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VOLUME 14

THE BIOSYNTHESIS
OF PROTEINS

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THE BIOSYNTHESIS OF PROTEINS

by

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au Professeur Jean BRACHET,
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Preface

KNOWLEDGE on the biosynthesis of proteins is increasing so rapidly, and relevant data are obtained from such a variety of approaches that it is more and more difficult to keep abreast with the progress of research. Chemistry, crystallography, genetics, cytology, cellular physiology, embryology, immunology, microbiology, enzymology all contribute to the analysis of the process. It is obviously impossible to master all these sciences, and each worker must go his own way, trying his best with what he can do. But he must from time to time stop and look around to appreciate the advances made along the other lines of research, lest he might lose contact altogether with the other approaches. The author felt this necessity for himself and for the students in his laboratory, and he tried to outline a picture of the whole field of protein biosynthesis as he sees it presently. The outcome of his attempt was this little book, in which well established facts were merely summarized, and greater emphasis was laid upon recent developments and new perspectives. It is realized that the discussions and interpretations of recent data that this book contains will soon become obsolete: this is unavoidable in a field of research which is developing so rapidly. The picture presented here should be regarded as a snapshot taken at the end of 1960; blurred spots on the picture are due in part to the lens, and in part to the fog that still covers large regions of the field.

The present book has profited very much by frequent conversations with Professors R. Jeener, J. Brachet and M. Errera, and with Dr R. Thomas and Dr M. De Deken. The author wishes to express his sincere thanks to Dr G. Palade who kindly gave beautiful electronmicrographs, to Mrs Bonnami and Mrs Hamers for their help in the publication of the manuscript, and to Mrs Chantrenne who prepared the index.

H. CHANTRENNE

Introduction

REMARKABLE advances in the knowledge of protein structure have been made during the last decade, and although many aspects of protein structure still raise some difficult problems, essential features are now clarified.

Protein molecules are made of one single polypeptide, or of a few polypeptidic chains associated together in a specific manner. The backbone of polypeptides is a quite regular structure in which one nitrogen and two carbon atoms alternate; one of these carbons carries an oxygen, the other one, which is asymmetrical, carries a hydrogen and a radical R.

FIG. 1

There are twenty common varieties of R radicals, and the sequence of these confers their individuality to the polypeptides, which would otherwise be all alike. Hydrolysis splits the peptide bonds —CO—NH— and the chains thus break down into a mixture of some twenty α -amino acids. All belong to the L series; this means that the relative positions of —NH_2 , —COOH , —R and —H around the asymmetric carbon is the same whatever the nature of the R radical. A very important consequence of the identical steric configuration of all the amino acids is that certain regular types of folding of the chains tend to form, whatever the nature of the R radicals. Among the possible types of folding a few are privileged; especially a certain type of helical folding, the α helix (Pauling *et al.*, 1951), which is found in many proteins. In this structure, each —CO— of one spire is hydrogen bonded with an —NH— in the next one; this confers a great stability and some rigidity to the framework.

Protein molecules, however, should not be visualized as straight and perfectly regular helices. Some R side chains may interact and thus cause deformations of the helices. Proline, one of the natural protein constituents, is actually an α -imino acid, and its presence causes the helix to bend sharply. Besides, extensive regions of polypeptides often are not in the form of perfect helices. As a result, the folded polypeptide is not contained within a straight cylinder as would be a perfect α helix; it is actually much distorted, and it must look like an oddly contorted piece of tubing, as depicted

in the model of myoglobin (Kendrew, 1959) (Fig. 4). This picture also shows the position of the haematin prosthetic group within the molecule.

The haemoglobin molecule is made of four chains, of two different compositions, each of which very much resemble myoglobin by the general shape (Perutz *et al.*, 1960). They are not very strongly bound with one another and they can be made to separate or reassociate rather easily.

Sometimes, protein molecules associate into structures of a higher order either with identical molecules or with others. For instance, many mole-

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Acetyl-Ser-Tyr-Ser-Ileu-Thr-Pro-Thr-Ser-GluNH ₂ -Phe-Val-Phe-Leu-Ser-													
15	16	17	18	19	20	21	22	23	24	25	26	27	28
Ser-Ala-Try-Ala-Asp-Pro-Ileu-Glu-Leu-Ileu-Asp*(CySO ₃ H,Thr,Asp*)-													
29	30	31	32	33	34	35	36	37	38	39	40		
Leu-Ala-Leu-Gly-AspNH ₂ -GluNH ₂ -Phe-Glu*-Thr-Glu-GluNH ₂ -Ala-													
41	42	43	44	45	46	47	48	49	50	51	52	53	
Arg-Thr-Val-Glu*-Val-Arg-GluNH ₂ -Phe-Ser-GluNH ₂ -Val-Try-Lys-													
54	55	56	57	58	59	60	61	62	63	64	65	66	67
Pro-Pro-Ser-GluNH ₂ -Val-Thr-Val-Arg-Phe-Pro-Asp*-Ser-Asp-Phe-													
68	69	70	71	72	73	74	75	76	77	78	79	80	81
Lys-Val-Tyr-Arg-Tyr-Asp*-Ala-Val-Asp-Pro-Leu-Val-Thr-Ala-Leu-													
83	84	85	86	87	88	89	90	91	92	93	94	95	96
Leu-Leu-Gly-Ala-Phe-Asp*-Thr-Arg-AspNH ₂ -Arg-Ileu-Glu*-Val-Glu*-Asp*-													
98	99	100	101	102	103	104	105	106	107	108	109	110	111
(Ala,Glu*,Thr,Asp*,Pro)-Thr-Ala-Glu*-Thr-Leu-Asp*-Ala-Thr-Arg-													
112	113	114	115	116	117	118	119	120	121	122	123	124	125
Arg-Val-Asp*-Asp*-Ala-Thr-Val-Ala-Ileu-Arg-Ser-Ala-Asp*-Ileu-													
126	127	128	129	130	131	132	133	134	135	136	137	138	139
AspNH ₂ -Leu-Ileu-Val-Glu-Leu-Ileu-Arg-Gly-Thr-Gly-Ser-Tyr-AspNH ₂ -													
140	141	142	143	144	145	146	147	148	149	150	151	152	153
Arg-Ser-Ser-Phe-Glu*-Ser-Ser-Ser-Gly-Leu-Val-Try-Thr-Ser-													
154	155	156	157										
Gly-Pro-Ala-Thr													

Asp* = Asp or AspNH₂; Glu* = Glu or GluNH₂

FIG. 2. Amino acid sequence of the protein of tobacco mosaic virus (Anderer *et al.*, 1960).

cules of the tobacco mosaic virus protein unite in a tightly packed hollow rod.

Several levels of complexity can thus be considered in proteins. The polypeptidic backbone is made of amino acids which are firmly bound together and arranged in a strictly determined sequence (Fig. 2); this is the primary structure. Folding of the chains into a construction — helices or otherwise — held together by hydrogen bonds, creates the secondary structure. The tertiary structure results from the folding of the helices in space as in Fig. 4. Higher orders of organization make the transition between molecular and microscopic structures (Fig. 5).

FIG. 4. A three-dimensional model of myoglobin illustrating the tertiary structure. The grey disc is the haem group (Kendrew, 1959).

Each step of the synthesis of any cell constituent is controlled by a protein, the specific enzyme. This confers on proteins a unique position in the activity and in the formation of cell material. It is to be expected that

FIG. 3. A portion of an α -helix. The dashed lines figure hydrogen bonds (Pauling and Corey, 1951).

the biosynthesis of proteins will raise questions which are not encountered for the biosynthesis of other constituents, since it is the formation of the tools themselves which is now being considered.

At the time of writing, no single problem of protein biosynthesis is completely solved, but the problems at least are clearly stated. The technology of protein biosynthesis is partly understood; the systems which supervise and control the process are being eagerly investigated and some of the key principles upon which they operate are known already. Nucleic acids are all important in these systems. Here chemistry and genetics meet and a new fascinating field opens: molecular biology.

FIG. 5. Schematic representation of a tobacco mosaic virus. The protein units form a hollow rod with the nucleic acid fibre embedded in the protein material (Franklin *et al.*, 1957).

CHAPTER I

The Genetic Control of Protein Synthesis

A. THE ONE GENE-ONE ENZYME HYPOTHESIS

1. *Basic Observations*

At the beginning of this century, Garrod called attention to several hereditary abnormalities of man. These abnormal conditions are characterized by obvious biochemical defects, for instance the excretion of unusual substances in the urine, or changes in pigmentation. These traits are transmitted from parent to progeny as if they were controlled by single recessive alleles. A classical case of 'inborn error of metabolism' is alcaptonuria. Patients excrete in the urine homogentisic acid which gives rise to a black pigment. When the acid is fed to an alcaptonuric, it can be recovered in the urine, whereas it disappears in a normal individual. It was established as early as 1914 by Gross that normal human serum destroys homogentisic acid whereas this substance is not changed in alcaptonuric serum. Clearly, alcaptonurics suffer from a metabolic lesion, from the failure of some enzymic processes. Since the disease is controlled by a single gene, it looks as if genes could control enzymic processes in some specific way.

Indications in the same direction were provided by studies on the inheritance of flower pigments, which were initiated by Onslow and Bassett (1913, 1914) and brilliantly developed by Scott-Moncrieff (1930, 1939) and Lawrence (1935, 1950). These researches are well illustrated by the work on the Cape primrose *Streptocarpus*.

The chemical structure and the genetic control of the petal pigments were analysed in a series of hybrids. Each strain was found to contain essentially one single pigment. All the pigments are closely related substances belonging to the class of anthocyanidins; they differ from each other by the presence of a few glycosyl or methoxy groups on the hydroxyls of the basic anthocyanin structure. The pigments are inherited as if they were controlled by three pairs of alleles, with one dominant and one recessive in each pair, *Oo*, *Rr*, *Dd*. In the triple recessive genotype *ord*, the pigment is mainly pelargonin-3-pentose-hexoside. Replacement of the recessive allele *r* by the dominant *R* results in the substitution of a methoxy group at position 3'. When the dominant allele *O* replaces the recessive *o*, two

methoxy groups appear, at positions 3' and 5', giving malvidin. When the dominant allele *D* is present, a hexose is found at position 5, giving a 3,5-dihexoside in place of the 3-pentosehexoside.

The individual genes thus correspond to certain *chemical operations* in the synthesis of the pigments: methoxylation, glycosidation, at specified positions of the anthocyanin nucleus. Obviously, the genes control well defined steps in the enzymic processes involved in the synthesis of the petal pigments.

FIG. 6. Chemical structure of pigments from four *Streptocarpus* varieties, and corresponding genotypes (after Lawrence, 1950).

Brilliant studies by Beadle and Ephrussi on the development of the eye pigments of *Drosophila* provided further information on the genetic control of anabolic processes. The dark red colour of wild type eyes is due to the presence of two types of coloured granules, red and brown, in the pigment cells which surround the ommatidia. A series of mutants (vermillion, cinnabar, scarlet) which have various tinges of bright red eyes are deficient in the brown pigment. Ephrussi and Beadle (1935, 1937) transplanted eye disks of vermillion or cinnabar larvae into the abdomen of wild type larvae, and they observed that the mutant eye disks developed into adult structures with wild type pigmentation. The lymph of wild type flies therefore provided the mutant eye disks with substances which they did not contain

and which allow them to make the normal pigment. Implantation of cinnabar into vermilion and vice versa showed that the transformations are due to two substances: v^+ which causes the change from vermilion to cinnabar, and cn^+ from cinnabar to wild.

A series of remarkable studies by the groups of Ephrussi, Tatum and Butenandt led to the discovery that v^+ is kynurenine, a product of the oxidation of tryptophan; cn^+ was later identified as hydroxykynurenine, a further step in tryptophan oxidation. Both substances are used up during the transformation of the eye disks, they are intermediates in the biosynthesis of normal eye pigments (Kikkawa, 1941). It was clear that mutation of the two genes each blocks one specified step in the oxidation of tryptophan. Ephrussi suggested (1942) among other possibilities that the genes involved control the formation of specific oxidation enzymes.

Further study of eye pigment synthesis from the enzymological point of view looked very difficult, but it was clear that the type of approach was very promising and that great developments both in genetics and in biochemistry would follow once a material more amenable to enzymological studies is found.

FIG. 7.

Neurospora crassa was such a material. The vegetative form of this mould, the mycelium, which can be grown easily in fairly large batches, produces asexual spores, the conidia. These make it possible to keep collections of various strains and to grow unlimited amounts of a given mycelium, the enzymes of which will easily be studied by classical biochemical methods. On the other hand, *Neurospora* offers many features which are of great interest for genetical studies. The mycelium is haploid; therefore, problems of dominance in the expression of the genetic characters are avoided. Sexual reproduction is easily obtained by fusion of mycelia of opposed mating types; the zygote develops into an ascus containing eight haploid spores from which the mycelium is obtained (Beadle, 1947).

Auxotroph mutants, that is mutants which require for growth a substance that the wild strain can dispense with, proved especially suitable for

biochemical studies. Let us consider one classical case, that of some tryptophan-less mutants of *Neurospora*.

The wild type mould will grow readily on a very simple medium containing salts, sucrose as the carbon source, and biotin. The mycelium forms spores which, when transferred to fresh medium, will give a new mycelium identical to the original one, and thus indefinitely. However, from time to time a spore will not be able to grow on the basic medium, unless tryptophan is added. Genetic analysis shows that this tryptophan requirement is inherited as a single mendelian character; it is the result of a single mutation.

FIG. 8. The life cycle of *Neurospora crassa* (from Wagner and Mitchell, 1955).

Tryptophan is a constituent of the proteins of the mould, and it is normally produced in the wild type. If the mutant cannot grow in the absence of the exogenous tryptophan, this means that the mutant cannot make tryptophan for itself as the wild type used to do, and that it must depend on the medium for securing the amino acid. The mutation has probably blocked biochemical processes involved in tryptophan biosynthesis.

Certain tryptophan-less mutants were found to excrete indol into the

medium. Tryptophan is an indol derivative, and the production of indol by a mutant which is unable to make tryptophan strongly suggests that indol is an intermediate in tryptophan biosynthesis and that in the mutant strain the further utilization of the indol nucleus is impaired.

Biochemical studies on the wild type mycelium indeed showed that tryptophan is formed by the condensation of indol with serine (Tatum and Bonner, 1944). This last step of tryptophan synthesis can be observed *in vitro*; it is catalysed by an enzyme present in extracts of wild type mycelium, which was called tryptophan synthetase (Umbreit, 1946). The hereditary defect is thus located at the level of one well defined chemical reaction, catalysed by a known enzyme.

FIG. 9.

It may be assumed that in tryptophan-less mutants which do not further use indol, the mutation has resulted in the inhibition or destruction of the enzyme, or else that it has prevented the formation of the enzyme. Mitchell and Lein (1948) showed that the extracts obtained from the mycelium of such mutants do not catalyse the condensation of indol with serine; if tryptophan synthetase from a normal strain is added to the extract of the mutant, it works perfectly. This indicates that no inhibitor of the enzyme is contained in the extracts of the mutant. It is clear therefore that no enzyme activity can be detected, simply because the enzyme is not present in the extracts of the mutant mycelium.

It must be concluded that mutation of a mendelian gene has prevented the *synthesis* of an enzyme which is normally formed in the wild strain. The whole field of microbial genetics is an illustration of this strict control of enzyme formation by mendelian determinants. A list of some 50 examples of well studied cases of genetic control of enzyme formation in microorganisms will be found in a recent review by Fincham (1959).

In higher organisms, as well as in bacteria, enzymes depend on mendelian genes. Inborn errors of metabolism in man, pigment heredity in flies and flowers are explained by the genetic control of enzyme synthesis. Thus the metabolic error in alcaptonuria results from the lack of homogentisic acid oxidase (La Du *et al.*, 1958); all the other enzymes of tyrosine oxidation are present, only one is lacking as the result of one gene mutation. Congenital galactosemia is due to absence of galactowaldenase (Kalckar

et al., 1956). Various forms of glycogen deposition diseases are due to lack of glucose-6-phosphatase, amylo-1-6-glucosidase or muscle phosphorylase (Hers and Malbrain, 1959; Hauk *et al.*, 1959; Mommaerts *et al.*, 1959; Yi Yung Hsia and Kot, 1959; Larner and Villar Palasi, 1959; Gilles *et al.*, 1960). Pentosuria may be due to hereditary deficiency in xylulose dehydrogenase (Bozian and Touster, 1959). The eyes of vermilion mutants (v) of *Drosophila* are devoid of tryptophan peroxidase, the enzyme which makes kynurenine (Glass, 1957; Baglioni, 1959).

Mutation of a mendelian gene thus suppresses the formation of an enzyme, i.e. of a protein endowed with well specified catalytic properties. One may wonder whether the enzyme protein does not form at all in the mutant, or whether another protein lacking enzymic activity is made instead of the normal enzyme.

Yanofsky studied 25 independent mutants of *Neurospora crassa* all lacking tryptophan synthetase activity. He found for many of them, that each contains a protein which is closely related to the enzyme for it gives strong cross reactions with an antiserum prepared against normal tryptophan synthetase. Moreover, this cross reacting material behaves exactly like the normal enzyme in biochemical fractionation procedures. A few mutants, however, do not contain any such 'cross reacting material' (Suskind *et al.*, 1955; Yanofsky, 1956; Suskind, 1957).

In many cases, mutation thus results in the replacement of a normal enzyme by some closely related protein devoid of normal catalytic properties. Cases are also known in which the abnormal protein formed still possesses some enzymic activity, but it is more heat labile, or it is more sensitive to an inhibitor, or it has an altered coenzyme requirement, or it has an abnormal optimum of pH or temperature.

In the last case, the mutant may behave like an auxotroph at the usual temperature and be able to grow like the wild strain at a higher temperature (Horowitz and Fling, 1953; Fincham, 1957; Yura, 1959; Lerner and Yanofsky, 1957; Yanofsky and Stadler, 1958; Yanofsky, 1957; De Moss and Bonner, 1959).

A slight change in the properties of the protein produced may thus be brought about by gene mutation. The nature of the chemical change produced is not revealed by these studies: one may think of slight modifications in the folding of the polypeptide chains, or of breakages in the chains or of changes in the amino acid sequence.

Extremely interesting and illuminating data were obtained in this respect in studies on hereditary blood diseases of man. Genetic analysis, no doubt, is at a great disadvantage here, but the ready availability of the proteins involved and the ease of purification proved favourable to detailed chemical investigation.

Sickle cell anaemia, a blood disease found rather frequently in popula-

a

b

FIG. 10. Finger prints of trypsin digests of haemoglobins A and S
a: Haemoglobin A; *b*: Haemoglobin S.
 Notice the identity of the two patterns, except for peptide 4 (Ingram, 1958).

tions originating from Nigeria, is characterized by an anomalous behaviour of the red blood cells: when the oxygen tension is low, the blood cells become twisted and under the microscope they look somewhat like sickles. In 1949, Neel established that the disease is inherited in a mendelian manner. Pauling *et al.* (1949) showed that the blood cells of individuals carrying the sickle cell trait contain a haemoglobin which can be separated from normal haemoglobin by electrophoresis. Sickle cell haemoglobin is a little less negatively charged, and it is less soluble at low oxygen pressures than normal haemoglobin; this lower solubility explains gelation and sickling of the cells (Harris, 1950).

Mutant individuals containing the sickle cell gene make a slightly abnormal haemoglobin, just as some tryptophan-less mutants of *Neurospora* make a slightly abnormal protein which has lost the catalytic properties of tryptophan synthetase. Sickle cell anaemia manifests itself in the individuals who are homozygous for the sickle cell gene; these make only sickle cell haemoglobin, no normal haemoglobin. The disease is the consequence of an inborn defect in haemoglobin, one of the best known of all proteins. The molecule of haemoglobin is made of two identical halves (Perutz *et al.*, 1951, 1960), each half containing two different polypeptide chains α and β . The complete amino acids sequence in haemoglobin is not known. Nevertheless, Ingram (1956, 1957) has been able to compare amino acid sequences of haemoglobin S (sickle cell) and haemoglobin A (normal adult haemoglobin). Haemoglobin is split by trypsin into 26 different peptides, and a resistant core is left which can in turn be split into about as many peptides by chymotrypsin. The peptides can be separated by paper chromatography and the chromatograms display a pattern of spots which is sufficiently constant and reproducible to be considered characteristic of the protein. The 'finger prints' of haemoglobin A and S are nearly identical: no difference can be detected in the chymotrypsin digest of the trypsin resistant core (Hunt and Ingram, 1958, 1959). Among the peptides produced by trypsin hydrolysis, all except one are identical (Ingram, 1958) in both haemoglobins. In the one peptide which differs between the two haemoglobins, the only difference is the replacement of one glutamic residue by a valine residue in S haemoglobin.

More than 20 varieties of abnormal haemoglobins are known at present (Itano, 1957; Ager *et al.*, 1958) and many of them have been shown to be genetically determined (Benzer *et al.*, 1958). The finger print procedure again revealed a great similarity of structure between these variants and normal haemoglobin; in all the cases analysed so far, each haemoglobin differs from the others by the substitution of one amino acid for another one, as shown in Fig. 11.

These remarkable studies clearly show that mutation of a gene can result in the replacement of one amino acid by another in the protein, without any

other change in the rest of the chains. Comparison of haemoglobin G, A and S further indicates that two contiguous amino acids can be replaced independently as the result of two independent mutations, and that mutational changes can occur in different regions of the molecule (Hunt and Ingram, 1959, 1960; Hill and Schwarz, 1959).

Haemoglobin A	Val-His-Leu-Thr-Pro-Glu-Glu-Lys
Haemoglobin S	Val-His-Leu-Thr-Pro- <i>Val</i> -Glu-Lys
Haemoglobin C	Val-His-Leu-Thr-Pro- <i>Lys</i> -Glu-Lys
Haemoglobin G	Val-His-Leu-Thr-Pro-Glu- <i>Gly</i> -Lys

FIG. 11. Amino acid sequence of the terminal part of the β chain in four human haemoglobins (Hunt and Ingram, 1959; Hill and Schwartz, 1959).

This established that the genetic material controls the nature and position of certain amino acids in the polypeptide chains and makes one suspect that the position of each individual amino acid of a polypeptide chain might indeed be controlled by the gene. It would seem that the gene responsible for the production of a given protein contains a set of instructions concerning the nature and positions of the amino acids in the polypeptide chain, perhaps a complete blue print of the polypeptide chains. One must then examine closely the fine structure of the gene and try to decipher the indications it contains.

2. Fine Structure of the Gene

(a) *Evolution of the notion of gene.* Early studies in genetics revealed that the genes — i.e. the determinants of certain morphological characters — are often inherited in groups, as if they were linked together in some way. The physical basis of linkage in higher organisms is the chromosome. In *Drosophila*, for instance, there are four chromosomes in a haploid set, and the genes fall into four linkage groups. Linked genes are located on the same chromosome. The linkage is not absolute, however, for separation and recombination between usually linked genes occur in a certain fraction of the progeny.

