fermentations that existed in 1931⁴ is summarized schematically in Fig. 1. It was generally accepted without

1. $C_6H_{12}O_6 \longrightarrow 2C_3 (?) \longrightarrow 2C_2 (?) + 2C_1 (?)$
2. $2CH_3CHO (?) \longrightarrow CH_3CHOHCH_2CHO (?) \longrightarrow$
 $CH_3CH_2CH_2COOH$
3. $2CH_3COOH (?) \longrightarrow CH_3COCH_2COOH \longrightarrow$
 $CH_3COCH_3 + CO_2$
4. $CH_3CH_2CH_2COOH \xrightarrow{4H} CH_3CH_2CH_2CH_2OH$
5. $HCOOH (?) \longrightarrow H_2 + CO_2$

Fig. 1. Chemistry of Butyric Acid-Butanol Fermentation, 1931.

proof that the early stages of hexose decomposition followed the general pattern of alcoholic fermentation. This involved the fission of the hexose into two C_3 compounds each of which was converted to a C_2 and a C_1 compound. Butyric acid and acetone were believed to be formed by a condensation of C_2 units, followed in the case of acetone by the removal of one carbon as carbon dioxide. Reilly et al.⁵ and Speakman⁶ had provided evidence that acetate and butyrate are intermediates in the formation of acetone and butanol, respectively. The acids were shown to accu-

mulate in the early stages of the fermentation and then decrease simultaneously with the rapid formation of the solvents. Addition of acetate to a starch fermentation resulted in an almost theoretical increase in the yield of acetone, whereas addition of butyrate increased the yield of butanol. The suggested occurrence of acetoacetate as an intermediate between acetate and acetone was supported by the observation that added acetoacetate was converted to acetone and carbon dioxide.

Several parts of the 1931 picture were largely speculative. For example, the identity of the postulated C_3 compound was not known. Both methyl glyoxal and pyruvate were suggested as possibilities, but definitive evidence for either was lacking. Also the C_2 precursor of butyrate was not known. It was commonly assumed to be acetaldehyde because the aldol condensation in non-biological systems gave a product which might be converted to butyric acid. However, evidence for the occurrence of acetaldehyde as an intermediate in the fermentation was slight; neither added acetaldehyde nor acetaldol increased the yield of butyric acid. The identity of the primary C_1 compound was also uncertain. It was thought to be either formate or carbon dioxide. Kluyver⁴ favored the view that formate was formed first and then decomposed to hydrogen and carbon dioxide, but again the evidence was not conclusive.

This brief summary indicates some of the uncertainties concerning the chemistry of these fermentations that existed twenty-five years ago. Since that time considerable advances have been made in this field. In the following sections I shall try to review some of these developments and present the current picture of the chemistry of the butyric acid–butanol fermentations.

The Role of Acetate. One of the most significant developments in this field was the recognition of the key role of

acetate or acetate derivatives in the formation of butyric acid. The first demonstration that acetate can be a precursor of butyrate and butanol was made by Wood et al.⁷ They used *Cl. acetobutylicum* to ferment starch in the presence of acetate -1-C¹³ and determined the distribution of the C¹³ in the products.

They found, as was to be expected on the basis of earlier balance studies, that the isotope was present in carbon dioxide and the carbonyl group of acetone. This labeling is consistent with a pathway involving acetoacetate, shown in equation 3 of Fig. 1. The additional finding of C¹³ equally distributed between the 1 and 3 positions of butyrate and butanol was quite unexpected. It necessitated the conclusion that the C₄ compounds also are formed by a condensation of two molecules of acetate or some compound that is easily formed from acetate.

At about the same time a role of acetate in butyrate synthesis was established independently by nutritional and fermentation balance experiments carried out with two other butyric acid bacteria, namely *Cl. kluyveri* and *Cl. tyrobutyricum*.

Clostridium kluyveri is a somewhat atypical butyric acid bacterium, which was isolated from enrichment cultures containing ethanol as the only organic compound.⁸ This organism was shown to have unusual substrate requirements. It could not decompose carbohydrates, amino acids, or even pyruvate, but it grew well with ethanol and a com-

pound present in yeast autolyzate prepared in a special way.⁹ The concentration of yeast autolyzate necessary to support moderate growth was so high that it soon became evident that the essential compound probably was not an amino acid or a growth factor. Eventually, by fractionation of the yeast autolyzate the major active component was shown to be acetate.

Fermentation balance experiments then showed that the energy-yielding process used by *Cl. kluyveri* is a conversion of ethanol and acetate to butyrate and caproate.¹⁰ When acetate is present in excess, butyrate is the main product (equation 1), whereas when ethanol is in excess the main product is caproate (equation 2). It may be noted that

no ethanol is utilized under the anaerobic conditions suitable for growth of this organism unless acetate is also supplied. These balance experiments demonstrated by direct chemical analysis the conversion of acetate to the C₄ and C₆ fatty acids.

Cl. tyrobutyricum is a species originally isolated by van Beynum and Pette¹¹ from low-grade silage. It was believed to be responsible for the conversion of lactate to the butyrate which is commonly present in silage that has not developed a sufficiently high acidity to inhibit spoilage organisms. When mixed cultures of *Cl. tyrobutyricum* and other silage bacteria were allowed to grow in a complex medium containing lactate and yeast extract, the organism appeared to be responsible for a vigorous fermentation of lactate. However, pure cultures fermented lactate very

feebly and then only when the medium was supplied with a very high concentration of yeast extract.

The high yeast extract requirement for the growth of *Cl. tyrobutyricum* on lactate was reminiscent of the similar requirement found earlier with *Cl. kluyveri*. Consequently Bhat and I¹² reinvestigated the substrate requirements of *Cl. tyrobutyricum* (*Cl. lactoacetophilum*) particularly in relation to a possible role of acetate. We found that glucose or pyruvate is readily fermented when supplied alone in a medium containing only the usual nutrients required by butyric acid bacteria. In contrast lactate or glycerol cannot be fermented in such a medium unless a roughly stoichiometric amount of acetate is also provided. When this is done, lactate or glycerol is fermented almost as readily as glucose. Balance experiments showed that acetate is actually consumed during the fermentation of lactate and is converted to butyrate just as in the *Cl. kluyveri* fermentation of ethanol and acetate.

A schematic explanation for the necessity of additional acetate in the lactate fermentation is given by the following equations.

The oxidation of lactate to acetate (equation 3) makes available four equivalents of hydrogen for the reduction of acetate or its derivations to butyrate. Two moles of acetate are required for reaction 4, whereas only 1 mole of acetate is formed. Therefore, a second mole of acetate must be supplied in the medium as a source of an oxidant so that

the fermentation can proceed according to reaction 5. Actually the stoichiometric relations are not quite so simple because some hydrogen gas is evolved; consequently somewhat less than 1 mole of acetate is consumed per mole of lactate.

Acetate is usually required in butyric acid fermentations of lactate, glycerol, and other compounds more reduced than carbohydrate. Whenever the ability of an organism to ferment such compounds is to be tested acetate should be supplied in the medium. In the past, failure to do this has resulted in erroneous conclusions concerning substrate utilization and the taxonomic relations of certain butyric acid bacteria.

Although acetate is usually required for the butyric acid fermentation of lactate, at least one exception to this generalization is known. *Butyribacterium rettgeri* readily ferments lactate in the absence of added acetate; in fact both acetate and butyrate are produced.¹³ This is possible because *B. rettgeri* possesses an additional oxidative mechanism, by which part of the carbon dioxide it forms is reduced to acetate.^{14,15} This type of oxidative mechanism occurs in several anaerobic bacteria.^{16,17,18}

Use of Enzyme Preparations. Most of the early work on the chemistry of butyric acid fermentations was done with growing cultures or washed cell suspensions. Experiments with such material provided information concerning the over-all chemical changes in these fermentations and permitted some rough conclusions about the course of the metabolic pathways. However, it eventually became apparent that a complete elucidation of the fermentation reactions could only be achieved by the use of cell-free extracts and purified enzyme systems.

The first extensive use of cell-free extracts in the study of the butyric acid fermentation was made by Koepsell and

Johnson.¹⁹ They found that when a cell paste of *Cl. butylicum* was kept frozen for a week or more and then allowed to thaw, a soluble enzyme preparation was obtained that catalyzed the conversion of pyruvate to acetate, carbon dioxide, and hydrogen. Phosphate was required for pyruvate decomposition, and later²⁰ evidence was obtained for the formation of acetyl phosphate (equation 6). This pro-

vided an indication that an acetyl compound might be a precursor of butyrate. Actually the extracts used by Koepsell and Johnson were unable to form butyrate from pyruvate under the conditions used. However, they did catalyze a reaction by which acetyl phosphate and butyrate were apparently converted to acetate and butyryl phosphate (reaction 7), thus opening the possibility that the latter

compound was involved in butyrate or butanol synthesis. The origin of the hydrogen, which is a conspicuous product of most butyric fermentations, was somewhat clarified by this work. Extracts that produced hydrogen readily from pyruvate (equation 6) were found to be unable to decompose formate. This eliminated the theory that formate is an obligatory intermediate in the formation of hydrogen from pyruvate.

The success of Koepsell and Johnson in obtaining enzymatically active extracts from *Cl. butylicum* suggested that similar methods might be used with other species. Consequently Stadtman and I²¹ tried preparing cell-free extracts of *Cl. kluyveri* by drying the cells and then extracting with

dilute buffer. This simple procedure gave a soluble preparation that contained an amazingly complete fermentation system. *Clostridium kluyveri* extracts were found to catalyze the oxidation of alcohol via acetaldehyde to acetyl phosphate (equation 8), the oxidation of butyrate to acetyl phosphate and acetate (equation 9), and the synthesis of butyrate from acetyl phosphate and acetate using hydrogen as the reducing agent (equation 10).