Crossing over can take place at many different points along a chromosome. If the chances of crossing over were equal all along the chromosome, then the frequency of recombination of two genes would be a simple function of their distance on the chromosome: the further apart they would be located, the more often would crossing over occur between them and the more frequently would recombination be observed. It would be possible to compute relative 'distances' between genes and the relative positions of the genes

within a linkage group, from the frequency of recombinations. A most remarkable outcome of studies based on such reasoning is that all the experimental data could be accounted for by a linear arrangement of the genes. Cytological observations revealed changes in the bands of giant chromosomes of salivary glands of diptera at corresponding positions (Bridges and Bridges, 1939; Demerec, 1941; Lewis, 1945).

In this outlook, crossing over between the members of a pair of homologous chromosomes at meiosis involves breakage and reassociation of the chromosome at the level of intergenic material; the genes are implicitly considered as discrete units which are small compared to the distance between them.

Developments of the genetics of micro-organisms made it possible to go one step further in the analysis of the structure of the genome.

Moulds and yeasts form zygotes by fusion of haploid forms. In bacteria, three processes have been discovered in which part of the genome of a bacterium can be introduced into another and give rise to recombinants. Thus in 'conjugation', part of the genome of the 'male' Hfr bacterium enters the F⁻ recipient cell (Lederberg and Tatum, 1946; Wollman *et al.*, 1956). In 'transduction' (Zinder and Lederberg, 1952), a fragment of the bacterial genome is carried over from one bacterium to another by a bacteriophage produced in the donor bacterium. In 'transformation', a piece of DNA extracted from a bacterium by the experimenter, is absorbed by a receptive bacterium and thus a new piece of genetic material is introduced which may eventually be integrated in the genome. In all these cases, the transfer is unidirectional and only part of the genome is usually carried over from the donor to the recipient bacterium. In bacteriophage, recombination of genetic markers is observed when two related phages develop within the same bacterium. (For a brief review, see Braun, 1953.)

The genome of bacteria, to say nothing of bacteriophage, is much smaller than that of higher organisms. Bacteria and bacteriophages do not possess chromosomal structures comparable to those found in animals or plants, and recombination in these micro-organisms probably occurs by a process which is quite different from crossing over as observed in higher organisms (Hayes, 1960). Nevertheless, application of the same mapping principle as used for higher organisms again led to results compatible with a congruent linear arrangement of the genes. The linear order of the genes was moreover confirmed by the observation of a progressive linear transfer of the genetic markers during bacterial conjugation (Jacob *et al.*, 1956; Jacob and Wollman, 1958; Wollman and Jacob, 1958).

With moulds, yeast, bacteria and bacteriophages it was possible to increase the resolving power of mapping procedures enormously. As many as 10^8 individuals can be handled in one experiment. With well chosen selective media, extremely rare recombinants can be recovered and isolated. It is

thus possible to determine rapidly the recombination frequency of series of mutations, and to study quantitatively recombinations which occur with a very low frequency, that is — according to the basic assumption — recombinations between closely located sites.

When several mutants all lacking the same enzyme activity were isolated and mapped, it was found that all the mutations are located within a narrow region of the genome. This is the genetic region corresponding to the enzyme in question. Crosses between these various mutants, however, may give rise — with a very low frequency — to wild type organisms which produce the normal enzyme, as if recombination had taken place between different mutation sites within the genetic region corresponding to the enzyme (locus).

Mapping of several such mutants on the basis of the frequency of recombination again led to their arrangement in a linear order, this time within the locus of a single enzyme. Although deviations from the proportionality of frequency with distance are often observed for very short distances, the linear order is maintained. The linear arrangement of the mutation sites within a locus found strong confirmation in a recent analysis of the topological relationships between markers in a bacteriophage, which was based on a completely different principle (Benzer, 1959).

Each mutation thus corresponds to an alteration at one of many possible sites within the linear array which makes up the genetic region corresponding to a given enzyme protein (Pontecorvo, 1958; Demerec, 1956; Demerec *et al.*, 1958; Hartman, 1958). The linear structure of this locus is of fundamental significance for the mechanism of protein synthesis.

Recombination after transduction in *Salmonella* showed that two mutation sites corresponding to two different enzymes may not be more distant from one another than two mutation sites within the same locus. This indicates that two loci can be very close to one another, and most probably contiguous (Hartman, 1957; Demerec *et al.*, 1958).

The elementary units of heredity cannot be visualized any more as well separated beads on a string; they are part of a much more continuous structure. It is obvious that the use of the term 'gene' to designate any specific piece of genetic material would now lead to confusion, and there has been a great deal of apparent conflict during the last few years, due to the fact that the same words were being used with different meanings by different authors. Benzer (1957) has greatly helped to clarify the field by introducing new terms for the elementary units of heredity:

The *muton* is the smallest unit the alteration of which results in a mutation. There is space for many mutons within the locus of an enzyme. The muton is of great interest for protein synthesis, for it is directly related to the smallest modification of the genetic material which causes a change in the structure of the protein produced. It will be very interesting to estimate

the size of a muton, how close together two different mutons can be located, and finally to translate this genetic concept into chemical terms. This is already partly accomplished, as we shall see later.

The *recon* is the smallest element which can be interchanged, as a unit, by genetic recombination. It is of interest for the analysis of recombination processes and it is related to the limit of divisibility of the genetic material. It has, so far, little bearing on studies of protein synthesis.

The *cistron*, which will be considered more closely in the following pages, is the unit of function. It can be defined as a piece of genetic material which must be present as a unit to accomplish its function. This function is the control of a certain observable character or phenotype. The character considered may be e.g. a morphological feature, or the capacity of growing on a defined medium, or the production of a pigment, etc. The observable character may depend on the genetic material in a simple way or in a very indirect way; it is clear therefore that the meaning of the 'genetic unit of function' will depend on the character which is being considered. The character we are interested in is the synthesis of a specific protein. The *cistron*, in this particular case, is a piece of genetic material which specifically controls the synthesis of this particular protein, and which *ceases to fulfil this function if it is fragmented*. The size of this unit has deep implications for the mechanism of protein synthesis. Let us examine how the *cistron* can be studied experimentally.

In the process of recombination, whatever the underlying mechanism, a unique genetic structure is reconstituted or copied from two pieces of genome which come each from one of the parents.

But it is also possible to confront within the same cell two genomes or parts of genomes coming from two different cells, under such conditions that they do not recombine or that recombination is negligible. This occurs for instance in the formation of a zygote by fusion of two gametes. In moulds, two haploid hyphae of the same mating type can fuse and form a heterocaryon, in which each cell contains two types of nuclei, one type coming from each organism (Beadle and Conratt, 1944). When a piece of an Hfr genome enters a F^- bacterium, a system resembling a zygote is formed. Again when two different bacteriophages enter the same bacterium, they multiply mostly independently, this is somewhat comparable to a heterocaryon. Also in abortive transduction, part of the genome is introduced into a bacterium and coexists with the bacterial genome without recombining (Demerec and Ozeki, 1959).

The study of such heterozygotes or heterocaryons makes it possible to find out whether two different mutations concern the same functionally indivisible unit of genetic material or whether they affect two units which can operate separately.

Let us consider two mutation sites A and B on the same linkage group

(chromosome or linear set of genes). Each site can exist in two alternative forms: normal (+) or mutated (-).

FIG. 12.

If the two mutants $A^+ B^-$ and $A^- B^+$ are crossed and if recombination occurs in between the two sites, a pair of recombinants will be obtained: $A^+ B^+$ and $A^- B^-$. Obviously $A^+ B^+$, which possesses the two normal sites, is normal ('wild type'), $A^- B^-$ to the contrary is a double mutant.

Instead of looking for recombinants between the two strains $A^+ B^-$ and $A^- B^+$, let us introduce these genomes within the same cell under conditions where there is no recombination (as in heterozygotes, heterocaryons, etc.). Each cell contains a sample of the normal site A^+ and a sample of the normal site B^+ , but these are located on two separate pieces of genetic material. If A and B belong to regions of the genome *which can each perform their function independently of the other*, the function of both A^+ and B^+ will be accomplished and the heterozygote or heterocaryon will behave as the wild type, or in a very closely similar way. *Complementation* will be observed.

For instance, if a heterocaryon is made between two mutants of *Neurospora*, one tryptophan-less and one adenine-less, the heterocaryon will have no requirement, because the nuclei which cannot control the synthesis of tryptophan synthetase cause the formation of the enzyme of adenine metabolism that the other type of nuclei cannot control, and vice versa.

But if the two mutation sites A and B both belong to the same functional unit of genetic material, this unit is bad in both sets of genes: in one set it does not work properly because site A is bad, in the other set, because site B is bad. There is no complementation if the two mutation sites belong to the same functional unit of genetic material.

Two mutations which do not show complementation when they are on two separate pieces of genome (trans), although they can restore the wild type when they are recombined within the same piece of genetic material (cis) are said to be comprised within the same 'cistron' (Benzer, 1957).

If we are studying the genetic control of synthesis of a particular protein, a *cistron* is thus a unit of nuclear genetic material which accomplishes one indivisible function required for the synthesis of that specific protein.

It could be for instance a segment of genetic material which controls the structure of the smallest piece of a protein which can be made independently and which can later be integrated into the finished protein.

In most cases, all the mutants affecting the synthesis of a specified enzyme are found to be in the same cistron. This means that the genetic information relative to these enzymes cannot be divided, it must be used in one piece. *It looks as if most enzymes could not be made piecemeal.*

However, a few very interesting exceptions to this rule have been found recently (Giles *et al.*, 1957; Woodward *et al.*, 1958, 1959; Case and Giles, 1958, 1960). Heterocaryons obtained from two mutants both lacking the same enzyme, glutamic dehydrogenase, as the result of closely located mutations showed complementation i.e. partial recovery of the enzyme production (Fincham and Pateman, 1957; Pateman and Fincham, 1958; Catcheside and Overton, 1958). This means that the two mutations are located in two different cistrons. However, the enzyme level in the heterocaryon reached at most 25 per cent of that in the wild strain. The low level of glutamic dehydrogenase could possibly be explained by assuming that the enzyme is composed of two pieces, namely two polypeptide chains A and B which are made independently, each under the control of one cistron. In the heterocaryon, the nuclei of one type would provide for the synthesis of normal A chains and abnormal B chains, whereas the other type of nuclei would cause the formation of abnormal A and normal B chains. Random association of A and B polypeptides would produce about 25 per cent of all good AB protein molecules and 75 per cent of protein molecules in which at least one of the constituent chains is bad.

Association of polypeptides into a finished protein can indeed occur spontaneously *in vitro*. Haemoglobin for instance can be made to dissociate into two α and two β chains. Under suitable conditions, these polypeptides will reassociate correctly and reconstitute normal haemoglobin molecules (Itano and Singer, 1958, 1959; Itano and Robinson, 1959; Jones *et al.*, 1959; Vinograd *et al.*, 1959). Studies by Hunt (1959) on foetal haemoglobin support the idea that the α and β chains might be made separately under the control of two functionally independent pieces of genetic material.

That complementation in certain heterocaryons occurs by association of polypeptides has received a convincing demonstration in Woodward's experiments (1959). By mixing extracts of two auxotroph mutants of *Neurospora* both lacking adenylosuccinase, some enzyme activity was restored. As the mixing took place at a low temperature under such conditions that no metabolic process could take place, it must be admitted that an active enzyme has been formed by spontaneous association of at least two parts, one coming from each auxotroph, by some process akin to the reassociation of α and β chains in the case of haemoglobin. The formation

of a hybrid enzyme in addition to the two parental types in a maize heterozygote is most probably another example of the same phenomenon (Schwartz, 1960).

To summarize, genetic evidence so far indicates that the information necessary for the synthesis of a protein molecule is contained in one or possibly in a very small number of cistrons.

It should be realized that the analysis has reached a point where the definition of the genetic unit of function, that of protein molecule and that of enzyme are at stake or raise difficulties. This is illustrated by recent studies on tryptophan synthetase in *Escherichia coli* (Lerner and Yanofsky, 1957; Crawford and Yanofsky, 1958; Yanofsky and Stadler, 1958; Yanofsky, 1959).

The normal enzyme as purified and isolated from the wild strain catalyses three different reactions:

Some mutants lose activity (1) and (2) but keep the other catalytic activity (3). On the other hand, the mutants affecting tryptophan synthetase belong to two contiguous pieces of genetic material. The normal protein tryptophan synthetase can be split into two inactive moieties which recombine *in vitro* into a complex having all the activities of tryptophan synthetase. Thus an enzyme having several related but clearly distinguishable catalytic properties results in the present case from the association of two protein structures the formation of which is controlled by two contiguous genetic regions.

Going one step further in this direction, we will observe, as in *Salmonella*, clusters of contiguous genetic loci arranged in the same relative order as the steps in the pathway of biosynthesis they control (Demerec and Hartman, 1959; Yanofsky and Lennox, 1959; Hartman *et al.*, 1960).

The names we give to pieces of genetic material and what we call enzymes or an enzyme system is not very important. The fundamental facts are that the smallest piece of protein material which can be made independently is of the size of a protein molecule or of a large polypeptide chain, and that all the specific information concerning such a major protein piece is found within a unique and limited region of the genome.*

* An objection can be opposed to this conclusion (Atwood and Mukai, 1953). If many proteins should share a common intermediate, for instance a quite small peptide, a mutation which would prevent the synthesis of this small intermediate would almost certainly be lethal and the mutant could not be detected. A selection is therefore operated and the mutants recovered necessarily correspond to genes controlling a restricted function such as the formation of one elaborate piece of macromolecule. This is a purely theoretical objection, which it is important to keep in mind, but there is no positive evidence that this situation exists.

(b) *Difficulties in the use of the cistron concept.* Further studies on cases of complementation in heterocaryons between mutants deficient in the same enzyme showed that the application of the cistron concept as the unit of function of genetic material in enzyme synthesis can lead to absurdity.

Thus Giles *et al.* (1957) studied several mutants of the locus responsible for adenylosuccinase synthesis. Certain associations of two such mutants in a heterocaryon gave complementation, others did not. The puzzling situation which follows was observed: mutants A and B gave no complementation, which means that they were in the same cistron, by definition. In the same way, mutants B and C were also found to be in the same cistron. However, A and C gave complementation and — by definition — were *not* in the same cistron. These three conclusions are obviously incompatible.

Similarly, Pateman and Fincham (1958) found that out of eleven mutants in the glutamic dehydrogenase locus of *Neurospora crassa*, only two pairwise combinations produced enzyme activity in heterocaryons, whereas all the others gave no complementation. Again some pairs of mutants were therefore in the same cistron, according to certain experiments, and in different cistrons according to other experiments. In such cases, the notion of cistron breaks down. The reason is most probably that the operational definition of the cistron by the *cis-trans* test rests on the common sense idea that adding two bad things together does not make a good one. This assumption is not entirely justified. Fincham (1960) has shown that the functional glutamic dehydrogenases produced by complementation are not identical to the normal enzyme: they differ from the wild type dehydrogenase and from each other in their thermostability, and they have abnormal Michaelis constants. The co-operation of the genomes of these mutants therefore has not reconstituted the normal protein, it has produced doubly abnormal proteins. But these happen to have an enzymic activity sufficiently similar to that of the normal enzyme to be functional. The molecule of glutamic dehydrogenase results of the association of several identical polypeptide chains. It is conceivable that polypeptides with lesions in different places might be able to cover each other's deficiencies and to form an almost correctly folded protein structure with almost normal enzyme activity (Fincham, 1960; Catcheside, 1960).

Fincham's observations are of great interest for protein synthesis. They are compatible with the idea that the genetic locus strictly controls the primary structure of the polypeptide chains (the amino acid sequence), and they call attention to the fact that the appearance of the specific catalytic or serological properties associated with the protein produced is an epiphenomenon. This depends on eventual association of the polypeptide chains, on their folding into helices, and on the manner parts of helices twist around each other, thus bringing together the elements which constitute the active centre of the enzyme, or the particular surface of the antigen.

Cases of complementation should be analysed very closely before being taken as evidence for the resolution of the genetic locus of an enzyme into several cistrons. The notion of cistron would recover its full value in such cases if the 'function' considered was the production of a certain arrangement of amino acids, but it is not an easy matter to study such a function.

The conclusions from the data examined in the preceding pages can be summarized as follows: the genetic information which controls specifically the arrangements of the amino acids of an enzyme is contained in a unique segment of genetic material, the locus of the enzyme. This locus usually acts as a unit; it may, in some cases, be composed of two or a quite small number of functional units.

3. *On a few Complications on the One Gene–One Protein Relation*

The fact that two contiguous pieces of genetic material may be involved in the control of the structure of one single enzyme does not contradict the one gene–one protein hypothesis, provided one is ready to readjust the definitions of gene and enzyme adequately. But a few categories of experimental data seem to conflict with basic ideas contained in the hypothesis.

(a) *Pleiotropy*. In higher organisms, it is not exceptional that a single mutation changes several morphological or physiological characters. This has little impact on the hypothesis for it is highly probable that one single biochemical deficiency in the zygote will have several effects in the course of development and may in the end change the properties of the differentiated cells in various ways. But pleiotropy has been observed also for biochemical characters in micro-organisms. A single mutation can result in a double requirement.

In well analysed cases, however, the double requirement was shown to result from the lack of one single enzyme. For instance, the double requirement for methionine and threonine in a *Neurospora* strain results from a single genetic block in the pathway of synthesis of homoserine, which is a common precursor of threonine and methionine (Teas *et al.*, 1948; Horowitz, 1956). The double requirement for valine and isoleucine is due to the inhibition by a precursor of isoleucine of the transaminase which makes valine. The precursor accumulates when the transaminase which makes isoleucine is absent (Bonner, 1946). A mutant lacking the latter enzyme therefore requires both valine and isoleucine for growth. A comparable case has also been described for *Escherichia coli* (Myers and Adelberg, 1954; Rudman and Meister, 1953).

(b) *Suppression*. A more puzzling phenomenon is the suppression of a nutritional requirement by a mutation which occurs in a region of the genome completely unrelated to the locus in which the first mutation has occurred.

In certain cases, the suppressor mutation was shown to open a pathway of biosynthesis alternative to the one which was blocked in the auxotroph (Lein and Lein, 1952), e.g. by relieving an inhibition (Strauss and Pierog, 1954; Howarth, 1958).

But all suppressors do not act in this way. Auxotroph behaviour in mutants of the *td* locus (responsible for tryptophan synthetase) in *Neurospora* can be 'suppressed' by mutations occurring at a great distance from the *td* locus. With a mutant strain which produces a temperature sensitive enzyme, it was observed that the 'suppressed' mutant still displayed a temperature sensitivity which does not exist in the wild strain (Bonner, 1946; Yanofsky, 1956; Suskind, 1957). This suggested that the *td* locus still controlled the structure of the enzyme and that the suppressor probably affected the *operation* of the abnormal protein in some indirect way.

Again, for a tryptophan synthetase mutant of *Neurospora*, such a mechanism has been partly cleared up recently (Suskind and Kurek, 1959). The mutant considered contained an enzyme protein which was much more sensitive to inhibition by zinc ions than the normal enzyme. In a 'suppressed' mutant, in which wild type behaviour was more or less restored, the enzyme produced was actually the zinc-sensitive enzyme, but the suppressor gene in some way controlled the adjustment of zinc ions concentration in the mycelium. As a result, the abnormal protein could fulfil an almost normal enzymatic function in the 'suppressed' mutant, whereas it could not (due to zinc inhibition) in the non-suppressed auxotroph.

In all the cases considered above, suppressors affect the *operation* of enzymes or of enzyme systems, and their action is irrelevant to the control of the enzyme *structure*. These facts do not conflict with the idea that the information relative to the primary structure of an enzyme is located within one restricted piece of genetic material.

There are cases where the difficulties raised by suppression cannot be completely dismissed at present. For instance, a strain of *Escherichia coli* which lacks tryptophan synthetase and does not produce any corresponding cross reacting material as judged by the usual immunological criteria, can recover the capacity of making a protein having the immunological and enzymic properties of the normal enzyme, as the result of a 'suppressor' mutation in another region of the genome (Yanofsky, 1958; Yanofsky and Crawford, 1959).

In another mutant, which does produce an enzymically inactive cross reacting material, a suppressor mutation caused the production of a new enzymically active protein, in addition to the altered one. It was shown, besides, that the locus of the suppressor mutation in itself does not carry the information for making the normal protein.

These facts indicate that suppressor mutations can indeed change somehow the mode of expression of a gene. This could possibly mean that the

final shape of a protein is not completely determined by the corresponding locus and that factors which are independent of this locus may play a part in shaping the protein molecule. This calls for the same remarks as the cases of complementation between non-identical alleles (cf. p. 20). The genetic locus provides information — perhaps all the information — concerning the linear arrangement of the amino acids in the polypeptide chain (the so-called 'primary structure of the protein'). But the activity of an enzyme or the immunological properties of a protein depend on their tertiary structure. The folding and association of polypeptide chains are conditioned by the amino acid sequence, but they might also depend on contingent factors like the concentration of various ions and possibly on somewhat more specific actions. Certain suppressors might act by modifying such factors.

(c) *Controlling genetic units.* Another example of complication to the gene-enzyme relationship might be seen in the genetic control of β -galactosidase synthesis in *Escherichia coli*. The production of the enzyme is controlled by three independent and separable loci z , i , y . Only one of those, the z locus, provides *structural* information for β -galactosidase synthesis. The i locus controls the actual production of the enzyme, it is involved in the regulation of the synthesis. This locus does not carry any information as to the structure of β -galactosidase since mutation of this gene does not alter the structure of the protein produced. The third gene y also regulates the synthesis of β -galactosidase indirectly, by controlling the formation of a system which concentrates into the bacteria inducers of β -galactosidase synthesis (Jacob and Monod, 1959). A similar situation exists for tyrosinase in *Neurospora* (Horowitz *et al.*, 1960) and most probably for other inducible and repressible enzymes (see Chapter V).

Actually, these facts are in perfect agreement with the concept that all or at least an essential part of the structural information relevant to a specified protein is located in a restricted part of the genome, in the present case the z locus.

(d) *Concluding remark.* In discussing the value of the one gene-one enzyme hypothesis, it should be clearly realized that two different meanings can be attributed to this relation depending on the direction in which it is expressed. This relation can be taken to mean that the structure of a protein is specifically controlled by a restricted section of genetic material; we have seen that this statement is supported by numerous experimental data and contradicted by none, if we reserve a few cases that have not been thoroughly analysed yet.

The other acceptance of the gene-enzyme relationship concerns the mode of action of the genetic material; this side of the matter has not been discussed here. It should be made clear that there are reasons to doubt that the genetic material exerts its action on the cell exclusively by controlling the structure of specific proteins. The genetic control of differentiation in

higher organisms might involve other modes of action of the genes. Moreover, there is evidence that the *i* gene which controls the inducible or constitutive character of β -galactosidase can be expressed under such conditions that no protein formation can occur (Pardee *et al.*, 1959); it would seem therefore that the *i* gene does not act by controlling the structure of any protein (Jacob and Monod, 1959).

B. CHEMICAL NATURE OF THE GENETIC DETERMINANTS OF PROTEIN STRUCTURE

1. *Genetic Material of Bacteria and Bacteriophages*

Direct evidence that DNA is the genetic material of bacteria was provided by studies on bacterial transformation. Griffith (1928) had observed that material obtained from encapsulated *Pneumococci* can confer upon non-encapsulated strains the ability to make a capsule composed of specific polysaccharide. Avery *et al.* (1944) found that transforming activity is associated with DNA. The transforming properties of the DNA preparations are not affected by proteolytic enzymes or by ribonuclease, but they are extremely sensitive to deoxyribonuclease. The transforming properties also disappear under conditions which cause denaturation of DNA (Zamenhof *et al.* 1953; Zamenhof, 1956; Lerman and Tolmach, 1959; Lacks and Hotchkiss, 1960).

Many hereditary characters, including the capacity to make well defined enzymes, can be transferred from one bacterial strain to another by highly purified DNA. This DNA was shown to transfer for instance the capacity to oxidize glucose (Ephrussi-Taylor, 1954) or to make mannitolphosphate dehydrogenase (Marmur and Hotchkiss, 1955). In the transformed bacteria, this capacity is thereafter inherited like any other character and DNA extracted from the transformed bacteria is able to cause transformation. DNA thus performs the two functions which are characteristic of the genes: it brings to the bacterium the capacity of making a specific enzyme and it is duplicated and transmitted to the progeny together with the genetic determinants. The genetic characters introduced by the transforming DNA are also capable of recombination (Ephrussi-Taylor, 1951, 1954, 1955; Hotchkiss and Marmur, 1954).

Treatment of DNA by nitrous acid is known to deaminate guanine, adenine and cytosine; when transforming DNA is treated *in vitro* by this agent and used thereafter as transforming agent, many transformed bacteria have acquired mutated characters. Modifications of DNA brought about *in vitro* by a chemical agent can thus be expressed as mutations when

the DNA is introduced into a bacterium (Litman and Ephrussi-Taylor, 1959).

Experiments by Hershey on the genetic material of bacteriophage are almost as convincing. Bacteriophages are complex organisms made of packed DNA enclosed in an elaborate protein structure. Hershey and

FIG. 13. Schematic representation of the experiment of Hershey and Chase (1952) (Reproduced from Kozloff, 1959).

Chase (1952) grew bacteriophage in a medium containing either $^{32}\text{PO}_4$ or $^{35}\text{SO}_4$; the collected phage contained ^{32}P in DNA exclusively or ^{35}S in protein constituents only. When non-labelled bacteria were infected with labelled phages, it was found that ^{32}P (therefore DNA) enters the bacteria and is retained in the progeny whereas most of the ^{35}S stays outside (Hershey, 1955). Phage particles may be compared to small syringes which inject the DNA they contain into a bacterium. The injected DNA confers to the infected bacteria the capacity of making the phage proteins and several enzymes involved in the synthesis of phage DNA precursors (Flaks and Cohen, 1959; Flaks *et al.*, 1959; Kornberg *et al.*, 1959; Somerville *et al.*, 1959; Barner and Cohen, 1959; Bessman, 1959; Keck *et al.*, 1960).

Phage DNA multiplies within the bacterium, it is later enwrapped into its protein container. When two related phages infect the same bacterium, that is when two phages inject their DNA into the same bacterium, recombination of hereditary characters may be observed. Phage DNA contains all the information required for orienting the cell metabolism towards the synthesis of a set of specific foreign proteins. It is clear that phage DNA has all the features of the genetic material.

There is also good reason to believe that during bacterial conjugation it is essentially a DNA fibre which passes into the recipient bacterium, for decay of ^{32}P , which breaks the DNA molecule, causes breakages in the chain of genetic markers (Jacob and Wollman, 1958).

2. *Genetic Material of Ribonucleoprotein Viruses*

Many viruses of plants and animals contain no DNA; they are made of RNA enclosed in protein. Viruses certainly bring to the cell a set of instructions as how to assemble building blocks of proteins and nucleic acids into virus RNA and protein. Extensive modifications of the protein moiety of tobacco mosaic virus can be brought about by chemical treatment without adverse effect on virus infectivity. Substitution of 70 per cent of the protein NH_2 groups by chlorobenzoyl groups (Miller and Stanley, 1942), addition of leucyl residues (Fraenkel-Conrat, 1953), removal of the terminal threonine of the protein by carboxypeptidase (Harris and Knight, 1952) have no effect on infectivity, nor on the nature of the virus produced. A large part of the protein can be removed without killing the virus (Schramm *et al.*, 1955).

On the other hand, slight modifications to the virus RNA result in virus inactivation. Structural analogues of purines and pyrimidines (e.g. 8-azaguanine, 2-thiouracil or 5-fluorouracil) drastically reduce multiplication (Commoner and Mercer, 1951, 1952; Matthews, 1952; Gordon and Staehelin, 1959); this must be due to alterations of the virus RNA by the abnormal purines or pyrimidines, for a close correlation was found between incorporation of thiouracil into the virus RNA and the inhibition of virus multiplication (Jeener and Rosseels, 1953). If ribonuclease is introduced into the cells of a tobacco plant at the beginning of infection, virus multiplication is blocked (Hamers-Casterman and Jeener, 1957; Benda, 1958). Multiplication of influenza virus is also inhibited by ribonuclease (Le Clerc, 1957).

Such experimental data indicated that the integrity of RNA is more essential to virus multiplication than integrity of the protein sheath (Jeener, 1956). Fraenkel-Conrat and Williams (1955) were able to separate the RNA from the protein and to reassociate them into virulent particles. Virus reconstituted by mixing RNA and protein from different strains always caused lesions which resembled very much those corresponding to the virus from which the RNA had been obtained (Fraenkel-Conrat, 1956; Fraenkel-Conrat *et al.*, 1957). Numerous mutants were formed, however, as if the genetic material of the virus had been slightly damaged during the process (Commoner *et al.*, 1956; Fraenkel-Conrat and Singer, 1957; Bawden and Pirie, 1957). Ribonuclease does not kill the intact virus, but the slightest action of the enzyme upon the naked RNA leads to complete inactivation of the reconstituted nucleoprotein (Fraenkel-Conrat and Williams, 1955).

Finally, Gierer and Schramm (1956) were able to isolate from tobacco mosaic virus, by phenol extraction, RNA which could transmit infection. This fundamental observation has now been confirmed by work from many laboratories and extended to other viruses. Control experiments of various

kinds make it quite clear that the infectivity of the RNA preparations is not due to traces of contaminant virus (Fraenkel-Conrat *et al.*, 1958; Bawden and Pirie, 1957; Staehelin, 1959; Engler and Schramm, 1959).

Infectious RNA has been isolated by phenol or detergent extraction from turnip yellow mosaic virus (Fraenkel-Conrat *et al.*, 1957; Cohen and Schachman, 1957), from tobacco Ring Spot (Kaper and Steere, 1959), from the virus of necrotic Ring Spot of cucumber (Diener and Weaver, 1959) and tomato Bushy Stunt (Rushizky and Knight, 1959). Ribonucleoprotein viruses which infect animal cells also provide infectious RNA. This was established for poliomyelitis virus, Encephalitis viruses and other neurotropic viruses by numerous workers: Alexander *et al.*, 1958; Huppert and Sanders, 1958; Ada and Anderson, 1959; Franklin *et al.*, 1959; Cheng, 1958; Wecker, 1959; Mountain *et al.*, 1959; Gerber and Kirschstein, 1960. Infectious RNA was also isolated from animal cells infected with neurotropic viruses (Colter *et al.*, 1957; Wecker and Schäffer, 1957; Sokol *et al.*, 1959), with Influenza virus (Maassab, 1959) or with Foot and Mouth disease (Brow and Stewart, 1959; Mussgay *et al.*, 1959) and also from an insect virus (Krieg, 1959).

It is now possible to study directly the effects of chemical or enzymic modifications of the isolated RNA upon its infectivity. Treatment of the isolated RNA by nitrous acid under very mild conditions rapidly inactivates the RNA. Nitrous acid acts upon adenine, guanine and cytosine and replaces their amino groups by a hydroxyl. It has been estimated that the modification of one nucleotide out of some three thousand can inactivate a virus particle (Schuster and Schramm, 1958); this amounts to one or two changes per virus particle. It was also observed (Gierer and Mundry, 1958) that the fraction of mutants recovered is increased very much after limited treatment of the RNA by nitrous acid. This suggests that replacement of, for example, a cytosine residue by its product of deamination uracil might lead to viable mutated forms of the virus (Gierer and Mundry, 1958).

RNA of the ribonucleoprotein viruses must carry the hereditary characteristics of the virus, since it is able to cause the complete process of infection, including the synthesis of the specific protein coating. It would seem that the protein moiety is a sheath which protects (Siegel *et al.*, 1956) RNA in the resting extracellular form of the virus. In normal infection, the RNA is indeed liberated into the cell where it reproduces first (Engler and Schramm, 1959); the unprotected RNA is then very sensitive to ribonuclease action within the cell (Hamers-Casterman and Jeener, 1957; Benda, 1958). The specific virus protein is made later and it associates into the typical rods in which one RNA fibre is enwrapped.

The protein moiety plays, nevertheless, an important part in virus infection. The host range of viruses is often rather restricted. Recent observations indicate that the susceptibility or resistance of animal or plant

cells to certain viruses depend on specific interactions between the surface of the cell and the virus protein (De Somer, *et al.* 1959; Holland *et al.*, 1959; Gordon and Smith, 1960). Only primate cells are susceptible to poliomyelitis virus. But cells of rabbit, pig, mouse and chicken can be infected by RNA extracted from the virus. It is striking that the virus thus produced in non-primate cells shows the typical host range and serological specificity of poliomyelitis virus. Since the structure of the protein coat is not influenced by the cell in which it is produced, it must have been strictly controlled by information contained in the virus RNA.

There is a complete analogy between virus infection by RNA and bacteriophage infection, which normally occurs by injection of DNA into the recipient cell. Virus RNA is the carrier of information for the production of at least the virus protein, just as DNA is the carrier of the information which controls the synthesis of an array of phage proteins.

3. *Genetic Material of Higher Organisms*

Sperm heads and chromosomes are two structures known to contain mendelian genes. Comparison of their constitution provides a first indication about the nature of the genetic material in animals. Chromosomes are made of DNA, histones, 'residual' acidic protein and RNA (Brachet, 1942). Residual protein may be absent from metaphase chromosomes (Caspersen, 1941; Bloch and Godman, 1955). Sperm head in fishes contains no RNA and no histones; it is made of DNA associated with protamines. The only constituent that fish sperm head and chromosomes have in common therefore seems to be DNA which accordingly must be the carrier of genetic determinants.

Other indications in the same direction are found in the constancy of the DNA content per cell in any given species. Moreover, many mutagenic agents act upon animals and plants in the same way as on bacteria and most of them are known to react easily with DNA. The ultraviolet action spectrum for the production of mutations in higher organism as well as in micro-organisms resembles very much a nucleic acid absorption spectrum. DNA from different species have different compositions: the adenine/guanine ratio seems to be characteristic of the species (Vischer and Chargaff, 1948; Chargaff *et al.*, 1949; Chargaff, 1956). DNA is also one of the most stable compounds of the animal cell and this quality is well suited to a guardian of hereditary characters.

DNA therefore appears as the most probable carrier of genetic information in higher organisms and although as direct an evidence as in bacterial transformation has not been clearly obtained so far, it is the common belief that DNA is at least the main genetic material in higher organisms.

Extensive discussions about the nature of the genetic material will be found in reviews by Hotchkiss (1955) and by Brachet (1957).

4. *The Structure of DNA*

DNA as isolated from animal tissues or from micro-organisms is a macromolecule with a molecular weight in the range of $6 \cdot 10^6$ (Sadron, 1959). Chemical studies (Brown and Todd, 1952; Michelson and Todd, 1953; Dekker *et al.*, 1953; Carter, 1951) showed that DNA is made of a linear backbone in which phosphoric groups and deoxyribose residues follow each other in a quite regular manner. Each phosphate group is bound to the C₅ position of a deoxyribose and to the C₃ of the next one. These long chains of two alternating elements are identical in all the DNA samples studied, they are perfectly monotonous and probably void of information. But each deoxyribose residue of the chain carries on the C₁ a purine or a pyrimidine bound in N-glycosidic linkage. Adenine, guanine, thymine and cytosine account for all but a small percentage of the bases. 5-Methylcytosine (Laland *et al.*, 1952; Wyatt, 1951), 6-methylaminopurine (Dunn and Smith, 1955, 1960) have been found in smaller amounts in DNA.

In a few exceptional but very interesting cases, large amounts of an unusual pyrimidine enter in DNA composition, thus T-even bacteriophages contain 5-hydroxymethylcytosine and a large part of it carries one or two glucose residues on the CH₂OH (Wyatt and Cohen, 1952, 1953; Sinsheimer, 1956; Loeb and Cohen, 1959).

The base composition of DNA proved quite constant within each species, and no significant differences were found between DNA samples obtained from different organs of a given species (Chargaff and Lipshitz, 1953). On the contrary, DNA specimens from various organisms were found to differ considerably in base composition (Chargaff, 1950, 1958; Wyatt, 1952) and in their chromatographic behaviour (Brown and Watson, 1953; Bendich *et al.*, 1956) which indicates that DNA preparations are heterogeneous populations of molecules of different composition. It has not been possible so far to unravel the arrangement of the purines and the pyrimidines in the chains, but some indications were obtained by the study of breakdown products formed by controlled degradation of DNA. Purines and pyrimidines do not follow each other at random: for instance clusters of three or more pyrimidines are more frequent than expected from a completely random distribution (Shapiro and Chargaff, 1957; Burton and Peterson, 1960). Differential distribution analysis of the split products, moreover, showed marked differences between DNA specimens of different origins which had almost indistinguishable gross composition (Shapiro and Chargaff, 1957, 1960).

DNA of different species thus differ in the proportions and in the arrangement of the bases along the common phosphate-carbohydrate backbone. If genetic information is a modulation of the DNA constitution, the arrangement of the bases along the backbone must be the language in which this information is recorded.

FIG. 14. Chemical structure of a section of a DNA chain.

Comparison of the analytical data obtained on a great many specimens of DNA brought to light a striking regularity or common principle of DNA composition: whatever the origin of the DNA samples, the number of adenine molecules was found to be equal to the number of thymine residues, and guanine and cytosine on the other hand were always present in equimolecular amounts (Chargaff, 1950). The fundamental significance of this rule was fully realized only when a satisfactory model of macromolecular structure was worked out for DNA.

Gulland *et al.* (1947) had observed that DNA solutions undergo irreversible changes in viscosity and in ionization characteristics outside a certain pH range. This made it probable that DNA as isolated contains labile bonds, e.g. hydrogen bonds, between ionizable groups. Since titration hysteresis persists at very low DNA concentrations, the labile bonds are probably located within each molecule, not between molecules (Jordan *et al.*, 1956). A considerable irreversible increase in ultraviolet absorption is brought about by lowering the pH for a short time or by heating DNA especially in solutions of low ionic strength (Thomas, 1951, 1953, 1954), indicating irreversible changes in electronic state of the heterocycles.

It is clear that DNA, like proteins, can be denatured by rather mild treatments and that its macromolecular structure is held together by labile bonds in which ionizable groups of the ultraviolet absorbing heterocycles are involved. DNA denaturation has been extensively studied, it manifests itself by changes in viscosity (Doty and Rice, 1955), streaming birefringence (Mathieson and Matty, 1955), rotatory dispersion (James and Levendahl, 1955), infrared absorption (Frick and Rosenberg, 1954; Blow and Lenormant, 1955), UV absorption (Thomas, 1951, 1953, 1954), affinity for certain dyes (Thomas, 1953), and sedimentation characteristics (Alexander and Stacey, 1955, Oth, 1959).

X-ray diffraction studies indicated a helical structure of DNA molecules (Wilkins *et al.*, 1953; Franklin and Gosling, 1953). The size of the structural unit and the density of DNA suggested that there must be two chains in the unit (Crick, 1954). Model building showed that the chains are probably held together by bonds between pairs of bases, and the best fit is obtained by pairing adenine with thymine and guanine with cytosine (Pauling and Corey, 1956). This was in agreement with the experimental fact that adenine and thymine on one hand and guanine and cytosine on the other are always found in equimolecular proportions in DNA.

Watson and Crick (1953) thus proposed the now famous double helix structure for DNA in which the two chains run in opposite directions; each adenine in one chain is linked by hydrogen bonding to a thymine in the other, and each guanine to a cytosine (or a cytosine derivative). The parameters of this structure or of a slightly modified version of it (Feughelman *et al.*, 1955; Langridge *et al.*, 1960) are in good agreement with X-ray

diffraction data (Wilkins *et al.*, 1953; Franklin and Gosling, 1953). The rigidity and the shape of such a macromolecule fit with the hydrodynamic properties of DNA solutions (Sadron, 1959). Denaturation is explained by breakage of the purine-pyrimidine hydrogen bonds.

FIG. 15. Hydrogen bonding of bases in DNA (Watson and Crick, 1953).

An extremely interesting feature of the Watson-Crick model is that it provides a ready made intuitive answer to the problem of replication of the detailed arrangement of the nucleotides during duplication. The genetic information is contained in each chain in two complementary sets of symbols; it can be visualized that during duplication the chains somehow separate and that each serves as a template for building a copy of the other. This remarkable hypothesis cannot be considered as established; it is at least compatible with the experimental facts at the present time.

The DNA macromolecule seems indeed admirably adapted to carrying coded information. If the information resides in the sequence of the four nucleotides, a linear chain of some ten thousand nucleotides can store an enormous amount of information. The double strand system keeps the information in a state which prepares it for duplication. DNA is chemically stable at temperatures compatible with life and indeed more stable than many essential cell constituents and it is metabolically inert.

Part of the DNA only exists as double helices and part in a less orderly structure. DNA fractionation indicates that a small fraction of DNA might exist as single polynucleotide strands (Lucy and Butler, 1954; Bendich *et al.*, 1956). In bacteriophage Φ X 174, DNA is not a double helix but a single polynucleotide chain (Sinsheimer, 1959). Studies on this particular system will probably help to clarify the duplication process, and the mechanism of information transfer.

5. Structure of Virus RNA

The chemical constitution of RNA is very similar to that of DNA. The phosphate-carbohydrate backbone is identical to that of DNA, except for the presence of a hydroxyl group instead of a hydrogen on the C₂ of the carbohydrate which is thus ribose instead of deoxyribose. The presence of this hydroxyl group next to a phosphodiester makes the ribonucleic acid backbone much more susceptible to acid and alkali hydrolysis than DNA.

The sequence of the bases, adenine, guanine, cytosine and uracil along the phosphate-carbohydrate backbone is not known for any RNA. There is evidence, nevertheless, that it is different in the RNA of different viruses (Reddi, 1959). In contrast with the data on DNA composition, no rule or regularities have been found for virus RNA, although for the mixture

FIG. 16. Molecular model of a two-strand helix of DNA (Feughelman *et al.*, 1955).

of RNAs extracted from animal or plant cells and from bacteria, the number of 6-keto groups was found to be close to the number of 6-amino groups.

X-ray studies on tobacco mosaic virus indicate that the virus RNA is made of a single polynucleotide chain of about 6000 nucleotides which rests in a spiral groove formed in the regularly packed protein units. This RNA

FIG. 17. Chemical structure of a section of an RNA chain.

spiral has nothing in common with the double helix structure of DNA, the spires of the RNA strands are far apart and separated by protein material.

For virus RNA as for DNA, it is reasonable to assume that whatever genetic information the linear chain of RNA can carry must be recorded in the molecule as a particular arrangement of the purines and pyrimidines. This idea is supported by experimental facts. For instance, a very mild treatment of the virus by nitrous acid causes the appearance of mutants (Gierer and Mundry, 1958; Boeyé, 1959; Schäfer, 1959; Mundry, 1959) and it would seem that the appearance of a given mutant results from deamination of a single base (Gierer and Mundry, 1958; Boeyé, 1959).

Tsugita and Fraenkel-Conrat (1960) have isolated a mutant of tobacco mosaic virus which had been obtained after treatment of the isolated virus RNA by nitrous acid. The amino acid composition of the protein coat of this mutant seems to differ from that of the original wild strain by the replacement of three amino acid residues — one proline, one aspartic acid and one threonine — by one residue each of leucine, alanine and serine. Moreover, the amino acid sequence of the last 17 amino acids of the N-terminal end of the protein has been established for both the original virus and the mutant. They are shown hereafter:

parent original strain:
-Ser-Ser-Phe-Glu-Ser-Ser-Ser-Gly-Leu-Val-Try-Thr-Ser-Gly- <i>Pro</i> -Ala-Thr-OH
mutant:
-Ser-Ser-Phe-Glu-Ser-Ser-Ser-Gly-Leu-Val-Try-Thr-Ser-Gly- <i>Leu</i> -Ala-Thr-OH

FIG. 18.

In the mutant, one proline has been replaced by one leucine, the rest of the sequence remaining unchanged. It would seem that deamination of a base in the virus RNA has caused the substitution of one amino acid for another at a specified point within the polypeptide sequence. This is strikingly similar to the modifications caused in human haemoglobin by spontaneous mutations. Just as human genetic material contains information relevant to the amino acid sequence of haemoglobin, virus RNA contains information which controls the amino acid sequence in viral protein. This information is modified when an amino group of certain bases within the RNA is replaced by a keto group.

Both DNA and RNA can be visualized as long strands of undifferentiated material, the phosphate-carbohydrate backbone, upon which purines and pyrimidines of a few different types (usually 4 different types) are fixed at regular intervals. The sequence of these constitutes genetic information,

just as proper linear sequences of letters on a white sheet constitute written language.

C. THE COLINEARITY HYPOTHESIS

1. *Principle*

Results reviewed in the preceding pages show that the genetic material controls the nature and the position of individual amino acids in the protein polypeptide chains. Since nucleic acids as well as proteins are linear polymers and since nucleic acids differ from one another by the arrangement of the bases just as polypeptides differ by the order of their amino acids, it seems logical to assume a point to point correspondence between the two linear sequences.

To test this hypothesis directly, one might think of isolating a specified protein and the corresponding piece of DNA. Determination of the complete amino acids sequence in the protein and of the nucleotides sequence in the DNA molecule would tell to what extent and in which way they are correlated.