The formation of acetyl phosphate by the oxidation of acetaldehyde, which was comparable to its formation from pyruvate by *Cl. butylicum* extracts, indicated that acetyl phosphate might play a key role in butyric acid fermentations. This idea was supported by the observation that under the conditions of these experiments acetyl phosphate was required for butyrate synthesis; it could not be replaced by acetate. A comparison of reactions 9 and 10 also indicated that the oxidation and synthesis of butyrate probably involve the same sequence of reversible reactions.

Early Studies on the Path of Butyrate Synthesis in *Clostridium kluyveri*. After the discovery of the enzymatic synthesis of butyrate from acetyl phosphate and acetate, the mechanism of this synthesis became the central problem of the butyric acid fermentation and indeed of fatty acid metabolism in general. The formation of butyrate from C_2 precursors was obviously a complex reaction

since it must involve a minimum of three reactions: a condensation and two reduction steps. The simplest possible mechanism of butyrate synthesis and oxidation, used as a working hypothesis in subsequent experimentation, is shown in the following reaction sequence:

In this sequence acetyl phosphate was postulated to condense with acetate to form acetoacetate which was reduced first to β -hydroxybutyrate and then to butyrate. Both of the postulated intermediates were known to accumulate during the oxidation of fatty acids in animals under certain circumstances.

The above sequence could readily be tested with *Cl. kluyveri* extracts by finding out whether acetoacetate and β -hydroxybutyrate react as the scheme predicts. Stadtman²² found that they do not so react. β -Hydroxybutyrate could not be reduced to butyrate nor oxidized to acetyl phosphate. Acetoacetate could undergo a phosphoroclastic conversion to acetyl phosphate and acetate, but this reaction was too slow and occurred under the wrong conditions to participate in butyrate oxidation. Furthermore, although acetoacetate could be reduced enzymatically with molecular hydrogen as the scheme predicts, the product was not butyrate but β -hydroxybutyrate, which could not be further reduced. These observations eliminated acetoacetate and β -hydroxybutyrate as intermediates in butyrate synthesis.

A variety of other four-carbon acids at the oxidation levels of acetoacetate and β -hydroxybutyrate were then tested.^{22, 23} The only one of these acids that proved to be

reactive in the *Cl. kluyveri* system was vinylacetic acid (3-butenic acid). This compound at first showed the reactions expected of an intermediate. Like butyrate, it was oxidized to acetyl phosphate and acetate (reaction 11) or, in the absence of orthophosphate, to acetoacetate (reaction 12).

In the presence of hydrogen, vinylacetate was reduced to butyrate (reaction 13) and in a nitrogen atmosphere it underwent a typical dismutation (reaction 14). Furthermore, all of these reactions occurred at rates equal to or greater than the over-all rates of butyrate synthesis and oxidation. However, all attempts to demonstrate the actual formation of vinylacetate during the synthesis or oxidation of butyrate were decisively negative. For example, when C^{14} -butyrate was oxidized in the presence of a pool of unlabeled vinylacetate, essentially no C^{14} was incorporated into the vinylacetate. This and other experiments excluded vinylacetate as a normal intermediate in butyrate metabolism.

The negative results obtained with vinylacetate, acetoacetate, and other acids at the same oxidation levels, appeared to exclude all C_4 acids as intermediates in butyrate metabolism. This led inevitably to the hypothesis that the actual intermediates are compounds in which the

C₄ acids are combined with another substance, possibly coenzyme A.^{23,24}

Role of Coenzyme A in Fatty Acid Activation. The suggestion that the intermediates in butyrate metabolism are coenzyme A derivatives was based on the work of Lipmann, Lynen, and others on the role of this cofactor in enzymatic acetylation reactions in animal systems, and on the work of Stadtman and his associates on several reactions catalyzed by *Cl. kluyveri* extracts.

Coenzyme A had been shown to be required for the acetylation of sulfanilamide by pigeon liver extracts in a system containing acetate and ATP.²⁵ Methods for the purification of coenzyme A had been developed, and the structure of the compound had been studied. Not all the structural details had been worked out at this time, but coenzyme A was known to contain adenosine-5'-phosphate, two additional phosphate groups, pantothenic acid, and thioethanolamine in a terminal position.²⁶ The mode of action of coenzyme A in acetate activation was discovered by Lynen et al.,²⁷ who isolated acetyl coenzyme A from yeast, demonstrated that the acetyl group is attached to the sulfur atom, and showed that acetyl coenzyme A acts as an enzymatic acetylating agent.

This new information concerning coenzyme A was soon applied to the *Cl. kluyveri* system. Stadtman et al.^{28,29} showed that extracts of *Cl. kluyveri* contain an enzyme called phosphotransacetylase that catalyzes a reversible transfer of the acetyl group from acetyl phosphate to coenzyme A. This enzyme was first detected as an activity that caused a rapid decomposition of acetyl phosphate in the presence of inorganic arsenate ("arsenolysis") and also catalyzed a rapid exchange between the phosphoryl group of acetyl phosphate and orthophosphate. When these reactions were found to be completely dependent upon the

presence of coenzyme A, the phosphotransacetylase reaction was formulated as follows:

This reaction accounts for the observed phosphate exchange. Since arsenate and phosphate have similar properties the reverse reaction in the presence of arsenate would be expected to give acetyl arsenate. This hypothetical compound, which has never been prepared or isolated, presumably hydrolyzes rapidly to acetate and arsenate and thus accounts for the arsenolysis reaction. Further evidence for the correctness of reaction 15 was provided by the use of substrate amounts of coenzyme A and the identification of the product with Lynen's acetyl coenzyme A. This was done in part by showing that the product of reaction 15 could serve as an acetyl donor in the enzymatic acetylation of sulfanilamide and in the condensation with oxaloacetate to give citrate.

The easy interconversion of acetyl phosphate and acetyl-SCoA by phosphotransacetylase opened the question as to which of these compounds is the immediate product of ethanol and acetaldehyde oxidations. Burton and Stadtman³⁰ investigated these reactions and found that ethanol oxidation involves two enzymes. Reaction 16 is catalyzed

by a DPN-specific alcohol dehydrogenase. The further oxidation of the resulting acetaldehyde by acetaldehyde dehydrogenase (reaction 17) is dependent upon DPN and

coenzyme A but not upon orthophosphate. The product of this reaction is acetyl-SCoA which accumulates when substrate amounts of coenzyme A are used. With only catalytic amounts of coenzyme A acetaldehyde oxidation ceases as soon as the coenzyme is mostly converted to the acetylated form. The further addition of orthophosphate in the presence of phosphotransacetylase permits the transfer of the acetyl group from SCoA to phosphate, giving acetyl phosphate and regenerating free coenzyme A so that the oxidation of acetaldehyde can continue. Of course, any other reaction that will consume acetyl-SCoA and regenerate coenzyme A will serve the same function. As we shall see, the normal acetyl-SCoA-consuming reaction in the *Cl. kluyveri* fermentation is that leading to the formation of acetoacetyl-SCoA. The formation of acetyl phosphate appears to be a side reaction which may be useful in the storage of acetyl groups and in the generation of ATP by the action of the enzyme acetokinase³¹ (reaction 18).

The formation of acetyl-SCoA by oxidation of acetaldehyde is a peculiarity of *Cl. kluyveri*. Other sugar-fermenting butyric acid bacteria probably form acetyl-SCoA during the phosphoroclastic decomposition of pyruvate (reaction 6). This reaction in *Cl. butyricum* is evidently quite complex since it requires at least three cofactors: cocarboxylase, coenzyme A, and ferrous ions.³² The requirement for coenzyme A, the eventual formation of acetyl phosphate, and the presence of phosphotransacetylase in the enzyme extracts indicate the intermediate formation of acetyl-SCoA. This compound is known to be a product of pyruvate oxidation by *Escherichia coli*.³³

The role of coenzyme A in the activation of fatty acids other than acetate has been studied in some detail with

butyric acid bacteria. It has already been mentioned that Koepsell et al.²⁰ obtained evidence that extracts of *Cl. butylicum* catalyze the synthesis of butyryl phosphate from acetyl phosphate and butyrate (reaction 7). *Clostridium kluyveri* extracts catalyze a similar reaction in which the phosphoryl group is transferred from acetyl phosphate to other fatty acids containing from three to eight carbon atoms.³⁴ In a further study of this reaction using acetyl phosphate and propionate, Stadtman³⁵ established that it is completely dependent upon the presence of coenzyme A and phosphotransacetylase. These requirements suggested that the apparently simple phosphoryl transfer (reaction 22) actually involves three distinct enzymatic steps shown in the following equations:

Reaction 19 is the usual phosphotransacetylase reaction, and reaction 21 is the analogous reaction involving propionyl phosphate, catalyzed by the same enzyme. Reaction 20 represents a reversible transfer of the SCoA group from acetate to propionate. This transfer results in the activation of propionate and the deactivation of acetate. The enzyme catalyzing this reaction has been partially purified and named coenzyme A-transphorase. In the presence of this enzyme the SCoA group may be transferred to several

carboxylic acids in addition to acetate and propionate, namely formate, *n*-butyrate, *n*-valerate, *n*-caproate, lactate, and vinylacetate. These include all the compounds that are formed or readily metabolized by *Cl. kluyveri* extracts. In contrast, crotonate, β -hydroxybutyrate, and acetoacetate, which are relatively unreactive in the *Cl. kluyveri* system, do not serve as substrates for coenzyme A-transphorase. This indicates that only those acids which can be converted to their coenzyme A derivatives are highly reactive in this system.

Further evidence for the participation of the coenzyme A derivatives of the four carbon acids in the metabolism of *Cl. kluyveri* has been obtained by studying the oxidation of butyrate and the reduction of vinylacetate. Crude extracts of *Cl. kluyveri* readily oxidize butyrate. However, Stadtman²¹ found that after these extracts were dialyzed to remove soluble cofactors, butyrate was not oxidized unless catalytic amounts of acetyl phosphate, a source of coenzyme A, and other cofactors were added. This suggested that butyrate had to be activated by conversion to either butyryl-SCoA or butyryl phosphate before it was susceptible to oxidation. Butyryl phosphate was excluded as an intermediate by the observation that it was oxidized more slowly than butyrate. Finally Stadtman³⁶ prepared butyryl-SCoA and showed that it was oxidized rapidly in the absence of free coenzyme A to acetoacetyl-SCoA, and in the presence of coenzyme A to acetyl-SCoA.

The evidence regarding the role of coenzyme A in the reduction of vinylacetate to butyrate is similar though less extensive. Peel³⁷ has found that a catalytic amount of either acetyl phosphate or acetyl-SCoA is essential for vinylacetate reduction. This points to the participation in this reaction of vinylacetyl-SCoA which is known to be formed from acetyl-SCoA and vinylacetate in the SCoA-transphorase

reaction. Later experiments by Bartsch³⁸ have shown that *Cl. kluyveri* extracts contain an enzyme called vinylacetyl isomerase which converts vinylacetyl-SCoA to crotonyl-SCoA.

The latter compound may be the substance actually reduced, as it appears to be in animal systems.^{39,40}

A key reaction in the synthesis of butyrate is the reversible formation of the C₄ compound at the oxidation level of acetoacetate from its C₂ precursor. The elucidation of this reaction required a series of investigations most of which were done with animal enzyme preparations. Studies carried out in Lipmann's laboratory with a soluble multi-enzyme preparation from pigeon liver showed that ATP, coenzyme A, and acetate are required for acetoacetate synthesis.⁴¹ This suggested that a coenzyme A derivative of acetate was a precursor of acetoacetate, but it was not known whether one molecule of "active" acetate condensed with a molecule of acetate to give acetoacetate directly or whether more complicated reactions were involved. This situation was clarified by Stadtman et al.,⁴² who studied acetoacetate synthesis in a system containing acetyl phosphate, acetate, coenzyme A, phosphotransacetylase from *Cl. kluyveri*, and a pigeon liver extract. In separate experiments, either the acetyl phosphate or the acetate was labeled with C¹⁴ and the incorporation of the isotope into acetoacetate was studied. The data showed that free acetate was not used, but on the contrary both halves of the acetoacetate molecule were derived from acetyl phosphate presumably via acetyl-SCoA (reaction 24).

Now the above reaction was catalyzed by a relatively crude pigeon liver extract, and there was no certainty that it represented a single enzymatic step. Another possibility^{24,43} was a two-step reaction sequence in which one enzyme catalyzed the reversible transfer of an acetyl group from one molecule of acetyl-S-CoA to another to give acetoacetyl-S-CoA plus free coenzyme A (reaction 25), and another enzyme hydrolyzed acetoacetyl-S-CoA to acetoacetate (reaction 26).

The postulated formation of acetoacetyl-S-CoA was consistent not only with all the available information concerning the pigeon liver acetoacetate-forming system but also with certain observations made on the *Cl. kluyveri* system. For example, at that time the necessity of the activation of butyrate prior to its oxidation was known and the activated compound was suspected to be butyryl-S-CoA. The oxidation of butyryl-S-CoA would be expected to give acetoacetyl-S-CoA, and the reverse reaction would also be anticipated.

More direct evidence for the reversible formation of acetoacetyl-S-CoA from acetyl-S-CoA was first obtained by Lynen et al.,^{44,45} with soluble enzymes from sheep liver. This was made possible by the discovery that thioesters of acetoacetate such as S-acetoacetyl-N-acetyl-thioethanolamine and acetoacetyl-S-CoA show a strong ultraviolet absorption peak at 303 m μ in alkaline solution. By means of this optical property the formation and decomposition of acetoacetyl-S-CoA according to reaction 25 were demonstrated. The enzyme catalyzing this reaction, called acetoacetyl thiolase

or just thiolase for short, was later purified from animal sources and studied extensively.^{46,47,48} Several other enzymes catalyzing specific reactions in the reversible conversion of acetoacetyl-S-CoA to butyryl-S-CoA have also been purified from animal tissues during the last few years.^{46,49,50,51,52}

Current Knowledge of the Path of Butyrate Synthesis in Bacteria. The accumulated information obtained with animal and bacterial enzyme systems indicates that butyrate formation in *Cl. kluyveri* involves the reactions shown in Fig. 2. Reactions I, II, and III have already been dis-

Fig. 2. Butyric Acid Synthesis in *Clostridium kluyveri*.

cussed; reaction IV is the reduction of acetoacetyl-S-CoA to β -hydroxybutyryl-S-CoA catalyzed by the enzyme β -hydroxybutyryl-S-CoA dehydrogenase; reaction V is the dehydration of β -hydroxybutyryl-S-CoA to crotonyl-S-CoA catalyzed by the enzyme crotonase; reaction VI is the reduction of crotonyl-S-CoA to butyryl-S-CoA by the enzyme butyryl-S-CoA dehydrogenase; and reaction VII is the transfer of the S-CoA group from butyrate to acetate catalyzed by the fatty acid S-CoA-transphorase.

Some evidence for the occurrence of these reactions in *Cl. kluyveri*, in addition to that already discussed, has accumulated. The oxidation of butyryl-S-CoA to aceto-

acetyl-SCoA in the absence of free coenzyme A (reactions VI, V, and IV) has been demonstrated by spectrophotometric methods.^{36,53} When free coenzyme A was added to such a reaction mixture, the accumulated acetoacetyl-SCoA was rapidly converted to acetyl-SCoA by reaction III. Unfractionated *Cl. kluyveri* extracts were found by Bartsch³⁸ to possess acetoacetyl thiolase activity corresponding to the decomposition of approximately 2 micromoles of acetoacetyl-SCoA per minute per milligram of protein. β -hydroxybutyryl-SCoA dehydrogenase activity (reaction IV) has also been demonstrated qualitatively by Bartsch by using a DPNH-generating system to reduce the N-acetyl thioethanolamine analogue of acetoacetyl-SCoA. The reaction was followed by the decrease in ultraviolet absorption of the analogue at 303 m μ . The reduction product has not been characterized in this system. The corresponding enzyme from rat liver mitochondria has been shown to produce L(+)- β -hydroxybutyryl-SCoA.^{54,55}

Crotonase (reaction V) activity is very high in *Cl. kluyveri* extracts.³⁸ Using crotonyl-SCoA as a substrate and following the hydration of the double bond by means of its ultraviolet absorption at 263 m μ , the crotonase activity was shown to correspond to the utilization of 570 micromoles of crotonyl-SCoA per minute per milligram of crude protein. This is roughly 20 times the specific activity of ox liver extracts, a rich animal source of the enzyme.⁴⁰

Butyryl-SCoA dehydrogenase activity (reaction VI) has been observed by coupling the oxidation of the pantotheine analogue of butyryl-SCoA with the reduction of indophenol,³⁸ and by coupling the reduction of crotonyl cystamine with the oxidation of leucosafranine.⁵⁶ By means of the latter technique it was found that crude extracts of *Cl. kluyveri* were twice as active with respect to butyryl-SCoA dehydrogenase as the corresponding animal enzyme

which had been purified 350-fold from pig liver extracts.

The available evidence indicates that butyrate synthesis in *Cl. kluyveri* involves reactions similar to or identical with those shown in Fig. 2. However, the precise identities of the postulated unsaturated and β -hydroxy compounds, here referred to as crotonyl-SCoA and β -hydroxybutyryl-SCoA, have not been established. The unsaturated compound might be crotonyl-, isocrotonyl-, or vinylacetyl-SCoA; and the hydroxy compound might be L(+)- or D(-)- β -hydroxybutyryl-SCoA.

Despite the considerable information that is available concerning the chemistry of the *Cl. kluyveri* fermentation, at least one major problem remains unsolved, namely the nature of the mechanism by which the organism obtains useful energy from the conversion of ethanol and acetate to butyrate and caproate. The oxidation of ethanol to acetyl-SCoA provides an energy-rich compound which might be used to generate ATP by means of the phosphotransacetylase and acetokinase reactions. However, most of the acetyl-SCoA is converted to butyrate and caproate, and therefore is not directly available as a source of energy for ATP synthesis. Indeed, acetyl-SCoA must be used in this way in order to produce the electron acceptors, acetoacetyl-SCoA, crotonyl-SCoA, and their six carbon analogues, without which the oxidation of ethanol and acetaldehyde would be impossible.

Since acetyl-SCoA seems to be excluded as a primary energy source for synthetic reactions, some other source of energy must be available to the organism. One possible method of providing ATP is by the coupling of an electron transport reaction with a phosphorylation as occurs in the so-called oxidative phosphorylation in aerobic organisms. In the *Cl. kluyveri* system the most suitable electron transport system for this purpose is that between ethanol or

acetaldehyde and crotonyl-SCoA. The potential span between the ethanol-aldehyde couple [E'_0 (pH 7) = -0.204 volt] and the crotonyl-SCoA-butyryl-SCoA couple [E'_0 (pH 7) = 0.19 volt], for example, is 0.39 volt which corresponds to a free-energy change of approximately -18 kcal. under physiological conditions. This is more than sufficient to provide the energy (8 to 10 kcal.) needed for the synthesis of 1 mole of ATP from ADP and orthophosphate. However, so far attempts to demonstrate a coupling of phosphorylation with crotonyl-SCoA reduction by a DPNH-generating system have not been successful.