One-half of such an experiment is feasible presently. The complete sequence of amino acids has been worked out for insulin, for several pituitary hormones, and for ribonuclease (Spackman *et al.*, 1960). Partial sequences are known for several other proteins. With skill and patience, the structure of a polypeptide can now be resolved by the methods developed by Sanger and his followers. But the other half of the experiment seems hopeless. No ways are known to recognize by physicochemical means the piece of DNA corresponding to a given protein and to separate it from the rest of the macromolecules. No methods are available either for determining the sequence of nucleotides in a polymer containing more than a few elements.

However, although the fine chemical structure of a nucleic acid is still beyond our reach, genetic analysis can locate with extreme accuracy mutation points within a genetic locus. It should be possible then to isolate several mutants of the locus corresponding to a given enzyme and to locate the mutation points within the locus by genetic analysis. The abnormal proteins corresponding to each mutant could be isolated and their structures compared. If the hypothesis is correct, the locations of the changes in the amino acid sequence should be correlated with the positions of the mutations within the gene.

Such an experiment will involve a considerable amount of skilled work. It looks feasible with a suitable material: a small protein molecule easy to isolate, produced by a micro-organism in which mapping can be performed down to very narrow regions of the genome.

A few laboratories are at present endeavouring to do such experiments, the results of which will be watched with great curiosity (Levinthal, 1959; Garen, 1960).

2. Coding Problems

Although the colinearity principle is nothing more than pure hypothesis, it looks so logical that several studies have already been devoted to the type of correlations which might exist between the arrangement of the nucleotides in DNA and the amino acid sequence in a polypeptide.

The problem is to discover and to decipher the language in which genetic information is written. These studies are based on abstract argument and often resemble mathematical recreations. But some of these are very ingenious and they may be fruitful if their conclusions are amenable to experimental test or if they suggest clear experiments.

Since there are only (essentially) 4 different nucleotides for controlling the arrangement of 20 amino acids, it is obvious that individual amino acids cannot correspond to individual nucleotides; they might correspond to *groups* of nucleotides. There are 16 possible oriented pairs of nucleotides, which is not enough either. The number of different sequences of three nucleotides is 64, which is much more than what is needed.

Gamow (1954) proposed a coding system in which triplets of nucleotides in a double helix of DNA would correspond to individual amino acids. These triplets are overlapping, i.e. each nucleotide is part of 3 triplets and concerns therefore 3 amino acids.

Crick *et al.* (1957) considered another coding principle also involving partly overlapping triplets. Such systems would make it possible to have about the same number ($n+2$) of nucleotides in the DNA chain as there are amino acids in the corresponding polypeptide. But the overlapping triplets would impose restrictions on the possible sequences of amino acids.

Systems have also been proposed in which only a few amino acids, e.g. the aromatic amino acids, are controlled by the gene (Schwartz, 1955), whereas the sequences in between are not.

The most ingenious system presented so far is the 'code without commas' (Crick *et al.*, 1957; Crick, 1957). It is assumed that a sequence of three nucleotides in the DNA chain corresponds to a single amino acid and that the trios of nucleotides are contiguous in the chain, but non-overlapping. Since in DNA thousands of nucleotides follow each other in a regular linear sequence, each occupies equivalent positions in the chain. A difficulty therefore arises for reading the information. One does not know how to cut the series of nucleotides into trios. A way out of this difficulty is to further assume that certain trios 'make sense', i.e. correspond to an amino acid, whereas others do not, just as certain groups of letters make a word and others are meaningless. The information will be readable 'without commas' if each triplet which makes sense can only produce with its neighbours overlapping triplets which are meaningless. A fascinating feature of this system is that the maximum number of trios which fulfil these conditions is exactly 20, i.e. exactly the number of the amino acids species to

be arranged. There are many different languages operating on this principle, and several types of comma-less codes have been examined (Freudenthal, 1958; Golomb *et al.*, 1958). Such systems could code any sequence and would impose no constraints or restrictions upon the sequence of amino acids. But strong restrictions would exist in the arrangement of the nucleotides in DNA.

It should not be forgotten that this is pure speculation. If taken too seriously these entertaining schemes might obscure the situation rather than to clarify it: the more clever they look, the greater is the danger that they might blind us to reality. On the other hand schemes of this sort will help to discuss and analyse the experimental data which will soon be obtained.

Data relevant to the coding problem already emerge from the comparative study of the fine structure of proteins. Thus Brenner (1957) showed that among the sequences of amino acids known to exist in proteins, there are more possibilities than an all overlapping code could allow (Gamow *et al.*, 1956). Morowitz (1959), studying the amino acids which are found in the protein of *E. coli* next to a given amino acid, concluded that there are no constraints as to the nature of the amino acid which is contiguous to glutamic or aspartic acid or which precedes arginine or lysine. But leucine or isoleucine are found next to arginine and lysine less frequently than would be predicted from equally probable distribution (Morowitz and Barra, 1959). These limited constraints may or may not be due to the coding system.

Comparison of abnormal haemoglobins in man shows that in mutations which result in the production of Hb S or Hb C instead of Hb A, one glutamic acid is replaced by valine or lysine respectively, and the contiguous amino acids are not affected. In the mutation of Hb A to Hb G, the amino acid which is replaced is the one next to the glutamic acid residue which was replaced in the mutation of Hb A to Hb S or Hb C. These facts are not compatible with an overlapping code; they show that the actual system allows for a large degree of independence of contiguous amino acids.

In haemoglobin again, single mutations can cause the replacement of glutamic acid by either valine, lysine or glycine (see Fig. 11). This suggests that the ciphers for these four amino acids differ only slightly from one another, perhaps by one nucleotide in each case. The number of different substitutions of one given amino acid, e.g. glutamic acid, by another as a result of single mutations might also be a test for certain coding systems; for instance, in the 'code without commas' the number of possible substitutions which still 'make sense' is very limited (Levinthal, 1959). The discovery of Tsugita and Fraenkel-Conrat (1960) that a mutation brought about by deamination in virus RNA can cause the substitution of leucine

for proline in the polypeptide also suggests that the coding sequences on RNA for leucine and proline are closely related.

Data which may be of crucial significance for the coding problem have been obtained from comparative studies on DNA. Two DNA species of slightly different densities can be separated by sedimentation equilibrium in a gradient of caesium chloride (Meselson *et al.*, 1957). Sueoka *et al.* (1959) found it possible to separate by this method DNA preparations differing in their A-T/G-C ratio. It was observed that DNAs from various bacteria differ very much from one another in their base composition but that within one species the characteristic composition is maintained even within rather small fragments of DNA (Rolfe and Meselson, 1959). If, as it would seem, the gross composition of the proteins is not very different between the various species, the very marked differences in DNA composition would indicate that the code is not the same for all the bacterial species. Another possibility is that the code does not use 4 letters but only 2. It is conceivable for instance that as far as coding is concerned the important matter is the presence of either NH₂ or OH in position 6 of the purines or the corresponding position of the pyrimidines; adenine and cytosine would then have the same meaning in terms of amino acid (Sinsheimer, 1959). Vielmetter and Schuster (1960) were able to deaminate preferentially the individual amino bases of the DNA of T₂ phage. They came to the conclusion that deamination of adenine and hydroxymethylcytosine can cause mutations, whereas deamination of guanine can be lethal but does not cause mutations. It is striking that the NH₂ of guanine is in position 2 whereas that of adenine and of cytosine is in position 6. This indicates that substitution of a keto group for an amino group in position 6 changes the information, and since deamination of adenine gives hypoxanthine which is not found in DNA, this strongly suggests indeed that the information might be written in a two-digit system: 6-keto or 6-amino. In such a case, each coding unit should contain 5 nucleotides for coding one amino acid.

Another question which can be raised in connexion with coding, is whether there are enough nucleotides in a genetic locus to code for all the individual amino acids of a protein molecule. Benzer tried to translate the distances between mutation sites, as derived from genetic recombination experiments, into number of nucleotides in DNA. He found that the elements separable by recombination in bacteriophage T₄ might be as small as two nucleotide pairs, and that a 'cistron' might contain of the order of 400 nucleotide pairs. Pontecorvo and Roper (1956) made similar estimations for several loci of *Aspergillus* and of *Drosophila*, and they found, in each case, values ranging from 1000 to 8000 nucleotide pairs. The calculation involves assumptions which cannot be completely checked at present, and the results must of course be considered a rough estimate.

If 3 or 5 nucleotide pairs* are required for coding each amino acid, a locus seems to be large enough for controlling the arrangement of 200–2000 amino acids. This corresponds to polypeptides weighing 20,000 to 200,000, which is in the range of molecular weight of proteins. In the light of these estimations, it does not appear unreasonable to assume that each amino acid in a polypeptide might be controlled genetically. The gene might thus contain a complete blue print of the amino acid sequence, and control all the details of the primary structure of the corresponding protein.

* One nucleotide pair in the double helix does not carry more information than one single nucleotide in a single strand.

CHAPTER II

The Sites of Protein Formation within the Living Cell

A. EARLY CYTOCHEMICAL DATA

The first data on the sites of protein synthesis in the cell emerged from cytochemical studies by Brachet and by Caspersson. Brachet (1933) had observed that the variations of DNA content during the development of the sea urchin egg do not parallel the changes of 'nucleic phosphorus'. This called his attention upon the possible existence of other nucleic acids beside DNA in this material. But at the time, there was no method available for detecting or for determining ribosenucleic acids. Plant nucleic acid, as RNA was then usually named, was regarded as a biochemical curiosity, so rich in phosphate that it was probably a phosphate storage form, which occurs in wheat germ, in yeast, and curiously enough also in pancreas. A few years later, the purification and isolation of pancreatic ribonuclease (Kunitz, 1940) provided a means of destroying RNA specifically. Making use of this new tool, Brachet tried to see which structures or regions of animal cells would be affected by ribonuclease. Brachet (1941) established that the basophilic substance of the cytoplasm of animal tissues is specifically removed by pancreatic ribonuclease. The substance responsible for cytoplasmic basophilia was thus clearly identified as RNA, and at the same time a very simple method for the detection and localization of RNA in tissue sections was introduced. A screening of animal tissues by this technique demonstrated the presence of RNA in all types of cells and showed that RNA is responsible for the basophilia of ergastoplasm and nucleoli. During cell division, RNA is also found in chromosomes and in the spindle. But by far the largest amount of RNA is in the cytoplasm.

Caspersson, on the other hand, had developed a microspectrophotometer with which he was able to measure light transmission at selected wave lengths in the ultraviolet on small regions of a cell. With this apparatus, Caspersson (1941) observed that the cytoplasm of animal cells contains substances which strongly absorb ultraviolet light, with an absorption spectrum similar to that of nucleic acid. Since well-known cytochemical tests indicated that DNA was not in the cytoplasm, it was inferred that the cytoplasm contains RNA. Later, the combined use of basic dyes, ultra-

violet absorption and ribonuclease test definitely confirmed that RNA is responsible for both the basophilia and the ultraviolet absorbancy of the cytoplasm (Gersh and Bodian, 1943; Davidson and Waymouth, 1946).

It had been known for a long time that the intensity of basophilia varies considerably from one type of cell to another. Chemical determination of RNA on various tissues confirmed that basophilia and gross content in RNA go together. Animal tissues can be arranged in the following way according to decreasing basophilia or RNA content : pancreas > intestinal and gastric mucosa > liver > spleen > lymph nodes, testis > kidney, muscle, heart, lung (Brachet, 1941b; Davidson and Waymouth, 1946). Brachet (1941) and Caspersson (1941) both called the attention upon the fact that the cells which contain large amounts of RNA are cells which produce large amounts of proteins. Systematic studies on specialized cells which are making protein, like silk gland or frog oocytes at the time of synthesis of yolk platelets, confirmed this rule which suggested that somehow RNA must be involved in protein synthesis. This hypothesis exerted a very stimulating effect on research in cytochemistry and biochemistry, and much of the development of RNA studies in the past twenty years is due to the recognition of a link between RNA and protein synthesis. An immediate consequence of this idea was to suspect that the ergastoplasm is the main site of protein synthesis.

B. FRACTIONATION OF CELL ORGANELLES

1. *Basic Observations*

The work of Claude (1939) on the purification of the agent of Rous papilloma led him to isolate from chick embryos ultramicroscopic particles which contained RNA, lipid and protein (Claude, 1940). A survey of many different organs of vertebrates and invertebrates, as well as plants and yeast showed that particles comparable to those isolated by Claude are present in all kinds of cells (Claude, 1943, 1944, 1946; Brachet and Jeener, 1944). It was further established in these researches that the bulk of cellular RNA is associated with these small particles. When animal tissues are homogenized in dilute salt solution, as was commonly done in these earlier works, a great variety of particles of different sizes are obtained; all of them seem to contain RNA and the smaller the particles, the greater is their RNA content (Chantrenne, 1947). The fractionation procedures used at the time were not very satisfactory. None of the well-known cell constituents, except damaged nuclei, could be recognized in the homogenates and no clear connexions were established between the fractions obtained by centrifugation and cell structures. A great progress was made when it was observed

that high sucrose concentrations in the extracting medium protect osmotically sensitive structures which would swell and be disrupted if transferred into dilute salt solutions (Claude, 1946; Hogeboom *et al.*, 1948). Under appropriate conditions it was possible to isolate from liver homogenates beside the nuclei, large particles which were easily identified as the mitochondria by their shape and their staining properties. From the supernatant, a clearly different particulate fraction was obtained; it was made of smaller particles or vesicles (Porter *et al.*, 1945) of about 200 $m\mu$ in diameter which were called microsomes. From then on, it became a common usage to fractionate tissue homogenates by centrifugation into four fractions, the nuclear, mitochondrial and microsomal fractions, and a supernatant containing the substances which are not sedimentable in one hour in the Spinco centrifuge.*

The microsomal fraction contained most of the RNA of the homogenate. As histological studies had shown that the bulk of cellular RNA is in the ergastoplasm or cytoplasmic ground substance, it was clear that the microsomal fraction was derived, for a large part at least, from the cytoplasm. High speed centrifugation of intact liver tissue indeed confirmed that RNA was bound to a sedimentable structure in the cell (Chantrenne, 1943; Claude, 1943; Brachet and Jeener, 1944; Brenner, 1947).

Fractionation of cell particulates and the availability of labelled amino acids opened a new approach to the study of the sites of protein formation within the living cell.

Several laboratories undertook kinetic studies on the incorporation of labelled amino acids into the proteins of fractions isolated by centrifugation. Labelled amino acids were injected into rats; the animals were killed 15–60 min after the injection, the liver was rapidly removed, chilled, homogenized and the suspension was separated into nuclear, mitochondrial, microsomal and supernatant fractions. Labelled amino acids were looked for and determined in the proteins of these fractions. In such experiments, Borsook *et al.* (1950), Hultin (1950), Lee *et al.* (1951, 1953), Tyner *et al.* (1953), Khesin (1954) established that all the fractions incorporate amino acids, but that liver microsomes are labelled more intensively than any of the other fractions. Allfrey *et al.* (1953) obtained similar results with mouse pancreas.

More striking were later experiments in which the liver was chilled and fractionated a few minutes after the injection of labelled amino acids (Keller *et al.*, 1954; Hultin, 1955; Loftfield, 1957). For the first ten minutes

* It should not be overlooked that these are extremely crude fractions which are not made of pure nuclei, pure mitochondria or pure microsomes. Moreover, conditions of fractionation which have been devised for rat liver, for instance, do not necessarily apply to other tissues. (For a discussion of fractionation procedures, see de Duve and Berthet, 1954.)

after injection of labelled alanine, leucine or valine, the specific activity of these amino acids in microsomal proteins was four times as high as in the supernatant proteins and eight times as high as in mitochondrial proteins. Quite similar results were obtained with plant tissues (Stephenson *et al.*, 1956). Obviously, the microsomal fraction derives from cellular structures which are more actively engaged in protein synthesis than the rest of the cell.

Several workers later succeeded in subfractionating the microsomal fraction into components which have different metabolic activities. By treating microsome preparations with deoxycholate, Littlefield *et al.* (1955) separated a nucleoprotein containing most of the RNA and a fraction made of protein and lipid. Allfrey *et al.* (1953), Daly *et al.* (1955) used ribonuclease and salt extraction to obtain microsomal subfractions, Hultin used extraction and reprecipitation with salt solutions. By a similar but somewhat more elaborate procedure, Simkin and Work (1957) separated several protein and nucleoprotein fractions. In every case, the most rapid incorporation of amino acids is observed in a protein material which remains closely associated with RNA. Moreover, during incorporation of labelled amino acids, the specific activity of the ribonucleoproteins very rapidly reaches a plateau whereas that of the other protein fractions keeps increasing slowly (Fig. 19).

If a tracer amount of radioactive amino acid is injected so as to present the tissue with a highly radioactive precursor for a very short period only, followed by dilution of the tracer, the specific activity of the ribonucleoprotein particle rises abruptly during the first few minutes and then decreases. To the contrary, the specific activity of the other microsomal subfractions (e.g. the lipoproteins) and of the soluble fraction keeps increasing for a rather long time. This is, qualitatively at least, what one would expect if a microsomal ribonucleoprotein was an obliged intermediary stage through which other protein fractions must pass (Littlefield *et al.*, 1955; Littlefield and Keller, 1957). Very clear evidence for a microsomal ribonucleoprotein intermediate in the synthesis of globin by rabbit reticulocyte was also presented by Rabinowitz and Olson (1956, 1959) and by Kruh *et al.* (1960). Similarly, serumalbumin appears first in liver ribonucleoproteins (Peters, 1957, 1959; Takanami, 1960) and it is later released as soluble protein. Antibodies are first detected in the particles of lymph nodes (Kern *et al.*, 1959).

Soluble proteins have often been found in a hidden form in the ribonucleoprotein particles of the tissue which produces them. Earlier observations of this type were made by Jeener and Brachet (1944) for haemoglobin in the 'small particles' of bone marrow cells and for the melanophore expanding hormone in particles from hypophysis. More recently, Peters (1957) and Elson (1958, 1959) found that newly made proteins can be set free by destruction of the particles and Feldman *et al.* (1960) separated

antibody from ribonucleoprotein particles of lymph nodes or spleen of immunized animals, by destroying the RNA with ribonuclease.

All the experiments reviewed above point to the microsomes and more especially to the ribonucleoprotein they contain as important centres of synthesis of soluble cell proteins within the living cell. It must be emphasized that 'microsome' is not the name of any intact cell structure. This term simply refers to a certain type of small particles which can be separated from cell homogenates by high speed centrifugation in an appropriate medium. Microsome pellets are jelly-like, transparent and slightly coloured. When redispersed in aqueous medium they form an opalescent suspension in which regular light microscopy or even phase contrast does not reveal

FIG. 19. *In vivo* incorporation of ^{14}C leucine into microsomes of rat liver fractionated with sodium deoxycholate.

(a) Saturating dose of ^{14}C leucine.

(b) Tracer dose of ^{14}C leucine.

(Littlefield *et al.*, 1955).

any structure. Dark field examination shows a great number of particles animated with intense brownian motion.

No such particles can be observed in living cells and it has long been suspected that microsomes form after disruption of the cell, during the dispersion of the cell contents in the extracting medium (Brachet and Jeener, 1944; Claude, 1947; Porter, 1953). The fact that they contain most of the cellular RNA indicated that microsomes originate from the ergastoplasm, also called cytoplasmic ground substance.

Thanks to the development of electronmicroscopy of ultrathin tissue sections (Porter and Blum, 1953; Sjöstrand, 1953), knowledge of the structure of cytoplasm made great progress during the last few years. A network of lamellar formations was observed in the ground substance of

many various cells; it is especially developed in secretory cells like liver or pancreas. This structure was described as a system of double lamellae (Sjöstrand and Hanzon, 1954; Bernhard *et al.*, 1954) or of elongated vacuoles forming a reticulum (Porter, 1953). Palade and Porter (1954) observed that the membranes of the reticulum are lined with small granules of a rather uniform size (100–150 Å in diameter). The number of these granules seems to be correlated with the basophilia, i.e. the RNA content of the tissue.

By electronmicroscopy, Slautterback (1953) observed that isolated mouse liver microsomes contain essentially two types of structures: rather large formations of about 0.1 μ and smaller dense particles associated with the larger ones. Better pictures of microsomal fractions isolated from rat liver showed that they were made of more or less swollen vesicles of various sizes (Kuff *et al.*, 1956) corresponding probably to the larger of Slautterback's particles. Littlefield *et al.* (1955) examined microsomal preparations with the electron microscope before and after deoxycholate treatment. Although the pictures they obtained were rather poor, it was clear that deoxycholate treatment destroys or dissolves the larger components and leaves the smaller particles. Parallel centrifugation experiments indicated that the smaller particles were the ribonucleoproteins which had been shown to be the most active in protein synthesis. Excellent systematic studies by Palade and Siekevitz (1956), in which centrifugation, electron microscopy and biochemical methods were combined, definitely established that the so-called microsomal fraction of liver results from the disruption and dispersion of the ergastoplasm or endoplasmic reticulum into the homogenization medium. The trabeculae and cisternae observed on sections of osmic acid fixed tissue are found in the homogenates as vesicles in the microsomal fraction. These vesicles react like osmometers to changes of sucrose or salt concentration, and they dissolve in deoxycholate. The Palade granules which line the lamellae of the reticulum do not dissolve; these are the ribonucleoprotein particles, most of which are normally attached to the lamellae *in vivo*. It is possible, however, that the membranes of the microsomal vesicles also contain some RNA (Moulé *et al.*, 1960).

In guinea pig pancreas, the picture is similar, except that many free ribonucleoprotein particles are observed between the lamellae, outside the cisternae. Very large dense granules are seen within these cavities; these are secretory granules at different stages of formation, i.e. packages of zymogens which are the main proteins produced and excreted by the pancreas (Siekevitz and Palade, 1958). In kinetic experiments in which labelled amino acids are injected to the animal, the ribonucleoprotein particles are the first to contain labelled polypeptides and also labelled zymogens. Siekevitz and Palade (1958) have very good evidence that in

the pancreas the finished proteins arise in the ribonucleoprotein particles attached on the outer surface of the cisternae, and that these proteins pile up as zymogen granules inside the cisternae (Siekevitz, 1959; Hirsch, 1960). The changes in intracellular distribution of pancreatic amylase during the secretion cycle observed by Laird and Barton (1958) are in agreement with this picture of the secretory process.

The ergastoplasm of secretory cells can be visualized as a highly organized system for the manufacture, storage and delivery of the protein secretion.

It is important to realize that ribonucleoprotein particles are only one of the constituents of microsomal fractions, and of the ergatoplasm. In order to avoid a very frequent confusion between 'microsomes' and 'ribonucleoprotein particles', it has been proposed to reserve the name 'ribosomes' for the discrete ribonucleoprotein particles (Roberts, 1958). This is the more justified in that ribosomes seem to be a fundamental constituent of all cells, whereas the structure of the ergastoplasm and the properties of the 'microsomal fraction' *in vitro* differ considerably from tissue to tissue, and are not found in bacteria.