The enzymatic reactions involved in butyrate formation from acetyl-SCoA in other butyric acid bacteria are probably similar to those demonstrated in *Cl. kluyveri* and animal tissues. Although relatively little specific information has been obtained with other species, Stern et al.⁴⁹ have recently reported that extracts of *Cl. acetobutylicum* and *Cl. sticklandii* (strain HF) show enzymatic activities attributable to crotonase, L(+)- β -hydroxybutyryl-SCoA dehydrogenase, and thiolase.

Formation of C₅-C₇ Fatty Acids. *Clostridium kluyveri* forms *n*-caproate from butyrate and ethanol, and forms *n*-valerate and *n*-heptanoate from propionate and ethanol.⁵⁷ The formation of caproate undoubtedly starts with a condensation of butyryl-SCoA, derived from butyrate or crotonyl-SCoA, with acetyl-SCoA derived from ethanol to give β -ketocaproyl-SCoA by a reaction analogous to the formation of acetoacetyl-SCoA from acetyl-SCoA. The further enzymatic steps in the formation of caproate are probably the same as those involved in butyrate synthesis. Experimental evidence for the participation of a C₆ β -keto acid derivative was obtained by showing that the oxidation of caproate, under conditions which prevent the thiolase reaction, results in the accumulation of β -ketocaproate.⁵⁸

The formation of the C_5 to C_7 fatty acids involves a condensation between acetyl-SCoA and the thioester of a longer-chain fatty acid. In these condensations the C_2 moiety always appears at the carboxyl end of the resulting fatty acid.⁵⁷ This result is consistent with a synthetic path involving β -keto acid derivatives.

Formation of Acetone and Butanol. Acetone and butanol are formed from carbohydrates by *Cl. acetobutylicum* and other butyric acid bacteria mainly toward the end of the fermentation when the pH of the medium drops to about 4.0. As we have seen, the accumulation of solvents coincides with a decrease in the concentration of volatile acids. The conversion of acetic acid to acetone and butanol and the conversion of butyric acid to butanol are clearly established.

Acetone is undoubtedly formed by the decarboxylation of acetoacetate (reaction 27). Johnson et al.⁵⁹ first demon-

strated this reaction with growing cultures and cell suspensions; later Davies⁶⁰ extracted and partially purified the decarboxylase responsible for the reaction and showed that it is most active at pH 5.

Acetoacetate is almost certainly formed from acetoacetyl-SCoA, although with bacteria only indirect evidence for this is available. In some *Cl. kluyveri* extracts acetoacetate accumulates during the oxidation of butyrate when the normal conversion of acetoacetyl-SCoA to acetyl phosphate is prevented by a lack of either coenzyme A or orthophosphate.^{23,36} This can be interpreted to mean that acetoacetate is formed from its coenzyme A derivative. The nature of the reaction by which acetoacetyl-SCoA is converted to acetoacetate in *Cl. kluyveri* has not been investigated in detail but it appears to be an hydrolysis. This is

very wasteful of energy since the hydrolytic cleavage of the thioester bond involves a free-energy change of about -8 kcal. In *Cl. kluyveri* this is unimportant since little or no free acetoacetate is formed during its normal metabolism. A more efficient way of forming acetoacetate would be a transfer of SCoA from acetoacetyl-SCoA to acetate (reaction 28) to give acetoacetate and acetyl-SCoA. The latter

compound can be used to make more acetoacetyl-SCoA without a further expenditure of energy. The SCoA transferase of animal tissues catalyzes a reversible reaction of this type between acetoacetyl-SCoA and succinate.⁶¹ However, this enzyme probably does not occur in butyric acid bacteria, and the bacterial fatty acid SCoA transphorase, at least that which occurs in *Cl. kluyveri*, does not react with acetoacetate.³⁵ Nevertheless, it seems probable that bacteria like *Cl. acetobutylicum* which form large amounts of acetone via acetoacetate will be found to conserve the thioester bond energy of acetoacetyl-SCoA by making use of reactions similar to reaction 28.

The formation of *n*-butanol from butyrate undoubtedly involves butyryl-SCoA as an intermediate. Butyryl-SCoA can be formed from butyrate and acetyl-SCoA, and probably also by the reaction of butyrate with ATP and HSCoA.³⁴

In the first demonstration of the enzymatic formation of butanol with *Cl. kluyveri*, butyryl phosphate was the substrate. The path of conversion of butyryl phosphate to butyryl-SCoA has not been determined, but a possible

reaction sequence would be a transfer of the phosphoryl group of butyryl phosphate to ADP (reaction 30) followed

by a reaction of the resulting ATP with butyrate and coenzyme A to give butyryl-SCoA as shown in reaction 29. It may be noted that phosphotransacetylase cannot catalyze a direct transfer of the butyryl group from butyryl phosphate to coenzyme A.

The reduction of butyryl-SCoA to butyraldehyde by DPNH (reaction 31) is catalyzed by the aldehyde dehydrogenase of *Cl. kluyveri*³⁰ and similar enzymes probably occur

in other butyric acid bacteria. The *Cl. kluyveri* enzyme also reacts with acetyl-SCoA and propionyl-SCoA to give acetaldehyde and propionaldehyde respectively. The aldehydes are finally reduced to the corresponding alcohols by DPNH under the influence of alcohol dehydrogenase (reaction 32). In carbohydrate fermentations the DPNH is probably supplied by the oxidation of triose phosphate.

Certain interrelations between acetone and butanol formation are of some interest. In the fermentation of carbohydrates by *Cl. acetobutylicum* acetone, butanol, and ethanol are formed simultaneously.³ The reason for this is not hard to find. The conversion of acetoacetyl-SCoA to acetone results in the loss of two electron-accepting reactions,

namely, the reduction of acetoacetyl-S-CoA to β -hydroxybutyryl-S-CoA and the reduction of crotonyl-S-CoA to butyryl-S-CoA. If the oxidation of triose phosphate is to continue, these reactions must be replaced by other electron-accepting reactions. The only alternative reactions of this type available to these organisms appear to be the reductions of acetyl- and butyryl-S-CoA and the corresponding aldehydes to alcohols. Therefore, the formation of acetone is necessarily linked with the formation of alcohols. The trigger mechanism for solvent formation must be a step in the conversion of acetoacetyl-S-CoA to acetone. If the conversion of acetoacetyl-S-CoA to acetoacetate is in fact a reversible reaction, the irreversible decarboxylation of acetoacetate is probably the key reaction. The observation of Davies⁶⁰ that solvent formation is associated with a rapid increase in the level of acetoacetate decarboxylase in *Cl. acetobutylicum* supports this conclusion.

The material presented in this chapter shows that substantial progress has been made in understanding the complex chemical reactions involved in butyric acid-butanol fermentations. However, despite an early start and rapid initial progress, knowledge of the enzymatic reactions of fatty acid metabolism in bacteria is now less complete than that of similar processes in animal tissues. Only a few bacterial enzymes concerned with fatty acid metabolism have been purified fairly extensively and studied in some detail. More information is needed concerning the enzymes and intermediates functioning between acetoacetyl-S-CoA and butyryl-S-CoA in butyrate synthesis, and between acetoacetyl-S-CoA and acetoacetate in acetone formation. We need to learn something about the mechanisms by which the energy derived from butyrate synthesis and associated reactions is made available to the cell. Also in the carbohydrate fermentations, the steps in the conversion of sugars

to pyruvate need to be carefully investigated in the light of the recent discovery of multiple pathways of carbohydrate breakdown. These are all problems for the future.

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FERMENTATIONS OF NITROGENOUS COMPOUNDS

In 1934 the biochemical transformations involved in the anaerobic breakdown of nitrogenous compounds had not been extensively studied. Various proteinaceous materials were known to undergo extensive decomposition in the absence of oxygen with the formation of ammonia, carbon dioxide, fatty acids, hydrogen sulfide, and an assortment of other volatile substances frequently having unpleasant or repulsive odors. Such "putrefactive" processes had been found to be caused mainly by clostridia, several species of which had been shown to grow in media containing proteins and amino acids but no carbohydrates. However, very little information was available concerning the specific reactions catalyzed by individual species. The reasons for this are not hard to find. With some conspicuous exceptions, most of the studies in this area had been done either by chemists who disregarded one of the basic requirements for sound microbiological work, namely the use of pure cultures, or by bacteriologists who were unequipped to handle the fre-

quently difficult problems of chemical analysis. The chemists typically inoculated a nutrient solution containing a relatively pure amino acid with a piece of decaying meat, waited several months for the microbial activity to cease, and then identified the products. The bacteriologists typically inoculated a pure culture of a clostridium into a complex protein hydrolyzate and in the end were unable to identify either the specific amino acids decomposed or all of the products formed.

One of the most substantial contributions in both a chemical and microbiological sense to knowledge of the anaerobic decomposition of amino acids was made by Stickland in 1934.¹ He studied the action of washed suspensions of *Clostridium sporogenes* on amino acids. By the use of both manometric and dye-coupling techniques he demonstrated that, whereas most single amino acids are not readily attacked by *Cl. sporogenes*, certain pairs of amino acids are decomposed rapidly by a coupled oxidation-reduction reaction; one member of the pair is oxidized while the other member is reduced. The simplest example of this type of reaction is that involving alanine and glycine. As the following equations show, the oxidation of alanine to acetic acid, carbon dioxide, and ammonia is coupled with the reduction of 2 moles of glycine to acetic acid and ammonia.