2. General Occurrence of Ribosomes

Electronmicroscopical studies on some forty types of mammalian tissues (Palade, 1955) revealed the presence of these dense granules in all the cells examined except mature red blood cells of the rat which, incidentally, make no protein. Part of the ribosomes are associated with the ergastoplasmic lamellae, but part are free. The distribution of ribosomes is largely similar to that of cytoplasmic basophilia, i.e. to the distribution of RNA. As the size and composition of the ribosomes is very uniform, the general relationship between the amount of RNA and the intensity of protein synthesis, which was pointed out by Brachet and by Caspersson, can now be expressed in newer terms as follows: *In animal tissues, the intensity of protein synthesis is related to the number of ribosomes. These are sites of protein formation.*

Nucleoprotein particles similar to the Palade granules have been isolated from the cytoplasm of plant tissues (Ts'O *et al.*, 1956; Ts'O, 1958) and from micro-organisms.

The association of part of the RNA of yeast with small particles has been known for a long time (H. Chantrenne, 1943, 1944; Schachman *et al.*, 1952). Yeast ribosomes were purified and studied more recently by physico-chemical methods (Chao and Schachman, 1956). They make up a population of nearly spherical particles with a sedimentation constant of about 80 S and a molecular weight in the range of $4 \cdot 10^6$. About 40 per cent is accounted for by RNA, the rest is protein. The stability of the particles depends very much on salt concentration, and especially of the presence of

FIG. 20. A. Section through a guinea pig pancreatic cell showing a few mitochondria with their inner cristae, and the endoplasmic reticulum lined with ribosomes. Free ribosomes can be seen between the cisternae. The large intracisternal granules are the secretory products. Magnification: $\times 22,000$ (courtesy Dr G. E. Palade).

FIG. 20. B. Microsomes from the guinea pig pancreas, showing vesicles and ribosomes. Magnification: $\times 56,000$ (courtesy Dr G. E. Palade).

FIG. 20. C. Rat liver ribosomes detached from microsomes by deoxycholate treatment. Magnification: $\times 84,000$ (Kirsch *et al.* 1960) (courtesy Dr G. E. Palade).

magnesium ions. At low magnesium concentrations, the particles dissociate into nucleoprotein fragments (Chao, 1957).

Yeast and moulds contain nuclei, mitochondria and ribosomes which resemble those of higher organisms (Yotsuyanagi, 1955, 1956; Shatkin and Tatum, 1959; Blondel and Turian, 1960). In bacteria, no mitochondria have been recognized, the nuclear apparatus is not very sharply separated from the surrounding material, no nuclear membrane and no structure resembling the endoplasmic reticulum has been found. However, electron microscopy on thin sections of bacteria shows a very dense population of particles which resemble the ribosomes of animal cells (Bradfield, 1956). In bacterial extracts, up to 85 per cent of the RNA is associated with sedimentable particles (Schachman *et al.*, 1952). Much attention is now being paid to these long neglected bacterial particles (see Roberts, 1958).

The ribosomes isolated from *Azotobacter vinelandii* (Gillchriest and Bock, 1958) or from *E. coli* (Bolton *et al.*, 1958; Wagman and Trawick, 1958; Tissières *et al.*, 1959; Spahr and Tissières, 1959) are very similar to the yeast particles. Ribosomes from liver and from micro-organisms are quite comparable in size range, composition, response to magnesium ion concentration and behaviour in the ultracentrifuge (Hall and Doty, 1958; Peterman *et al.*, 1958). Cheng (1957) remarked that the RNA content of a ribosome of rat liver, pea seedlings and yeast as well, can be estimated as equal to $1.7 \cdot 10^6$ as expressed in molecular weight units. The same figure was later found for certain ribosomes of *E. coli* (Tissières and Watson, 1958), tobacco plant (Gierer, 1958) and mouse brain (Cheng, 1960). It would seem therefore that a class of ribosomes might all contain about the same absolute amount of RNA. Another striking coincidence is that the ribonucleoprotein viruses so far studied in this respect also contain one RNA molecule with a molecular weight of about $2 \cdot 10^6$ per virus particle. One should probably not give too much weight to a coincidence of numbers which may still be fortuitous. The more so, in that ribosomes can dissociate into smaller particles (Elson and Tal, 1959; Hall and Slayter, 1959; McCarthy, 1960; Huxley and Zubay, 1960) and sometimes constitute a physiologically heterogeneous population (Siekevitz and Palade, 1959). Nevertheless, Chen's observations raise the question of the existence of a RNA 'quantum' and make one suspect similarities of structure and function between ribosome RNA and virus RNA.

In animal cells, the ribosomes of the cytoplasmic ground substance are certainly an important part of the protein-making system, they are most probably the sites where polypeptides arise. Their function in bacteria was more difficult to ascertain. The total amount of ribosomes is greatest when the bacteria are in the logarithmic phase of growth (Mendelsohn and Tissières, 1959). But no correlation was found between the concentration

of the 40 S compound and enzyme synthesis (Wade and Morgan, 1957; Bowen *et al.*, 1959). Protein synthesis is so fast in growing bacteria (Roberts, 1957) that detailed kinetic studies on the incorporation of amino acids into bacterial fractions comparable to the studies which were so successful with rat liver, at first failed to show any difference in rate of protein synthesis between ribosomal and soluble proteins, and many workers thought that ribosomes in bacteria are of no great interest and even that their RNA might be a waste product. Finally, McQuillen *et al.* (1959) clearly established that when radioactive methionine is added to exponentially growing *E. coli*, the radioactivity of the 80 S ribosomes is built up to saturation within 5 sec, perhaps less, and that it disappears equally rapidly when the tracer amino acid is diluted out by addition of non-labelled methionine. The lost radioactivity appears in soluble proteins. The substance which is rapidly labelled in the microsome has the chemical properties of a polypeptide and it behaves like a precursor of soluble protein. Beside this transient protein which is continuously chased by nascent molecules, another ribosome protein forms more slowly; it is a permanent constituent of the ribosome structure the synthesis of which reflects ribosome formation in the growing bacterial population.

Ribosomes can now be regarded as an essential piece of the protein forming system in bacteria and in the cytoplasm of mould, plant and animal cells. It is quite possible that no protein synthesis takes place except on ribosomes, for particles which behave like ribosomes have been isolated from nuclei (Frenster *et al.*, 1960), and there are indications of their being responsible for protein synthesis in these organelles as well. The existence of ribosomes within mitochondria, however, is not quite clearly established, although mitochondria do make proteins (see p. 53).

C. PROTEIN SYNTHESIS IN ISOLATED CELL FRAGMENTS AND ORGANELLES

1. *Protein Synthesis in Enucleate Cytoplasm*

It is clear from the experiments which have been reported above that the cytoplasmic ground substance is the main site where proteins arise in most animal cells. On the other hand, it is well established that the primary structure of proteins is controlled by the nuclear genes. One then wonders how immediate is the nuclear control upon protein synthesis.

In all the *in vivo* experiments that have been considered so far, protein synthesis took place in the intact cell; and even though the cytoplasm was the first site of protein appearance, the nucleus was always present, and one may wonder to what extent it was involved in the process. This problem

could be partly solved by studying protein synthesis in certain types of cells which can easily be cut into two parts, one of which is devoid of nucleus. The enucleate fragments survive for some time and it is possible to establish directly whether protein synthesis can take place in the absence of a nucleus.

Amoeba proteus was used by Brachet (1955) and by Mazia and Prescott (1955) for such experiments. The nucleus of this *Amoeba* is usually located in the rear part of the cell when the animal is moving about on a glass surface. When the cell is cut into two approximately equal parts by means of a thin glass rod, the fragment which contains the nucleus retains all the morphological characters and the habits of a normal *Amoeba*. Nucleate fragments thrust out pseudopodia, crawl about and catch ciliates or other prey upon which they feed. To the contrary, enucleate moieties are rather inert; they assume a nearly spherical shape shortly after sectioning, lose the ability to form pseudopodia and to secure food. Actually, enucleate amoeba fragments are always starved; they survive nevertheless for 10–14 days, before collapsing. In all the experiments with *Amoeba* fragments, the nucleate part to which the enucleate halves are compared must naturally also be deprived of food.

Starvation is definitely not favourable to protein synthesis. In spite of this, it was possible to obtain very interesting information on the presently discussed question with this material. As can be expected, the total amount of protein decreases in starved *Amoeba*. Brachet (1955) observed that total protein diminishes more rapidly in enucleate fragments than in starved whole *Amoeba* or in starved nucleate fragments. By following, in the course of time, the changes in several individual enzymes in both types of fragments, Brachet showed that the removal of the nucleus results in widely different effects in the case in the individual enzymes studied.

Thus an acid phosphatase, a dipeptidase and an esterase decreased much faster in enucleate fragments than in nucleate parts, but other enzymes, like a protease, enolase, adenosine triphosphatase and the respiratory system were not more labile in the absence of the nucleus than in its presence during starvation. It would seem therefore that the maintenance of the individual cytoplasmic proteins is not equally dependent on the presence of the nucleus (Brachet, 1955, 1956, 1957).

Although a net decrease of protein material is always observed in starved *Amoebae*, some anabolic processes can nevertheless be observed. Mazia and Prescott (1955), Mazia (1956), Ficq (1955) and Brachet and Ficq (1956) showed that labelled methionine or phenylalanine are incorporated into cytoplasmic proteins. The incorporation is 50 per cent lower in enucleate fragments than in nucleate moieties or in intact *Amoebae*. However reduced, the incorporating capacity is maintained for up to 10 days after

enucleation. This clearly indicates that the cytoplasm contains a complete system for incorporating amino acids into protein material and that the pathway for this residual incorporation does not go through the nucleus.

The drop in incorporating activity after enucleation might be due to indirect effects, irrelevant to a nuclear control of cytoplasmic protein synthesis, for many changes occur in the general metabolism of the *Amoeba* as a result of enucleation. The enucleate part loses, for instance, the capacity to use its lipid and glycogen stores, and it is heavily handicapped as against nucleate parts for so basic a function as glycolysis (Brachet, 1955, 1956, 1957; Brachet and Chantrenne, 1956; Chantrenne, 1958). On the other hand, the fact that the maintenance of various proteins is not equally dependent on the presence of the nucleus makes one suspect that the cytoplasm can make proteins but that a considerable fraction of the proteins normally present in the cytoplasm of the amoeba are made under very close nuclear control and possibly within the nucleus itself.

Another material which is very well suited for merotomy experiments is the green marine alga *Acetabularia mediterranea*, which belongs to the class of Dasycladaceae. During most of its life, the alga consists of a rather thick filament or stalk attached to the rocks at one extremity by rhizoids. The stalk can be 1–2 in. long; it is made of one single cell, the nucleus of which is always located in one of the rhizoids. At the end of the life cycle, the tip of the stalk develops into an umbrella. When this is fully grown, the big nucleus divides into many small nuclei which invade the cytoplasm and finally reach the umbrella where they become enclosed in the cysts. These are the resistant form of the organism; each contains a few nuclei. After a maturation period, the cysts can germinate and liberate flagellate gametes. Conjugation of two gametes gives a uninucleate zygote. This grows into rhizoids and a stalk, which will later develop an umbrella (see e.g. Kühn, 1955). The complete cycle takes one year in nature and about six months in the laboratory.

During the part of the life cycle which extends from the formation of the zygote up to the complete development of the umbrella, *Acetabularia* is a single cell; the nucleus is located in a rhizoid and the cytoplasm is uniformly filled with chloroplasts. The cell is surrounded by a tough polysaccharide casing. If the alga is cut across the stem, both fragments recover very well and can be kept alive for more than two months. The fragment with the rhizoids contains the nucleus, the other one is purely cytoplasmic. Both fragments are amply provided with chloroplasts, and photosynthesis is not impaired by enucleation (Brachet *et al.*, 1955). Extremely interesting experiments were performed on *Acetabularia* by Hämmerling (1934, 1946, 1953) and by his collaborator Beth (1943). If the nucleate part of one strain is grafted on an enucleate part of another strain of *Acetabularia*, the

morphology of the umbrella which develops corresponds to the strain of the nucleate part. Since the structure of the umbrella is a hereditary character, it is quite clear that genetic determinants are provided by the nucleate moiety. This is important for the present discussion.

In his early studies, Hämmerling (1934) had already observed that enucleated fragments can elongate for some time and that the number of chloroplasts increase. This made it probable that proteins were formed in

FIG. 21. Life cycle of *Acetabularia mediterranea* (courtesy of J. Brachet).

cytoplasmic fragments. Direct biochemical studies by Brachet and his collaborators established that the rate of incorporation of $^{14}\text{CO}_2$ or of labelled glycine into the proteins of *Acetabularia* is not changed in the enucleate fragment. The rate of incorporation is the same in both halves at least during the first two weeks after cutting (Brachet and Chantrenne, 1951, 1952; Chantrenne *et al.*, 1953). The total protein content increases at the same rate in nucleate as in non-nucleate fragments (Vanderhaeghe,

1954; Brachet *et al.*, 1955; Clauss, 1958). This means that in *Acetabularia* a complete system for making protein material is contained in the cytoplasm and can operate in the absence of the nucleus.

Studies on non-nucleate fragments of sea urchin eggs (Malkin, 1954) or of newt eggs (Tiedemann and Tiedemann, 1954) also showed that various labelled protein precursors can be incorporated into proteins in a cytoplasm without a nucleus. Rabbit reticulocytes is another example of an enucleate cell which can incorporate labelled amino acids into its proteins (London *et al.*, 1950; Borsook *et al.*, 1952; Koritz and Chantrenne, 1954; Nizet and Lambert, 1953).

However, in all the experiments mentioned above there was no evidence that the protein material synthesized in the absence of the nucleus was made of normal protein. Some data on reticulocytes made it probable that regular haemoglobin was made in these enucleate cells (Hämmarsten *et al.*, 1953). But this important question has been answered only recently in studies on the net synthesis of specific enzymes in fragments of *Acetabularia*. Thus enolase is made in the absence of the nucleus at the same rate as total protein material for at least eleven days after enucleation (Baltus, 1959) and net synthesis of phosphorylase and of invertase goes on at an almost normal rate for two or three weeks in enucleate cells (Clauss, 1959). *This means that cytoplasmic fragments of Acetabularia contain a perfect and complete system for making certain proteins, including the genetic information required for the synthesis of active enzymes.*

This conclusion must, however, be qualified. Protein synthesis is independent of the nucleus *for some time only*: a change occurs in the non-nucleate fragments about two weeks after the algae have been cut. Between the twelfth and the fifteenth days after section, the rate of incorporation of $^{14}\text{CO}_2$ into the non-nucleate fragments drops to about 30 per cent below that of the nucleate halves, and net synthesis of proteins stops altogether, although the enucleate fragments can survive for at least two months thereafter (Brachet and Chantrenne, 1951; Vanderhaeghe, 1954; Brachet *et al.*, 1955; Richter, 1959). Protein synthesis in older algae is more independent of the nucleus than in younger ones (Hämmerling, 1956). It would appear that some substance produced by the nucleus is required for maintaining protein synthesis and that this substance is slowly exhausted when the cytoplasm is deprived of the nucleus. There is, however, no evidence that this hypothetical substance is a specific gene product: it might be quite a common biochemical originating in the nucleus (Brachet, 1952, 1954; Brachet and Chantrenne, 1956).

Moreover, in *Acetabularia* as well as in *Amoeba*, the formation of certain proteins, e.g. acid phosphatase (Keck and Clauss, 1958) appears to depend more directly on the presence of the nucleus; for it stops shortly after enucleation, i.e. at a time when the bulk of cytoplasmic proteins and

enzymes are still being synthesized at a normal pace. Some other species of algae, e.g. *Spirogyra* (Van Wysselingh, 1908) or *Elodea* (Yoshida, 1959) behave probably very much like *Acetabularia*; others, like *Micrasterias* (Waris, 1951), do not stand enucleation as well.

It remains that enucleation experiments establish that in several of the cell types examined a large part of the cytoplasmic proteins can be made in the absence of the nucleus. The cytoplasm must therefore contain centres of synthesis which can produce perfect protein molecules and are able to operate in the absence of the nucleus for a considerable time period. This conclusion has received new support from recent experiments on isolated mitochondria, which will be examined presently.

2. Protein Synthesis in Isolated Mitochondria

Kinetic studies on amino acid incorporation into cellular components *in vivo* pointed to the microsomes as the most active centres of protein synthesis. In liver, kidney or pancreas, amino acid incorporation into proteins of the mitochondria represents but a minor fraction of total incorporation and for a long time mitochondria did not retain the attention as sites of protein synthesis. In muscle, however, the picture is different. Mitochondria come close to microsomes in rate of amino acid incorporation *in vivo*. Indeed, for the first five minutes, incorporation into the mitochondrial fraction is somewhat higher than in the microsomes (Simpson and McLean, 1955).

Thanks to studies on oxidative phosphorylation in isolated mitochondria, conditions have been worked out for isolating and preserving mitochondria *in vitro* in relatively good conditions (e.g. Slater and Holton, 1954). In such preparations from muscle and even from liver, a fairly rapid incorporation of amino acids into protein material could be observed (McLean *et al.*, 1958; Campbell and Greengard, 1959; Reiss *et al.*, 1959). Incorporation was later shown to take place in well defined proteins, like cytochrome-*c* (Bates *et al.*, 1958, 1960) and this line of research culminated in the observation of the net synthesis of cytochrome-*c* in isolated heart muscle mitochondria (Bates and Simpson, 1959). A weighable amount of cytochrome-*c* was actually synthesized *in vitro* in these experiments, and good evidence was obtained that a synthesis of the protein moiety was taking place in the process.

This is a very important achievement for several reasons. It shows that the ergastoplasm is not the only centre of cytoplasmic protein synthesis. On the other hand, these experiments, like those on enucleate cytoplasm, show that complete systems for making specific proteins exist in cytoplasmic organelles and can accomplish their function without the direct participation of the nuclear genetic material. Liver or muscle mitochondria are relatively large elaborate structures and attempts have been made at

locating, among the mitochondrial components, the first centres of protein synthesis. The results at the time of writing are not quite clearcut. Rendi (1959, 1960) has isolated from disrupted mitochondria a sedimentable fraction which contains most of the RNA of the mitochondria and may have some similarity with ribosomes. On the other hand, Kalf and Simpson (1959) showed that when mitochondria are exposed to sonic vibrations followed by differential centrifugation, components which are not sedimented in 8 hrs at 105,000g possess a pronounced capacity to incorporate amino acids into protein material. The protein synthesizing system in this supernatant must comprise RNA as an essential part, for ribonuclease suppresses the amino acid incorporation (see also Kalf *et al.*, 1959). The discrepancy between these two results probably reflects differences in degree of disorganization of the preparations used. Both groups of work, however, point to some RNA containing element as being again at the heart of the process of protein synthesis.

3. *Protein Synthesis in Chloroplasts*

In green plant tissues, much less information has been obtained so far on the kinetics of amino acid incorporation. Stephenson *et al.* (1956) and Sissakian (1957) studied the incorporation of labelled amino acids *in vivo* into various cell constituents of tobacco leaves and found that microsomes are preferentially labelled and contain the bulk of radioactivity for the first few minutes, but that chloroplasts thereafter incorporate amino acids at a fairly high rate, as if they were also important centres of protein synthesis. Plashkov and Ivanko (1956) found the highest *in vivo* incorporation of methionine into chloroplasts and mitochondria. Racusen and Hobson (1959) using $^{14}\text{CO}_2$ as the precursor found no significant difference between the synthesis of chloroplast protein and the other proteins, in swiss chard leaves.

That chloroplasts and microsomes contain independent centres of protein synthesis was actually established by earlier data which passed unnoticed (Chantrenne *et al.*, 1953; Brachet *et al.*, 1955). In parallel experiments in which $^{14}\text{CO}_2$ and $^{14}\text{glycine}$ were used as precursors, it was found that glycine incorporation is more rapid in microsomes than in chloroplasts, whereas $^{14}\text{CO}_2$ to the contrary is more rapidly incorporated into the proteins of the chloroplasts than into microsomal protein. This clearly indicates that amino acids which arise within the chloroplasts as a result of photosynthetic carbon dioxide fixation can be used for protein synthesis directly within the chloroplast without going through the microsomes. Since, on the other hand, an exogenous amino acid is incorporated more readily in the microsomes than into the chloroplasts, it must be concluded that microsomes and chloroplasts are two independent sites of protein synthesis.

4. *Synthesis of Protein in the Cell Nucleus*

Cytochemical data, especially observations of amino acid incorporation into cell protein by autoradiographic techniques, indicated that the nucleus is not as active a centre of protein synthesis as the ergastoplasm. Nevertheless, labelled amino acids were found within a relatively short time in the nuclear protein; and in liver and lymph nodes, the *in vivo* incorporation of labelled phenylalanine into the nucleus, as judged from radioautography experiments, represents a considerable fraction of total incorporation (Ficq and Brachet, 1956; Ficq, 1959; Ficq and Errera, 1959; Gavosto and Ficq, 1954; Zalokar, 1960b). More recent observations (Zalokar, 1960a) on *Drosophila* show that protein synthesis begins a little later in the nucleus than in the cytoplasm.

The drop in amino acid incorporation following enucleation of *Amoeba* (Mazia and Prescott, 1955), the decrease of certain enzymes under the same conditions (Brachet, 1955–1957) or the passage of ^{35}S labelled proteins from a grafted labelled nucleus to the unlabelled nucleus (Goldstein, 1959) could be interpreted as indications that certain proteins were made in the nucleus. Daly *et al.* (1952) showed that certain nuclear proteins of various mouse tissues incorporate labelled glycine *in vivo* at a rate comparable with the average cytoplasmic proteins. Similar results were later obtained by Smellie *et al.* (1953) for rat liver. It is not quite clear, however, whether the labelled proteins found in the nuclei were made within the nuclei, or whether they originated in the cytoplasm.

Allfrey (1954) succeeded in isolating nuclei of thymus which are able to incorporate amino acids *in vitro* into their proteins. Adequate preparations can be obtained by fractional centrifugation of a calf thymus homogenate in sucrose solutions. Allfrey *et al.* (1955, 1956, 1957) thoroughly studied the requirements for the incorporation of labelled amino acids and established convincingly that labelled alanine, glycine and lysine are incorporated to various extents into several typical nuclear proteins in isolated thymus nuclei. Radioautography experiments (Ficq and Errera, 1958, 1959) confirmed that the incorporation (of phenylalanine) really occurs in clean nuclei and is not due to contamination with remnants of cytoplasm or with thymocytes which are usually present in small numbers in the preparations. Since only the L-amino acid isomers are taken up, and since an amino acid once incorporated into protein is not exchanged with free amino acids in the medium, it can be taken as most probable that the incorporation represents true synthesis of nuclear proteins (Allfrey *et al.*, 1957).