Certain other amino acids react in similar ways. Other

amino acids that can be oxidized include leucine and valine which are converted to the branched-chain fatty acids with one less carbon atom. Some of the amino acids that serve as oxidants, such as arginine, are reduced like glycine with the formation of ammonia. Others do not give ammonia but are reduced with the opening of a ring; proline for example is converted to δ -aminovaleric acid (equation 4).

These reactions provide mechanisms for the decomposition of a variety of amino acids. Stickland in fact suggested that they could account for most if not all of the anaerobic decomposition of amino acids. In support of this idea, Clifton, Cohen, Nisman, and others² later showed that some fifteen species of clostridia, including *Cl. botulinum*, *Cl. butyricum*, and *Cl. histolyticum*, make use of the Stickland reaction. However, the occurrence of this type of amino acid decomposition in many clostridia in no way excluded the possibility that other mechanisms for amino acid breakdown could also be operative and have quantitative significance.

Considerably before the work of Stickland, the fermentability of single amino acids had already been observed by Naviasky, by Brasch, and by Liebert. Naviasky³ was perhaps the first investigator to observe the anaerobic decomposition of a purified nitrogenous compound by a single bacterium. In 1908 he showed that a pure culture of *Bacillus proteus vulgaris* could ferment asparagine with the for-

mation of several products among which succinate, acetate, carbon dioxide, and ammonia were positively identified. Two years later, Brasch⁴ isolated from a medium containing glutamate as the only carbon and nitrogen source an obligate anaerobic bacterium which he identified with Bienstock's *Bacillus putrificus* and reported that it could ferment glutamate with the formation of ammonia, formate, and butyrate. At about the same time Liebert,⁵ working in Beijerinck's laboratory, described a fermentation of uric acid by an organism he had isolated from a uric acid enrichment culture and named *Bacillus acidi-urici*. Other experiments on the anaerobic decomposition of single nitrogenous compounds made during the same period were mostly carried out with mixed cultures. They showed that several other amino acids including lysine, arginine, aspartate, serine, and alanine probably can be fermented.

Following this flurry of research on amino acid fermentations, interest in this subject subsided and was not reawakened until some twenty-five years later when van Niel⁶ began to investigate the possibility that amino acids and purines can serve as energy sources for some fermentative bacteria just as carbohydrates do for others. He also made use of the enrichment culture method in a search for organisms that can attack various nitrogenous compounds in the absence of oxygen, and he found, in confirmation of Brasch and Liebert, that glutamate and urate are particularly susceptible to decomposition. With both substrates clostridia appeared to be the predominant organisms in the enrichment cultures.

My associates and I continued the study of the anaerobic bacteria that can be obtained from soil and decomposing organic materials by the use of enrichment cultures supplied with single nitrogenous compounds, and over a period of years we isolated a number of species capable of fermenting

several such compounds. A list of these species and the substrates used for their isolation are given in Table 1.

As has already been mentioned, glutamate is readily fermented by clostridia which are abundant and widely distributed in soils.⁷ All of the glutamate-fermenting strains we have isolated belong to one of two closely related species

TABLE 1

Some Amino Acid-, Purine-, or Pyrimidine-Fermenting Bacteria
Isolated by the Enrichment Culture Method

Enrichment Substrate	Species
Glutamate	<i>Cl. tetanomorphum</i> <i>Cl. cochlearium</i>
Histidine	<i>Cl. tetanomorphum</i>
Glycine	<i>Diplococcus glycinophilus</i>
Alanine	<i>Cl. propionicum</i>
Uric acid	<i>Cl. acidi-urici</i> <i>Cl. cylindrosporum</i>
Allantoin	<i>Streptococcus allantoicus</i>
Orotic acid	<i>Zymbacterium oroticum</i>

which have been known for a long time, namely *Cl. cochlearium* and *Cl. tetanomorphum*. The latter species has been isolated also by enrichment with histidine.⁸ Besides decomposing glutamate and histidine, *Cl. tetanomorphum* is also known to ferment cysteine, serine, aspartate, and tyrosine,⁹ but the efficacy of these compounds as enrichment substrates has not been tested.

Enrichment with glycine¹⁰ has given cultures in which a non-motile coccus *Diplococcus glycinophilus* is the predominant organism. This species is highly specialized with respect to its energy source since it appears to be limited to the use of glycine and some simple peptides containing glycine; it is unable to attack a variety of other amino acids

or carbohydrates. Such a restricted diet is unusual even among bacteria. Almost nothing is known about the distribution of this organism.

Alanine is a good substrate for the enrichment of an anaerobic spore former which we have called *Cl. propionicum*.¹⁰ This organism, which also ferments serine, threonine, lactate, pyruvate, and acrylate but not glucose, appears to be rather widely distributed in soils since it has been isolated in England, New Zealand, and several parts of the United States.

Uric acid enrichments from soil or mud invariably lead to the development of clostridia similar to Liebert's *Bacillus aciduri*. Out of thirteen strains isolated from different soils,¹¹ twelve proved to be indistinguishable from Liebert's organism and therefore were called *Clostridium aciduri* in accordance with modern nomenclature. The remaining strain was physiologically similar to the other twelve but was quite different morphologically, and therefore was called *Cl. cylindrosporum*. Both species decompose uric acid and certain other purines with great facility but do not attack carbohydrates or amino acids, except glycine and serine. In view of their highly developed ability to attack purines and their wide distribution in soils, these species may be largely responsible for the anaerobic decomposition of uric acid in nature.

Several attempts to obtain anaerobic bacteria capable of growing on allantoin from several soil and mud samples were unsuccessful, indicating that such organisms are not common in nature; but eventually a vigorous fermentation of allantoin was obtained starting with an inoculum of black mud, and the causative organism was found to be a large streptococcus which we have called *S. allantoicus*.¹² This is one of the few species of the genus that is known to obtain energy for growth from a nitrogenous compound.

Zymobacterium oroticum was isolated from mud by Kornberg¹³ by enrichment with orotic acid. This organism grows slowly at the expense of orotic acid in a relatively complex medium containing tryptone and yeast extract. It also ferments glucose and several other sugars, but not glutamate or lactate. *Zymobacterium oroticum* has been isolated only once, so nothing can be said about its natural occurrence. This organism is of special interest because it has been used by Lieberman and Kornberg^{14,15} to elucidate some of the enzymatic steps in pyrimidine biosynthesis.

The application of the enrichment culture technique has clearly established the existence of a variety of bacteria capable of fermenting single nitrogenous compounds. Several of these organisms were not previously known. In general, these bacteria show a considerable specialization for the utilization of one or a few structurally related nitrogenous substrates. This specialization is most highly developed in *D. glycinophilus* and the purine fermenting clostridia, and least developed in *Cl. tetanomorphum*, which ferments a number of amino acids and other compounds.

The substrates mentioned in Table 1 are not the only nitrogenous compounds suitable for the enrichment of anaerobic bacteria. Serine and threonine are readily fermented in enrichment cultures, although the species mainly responsible for their decomposition under such conditions have not as yet been identified. We have also observed that leucine, valine, cysteine, aspartate, uracil, and thymine can serve as anaerobic enrichment substrates. Probably still other compounds would also be suitable. Further exploration of amino acid, purine, and pyrimidine decomposition by the enrichment culture technique will undoubtedly lead to a better understanding of both the biochemistry and microbiology of fermentative processes.

Of course, knowledge of amino acid fermentations has

not been obtained only by the study of organisms isolated from highly selective enrichment media. Several investigations have been carried out on anaerobic bacteria obtained by direct isolation from soil, spoiled foods, infected wounds, and similar sources. Species that apparently derive most of their energy from the fermentation of single amino acids include *Cl. tetani*,^{16,17} *Cl. saccharobutyricum*,¹⁸ *Micrococcus aerogenes*,¹⁹ and *Fusobacterium nucleatum*,²⁰ which use glutamate, histidine and serine, sometimes threonine and cysteine, and occasionally aspartate, lysine, and tyrosine. Other species in this group are *M. anaerobius* and *M. variabilis*,²¹ which like *D. glycinophilus* appear to be restricted to the utilization of glycine. In addition, a number of bacteria, both obligate and facultative anaerobes, are able to decompose some or all of the amino acids which readily undergo a non-oxidative deamination, namely serine, cysteine, threonine, and arginine. These bacteria include *Cl. welchii*,²² *Cl. sporogenes*,²³ *Cl. botulinum*,²⁴ *E. coli*,^{25,26} *Proteus vulgaris*,^{27,28} *P. morgani*,²⁹ and an anaerobic coccus isolated from the rumen of the sheep.³⁰ A conversion of lysine to acetate, butyrate, and ammonia by *Cl. sticklandii* has been reported by Stadtman,³¹ but this process alone does not support growth.

Chemistry of the Fermentations of Nitrogen Compounds

Fermentations of Histidine and Glutamate by *Clostridium tetanomorphum*. *Clostridium tetanomorphum* ferments histidine and glutamate with great facility. In view of the evidence that the decomposition of histidine goes by way of glutamate or glutamate derivatives in both aerobic bacteria³² and mammalian liver³³ it seemed desirable to find out whether the same or similar reactions are utilized by anaerobic bacteria.