Thymus nuclei were for several years the only type of nuclei with which incorporation could be observed *in vitro*. Logan *et al.* (1959) recently obtained similar results with isolated nuclei from rat liver. All these observations make it clear that nuclei contain all that is necessary for

incorporating amino acids into proteins and most probably possess a complete system for the synthesis of proteins.

Summing Up

From the results reviewed in the foregoing pages, it is clear that there are many sites of protein synthesis in the living cell. Nuclei, mitochondria, chloroplasts make their own protein constituents. In bacteria, the cell membrane appears as a site of intense protein formation, but the ribosomes are the most actively engaged in protein synthesis. The cytoplasmic ground substance of higher organisms is the site of synthesis of the bulk of the proteins of many cells. In the ground substance, protein appears first in the ribonucleoprotein particles (ribosomes) which are responsible for the production of the soluble cytoplasmic proteins. In secretory cells, the ergastoplasm has a remarkably elaborate structure which probably plays a part in the process of secretion and with which many ribosomes are associated.

Ribosomes are in all cases an essential part of the protein-making machinery.

CHAPTER III

Nucleic Acids and Protein Synthesis

A. THE POSITION OF DNA IN PROTEIN SYNTHESIS

For reasons which have been considered in Chapter I of the present book, it is almost certain that DNA ultimately controls the primary structure of the individual proteins. A question then arises — are the amino acids arranged in the correct sequence under the immediate action of the gene, is specific DNA the protein-forming system?

At the present time it is convenient to examine separately the data obtained with higher organisms and with bacteria.

1. *Higher Organisms*

In animal or plant tissue, asking whether DNA is directly and 'personally' involved in assembling the proteins amounts to asking whether all proteins are made in the chromatin. From what we have seen in the preceding chapters, the answer to this question is *definitely negative*. Complete proteins endowed with their normal biochemical properties or enzymic activity can be made in the absence of the nucleus, e.g. in isolated pieces of cytoplasm or in isolated mitochondria, which contain no DNA. Most cytoplasmic proteins are made in the cytoplasm, and DNA is not a constituent of the system which is immediately involved in their making.

The synthesis of nuclear proteins must be examined more closely. Fractionation of isolated nuclei after incorporation of amino acids indicated that the rate of labelling differs considerably among the various nuclear proteins. Histone is almost completely inert, whereas two protein fractions which are found in close association with DNA and with RNA respectively incorporate amino acids very rapidly.

In vitro, the incorporation is inhibited and finally suppressed in the presence of deoxyribonuclease, which destroys DNA. The degree of impairment becomes greater as more and more of the DNA is depolymerized and removed from the nucleus. However, the amino acid incorporating ability is not irreversibly destroyed by treatment with deoxyribonuclease, for it can be largely restored by the addition of a DNA supplement (Allfrey *et al.*, 1955; Mirsky *et. al.*, 1956).

These very striking results at first were taken as direct evidence that

DNA is required for protein synthesis in isolated nuclei, and that DNA is involved personally in the process. However, further data from the same laboratory (Allfrey and Mirsky, 1957) showed that after inhibition by deoxyribonuclease, amino acid incorporation into nuclear proteins can be restored by partially degraded DNA just as well as by native DNA. Even RNA would restore the amino acid uptake. More strangely still, polyadenylic acid, heparin, chondroitinsulphate and as unnatural a polyanionic compound as polyethylene sulphonate could restore much of the incorporating capacity of deoxyribonuclease inactivated nuclei. If polyethylene sulphonate is added immediately after the depolymerization of DNA has occurred, it is possible to remove 75 per cent of the DNA without change in nuclear ability to incorporate amino acids into protein (Allfrey and Mirsky, 1959).

Moreover, protein synthesis is not the only function of isolated nuclei to be impaired by deoxyribonuclease; the enzyme deeply damages other metabolic processes in nuclei and especially oxidative phosphorylation (Allfrey and Mirsky, 1959). Again, exogenous DNA, RNA and polyanionic substances restore ATP production. The effects on amino acid incorporation might thus be a mere consequence of the inhibition of phosphorylation.

The experiments with isolated nuclei cannot be taken any more as evidence of DNA being directly involved in the synthesis of nuclear proteins. To the contrary they underline that the presence of intact DNA is unimportant for the incorporation process, since DNA can be substituted for by any one of several polyanionic substances including synthetic resins.

It is not known how deoxyribonuclease inhibits phosphorylation. A possibility is that deoxyribonuclease would bind reversibly some negatively charged compounds like phospholipids or perhaps some enzyme involved in the process, and could be displaced by an excess of exogenous polyanionic compounds (cf. Sekiguchi and Sibatani, 1958).

Whatever the explanation of deoxyribonuclease inhibition proves to be, one must admit that there is at present *no evidence* that DNA is directly involved in the synthesis of even nuclear proteins.

2. *Bacteria*

The most recent studies on DNA formation indicate that at the level of individual bacteria in exponentially growing cultures the synthesis of DNA is essentially continuous (Schaechter *et al.*, 1959; Young and Fitz-James, 1959; McFall and Stent, 1959). Under the usual laboratory conditions, protein synthesis and DNA synthesis thus occur simultaneously in bacteria. It is possible, however, to dissociate protein synthesis from DNA formation in a number of different ways. Thymidine starvation of

Lactobacillus acidophilus suppresses DNA synthesis, but the bacteria continue to make RNA and protein. No division occurs, and the bacteria grow into long filaments. Under conditions which prevent any measurable synthesis of DNA, the amount of protein can increase by a factor ten (Jeener and Jeener, 1952). Similar observations were made later by Cohen and Barner (1955) with thymidine requiring strains of *E. coli*. In these experiments the synthesis of enzymes was studied, and the results clearly showed that perfect enzymatically active proteins can be made in the absence of DNA synthesis. Very small doses of ultraviolet light (257 m μ) inhibit DNA synthesis without preventing protein formation (Kelner, 1953; Kanazir and Errera, 1954). High doses of X-rays can abolish DNA synthesis completely without inhibiting the formation of enzymes, in bacteria and yeast (Baron *et al.*, 1953; Chantrenne and Devreux, 1959). Mustard gas also inhibits DNA synthesis in *E. coli*, without impairing β -galactosidase formation (Pardee, 1954), and the slight inhibition of protein synthesis by N-mustard, which is observed occasionally, is due to general toxicity (Clark *et al.*, 1957).

All these data constitute good evidence that the *synthesis* of DNA is not necessary for protein synthesis in bacteria. This conclusion is an interesting one, but more important still would be to establish whether the structural integrity of the DNA gene is necessary for continued protein production, since information relative to protein primary structure is contained in DNA.

When micro-organisms are irradiated with heavy doses of X-rays, their DNA may be damaged. DNA extracted from yeast which had received 200,000 r was degraded in such a way that it could not be precipitated any more by acid (Chantrenne, 1958; Chantrenne and Devreux, 1959). This depolymerization of DNA may not be a direct effect of ionizing radiations; it may be due to the action of some nucleases released in the cell by membrane breakdown (F. Hutchinson, personal communication). Whatever the mode of action of the X-rays may be, yeast cells containing largely degraded DNA were able to produce proteins at a normal rate; actually the synthesis was somewhat stimulated.

The first studies on the effect of DNA removal or DNA destruction on protein synthesis in bacterial systems are due to Gale. *Staphylococcus aureus* cells disrupted by supersonic vibrations are able to make enzymes (Gale and Folkes, 1955). It is possible to remove nucleic acids (both RNA and DNA) from such preparation by M NaCl extraction at 37° and the degree of nucleic acids depletion can be controlled to a certain extent. It was found that removal of the nucleic acids progressively reduces enzyme production. The synthesis of various enzymes can be restored by adding RNA if the depletion has been moderate, but DNA becomes an additional requirement when nucleic acid depletion has been severe. This

would indicate that DNA is required for the synthesis of certain enzymes. The results, however, would be very interesting if the system required a quite specific type of DNA. In some experiments it looked as if DNA from the same bacterial species only was able to reactivate protein synthesis. However, substances were later found among the split products of yeast RNA which are able to reactivate the system just as well as nucleic acids. The substances involved have already been purified to a considerable extent by Gale but they are not as yet identified. They are low-molecular-weight substances of very high activity. The meaning of these very intriguing results is not clear at present (Gale, 1956, 1957, 1958); the situation in this system reminds one to some extent of the case of isolated thymus nuclei mentioned before; an agent which destroys DNA suppresses protein synthesis, but this process can be restored by other substances beside DNA. Other experiments which are in some way comparable to Gale's were made by Spiegelman on protoplasts of *B. megaterium* (Spiegelman, 1957; Landman and Spiegelman, 1955). Lysozyme catalyses the hydrolysis of cell walls of various bacteria. When living *B. megaterium* or *B. subtilis* are treated by lysozyme, their cell wall dissolves more or less completely; if the osmotic pressure of the medium is sufficiently high, the bacterial content is not dispersed in the medium and the bacterial body changes into spherical droplets, the protoplasts (Weibull, 1953), which preserve most of the biochemical activities of intact bacteria, including the synthesis of proteins (Lester, 1953; Beljanski, 1954; Bridoux and Hanotier, 1954; McQuillen, 1955), including active enzymes (Wiame *et al.*, 1955), and bacteriophage production (Brenner and Stent, 1955; Salton and MacQuillen, 1955). Treatment of the protoplasts by deoxyribonuclease under suitable conditions (Spiegelman, 1957) results in the removal of up to 99 per cent of the DNA; this has no adverse effect on enzyme synthesis, which is often stimulated. Since deoxyribonuclease splits the DNA molecule into very small pieces, it is obvious that the physical integrity of DNA is not required for enzyme production in these protoplasts. Spiegelman went a step further in the dissociation of the system in using protoplasts which had been broken by osmotic shock. From the disintegrated protoplasts, sedimentable systems were obtained which retained the ability to make enzymes. Like Gale's disrupted *Staphylococci*, these preparations consisted of membranous bodies containing only a small fraction of the original DNA of the protoplasts. Treatment by deoxyribonuclease removed most of the residual DNA and did not impair enzyme synthesis. It must be mentioned, however, that much DNA was reformed in these disrupted preparation at the same time as enzymes were produced.

It would seem that DNA is not a rate limiting factor of protein synthesis in disrupted bacterial preparation since DNA can be damaged and elimin-

ated almost completely without effects on enzyme synthesis. These works indicate that in bacteria as well as in higher organisms many proteins can be made in the absence of DNA.

A completely different approach to the same problem was that of Fuerst and Stent (1956), who studied the consequences of breakages in DNA chains caused by the decay of ^{32}P previously incorporated in the macromolecule. *E. coli* was grown in a medium containing phosphate of fairly high specific radioactivity. The bacteria were then frozen and kept at a very low temperature in order to interrupt all metabolic activities and let the ^{32}P decay. After periods of time sufficient for a notable fraction of the ^{32}P to have disintegrated, the bacteria were plated and tested for viability (ability to form a colony). The experiments showed that the bacteria lose their viability as a result of ^{32}P decay. With thymidine-less mutants in which the synthesis of DNA can be controlled, it could be shown that the loss of viability is largely due to the decay of ^{32}P atoms in DNA.

A very striking fact is that the capacity of making β -galactosidase is lost at the same pace as viability, although this enzyme is not necessary to cell maintenance or multiplication under the conditions used. Moreover, a few hundred disintegrations of ^{32}P per bacterium in the DNA are enough to reduce the fraction of viable bacteria, and the enzyme production to 1 per cent. Since *E. coli* contains several thousand ^{32}P atoms in DNA, this indicates that a relatively small damage to DNA chains can block enzyme production entirely (McFall *et al.*, 1958).

These results no doubt indicate a very close dependence of protein synthesis on the integrity of the genetic material and they are in sharp contrast with the data quoted previously. However, a very puzzling implication of these observations is that the synthesis of a specific protein, β -galactosidase for instance, is abolished as a result of the decay of ^{32}P atoms occurring outside the genetic locus of the enzyme. For ^{32}P decay is certainly random and there are at least a thousand genes; the chances that one ^{32}P decay occurs within the enzyme locus are much too small to explain the observed effects by local action within the locus of β -galactosidase (Pardee, 1959). Moreover, experiments by Jacob and Wollman (1958) on bacterial conjugation show that ^{32}P decay can break the linear structure which contains the genes without inactivating the individual loci.

Since in Stent's experiments destruction in other regions of the genetic material of the bacteria can suppress the synthesis of the enzyme, the effects of ^{32}P decay are not directly relevant to the problem that we are considering at present, namely the personal involvement of the gene in putting together the protein. The effects of ^{32}P decay obviously do not concern the control of the structure of an individual protein by a specific DNA region, they are probably more relevant to the control of the concerted operation of bacterial biosynthesis by the bacterial genome as a whole. One might be observing

here a very interesting aspect of the *regulation* of bacterial protein synthesis.

A direct attempt was made by Jacob and Pardee (1959) to find out whether genetic DNA participates in making the corresponding protein. They studied the kinetics of β -galactosidase appearance in bacteria which originally lack the corresponding gene, when the gene is introduced by bacterial conjugation. It was shown that the enzyme is produced at the maximal rate within a few minutes, and possibly immediately after the introduction of the piece of genome containing the competent gene. Moreover, the amount of β -galactosidase synthesized in a population of such zygotes is proportional to the square of time. This can be interpreted in the following way: as the number of zygotes formed is known to be proportional to time, the production of protein molecules must again be directly proportional to the time elapsed after zygote formation for the experimental quadratic function to be satisfied. This is exactly what one would expect if the gene would act directly as a catalyst in the production of the protein. If the gene were producing a stable intermediary catalyst at a constant rate, which in turn would make the enzyme at a constant rate, the enzyme production would be a function of the third power of time. The experimental data are therefore incompatible with the continuous production at a constant rate under the action of the genetic locus of a stable catalyst which in turn produces enzymes linearly (Jacob, 1959). They could not, however, be taken as evidence for the direct participation of DNA in the making of β -galactosidase. A few minutes is a long time for *E. coli*; which is able to make a protein within 5 sec (McQuillen *et al.*, 1959) and very complex processes may take place within the 5 min or so which follow conjugation. It is conceivable that some stable catalyst is being made rapidly in a limited number of samples during this period. Another possibility is that an unstable agent is produced continuously in the presence of DNA only.

3. Conclusion

There is no positive evidence at present for a direct participation of DNA in protein synthesis in any of the systems studied so far. On the other hand, there is ample and clear evidence that many proteins can be made without the direct participation of DNA.

This raises a fundamental question. If DNA carries coded information concerning the primary structure of proteins, and if its presence and integrity are not a prerequisite of protein synthesis, it must be concluded that the genetic information is transferred from DNA to some other cell constituent in which it can be preserved for some time and eventually used in protein synthesis. This is a logical necessity. But no one knows to what substance the information is transferred, neither in what form, nor when

the transfer occurs. Lack of experimental evidence has encouraged speculation. It seems obvious that a substance able to carry the information required for controlling the primary structure of a protein must be a rather large molecule, most probably with a linear structure. There are good reasons to suspect that the information carrier might be made of ribonucleic acid.

B. RIBOSENUCLEIC ACIDS AND PROTEIN SYNTHESIS

The first pieces of evidence for a participation of RNA in protein synthesis are found in the works of Brachet and of Caspersson on the distribution of RNA in various animal tissues. A striking correlation was shown to exist between the amount of RNA and the intensity of protein synthesis (cf. p. 41). The fact that newly-formed proteins are first detected in ribosomes (cf. p. 43) is further evidence for the participation of RNA in protein synthesis. Before considering what is known or suspected at present about the function of ribonucleic acids in protein synthesis, it is essential to be aware of their extreme diversity.

1. *Plurality and Metabolic Heterogeneity of Cellular RNAs*

It is known from cytological studies that RNA is present in several cell constituents: ergastoplasm, nucleoli, chromatin, mitochondria and certain membranes (Brachet, 1940, 1942, 1957; Caspersson and Schulz, 1939; Caspersson, 1941, 1950). During mitosis, RNA is also found in chromosomes and in the spindle (Brachet, 1942, 1957).

Autoradiographic studies show in the most striking manner that differences in metabolism exist between these differently located RNAs. Thus in the salivary glands of *Drosophila* (Herbert, 1954; McMaster-Kaye and Taylor, 1958) radioactive phosphate is incorporated more rapidly into the RNA of the nuclei and of the chromosomes than into cytoplasmic RNA. Tritiated cytidine is found in nucleolus RNA earlier and in greater amount than in any other cell constituent (Ficq, 1959; Perry, 1960). The loops of the lampbrush chromosomes of amphibian oocytes are the site of a rapid incorporation of adenine into RNA (Ficq *et al.*, 1959; Sirlin, 1960 a, b). In the giant chromosomes of diptera, labelled cytidine is incorporated into RNA at well-defined positions along the chromosomes, at certain times of the life cycle of the larvae (Rudkin and Woods, 1959). Differences in RNA metabolism are thus found between different cell regions, even within one subcellular structure.

Biochemical studies on isolated cell constituents had also revealed the metabolic heterogeneity of cellular RNA. Radioactive phosphate was injected into an animal, and the liver was isolated and homogenized. The

homogenate was fractionated by centrifugation into nuclear, mitochondrial and microsomal fractions, and a supernatant. All these fractions contain RNA, and the specific radioactivity of their RNA phosphorus showed marked differences: the nuclear RNA had always a higher radioactivity than the other fractions. Differences in rate of incorporation were also observed between the cytoplasmic fractions (Marshak and Calvet, 1949; Jeener and Szafarz, 1950; Barnum and Huseby, 1950; Jeener, 1952; Tyner *et al.*, 1953; Moldave and Heidelberger, 1954; Sacks and Samarth, 1956). The use of other precursors of RNA, like glycine, formate (Smellie and Davidson, 1956) and orotic acid (Hurlbert and Potter, 1952) led to similar observations. Changes in physiological conditions differently affect the metabolism of these various fractions (Jeener and Szafarz, 1950; Reid and Stevens, 1956; Moldave, 1954; De Lamirande *et al.*, 1958).

Even the more elaborate among the presently used fractionation procedures are still very crude, and each time it has been possible to further separate one of the cellular fractions into several subfractions, e.g. by combining centrifugation with extraction in salt, in phenol or in detergents, it was found that the RNAs contained in the subfractions again differ in their physiological or metabolic activity (Vincent, 1952, 1957, 1958; Bhargava *et al.*, 1958; Schneider and Potter, 1958; Logan, 1957; Osawa *et al.*, 1958; Sibatani *et al.*, 1959, 1960; Osawa, 1959; Goldthwait, 1959; Georgiev *et al.*, 1960; Antoni *et al.*, 1960; Scholtissek, 1960).

Among the various RNAs so far detected, special mention must be made of the so-called 'soluble RNA'. In their studies on incorporation of amino acid into proteins of liver homogenates, Hoagland *et al.* (1957, 1958) observed that labelled amino acids are bound to a special RNA fraction which is not sedimented with the particle bound RNA (see Chapter IV).

This 'soluble RNA' is not a single substance, but a mixture of several molecular species (Bloemendal and Bosch, 1959). It could be separated by chromatography on cationic starch exchanger or by counter-current distribution into fractions selectively binding certain amino acids (Smith *et al.*, 1959; Holley *et al.*, 1959).

It is quite possible that soluble RNA preparations contain, beside amino acid binding RNAs, other RNAs which play a different role or have a different type of metabolism (Canellakis and Herbert, 1960).

In bacteria also, RNA fractions differing in their metabolism have been detected and separated (Countryman and Volkin, 1959). Immediately after infection by a bacteriophage, a limited amount of a special high turn-over RNA is formed (Volkin and Astrachan, 1956; Astrachan and Volkin, 1958; Watanabe and Kiho, 1958). In ultraviolet irradiated (Suzuki and Ono, 1959) or in chloramphenicol treated bacteria (Neidhart and Gros, 1957), labile RNA fractions have been detected. Column fractionation of extracts of normal *E. coli* after short time incorporation of radioactive phosphate

shows unequal distribution of ^{32}P in the RNA of the collected fractions (Roberts *et al.*, 1958).

Differences in nucleotide composition have been observed between various RNA fractions isolated from a single tissue. For instance, the composition of RNA is not the same for all the fractions isolated from rat liver homogenate (De Lamirande *et al.*, 1955; Elson *et al.*, 1955). Vincent (1952) showed that RNA of isolated nucleoli of starfish oocytes contains more guanine and less uracil than the average cytoplasmic RNA (see also Harris, 1959; Vincent and Baltus, 1960).

In yeast and in pancreas, soluble RNA contains a 5-ribosyluridine nucleotide which is not found in particle RNA (Davis and Allen, 1957; Kemp and Allen, 1958; Osawa and Otaka, 1959; Yu and Allen, 1959; Cohn, 1959; Scannell *et al.*, 1959; Otaka *et al.*, 1959). Methylated purine are also more abundant in this RNA (Bergquist and Matthews, 1959).

Differential salt extraction, enzyme or acid hydrolysis (Jeener, 1948; Sacks *et al.*, 1955; Sacks and Samarth, 1956; Martin and Morton, 1956; Osawa *et al.*, 1958) also reveal the presence of RNAs which may differ in the way they are associated with other cell constituents (Martin and Morton, 1956; Brachet, 1959; Bell, 1959; Shigeura and Chargaff, 1960).

All these results leave no doubt that a living cell contains a population of many different molecular species of RNA which have independent physiological activities, just as a cell contains many protein species. Good methods of fractionation are urgently needed and there is little doubt that progress in the study of the function of RNA will be crippled until suitable methods are found. Attempts at fractionating ribonucleic acids by physicochemical methods have been made repeatedly (Chantrenne, 1945; Bacher and Allen, 1950; Ghuysen, 1950; Delcambe, 1950; Delcambe and Desreux, 1950; Desreux and Ghuysen, 1951; Ghuysen and Desreux, 1952; Mallette and Lamanna, 1953; Roberts *et al.*, 1958). The fractions thus obtained from yeast RNA differ in molecular weight but not much in composition (Ghuysen and Desreux, 1952; Miura and Suzuki, 1956; Miura *et al.*, 1958; Smith, 1960). By countercurrent distribution, RNA of different compositions can be partly separated (Kirby, 1960). Electrophoresis also permits some fractionation (Harris and Davies, 1960).

The development of methods for isolation of proteins has been facilitated by the fact that many proteins have individual features which make it easy to recognize and to determine them: typical solubility, characteristic colours, specific enzymic activity. Nothing quite comparable is known for RNA. Two recent developments indicate, however, that one might witness important progress in this field in the coming years: the so-called soluble RNAs contain abnormal nucleotides and bind amino acids by ester bond, whereas the bulk of RNA does not. It is to be expected that several RNAs will be characterized by their capacity to bind individual amino acids in this

fashion. On the other hand the isolation of infectious RNA from several viruses is a fundamental progress not only for the understanding of the process of virus multiplication, but also for the development of our knowledge of RNA in general. With these RNAs which are endowed with specific biological activities, biochemists will learn how to handle RNA without destroying the structural features which are essential to their biological activities.