The pathway of L-histidine decomposition in *Pseudomonas fluorescens*³² involves the following sequence:

Clostridium tetanomorphum carries out a similar sequence of reactions with certain modifications (Fig. 1). Urocanate has been shown to be formed from histidine and further decomposed by washed cells.^{34,35} The evidence for formiminoglutamate (formamidinoglutamate) is less conclusive, although possibly adequate. Both washed cells and an acetone powder have been observed to convert histidine to a compound which on alkaline hydrolysis yields 1 mole each of glutamate and formate and 2 moles of ammonia; these products are known to be formed under the same conditions from formiminoglutamate. When chromatographed on paper the compound has the same R_f as the formiminoglutamate formed enzymatically by liver enzymes, and gives the same color with a nitroprusside-ferricyanide reagent that reacts with the formimino group. The enzymatic decomposition of formiminoglutamate has been observed but has not been extensively investigated in *Cl. tetanomorphum*. Presumably this is a tetrahydrofolic acid-dependent reaction as are the analogous reactions in liver³⁶ and in *Cl. cylindrosporum*.³⁷ The immediate products of formiminoglutamate decomposition have not been determined. However, glutamate has been detected in small amounts during the fermentation of histidine, and formamide* accumulates as a major end product of the fermentations of both histidine and urocanate. Tracer experiments have demonstrated that formamide is formed

* Formamide is also formed from histidine by *Aerobacter aerogenes*.³⁸

from the δ nitrogen and carbon atom 2 of the imidazole ring of histidine.⁸ These results are consistent with the reaction sequence involving formiminoglutamate shown in Fig. 1.

Fig. 1. Conversion of Histidine to Glutamate by *Clostridium tetanomorphum*.

The available information indicates that the main difference in the paths of histidine decomposition by *Cl. tetanomorphum* and *Ps. fluorescens* probably lies in the reactions of formiminoglutamate. Whereas *Cl. tetanomorphum* removes the terminal nitrogen and carbon atoms of the formamidine side chain simultaneously, *Ps. fluorescens* removes them by successive hydrolytic reactions with formyl glutamate as an intermediate.

The final products of the fermentation of glutamate by *Cl. tetanomorphum* are butyrate, acetate, carbon dioxide, ammonia, and hydrogen.³⁹ These products suggest that the terminal stages of the glutamate fermentation are similar to those of the butyric acid fermentation of carbohydrates and other non-nitrogenous substrates. However, one unusual and significant feature appears when the quantitative relations are considered, namely, only 1 mole of carbon dioxide is formed per mole of glutamate decomposed. This is significant because in most organisms that have been studied glutamate is converted to acetate via α -ketoglutarate and the tricarboxylic acid cycle, and this reaction sequence produces not 1 but 3 molecules of carbon dioxide per molecule of glutamate decomposed; consequently the observed yield of carbon dioxide excludes the participation of the tricarboxylic acid cycle functioning in the forward or oxidative direction in the glutamate fermentation.

In order to define the path of glutamate fermentation more clearly, Wachsman investigated the fate of the individual carbon atoms of glutamate by means of tracer experiments.⁴⁰ Samples of glutamate labeled with C^{14} in specific positions were fermented by washed cells, and the products were isolated and degraded to locate the isotope. Figure 2 summarizes the results. Carbon atoms 1 and 2 of glutamate are converted mainly to acetate. Carbon atoms 3 and 4 are converted mainly to butyrate, carbon atom 3 appearing in the α and γ positions, and carbon atom 4 in the carboxyl and β positions; carbon atoms 3 and 4 also go to the methyl and carboxyl positions of acetate to a small extent. Carbon atom 5 of glutamate appears entirely in carbon dioxide. These results show that two types of C_2 units are formed from glutamate, one of which, derived from carbon atoms 1 and 2, is converted directly to acetate, whereas the other,

derived from glutamate carbon atoms 3 and 4, gives rise to an active acetyl group which is preferentially converted to butyrate. The acetyl group and carbon dioxide might well be formed from pyruvate derived from carbon atoms 5, 4, and 3. Therefore, a fission of the glutamate carbon

Fig. 2. Fermentation of C^{14} -Glutamate by *Clostridium tetanomorphum*.

chain between carbon atoms 2 and 3 to give acetate and pyruvate is consistent with the tracer experiments. The results exclude the occurrence of a tricarboxylic acid cycle in a reverse as well as a forward direction. The operation of the cycle in the reverse direction, i.e., from α -ketoglutarate to oxalacetate and acetate via citrate, would give an acetyl group in which the methyl carbon atom was derived from glutamate carbon atom 4, instead of carbon atom 3 as observed.

The further exploration of the pathway of glutamate fermentation has been done with enzyme preparations. Wachsman⁴¹ found that cell-free extracts of *Cl. tetano-*

morphum can readily be obtained which convert glutamate in the absence of oxygen to ammonia, carbon dioxide, hydrogen, acetate, and several minor products which have been identified as pyruvate, α -ketoglutarate, mesaconate, and citramalate.⁴² Mesaconate, which is a branched-chain unsaturated dicarboxylic acid, was first detected by paper chromatography as an acidic spot which also appeared as a dark ("quenching") spot when viewed by ultraviolet light. The characteristic ultraviolet quenching of mesaconate is attributable to a conjugated system of double bonds in the molecule. Subsequently both mesaconate and citramalate were isolated in quantity and characterized more fully.

A variety of enzymatic and tracer experiments have demonstrated that mesaconate and citramalate are intermediates in the fermentation of glutamate. As shown in Fig. 3, glutamate is converted to mesaconate and ammonia, the double bond of mesaconate is hydrated to give citramalate, and citramalate is converted to pyruvate and acetate. The formation of acetate, butyrate, carbon dioxide, and hydrogen from pyruvate presumably involves reactions of the types discussed in Chapter 2.

The formation of mesaconate from glutamate and its further decomposition have been shown to occur at rates as great as the over-all rate of glutamate fermentation by cell-free extracts. Under conditions which block its hydration, mesaconate can be made to accumulate in almost theoretical yield, i.e., 1 mole per mole of glutamate. The formation of mesaconate from glutamate is of considerable interest, because it involves the interconversion of straight- and branched-chain compounds without loss of carbon atoms. This process undoubtedly is the result of a fairly complex reaction sequence, the details of which have not yet been worked out. However, some information concerning the origin of the methyl group of mesaconate has been

obtained. Munch-Petersen⁴³ has studied the conversion of glutamate-4-C¹⁴ to mesaconate and has shown that the C¹⁴ is all present in the carbon atom adjacent to the methyl group (see Fig. 3). This means that the methyl carbon must be derived from carbon 3 of glutamate and excludes any mechanism of methyl group formation involving a

Fig. 3. Role of Mesaconate and Citramalate in Glutamate Decomposition.

transfer of the carboxyl carbon 5 of glutamate from carbon 4 to carbon 3, which would be similar to the interconversion of methyl malonate and succinate reported by Flavin et al.⁴⁴ The conversion of glutamate to mesaconate apparently involves a transfer of carbon atoms 1 and 2 of glutamate from carbon atom 3 to carbon atom 4. This would leave carbon atom 3 in the position occupied by the methyl group of mesaconate.

The conversion of mesaconate to citramalate involves a

hydration of the double bond. The product of the reaction has been identified by chemical and enzymatic methods as (+)-citramalate. Cysteine and Fe^{++} are cofactors for the reaction, and orthophenanthroline and other reagents that combine strongly with Fe^{++} are powerful inhibitors. As in the aconitase and fumarase reactions, the equilibrium favors the formation of the hydroxy acid.

Citramalate is cleaved by a reversible aldolase-type reaction to acetate and pyruvate. The enzyme citramalase which catalyzes this reaction shows a high degree of specificity since it attacks (+)-citramalate but neither the levorotatory isomer nor several structurally related compounds including citrate, isocitrate, and malate. The equilibrium of the reaction favors the cleavage of citramalate.

The analysis of the *Cl. tetanomorphum* system has established the existence of a new pathway of glutamate metabolism which may be regarded as a substitute for a part of the tricarboxylic acid cycle in the sense that it permits a conversion of glutamate to pyruvate, acetate, and carbon dioxide. All of the reactions between glutamate and pyruvate are readily reversible and involve only small free-energy changes. Therefore this sequence differs from the oxidative part of the tricarboxylic acid cycle in not providing energy for the synthesis of adenosine triphosphate.

The occurrence of the mesaconate pathway in other organisms has not yet been investigated. However, indirect evidence for this pathway in several anaerobic bacteria, including *Cl. botulinum*, *Cl. tetani*, *Cl. saccharobutyricum*, and *Fusobacterium nucleatum*, is provided by the observation that these species, like *Cl. tetanomorphum*, form 1 mole of carbon dioxide per mole of glutamate fermented. The occurrence of mesaconate in sugar cane and cabbage, and of (-)-citramalate in apples, indicates that these compounds also have a role in the metabolism of higher plants.

Glycine Fermentation by *Diplococcus glycinophilus*.

The fermentation of glycine⁴⁵ proceeds according to equation 5. In addition, a small amount of hydrogen is pro-

duced, but since the evolution of hydrogen is readily reversed by a partial pressure of the gas exceeding one-third of an atmosphere, the formation of hydrogen soon ceases in stationary cultures in which the medium quickly becomes saturated with hydrogen.

The glycine fermentation presents some interesting problems of intermediary metabolism which so far have been studied only by tracer methods. The results show plainly that the chemistry of glycine decomposition by *D. glycinophilus* is entirely different from that catalyzed by *Clostridium sporogenes*. Since the latter organism reduces glycine to acetate and ammonia, one might expect that the glycine fermentation by *D. glycinophilus* would consist of a complete oxidation of 1 mole of glycine to carbon dioxide coupled with reduction of 3 moles of glycine to acetate. This possibility was tested by fermenting glycine-1-C¹⁴, glycine-2-C¹⁴, and unlabeled glycine plus C¹⁴-carbon dioxide and determining the distribution of isotope in the products. The results show that the carbon dioxide is derived entirely from carboxyl carbon of glycine, whereas both carbons of acetate are derived partly from the methylene carbon of glycine and partly from carbon dioxide. These results exclude both a direct reduction of glycine to acetate and a complete oxidation of glycine to carbon dioxide. They suggest an oxidative conversion of glycine to acetate and carbon dioxide, possibly via serine, coupled with a reduction of carbon dioxide to acetate.