It is very important to keep in mind that so far all the studies on RNA (except a few recent works on virus RNA) have been performed on mixtures of various RNA fractions, which were most of the time partly degraded. This has been an inexhaustible source of confusion. Innumerable works on nucleic acid metabolism are extremely difficult to interpret for the results always concern a mixture of nucleic acids. When tracers are used, new sources of hidden difficulties are involved. Beside the familiar complications due to dilution of labelled precursors, existence of several pools of precursors, interconnexions between metabolic pathways, a very serious type of complication has been appreciated only recently (Vincent and Baltus, 1959). It was found that certain RNA fractions readily bind two molecules of cytidylic acid and one of adenylic acid at the end of their molecule (Heidelberger *et al.*, 1956; Canellakis, 1957; Edmonds and Abrams, 1957; Paterson and Lepage, 1957; Hecht *et al.*, 1958; Herbert, 1958; Allfrey and Mirsky, 1959; Harbers and Heidelberger, 1959; Okazaki and Okazaki, 1959). This process was often taken as reflecting nucleic acid synthesis; it now appears that it corresponds to a metabolic activity which might be connected with protein synthesis.

All this serves to emphasize the physiological heterogeneity of nucleic acids, the complexities of their metabolism, and the primitive stage in which we are still in our attempts to analyse the function of these substances in biochemical terms. It is important to keep this in mind and to avoid being too dogmatic in all interpretations of RNA metabolism in terms of RNA formation.

2. *Metabolism of RNA and Protein Synthesis*

One of the first working hypotheses retained by workers interested in the function of RNA in protein synthesis, was that RNA and protein must be made simultaneously or that the synthesis of protein depends on RNA formation. A very great amount of research has been devoted to testing these ideas, and it would be hopeless to try and analyse the results of all these contributions. It has repeatedly been reported, for instance, that in growing tissues or in growing bacteria, protein and RNA synthesis go together, or that the rate of incorporation of precursors into the two types of macromolecules depends on the same basic conditions, like the provision of energy, a carbon source, a nitrogen source and active phosphorylations.

Obviously, these facts are irrelevant to the existence of a connexion between RNA synthesis and protein formation. They are a mere reflexion of the general requirements of cells for growth or for any type of synthesis. In the same way, the fact that a population of growing bacteria usually make protein and nucleic acid simultaneously does not imply that the mechanism of protein synthesis is such that one type of macromolecule cannot be made without the other being made at the same time.

More significant are the attempts made at dissociating the two processes, e.g. by varying growth-rate, by using inhibitors or by causing specifically the synthesis of a protein. Let us consider briefly a few typical data.

Spiegelman and Kamen (1947) showed that the phosphorus of yeast RNA is renewed very slowly in actively respiring yeast which is deprived of nitrogen source. If ammonium salts are added, proteins are synthesized, the uptake of radioactive phosphate is increased in all phosphorus compounds of the cell, but much more so in RNA than in any other cellular constituent, as if the synthesis of RNA was indeed connected with protein synthesis. Abrams *et al.* (1949) made similar observations for the incorporation of precursors into RNA purines; however, it was later found that X-rays markedly reduce this RNA metabolism without inhibiting protein synthesis (Abrams, 1951). Grenson (1952) studied incorporation of phosphate into various types of animal tissues which have in common a high rate of protein synthesis, but which differ physiologically. She found no simple or uniform correlation between phosphorus incorporation into RNA and the production of protein. Similar observations were made for RNA synthesis during synthesis and secretion of amylase by pigeon liver pancreas (Hokin and Hokin, 1953, 1954, 1956), or during the period of recovery after starvation in muscle or liver (Laird *et al.*, 1955; Zak and Gutmann, 1960). In continuous cultures of a flagellate, a simple correlation was clearly observed between RNA synthesis and protein formation when the organisms were growing at a constant rate. However, strong disharmonies appeared as soon as the rate of growth was abruptly changed by a sudden modification of the medium composition. A net loss of RNA for instance could even be observed at times when protein synthesis was stimulated (Jeener, 1952). Price (1952) and more recently Magasanik *et al.* (1959), Peabody and Hurwitz (1960) observed comparable phenomena with bacteria: if the substance serving as carbon source is replaced by another one to which the organism is not adapted, growth slows down very much while the bacteria make enzymes which will metabolize the new carbon source. RNA synthesis is very much depressed during this period.

Direct comparison of RNA *formation* and protein synthesis thus led to conflicting results for many years: a rather convincing correlation was found in certain systems but it failed to appear in crucial cases. The synthesis of protein and of RNA may very often occur simultaneously, but

they are not necessarily connected. Moreover, the positive correlations which were occasionally observed between incorporation of precursors into protein and net RNA synthesis for instance have lost much of their interest since the plurality and the metabolic heterogeneity of cellular ribosenucleic acids have been recognized. If a cell contains quite an assortment of various ribosenucleic acids, data on total net synthesis of RNA, or incorporation into total cellular RNA give but average values; important changes involving certain RNA fractions might pass unnoticed because they can be masked by other processes occurring at the same time in other RNA fractions. It is clear that a direct comparison of the metabolism of total RNA and total protein synthesis cannot be very informative, and one should look for data on the possible connexions between RNA metabolism and protein synthesis at the level of different ribonucleoprotein fractions. A good start in this direction can be found in a metabolic investigation of the ribonucleoprotein particles of guinea pig pancreas by Siekewitz and Palade (1959). Five ribonucleoprotein fractions were isolated by fractional centrifugation and compared as for the rate of *in vivo* incorporation of adenine into RNA and of amino acids into proteins. The rates and the kinetics of incorporation differ very much between the five fractions. It is striking that fractions especially active in protein synthesis showed practically no incorporation of adenine into their RNA, whereas another fraction incorporated adenine very actively but was very poor in protein synthesis. This indicates that the synthesis of protein and that of nucleic acids are not associated at the level of nucleoproteins.

A completely different approach to the problem discussed in the present chapter consists in interfering specifically in various ways with the metabolism of RNA and in watching the effects on protein formation. The first attempt along this line was probably made by Jeener and Jeener (1952) with *Thermobacterium acidophilus*. This is an exacting bacterium which requires, among a score of various substances, uracil and thymidine for growth. Deprivation of thymidine stops DNA synthesis and bacterial division, but it has no immediate effect on protein synthesis. On the contrary, uracil starvation almost immediately stops protein formation. These early observations have been confirmed by Okazaki and Okazaki (1958). Similarly, in pyrimidine-less mutants of *E. coli* the synthesis of enzymes depends on the exogeneous supply of pyrimidines, except under special conditions when these are provided by the breakdown of certain RNA fractions (Pardee, 1954, 1955; Barner and Cohen, 1958). The ability of resting yeast to make the enzyme α -glucosidase depends on the level of the free nucleotide pool, and the best way of depleting this pool is to force yeast to produce proteins (Spiegelman *et al.*, 1955).

Deprivation of nucleic acid precursors, in all these cases, was thus found to be damageable to protein production. Conversely, the provision of

purines, pyrimidines and their derivatives favours it in some systems (Hancock, 1957; Reiner, 1960). In disrupted *Staphylococcus aureus*, a supplement of purines or pyrimidines stimulates protein and enzyme formation (Gale and Folkes, 1953; Creaser, 1955; Gale, 1956). A ribonuclease hydrolysate of RNA also contains substances which stimulate the incorporation of amino acids into the proteins of such systems; but this effect which was first attributed to oligonucleotides (Gale and Folkes, 1955) is in fact due to other substances which have not been identified yet (Gale *et al.*, 1958). Webster observed that the incorporation of amino acid into cytoplasmic particles from pea roots is markedly promoted by a mixture of the four ribonucleosides, whereas desoxyribosides are inactive. A mixture of the four ribonucleoside triphosphates ATP, GTP, CTP and UTP also stimulated amino acid incorporation (Webster and Johnson, 1955; Webster, 1957).

Thus protein synthesis can, in certain systems, be limited by lack of purine, pyrimidine and derivatives. As the latter substances can serve as precursors of RNA, these observations have often been taken as evidence that protein synthesis requires the synthesis of new RNA molecules. Apparent confirmation of this conclusion was found in the inhibition of protein synthesis by purine and pyrimidine analogues, and by the direct observation of an increased incorporation of labelled adenine or uracil into RNA during induced enzyme formation (Gale and Folkes, 1955; Chantrenne, 1956). It seemed highly probable therefore that the continuous synthesis of some RNA fraction was necessary for protein synthesis (see review by Spiegelman, 1956). Although this possibility is in no way excluded, it is realized at present that other interpretations of the experimental facts are equally probable. The fact that RNA precursors are required for some process does not prove that the synthesis of new RNA molecules is required. Many 'RNA precursors' also participate in the metabolism of low molecular weight material. As will be shown in the next chapter, purine and pyrimidine analogues do interfere with protein synthesis, but in most cases it is not by preventing RNA synthesis that they do so. The increased incorporation of adenine which accompanies enzyme induction in yeast is a rather involved process, in some way connected with the breakdown of certain RNA fractions (Chantrenne, 1958). The evidence for a synthesis of new RNA molecules linked to protein synthesis therefore is certainly not compelling. But it remains that *metabolic processes involving some RNA fractions* are most probably involved in protein synthesis. The nature and significance of these processes is at present anyone's guess. The results of recent investigations have indicated that soluble RNA can bind in succession at the end of its polynucleotidic chains two pyrimidine nucleotides followed by a purine nucleotide. The attachment of this terminal sequence of three nucleotides is a prerequisite for the fixation of activated

amino acids on to soluble RNA (Heidelberger *et al.*, 1956; Canellakis, 1957; Hecht *et al.*, 1958; Edmonds and Abrams, 1957; Paterson and Le Page, 1957; Okazaki and Okazaki, 1959; Harbers and Heidelberger, 1959; Vincent and Baltus, 1959). The requirement for purines and pyrimidines for protein synthesis might very well find its explanation in this end chain metabolism of soluble RNA, or in other reactions that are still to be discovered.

3. *Importance of the Structural Integrity of Ribonucleic Acids*

(a) *Effects of ribonuclease.* Highly purified ribonuclease from beef pancreas was found to inhibit completely the incorporation of labelled amino acids into proteins of homogenized pancreas or liver (Siekevitz, 1952; Allfrey *et al.*, 1953; Zamecnik and Keller, 1954). In similar preparations, ribonuclease was without any effect on other complex biosynthetic processes such as lipid formation. The selective action of ribonuclease upon amino acid incorporation thus provided a very direct evidence to the participation of some RNase sensitive substance, most probably RNA, in protein formation. Observations of the same kind were made on homogenates of animal or plant cells. Disrupted bacteria are able to incorporate amino acids into their proteins under appropriate conditions; here again the incorporating system is inactivated by ribonuclease, which destroys a large part of the RNA in the preparation (Gale, 1956, 1957; Gale and Folkes, 1955). Protoplasts (i.e. bacteria which have been stripped of their cell wall and protected from osmotic effects by an adequate concentration of sugar or salts) keep the capacity of making proteins, including inducible enzymes (Wiame *et al.*, 1955; Landman and Spiegelman, 1955; Spiegelman, 1957). In the presence of ribonuclease, incorporation of amino acids into protoplasts protein is completely inhibited (Lester, 1953; Beljanski, 1954). Protoplasts are very fragile structures, and ribonuclease easily causes their disruption, followed by dispersal of their content (Brenner, 1955). It can be shown, however, that the inhibition of protein synthesis occurs before the disruption of the protoplasts (Bridoux and Hanotier, 1956). Spiegelman and Landman (1955) established conditions which ensure stability and preserve the metabolic activity of the protoplasts in the presence of ribonuclease for a longer time. The enzyme invariably suppresses protein formation under these conditions, in a rather selective manner.

More striking still are the effects of ribonuclease on otherwise intact living cells. Crude ribonuclease preparations had been shown to inhibit cell division when injected into an amphibian egg (Thomas *et al.*, 1946). This experiment was later repeated with highly purified enzyme on dividing eggs at the two and four cells stages; it was observed that microinjection of ribonuclease into one blastomere inhibits the division not only of the injected cell but of the other blastomeres as well, as if ribonuclease

was able to pass from one cell into the next one (Ledoux *et al.*, 1954; Brachet and Ledoux, 1955). Indeed, injection of the enzyme is not necessary; ribonuclease readily enters the cells of amphibian embryos at the morula stage when these are merely immersed in a dilute ribonuclease solution in tap water. This offered an easy way of affecting RNA *in vivo* and of exploring the function of RNA in protein synthesis. Simple experiments that biochemists had never attempted because of their school years prejudices about permeability of cell membranes, now looked sensible and were soon performed; many types of cells were found to pick up ribonuclease. *Amoeba proteus* for instance rapidly takes up ribonuclease from the medium (Brachet, 1954-55; Schumaker, 1958), most probably by a process known as pinocytosis (Lewis, 1931; Holter and Marshall, 1954; Holter, 1959), which consists in the engulfment of droplets of medium; the basic proteins contained in the medium penetrate the cell for they are later found in the cytoplasm and even in the nucleus. In a ribonuclease solution, *Amoebae* lose part of their basophilia (i.e. RNA is partly destroyed) and at the same time their capacity for incorporating labelled amino acids is almost completely abolished; respiration is not affected and ATP production continues, an accumulation of labile phosphate esters is even observed (Škreb-Guilcher, 1955). Ribonuclease thus can inhibit protein synthesis under such conditions that the general metabolic processes are maintained.

Another type of ribonuclease sensitive system is the onion root. As observed by Kaufmann *et al.* (1954, 1957), the pancreatic enzyme causes mitotic abnormalities. The absorption of RNase and other proteins by onion root cells has been confirmed by direct experiments with ^3H labelled protein (Jensen and McLaren, 1960); labelled RNase was observed in association with the nucleolus. Brachet (1954) showed that incorporation of amino acids and growth of the root are stopped by ribonuclease. An important observation (Brachet, 1954, 1955, 1956) is that protein synthesis is almost completely suppressed by a rather short ribonuclease treatment (e.g. 30 min). Under these conditions, the extent of RNA degradation is very limited; even after 18 hrs action only 20 per cent of the RNA is removed. A limited damage to RNA or the destruction of a certain type of RNA only is enough to suppress protein synthesis. A quite similar conclusion was reached by Yakeyama *et al.* (1958) who observed a complete inhibition of fibroin synthesis by ribonuclease in isolated silk glands of *Bombyx mori* under such conditions that only a limited RNA destruction had occurred. Roots which had been treated by RNase for 30 min, and in which protein synthesis was thus almost completely suppressed, were homogenized and fractionated by high speed centrifugation. No significant difference in RNA content was found in any of the sedimentable fractions between RNase treated and control roots. But a 50 per cent decrease in the

RNA to protein ratio in the 'soluble' fraction was consistently observed. This indicates that the inhibition of protein synthesis in living onion roots caused by ribonuclease is probably due to partial degradation of a soluble RNA fraction by the enzyme (Brachet and Six, 1959).

The possibility, however, remains that RNase might also interfere with protein synthesis in other ways, for instance, by slowly damaging ribosome RNA or by forming complexes with it. Ribosome RNA is less sensitive to the lytic action of the enzyme than soluble RNA (Shigeura and Chargaff, 1960); other basic proteins like histone, salmine and to a lesser extent cytochrome-*c* indeed partly inhibit protein synthesis in roots (Brachet, 1956) and in ascites cells (Becker and Green, 1960) by combining with RNA.

Finally, in certain types of animal cells, ribonuclease causes deep changes in RNA metabolism. For instance, in ascites cells in a medium supplemented with free nucleotides, the enzyme first stimulates RNA synthesis (Ledoux, 1956; Pileri *et al.*, 1957), causing a rapid uptake of pyrimidine nucleotides, thus leading to the formation of abnormal RNA, which is later degraded (Ledoux and Vanderhaeghe, 1957). The effect on protein synthesis is not striking in this case, however, and respiration is depressed (Ledoux and Baltus, 1954).

Ribonuclease was also found to inhibit growth of *Bacillus megaterium* (Groth, 1956) and of *E. coli* (Jerne and Maaløe, 1957). In many cases, lysis of the bacteria occurs, but it is possible to isolate strains which are not lysed by the enzyme, and which will continue to grow in its presence (Jeener, 1959a). Using a lysogenic strain of *Bacillus megaterium* resistant to ribonuclease lysis, Jeener showed that the synthesis of phage protein is partly inhibited whereas bacterial growth is not. The phage proteins made in the presence of the enzyme have abnormal immunological and chromatographic properties (Jeener *et al.*, 1960). They can be integrated into phage-like structures, but these are poorly adsorbed on receptive bacteria, and they are also very fragile. Incomplete phage particles have indeed been isolated by serological precipitation (Jeener, 1959b). In these experiments, also, a small fraction only of the total RNA could have been degraded, for the RNA/DNA ratio was practically unaffected. But the composition of RNA was changed: a higher content in uridylic acid was consistently found in the bacteria which had been treated with ribonuclease. This suggests that the abnormalities observed in the proteins produced might result from a structural modification of some RNA fraction (Jeener, 1959b).

It should be mentioned again here that ribonuclease does not affect protein synthesis in intact mitochondria, but it suppresses completely amino acid incorporation into proteins in extracts obtained from mitochondria (Kalf and Simpson, 1959; Kalf *et al.*, 1959). It is probable therefore that protein synthesis in mitochondria can go on in a medium containing

ribonuclease simply because the enzyme cannot enter intact liver mitochondria.

The results reviewed above show that ribonuclease acting on living cells brings about various types of damages. In favourable cases, it can selectively inhibit the synthesis of protein or of certain proteins without acting upon general metabolism and energy production, or without affecting the synthesis of many various cell constituents. The mode of action of the enzyme is not completely analysed, but inhibition of protein synthesis coincides with the breakdown of certain RNA fractions especially soluble RNA.

It would seem that a limited degradation or modification of certain RNA fractions is enough to stop protein synthesis, or to cause modifications in the properties of the proteins produced.

(b) *Modification of RNA by analogues of purines and pyrimidines.* Many analogues of the natural purines and pyrimidines have been prepared by chemical synthesis. Some of them proved to be potent inhibitors of growth for certain animal or plant cells and for certain strains of bacteria (Elion *et al.*, 1954). It is likely that substances of this kind interfere with the metabolism, the synthesis or the function of nucleic acids or nucleotidic compounds. Present knowledge of the mode of action of these analogues is actually rather poor, restricted as it is to the case of a few compounds and a few organisms or biochemical systems. It is, however, sufficient already to show that purine and pyrimidine analogues can interfere in many ways with the metabolism of the natural parent products, and more indirectly with other metabolic processes as well. This is illustrated by the following examples: 5-fluoro-uracil inhibits methylation in the synthesis of thymine (Bosch *et al.*, 1958; Cohen *et al.*, 1958; Heidelberger *et al.*, 1957; Eidinoff *et al.*, 1957; Harbers *et al.*, 1959) and interferes with cell wall formation (Tomasz and Borek, 1959); 6-mercaptopurine affects acetate and formate utilization, probably by interfering with the formation of purine containing cofactors (Bolton and Mandel, 1957); 5-hydroxyuridine exerts several different effects including inhibition of purine utilization (Slotnick *et al.*, 1953); 8-azaguanine inhibits adenosine deaminase (Feigelson and Davidson, 1956); 6-azauracil causes the inhibition of polynucleotide phosphorylase (Škoda *et al.*, 1959) and it blocks the conversion of orotic acid into uracil (Škoda and Šorm, 1958, 1959; Sells, 1959–1960). Several analogues can substitute for the natural parent constituent in the feed-back control exerted by this compound upon its own synthesis (Gots and Gollub, 1959; Trudinger and Cohen, 1956; Smith and Sullivan, 1960).

Different types of cells also react differently or to various degrees to a purine or pyrimidine analogue, and a type or effect which is shown in one organism does not necessarily occur in others. Therefore the effect of an analogue on a given organism cannot be predicted, and careful analysis is

necessary in each case. This situation may look depressing; on the other hand, it makes it possible to find organisms and analogues which will be particularly suitable for the study of a given biochemical problem. Cases which are especially interesting for the problem considered in the present chapter, namely the importance of RNA integrity for protein synthesis, are those of organisms which incorporate purine or pyrimidine analogues into their nucleic acids. Thus 8-azaguanine is incorporated into RNA of various organisms (Mitchel *et al.*, 1950; Heinrich *et al.*, 1952; Bennett *et al.*, 1953; Mandel *et al.*, 1954; Matthews, 1953, 1957; Lasnitski *et al.*, 1954; Matthews and Smith, 1955, 1956; Mandel and Markham, 1958). 2-Thiouracil incorporation into RNA of tobacco mosaic virus (Jeener and Rosseels, 1953; Jeener, 1957; Matthews, 1956; Mandel *et al.*, 1957) and of *Bacillus megaterium* (R. Hamers, 1956; Amos *et al.*, 1958) has been established. 5-Fluorouracil can also be incorporated into RNA in several organs of rat and mouse (Heidelberger *et al.*, 1957; Harbers *et al.*, 1959), in *Escherichia coli* (Horowitz *et al.*, 1958; Horowitz and Chargaff, 1958; Gros, 1959; Brockman *et al.*, 1960) and in tobacco mosaic virus (Gordon and Staehelin, 1959).

As 2-thiouracil is being incorporated into RNA by *Bacillus megaterium*, the bacteria continue to grow and to make proteins, but growth becomes linear (Hamers, 1956). In *E. coli*, a similar change in the shape of the growth curve is observed, but the effect is not as rapidly established as in *B. megaterium*. Protein synthesis is but slightly inhibited by thiouracil in *E. coli*, but the production of certain enzymes is drastically reduced. The differential rate of increase in β -galactosidase activity, for instance, is reduced by about 90 per cent. The residual increase is due to the production of a protein material which is precipitated by an antiserum prepared against regular β -galactosidase. But titration by the immunological method of Cohn and Torriani (1952) shows that this material has less enzyme activity per unit of serologically precipitable protein than regular β -galactosidase. This indicates that a slightly abnormal enzyme is produced in the presence of thiouracil (Hamers and Hamers-Casterman, 1959).

Bacteriophage production by a lysogenic strain of *Bacillus megaterium* can also be inhibited by 2-thiouracil. The major action of the analogue in the present case is a drastic inhibition of phage DNA synthesis; the formation of phage proteins is also decreased, but to a lesser extent, and the protein produced in the presence of thiouracil retain normal immunological properties, as well as the capacity of fixation upon bacteria (Jeener *et al.*, 1959).