Alanine and Threonine Fermentations by *Clostridium propionicum*. *Clostridium propionicum* is able to ferment alanine, serine, threonine, lactate, pyruvate, and acrylate.⁴⁵ With all these substrates except threonine, the main products are acetate, propionate, and carbon dioxide; ammonia is also formed from the amino acids. Equation 6 describes

the fermentation of alanine. Except for ammonia, these products are the same as those formed by the propionic acid bacteria (genus *Propionibacterium*). Despite the similarities in the products, a considerable amount of evidence indicates that the chemical reactions in the *Cl. propionicum* and *Propionibacterium* fermentations are substantially different. Succinate is a characteristic product of the *Propionibacterium* fermentation, and Whiteley⁴⁶ has shown that succinate is converted to succinyl-CoA and then decarboxylated to propionyl-CoA, the immediate precursor of propionate. *Clostridium propionicum* on the contrary neither forms nor decarboxylates succinate.⁴⁷ Further evidence against the participation of succinate is the absence of randomization of the α and β carbons of lactate during its conversion to propionate.⁴⁸ Also Johns⁴⁷ has shown that *Cl. propionicum*, unlike the propionic acid bacteria, is unable to incorporate carbon dioxide into propionate.

Perhaps the first indication of a distinctive pathway of propionate formation in *Cl. propionicum* was provided by the discovery that acrylate is fermented at least as rapidly as alanine or lactate.⁴⁵ This led to the suggestion that the latter substrates might be converted by loss of ammonia or water to acrylate, which might then be reduced to propionate as follows:

The occurrence of such a sequence in the reverse direction was investigated by Stadtman⁴⁹ by studying the oxidation of propionate by cell-free extracts of *Cl. propionicum*. He found that propionate was oxidized only after being converted to propionyl CoA. The supposed product of the oxidation, acrylyl CoA, could not be directly identified. However, indirect evidence for this compound was provided by the finding that acrylyl thioesters react enzymatically with ammonium ion to form β -alanyl thioesters and ultimately β -alanine itself in this system (equation 8), and

that β -alanine is also formed in the oxidation of propionyl thioesters. Presumably the acrylyl thioester is so reactive that it cannot accumulate to an appreciable extent under the experimental conditions used. These experiments suggest that acrylyl CoA may be a precursor of propionate in *Cl. propionicum*. However, as yet a conversion of alanine or lactate to acrylyl CoA has not been demonstrated nor is there any indication of the role of β -alanine in these fermentations.

Threonine is exceptional among the substrates fermented by *Cl. propionicum* in that it gives rise to butyrate and propionate (equation 9) instead of propionate and acetate.⁵⁰ This difference is obviously dependent on the fact that threonine is the only four-carbon compound attacked. Evidently the carbon chain of 1 mole of threonine is reduced

to butyrate whereas 2 moles of threonine are oxidized to propionate and carbon dioxide. The specific reactions in-

volved in these processes have not been investigated but it is probable that α -ketobutyrate is an intermediate in the fermentation.

Allantoin Fermentation by *Streptococcus allantoicus*.

The decomposition of allantoin by *S. allantoicus* cannot be represented by a simple equation. The major products are ammonia, urea, oxamic acid, and carbon dioxide, and the minor products are formic, acetic, lactic, and glycolic acids.¹² Oxamic acid, the monoamide of oxalic acid, has not been found in any other fermentation.

The chemistry of the allantoin fermentation has not been extensively studied. The first step is known to be the opening of the ring with the formation of allantoic acid. Beyond this little is known with certainty except that the nitrogen

of oxamic acid is not derived from ammonia. This has been shown by experiments with N^{15} ammonia. Since ammonia is formed in the fermentation and the organism

cannot convert urea to ammonia, it may also be concluded that at least one of the ureido groups of allantoic acid is decomposed before being separated from the two-carbon chain. The latter is ultimately oxidized in part to oxamic acid, carbon dioxide, and formate, and partly reduced to glycolate and acetate by paths that are yet to be determined.

Orotic Acid Fermentation by *Zymobacterium oroticum*. Not all of the fermentation products of orotic acid have been identified, but ammonia, carbon dioxide, acetate, and one or more four-carbon dicarboxylic acids appear to be formed. The early reactions in the fermentation of orotic acid by this bacterium have been extensively studied by Lieberman and Kornberg^{14,15} by the use of cell-free extracts. The first reaction is the DPN-linked reduction of orotic acid to L-dihydroorotic acid. This is followed by a hydrolytic cleavage of the pyrimidine ring to give L-ureido-succinic acid.

The ureido group of the latter compound is degraded with the formation of ammonia, carbon dioxide, and L-aspartic acid. The further transformations of aspartic acid have not been analyzed in detail, but it is probable in view of the

nature of the observed fermentation products that aspartic acid is converted either to fumarate or oxalacetate which is then partly oxidized to acetate and carbon dioxide, and partly reduced to succinate. The energy-yielding steps could be the decomposition of the ureido group and the oxidation of pyruvate; however, no phosphorylation reactions have yet been demonstrated in this organism.

Both the reduction of orotic acid and the conversion of the latter to ureidosuccinic acid are readily reversible reactions and therefore permit the synthesis of the pyrimidine orotic acid from non-pyrimidine precursors. Considerable evidence now indicates that this reaction sequence represents a major path of pyrimidine synthesis in living organisms.⁵¹

Purine Fermentations by *Clostridia*. *Clostridium aciduri* and *Cl. cylindrosporum* readily ferment xanthine, uric acid, guanine, and 6,8-dihydroxypurine, and decompose hypoxanthine, guanosine, and inosine more slowly or after a period of adaptation.⁵² Chemical studies of purine fermentations by growing cultures have established that the main products formed by *Cl. aciduri* are ammonia, carbon dioxide, and acetate; in addition a small

amount of formate accumulates. If, as a first approximation, we disregard the formate, the decomposition of xanthine is represented by equation 11. With uric acid, the yield of carbon dioxide is increased and the yield of acetate

is decreased; with hypoxanthine the reverse is true. With all three substrates, the purine nitrogen is converted essentially quantitatively to ammonia, without any detectable accumulation of urea. The same products are formed by *Cl. cylindrosporum*, except that the yield of formate is higher, approximately 1 mole per mole of purine, and a considerable amount of glycine accumulates.⁵³

An early attempt to get some evidence concerning the path of uric acid fermentation was made by investigating the utilization of various compounds previously implicated in the biological oxidation of uric acid.⁵² In particular, allantoin and urea, products of uric acid oxidation by both animals and aerobic bacteria, were found not to be attacked by cell suspensions of *Cl. acidi-urici* which rapidly fermented uric acid. This appeared to exclude the only known pathway of uric acid decomposition via allantoin and indicated that the aerobic and anaerobic pathways must be fundamentally different.

An indication of the actual mechanism of the purine fermentation was provided by the discovery that glycine is not only formed by *Cl. cylindrosporum* but is also decomposed by both species under appropriate conditions. The activation of glycine was first shown by the methylene blue reduction technique. Of a considerable number of purines and amino acids tested as reducing agents, using cell suspensions, glycine was by far the most active. When cell suspensions were allowed to act upon glycine alone under anaerobic conditions, essentially no decomposition of the amino acid could be observed. However, when glycine and uric acid were supplied simultaneously, both compounds were decomposed and the amount of glycine consumed was dependent upon the amount of uric acid fermented. This relation, combined with the demonstration that glycine could act as a reducing agent for methylene

blue, suggested that the oxidation of glycine was coupled with the reduction of urate. Evidence was also obtained that the decomposition of uric acid under some conditions was dependent on the presence of glycine. Certain thoroughly washed and aged cell suspensions of *Cl. acidii-urici* could ferment uric acid only after a long lag period. The lag could be abolished completely by the addition of glycine.⁵⁴

Further investigation of the role of glycine in the purine fermentation was delayed for several years. During this interval, Sonne, Buchanan, and Delluva⁵⁵ studied the synthesis of uric acid in the pigeon and established by means of tracer experiments with C¹⁴ that various carbon atoms in the purine are derived from carbon dioxide, formate, and glycine. Uric acid carbon atoms 2 and 8 are derived from formate, carbon atoms 4 and 5 from the carboxyl and methylene groups of glycine respectively,⁵⁶ and carbon atom 6 from carbon dioxide (Fig. 4). The nitrogen in position

Fig. 4. Uric Acid Synthesis in Pigeons.

7 was shown also to be derived from glycine.⁵⁷ Therefore glycine appeared to be incorporated intact into the adjacent positions 4, 5, and 7 of the purine.

In view of the obvious similarities between the bacterial purine fermentations and purine synthesis in the pigeon, it seemed probable that the two processes would follow the same general pathway but in opposite directions, particu-

larly since fatty acid oxidation and synthesis in various organisms had been shown to be related in this way. Consequently we investigated the origin of the fermentation products by use of purines specifically labeled with C^{14} .

Fig. 5. Uric Acid Breakdown by Clostridia.

The results, summarized qualitatively in Fig. 5, show that the degradation of purines by clostridia bears a close resemblance to the general pattern of purine biosynthesis. Thus, carbon atoms 1 and 2 and the nitrogen atom of glycine are converted to purine carbon atoms 4, 5, and 7 respectively in the pigeon, and are formed from these same purine atoms in the fermentation. Carbon atoms 6 and 8 of purine arise from carbon dioxide and formate, respectively, in the biosynthesis and yield the same compounds in the fermentation. The only significant difference between the two processes is in the origin and fate of carbon atom 2 of purine; in biosynthesis this carbon atom is derived from formate, whereas in the fermentation it is converted to carbon dioxide. This difference is attributable to the fact that the primary synthetic product in the pigeon is hypoxanthine ribotide, which contains a reduced carbon atom in the 2 position, whereas the purine that actually undergoes cleavage in the fermentation is probably xanthine, which contains an oxidized carbon atom in this position.

Experiments on the fermentation of unlabeled purines in the presence of C^{14} -labeled glycine, formate, or carbon

dioxide have confirmed the conclusion, already reached by other methods, that these compounds are not stable end products but are extensively further metabolized.^{53,58} The carboxyl carbon of glycine is converted mainly to carbon dioxide, whereas the methylene carbon is extensively incorporated into both carbons of acetate. Formate is partly oxidized to carbon dioxide and partly incorporated into the methyl group of acetate. Finally, carbon dioxide is rapidly reduced to formate and is extensively incorporated into the carboxyl group of glycine and into both carbon atoms of acetate. Acetate itself is not metabolized to a significant extent. In addition to establishing the roles of carbon dioxide, formate, and glycine as intermediates, these observations also emphasize the complexity of the fermentation.

I have already mentioned that xanthine is the purine which probably undergoes ring cleavage in the purine fermentation. The evidence for this, which is now fairly conclusive, was obtained in several ways. The first definite indication for the key role of xanthine was obtained by Beck⁵⁹ in experiments on the influence of the growth substrate on the relative rates of decomposition of xanthine, guanine, uric acid, and hypoxanthine. He found that uric acid, hypoxanthine, and to a lesser extent guanine are decomposed by adaptive enzyme systems, whereas xanthine is rapidly utilized by cells grown on any of the four purines. This suggested that uric acid, guanine, and hypoxanthine are broken down via xanthine as follows:

In support of this scheme Beck showed that cells grown on guanine contain an active guanase capable of converting guanine to xanthine.⁶⁰ He also found that the decomposition of uric acid by cells grown on this purine is inhibited by 2,4-dinitrophenol without affecting the fermentation of xanthine. Radin and Barker,⁶¹ using both dried-cell preparations and cell-free extracts of *Cl. aciduri*, further showed that, whereas the decomposition of uric acid was increased by the presence of reducing agents and inhibited by oxidizing agents, the decomposition of xanthine was almost independent of such reagents. These and other types of evidence support the above scheme.

The study of the enzymatic steps in the decomposition of xanthine was initiated by Radin and Barker⁶¹ and independently by Bradshaw and Beck.⁶² Both groups found that crude cell-free extracts readily convert xanthine to ammonia, carbon dioxide, formate, and glycine, or products which yield these substances in the analytical procedures used. Radin also made the important observation that one or more compounds which react in the Pauly and Bratton-Marshall tests were formed from xanthine in small amounts. The nature of the tests indicated that they were probably aminoimidazoles. These observations suggested that the pyrimidine ring of xanthine was first attacked in such a way as to form an imidazole derivative which was then further degraded.

The individual enzymatic steps in the conversion of xanthine to glycine have been extensively studied by Rabinowitz and his collaborators, who have developed methods for the isolation, analysis, and characterization of three imidazole derivatives and have clarified the reactions involved in the tetrahydrofolic acid dependent formation of glycine and formate.

The decomposition of xanthine occurs by a hydrolytic

cleavage of the pyrimidine ring between positions 1 and 6 and results in the formation of 4-ureido-5-imidazolecarboxylic acid⁶³ (see Fig. 6). The accumulation of this com-

Fig. 6. Conversion of Xanthine to Formiminoglycine.

pound in crude cell-free extracts is made possible by the addition of sequestering agents such as ethylenediamine-tetraacetic acid which remove the Fe^{++} or Mn^{++} ions required for its further decomposition. The next enzymatic step is the cleavage of the ureido group to yield carbon dioxide, ammonia, and 4-amino-5-imidazolecarboxyl acid.⁶⁴ The latter compound accumulates in an alkaline reaction mixture. The mechanism of this reaction has not been examined in detail, but since orthophosphate is not required, the reaction appears to be hydrolytic. The aminocarboxy-

imidazole is enzymatically decarboxylated to 4-aminoimidazole which accumulates in the presence of ethylenediamine-tetraacetic acid. The aminoimidazole is readily decomposed to ammonia and formiminoglycine by crude extracts of *Cl. cylindrosporum*.⁶⁵ Formiminoglycine (FIG) is also the product accumulating in the decomposition of xanthine by crude extracts. In earlier experiments on xanthine decomposition by extracts, FIG was mistakenly identified as a mixture of glycine, formate, and ammonia, because these compounds are readily formed non-enzymatically from FIG under conditions commonly used for their estimation. The conversion of 4-aminoimidazole to FIG is apparently a complex reaction involving more than one step. The enzyme responsible for 4-aminoimidazole decomposition has been partially purified and shown not to form formiminoglycine directly, but an unidentified compound (X in Fig. 6) which can be hydrolyzed non-enzymatically to glycine in the presence of either acid or alkali. This compound is not an intermediate in the fermentation, since it is not converted to formiminoglycine by crude extracts. Probably it is formed from the true intermediate by a non-enzymatic side reaction.

One of the most interesting parts of the purine fermentation is the conversion of FIG to glycine, ammonia, and formate. This conversion, which evidently requires several enzymes, can provide useful energy to the organism in the form of ATP. Rabinowitz and Pricer⁶⁶ have shown that

under certain conditions the over-all reaction may be represented by equation 12. This reaction sequence is dependent upon the presence of tetrahydrofolic acid (THFA) or

other coenzyme form of folic acid. The initial step is a reversible transfer of the formimino group to THFA. The

first evidence for this reaction consisted in the demonstration by Sagers et al.⁶⁷ that certain cell-free extracts catalyze an exchange between C¹⁴-glycine and formiminoglycine which is more rapid than the formation of ammonia and formate. Recently Rabinowitz and Pricer⁶⁸ have purified the enzyme catalyzing this reaction and have partially characterized the product as 5-formimino-THFA. They have also shown that a second enzyme removes ammonia from 5-formimino-THFA to give 5,10-anhydroformyl-THFA (anhydroleuovorin) which in turn is converted by a third enzyme to 10-formyl-THFA.

The decomposition of 10-formyl-THFA can be coupled with the formation of ATP by the following reversible reaction:

Perhaps this reaction also consists of more than one step since it involves an activation of orthophosphate (P_i) and a transfer of the phosphate group to ADP.

The above reactions account satisfactorily for the decomposition of xanthine to glycine, formate, carbon dioxide, and ammonia. However, in the fermentation of purine by living cells of *Cl. acidu-urici* very little glycine and formate accumulate; the main organic product is acetate. Therefore it is necessary to account for the conversion of glycine and formate or their known precursors to acetate.

The most likely pathway for the formation of acetate is via serine and pyruvate as follows:

This scheme postulates an hydroxymethylation of glycine by an active formaldehyde group which could be derived from the formimino group of formiminoglycine via formyl-THFA. The formation of serine has not yet been experimentally demonstrated in this system. The conversion of L-serine to pyruvate and the oxidation of pyruvate to acetate and carbon dioxide are catalyzed by both intact cells and extracts.⁶⁹ Presumably the oxidation of pyruvate can be coupled with the formation of ATP although this has not yet been shown.

The above scheme is further supported by a variety of tracer experiments.^{69,70} The carboxyl groups of both glycine and formiminoglycine are converted to carbon dioxide; glycine-2-C¹⁴ yields pyruvate-2-C¹⁴; both glycine-2-C¹⁴ and formiminoglycine-2-C¹⁴ yield acetate-1-C¹⁴; and formiminoglycine-C¹⁴ labeled in the formimino group is converted to acetate-2-C¹⁴. All of these results and others which will not be mentioned are consistent with the above scheme.

We have seen that the formimino carbon of formiminoglycine can undergo two types of reactions. (1) It can be converted to formate via formyl-THFA with the simultaneous formation of ATP, or (2) it can be reduced and transferred, presumably via the THFA derivatives, to glycine to give serine or an analogous compound. The relative importance of these two processes obviously de-

pendes upon the relative activities of the enzyme systems. With *Cl. cylindrosporum*, glycine and formate accumulate in substantial amounts and therefore the enzyme system leading to ATP is probably more active. With *Cl. acidurici*, on the contrary, nearly all of the glycine moiety of formiminoglycine is converted to acetate and carbon dioxide, indicating a very active formyl transferring system.

The studies summarized above have established the general pathway of the clostridial purine fermentation, although obviously several aspects of the process, particularly the incorporation of carbon dioxide into acetate and glycine⁷¹ and the assumed formation of serine, need to be investigated more fully.

The ability to ferment purines is not restricted to clostridia. Two anaerobic micrococci, *M. aerogenes*¹⁹ and *M. lactilyticus*,⁷² are able to grow in a complex medium at the expense of certain purines and carry out a modified propionic acid-type fermentation. These purine fermentations have not been studied extensively but the experiments of Whiteley show that *M. aerogenes* forms considerable amounts of uracil and thymine during the decomposition of xanthine. This indicates that the initial enzymatic attack may be on the imidazole ring in contrast to what has been found with the purine fermenting clostridia.

These studies of the fermentations of nitrogen compounds have led to the discovery of several metabolic pathways not previously encountered in other organisms. There is no reason to believe that these pathways are restricted to anaerobic bacteria. Indeed the whole history of biochemistry indicates that any reaction discovered in one organism is ultimately found to occur in many others. Anaerobic bacteria obviously provide excellent and often neglected biological material for the study of certain types of meta-

bolic pathways. Consequently it can be anticipated that the further investigation of such bacteria will contribute significantly to a general understanding of the elaborate chemical machinery of living organisms.

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