5-Fluorouracil inhibits growth of *E. coli* by 50 per cent. Certain enzymes, like catalase, succinic dehydrogenase or phosphatase continue to be produced whereas β -galactosidase synthesis is almost completely stopped (Horowitz *et al.*, 1958; Horowitz and Chargaff, 1959; Gros, 1960). While

β -galactosidase production is thus drastically reduced, the synthesis of a protein closely related to the enzyme but catalytically inactive can be demonstrated by serological reactions (Bussard *et al.*, 1960).

The analogue inhibits growth of *E. coli* only when it can be incorporated into nucleotidic compounds, since mutants lacking the enzymes for making the nucleotide of fluorouracil are resistant to the analogue (Brockman *et al.*, 1960). As the effects of the analogue are observed without delay, the damage caused by fluorouracil incorporation must concern one of the RNA fractions which are rapidly renewed (Naono and Gros, 1960). The damaged RNA seems to be involved in the process of selection or of arrangement of the amino acids, for the proline and tyrosine content of the total bacterial protein material made in the presence of fluorouracil is lower than normal.

The reason why certain enzymes are deeply affected by such errors in amino acid selection, whereas other enzymes are rather insensitive, can be visualized easily. It is clear that the active centre of a given enzyme, or the folding which creates this centre, may eventually be affected by amino acid replacements. If, however, the number of changes in the amino acid sequence is not too large, it is conceivable that the enzyme retains a practically normal tertiary structure, together with its catalytic and serological properties. Ribonuclease, for instance, can stand a considerable amount of damage indeed, without losing its catalytic properties: certain peptide bonds can be broken (Richards, 1958), a whole section of polypeptide can even be removed (Anfinsen *et al.*, 1955; Rogers and Kalnitzky, 1957) without much change of activity. The resulting molecules are simply more fragile than the intact enzyme.

In contrast with thiouracil and fluorouracil, another analogue, 6-azauracil, which also inhibits bacterial growth, does not interfere with protein synthesis more than with the formation of other cell constituents (Sells, 1959). It is important to realize that 6-azauracil is not incorporated into RNA in any detectable amount (Handschumaker, 1957); it inhibits nucleic acid synthesis by blocking the pathways of uridylic acid synthesis (Škoda and Šorm, 1958, 1959).

The rate of incorporation of 8-azaguanine into the nucleic acids varies very much according to the organism (Matthews, 1957). *Bacillus cereus* has received special attention because it takes up quite a large amount of the analogue. Detailed studies by Smith and Matthews (1957), Mandel and Markham (1958) showed that 8-azaguanine replaces up to 40 per cent of the guanine in the RNA made in its presence, and that the composition of the nucleic acid is not otherwise changed. Synthesis of RNA continues at a somewhat increased rate in the presence of the analogue, DNA is made at a closely normal pace, but protein synthesis is drastically inhibited (Chantrenne, 1958, 1959; Chantrenne and Devreux, 1958, 1959, 1960; Mandel and Altman, 1960).

It must be emphasized that many cell constituents are still synthesized normally, which indicates that basic metabolism and energy production are not touched. The process of RNA synthesis also continues but confusion of azaguanine for guanine leads to abnormal nucleic acids. Under these conditions protein synthesis is obliterated, although the utilization of amino acids by the bacteria is not prevented, neither their condensation into peptides. Cell wall material for instance, including the peptides they contain, continue to be made at a normal rate (Chantrenne and Devreux, 1958; Richmond, 1959; Roodyn and Mandel, 1960).

In these cases, the analogue partly replaces the normal purine or pyrimidine compound, and this results in the formation of modified RNA. It is striking that in all the cases where such an incorporation of abnormal bases into RNA occurs, protein synthesis is interfered with. Let us consider these facts a little more closely.

There are good reasons to believe that the inhibition of protein synthesis is linked to the incorporation of azaguanine into nucleic acids. For instance mutant strains of *Streptococcus faecalis* resistant to azaguanine have been shown to differ from the wild strain in that the resistant mutant has lost the ability to incorporate azaguanine (and guanine) into nucleotidic compounds. The same difference was observed for sensitive and resistant lines of a leukaemia (Brockman *et al.*, 1957–58). In *Tetrahymena geleii*, inhibition of growth by azaguanine is observed only in the presence of uracil which is required for RNA synthesis in this organism (Kidder *et al.*, 1951; Heinrich *et al.*, 1952). Recent research in the author's laboratory has shown that this also applies to protein synthesis in the same organism. It is only when azaguanine can be incorporated into RNA that protein synthesis is inhibited by the analogue. On the other hand, kinetic studies on the incorporation of azaguanine into RNA and of amino acids into protein indicate that in *Bacillus cereus* the inhibition of protein synthesis is already fully expressed at a time when the amount of RNA made in the presence of azaguanine represents only about 10 per cent of the total RNA (Chantrenne, 1958). Fractionation of bacterial content by centrifugation shows that at that time the 'non-sedimentable' RNA is already heavily loaded with azaguanine, whereas ribosome RNA contains relatively little of the analogue. This observation, together with the facts reported above, suggests that the incorporation of azaguanine into a soluble RNA might be responsible for the general inhibition of protein synthesis caused by the analogue.

This is one more piece of evidence that the integrity of some soluble RNA is a requirement for protein synthesis.

The action of azaguanine on *B. cereus* can be completely prevented by several purines or purine derivatives (Mandel, 1957). Thus in the presence of an adequate concentration of guanosine, no azaguanine is incorporated into RNA and no inhibition of protein synthesis is observed. Guanosine

can even release the inhibition produced by azaguanine and completely restore protein synthesis and bacterial growth if added some time after the inhibition by azaguanine is established. Under those conditions, a large part of the azaguanine that had been incorporated into RNA is rejected and found in the medium mostly as azaguanosine (Matthews and Smith, 1956; Smith and Matthews, 1957; Mandel, 1957; Mandel and Markham, 1958). Restoration of protein synthesis by guanosine, however, becomes more and

FIG. 22. Restoration by guanosine of the synthesis of protein material (a) and of constitutive penicillinase (b), in *B. cereus* after azaguanine inhibition. The numbers at the end of the curves indicate the time in minutes elapsed between the additions of azaguanine and of guanosine (Chantrenne and Devreux, 1960).

more sluggish when the period of azaguanine inhibition increases. An unexpected feature of the recovery of protein synthesis under the action of guanosine is that the synthesis of an enzyme like constitutive penicillinase is not restored at the same time as net synthesis of the average protein material. This is illustrated in Fig. 22. For instance, when guanosine is added 45 min after the analogue, the synthesis of protein is rapidly restored, whereas penicillinase formation is not yet restored 2 hrs. later (Chantrenne, 1959; Chantrenne and Devreux, 1960). There is evidence that

proteins made during this restoration period are abnormal; catalase for instance has an abnormal temperature coefficient (Chantrenne, in press). Beside the lesion which blocks all protein synthesis, and which is easily repaired by an early addition of guanosine, continued action of azaguanine causes another type of lesions which do not affect the synthesis of all the proteins equally. These secondary lesions develop rather slowly, but they are also cured very slowly by guanosine. A closer study of RNA during azaguanine inhibition and of its reversal by guanosine showed that an increasing amount of azaguanine is incorporated into RNA 'irreversibly', in this sense that it fails to be displaced by guanosine. Identification of this fraction of RNA will eventually make it possible to locate the site of the secondary lesions which affect differently the synthesis of the individual proteins. The specificity of synthesis of the individual proteins depends probably on the integrity of this fraction of RNA.

However, this conclusion cannot be taken for certain at present, since DNA also has been shown to take up some azaguanine (Smith and Matthews, 1957; Mandel *et al.*, 1957). Although the amount incorporated into DNA is very small as compared to RNA, it might be enough to explain the specific effects on protein synthesis.

In *Bacillus cereus*, the uptake of azaguanine into soluble RNA is rapid and the degree of guanine replacement by azaguanine very high. This rapidly leads to an almost complete inhibition of all protein synthesis. It is only during the recovery caused by guanosine that differential effects on the synthesis of individual proteins can be observed. In other systems which are somewhat less sensitive to the guanine analogue, differential effects are observed during the action of the drug. Thus Creaser (1955-56) noticed that in a *Staphylococcus* azaguanine can inhibit the synthesis of β -galactosidase much more than that of catalase. Jeener *et al.* (1959) also observed that within a well-chosen range of concentrations, azaguanine depresses the synthesis of bacteriophage protein much more than that of bacterial proteins in *B. megaterium*. More recently Heyes (1959) reported that azaguanine inhibits protein synthesis in roots of pea seedlings only partially, and that the degree of inhibition is not the same for the four individual enzymes which were studied. Disturbances of the development of embryos (Waddington *et al.*, 1955; Nishimura and Nimura, 1958; De Vincentis, 1960) or of fern gametocyte (Hotta and Oswa, 1958; Hotta *et al.*, 1959), or of tumour growth (Kidder *et al.*, 1951; Mandel *et al.*, 1954) might be due to a more or less selective action of azaguanine upon the synthesis of individual proteins at certain stages of development (O'Brien, 1959). In other systems, general inhibition of protein synthesis prevails (Dutton *et al.*, 1958).

(c) *Summing Up.* It can be concluded from the foregoing that slight modifications of RNA brought about *in vivo* by ribonuclease or by the

fraudulent incorporation of abnormal purines or pyrimidines, result in deep changes in protein synthesis. The effects can be more or less drastic: protein synthesis can be stopped or only reduced to a certain extent. Under these latter conditions, different proteins can be affected to various degrees and abnormal proteins can be produced. This clearly indicates that the integrity of certain RNA fractions is a necessary requirement for the synthesis of normal protein. That changes in protein structure result from changes in RNA composition is probably the most convincing evidence for a control of protein structure by RNA. Of all cellular RNAs, soluble RNAs are the first to be affected by the destructive action of RNase and the first to be deeply changed by analogues. It would seem that modification of some soluble RNA fraction is enough to block or to upset the specificity of the process. Evidence from the restoration of protein synthesis after inhibition by ribonuclease or by azaguanine indicates, however, that soluble RNA is rather easily replaced by the cell when favourable conditions are returned. To the contrary, damage caused by the same agents, most probably to another type of RNA, which also affect the specificity of protein synthesis, becomes easily irreversible.

A thorough study of changes brought about in protein structure by modifications of RNA may afford a means of studying the most puzzling aspect of protein synthesis, namely the control of specificity.

Beside the agents mentioned above — purine and pyrimidine analogues and ribonuclease — the use of nitrous acid for the limited deamination of nucleic acid adenine, guanine and cytosine might prove very informative in this respect. Hexetidine has also been shown to alter the specificity of protein synthesis in a bacterium (Halvorson and Gorman, 1959).

Chloromycetine strongly inhibits protein synthesis in bacteria. The action of this antibiotic closely resembles the effects of azaguanine in many respects; it probably acts at about the same point upon the mechanism of protein synthesis. Closer analysis of the various substances which dissociate protein and RNA synthesis in different ways (Gale and Folkes, 1953; Gale, 1958) remains certainly one of the promising approaches to the understanding of the exact function of RNA.

4. *RNA as the Genetic Messenger*

At the end of Chapter II, it was concluded that DNA is not directly involved in the synthesis of most proteins, and that the genetic information it contains must be transferred to some other substance before serving in the synthesis of cytoplasmic proteins.

The common belief is at present that the substance which carries the genetic information from DNA to cytoplasmic centres of protein synthesis must be a RNA. This is one side of what Crick (1958) called the 'central dogma', thus making it clear that this opinion rests largely on *a priori*

considerations. The intermediate depositary of genetic information must be able to receive the information from DNA, to record it in some way and to convey it to the sites of protein synthesis. As we shall see, RNA seems to fulfil these *a priori* requirements.

(a) *Do RNA molecules carry genetic information?* Virus RNA can indeed transfer to a living cell the specific information required for making at least the protein of the virus (see Chapter I). It is therefore quite reasonable to suspect that some fraction of cellular RNA might play a similar role, without prejudice as to what fraction this may be.

There is every reason to believe that the genetic information contained in DNA or in virus RNA consists in a specific arrangement of the purines and pyrimidines on the phosphate-carbohydrate backbone. The two families of nucleic acids are so closely related, that they must speak closely similar languages. The translation of DNA information into RNA language must be a rather simple matter. The easiest way to visualize how RNA could receive information from DNA and store it in its own structure, is to assume that specific RNA is made under direct control of DNA. Interesting attempts have been made at formulating in terms of structural chemistry possible template processes by which a unique sequence of bases might be imposed upon a nascent RNA chain which would take shape in close contact with DNA. Thus Lockingen and De Busk (1956) developed this idea in rather general terms, and suggested that the strands of the DNA double helix could separate and a RNA chain would organize under the influence of the bases of single DNA strands. Although the DNA structure which was then assumed by the authors is probably outdated by now, the same idea might soon be considered again in a revised form since single strand DNA have been shown to exist (Sinsheimer, 1959).

Stent (1958) and Zubay (1958) developed models in which an RNA chain would be organized in the deep groove of a DNA double helix. The sequence in RNA is supposed to be imposed by that of the pairs of bases in DNA (Fig. 23).

Experimental results obtained by Rich and associates with synthetic polynucleotides make processes of this type rather plausible. When equivalent amounts of polyadenylic and polyuridylic acids are mixed together in dilute salt solution, a double helix forms in which each adenine in the polyadenylic is hydrogen bonded to a uracil in the polyuridylic chain (Rich, 1957; Felsenfeld, 1958; Steiner and Beers, 1959). The structure of this double helix is very similar to that of DNA. If enough magnesium ions or polyamines are added, the double helix is able specifically to bind another polyuridylic acid chain. A three-stranded molecule is thus formed, one component of which is loosely bound to a very stable double helix (Felsenfeld and Rich, 1957; Zubay, 1958; Rich, 1959a, 1959b).

(a) According to Stent (1958).

(b) According to Zubay (1958b).

FIG. 23. Purine-pyrimidine triplets which may be involved in directing the synthesis of an RNA chain upon a DNA double helix.

Hybrid helices can also form in which one strand with a DNA backbone wraps around another strand with a RNA backbone in such a manner that the strands are held together by complementary hydrogen bonds formed between purine and pyrimidine residues (Rich, 1960). Should RNA be copied from DNA by such a process, a complete correlation between RNA and DNA composition would be expected only if both DNA chains were copied and if each DNA unit would produce about the same number of RNA replicas.

Belozersky and Spirin (1958) determined the base composition of DNA and total RNA of nineteen bacterial species belonging to various groups. As shown in the tables, the composition of DNA varies widely, from species in which the Adenine Thymine pair accounts for 70 per cent of the bases, to types in which it accounts for about 25 per cent only. In the same organisms, the composition of total RNA differs much less from species to species. However, a certain correlation is observed between the composition of RNA and DNA: the ratio $\frac{(G+C)}{(A+U)}$ seems to vary as a linear function of $\frac{(G+C)}{(A+T)}$ in DNA (Belozersky, 1959).

Although the composition of global RNA does not correspond to that of global DNA of the same organism, the observed correlation together with the fact that DNA composition in a given species is rather homogeneous down to the level of smaller molecules (Rolfe and Meselson, 1959) suggests that a fraction of the RNA has indeed a composition which corresponds to that of DNA. Belozersky's results are therefore compatible with the idea that a fraction of bacterial RNA is organized under the control of DNA, and might possibly carry specific genetic information.

Volkin and Astrachan (1957) made very interesting observations on RNA synthesis at the beginning of phage infection. The distribution of ^{32}P incorporated into RNA indicates that the small RNA fraction which rapidly forms after infection has the same base composition as phage DNA (with uracil substituted for thymine). Using the same method, Yčas and Vincent (1960) reached the conclusion that yeast contains a relatively small fraction of RNA with a high turnover rate, whose composition is very similar to that of yeast DNA, with uridylic acid replacing thymidylic acid. The authors, for this reason, suspect that this fraction may be a primary gene product, a specific RNA copied from DNA and which carries information to centres of protein synthesis. A new RNA fraction which may deserve special attention has recently been isolated by Sibatani *et al.* (1960) from animal tissues. It seems to be made at the same time as DNA and to be metabolically as inert as DNA.

None of these observations can be taken as definite evidence that specific

ribosenucleic acids carrying genetic information for the synthesis of proteins do exist in cells. However, they point towards directions where such evidence should be sought.

DNA COMPOSITION IN DIFFERENT BACTERIA

Species	Content of bases in molar per cent				Pur.	G + T	G + C
	G	A	C	T	Pyr.	A + C	A + T
<i>Clostridium perfringens</i>	15.8	34.1	15.1	35.0	1.00	1.03	0.45
<i>Staph. pyogenes aureus</i>	17.3	32.3	17.4	33.0	0.98	1.01	0.53
<i>Pasteurella tularensis</i>	17.6	32.4	17.1	32.9	1.00	1.02	0.53
<i>Proteus vulgaris</i>	19.8	30.1	20.7	29.4	1.00	0.97	0.68
<i>Escherichia coli</i>	26.0	23.9	26.2	23.9	1.00	1.00	1.09
<i>Proteus morganii</i>	26.3	23.7	26.7	23.3	1.00	0.98	1.13
<i>Shigella dysenteriae</i>	26.7	23.5	26.7	23.1	1.01	0.99	1.15
<i>Salmonella typhosa</i>	26.7	23.5	26.4	23.4	1.01	1.00	1.14
<i>Salmonella typhi-murium</i>	27.1	22.9	27.0	23.0	1.00	1.00	1.18
<i>Ervinia carotovora</i>	27.1	23.3	26.9	22.7	1.02	0.99	1.17
<i>Corynebact. diphtheriae</i>	27.2	22.5	27.3	23.0	0.99	1.01	1.20
<i>Azotobacter agile</i>	28.3	21.4	26.5	23.8	0.99	1.08	1.21
<i>Azotobacter vinelandii</i>	27.4	22.1	28.9	21.7	0.98	0.97	1.28
<i>Azotobacter chroococcum</i>	28.7	20.5	28.5	22.2	0.97	1.04	1.34
<i>Aerobacter aerogenes</i>	28.8	21.3	28.0	21.9	1.00	1.03	1.31
<i>Mycobacterium vadosum</i>	29.2	20.7	28.5	21.6	1.00	1.03	1.37
<i>Brucella abortus</i>	29.0	21.0	28.9	21.1	1.00	1.00	1.37
<i>Alcaligenes faecalis</i>	33.9	16.5	32.8	16.8	0.98	1.03	2.00
<i>Pseudomonas aeruginosa</i>	33.0	16.8	34.0	16.2	0.99	0.97	2.03
<i>Mycobacter. tuberculosis BCG</i>	34.2	16.5	33.3	16.0	1.03	1.01	2.08
<i>Sarcina lutea</i>	36.4	13.6	35.6	14.4	1.00	1.03	2.57
<i>Actinomyces globisporus streptomycini</i>	36.1	13.4	37.1	13.4	0.98	0.98	2.73
<i>Actinomyces globisporus flavellus</i>	36.3	13.8	37.2	12.7	1.04	0.96	2.77
<i>Actinomyces griseus</i>	35.8	13.8	37.4	13.0	0.97	0.96	2.73
<i>Actinomyces viridochromogenes</i>	36.6	13.3	37.2	12.9	0.99	0.98	2.80
<i>Proactinomyces citreus</i>	35.3	14.2	36.7	13.8	0.98	0.96	2.57
<i>Micromonospora coerulea</i>	36.2	14.3	35.6	13.9	1.02	1.04	2.53

Abbreviations: G = guanine; A = adenine; C = cytosine; T = thymine; Pur. = purine bases; Pyr. = pyrimidine bases.

FIG. 24. (Belozersky, 1959).

(b) *Is cytoplasmic RNA made in close contact with DNA?* The pathways of RNA synthesis remain very obscure. It is true that Grunberg-Manago and Ochoa (1955) discovered an enzyme which catalyses the condensation

of ribonucleoside diphosphates into polynucleotides the basic chemical structure of which is identical to that of RNA (Grunberg-Manago *et al.*, 1956; Ochoa, 1959). DNA does not seem to be required for this reaction, but the enzyme also lacks specificity, and the nature of the polynucleotides which are made under its action depends essentially on the composition of the mixture of nucleoside diphosphates. It is only little influenced by the nature of the primer.

RNA COMPOSITION IN DIFFERENT BACTERIA

Species	Nucleotide content in molar per cent				Pur.	G + U	G + C
	G	A	C	U	Pyr.	A + C	A + U
<i>Clostridium perfringens</i>	29.5	28.1	22.0	20.4	1.36	1.00	1.06
<i>Staphyl. pyogenes aureus</i>	28.7	26.9	22.4	22.0	1.25	1.03	1.05
<i>Pasteurella tularensis</i>	29.8	27.3	21.0	21.9	1.33	1.07	1.03
<i>Proteus vulgaris</i>	31.0	26.3	24.0	18.7	1.34	0.99	1.22
<i>Escherichia coli</i>	30.7	26.0	24.1	19.2	1.31	1.00	1.21
<i>Proteus morganii</i>	31.1	26.0	23.7	19.2	1.31	1.01	1.21
<i>Shigella dysenteriae</i>	30.4	25.9	24.4	19.9	1.29	0.99	1.21
<i>Salmonella typhosa</i>	30.8	26.1	24.0	19.1	1.32	1.00	1.21
<i>Salmonella typhi-murium</i>	31.0	26.1	23.8	19.1	1.33	1.00	1.21
<i>Ervinia carotovora</i>	29.5	26.5	23.7	20.3	1.27	0.99	1.14
<i>Corynebact. diphtheriae</i>	31.6	23.1	23.8	21.5	1.21	1.13	1.24
<i>Azotobacter agile</i>	31.0	24.2	26.0	18.7	1.23	0.99	1.33
<i>Azotobacter vinelandii</i>	30.3	23.9	25.5	20.2	1.19	1.02	1.27
<i>Azotobacter chroococcum</i>	30.4	24.7	24.7	20.1	1.18	1.02	1.23
<i>Aerobacter aerogenes</i>	30.3	26.0	24.1	19.6	1.29	1.00	1.19
<i>Mycobacterium vadosum</i>	31.7	23.8	23.5	21.0	1.25	1.12	1.23
<i>Brucella abortus</i>	30.2	25.4	24.9	19.5	1.26	0.99	1.23
<i>Alcaligenes faecalis</i>	30.9	25.7	24.1	19.3	1.31	1.01	1.22
<i>Pseudomonas aeruginosa</i>	31.6	25.1	23.8	19.5	1.31	1.05	1.24
<i>Mycobacter. tuberculosis BCG</i>	33.0	22.6	26.1	18.3	1.25	1.05	1.45
<i>Sarcina lutea</i>	32.7	23.2	24.2	19.9	1.27	1.11	1.32
<i>Actinomyces globisporus streptomycini</i>	31.1	23.8	25.2	19.9	1.22	1.04	1.29

Abbreviations: G = guanylic acid; A = adenylic acid; C = cytidylic acid; U = uridylic acid; Pur. = purine nucleotides; Pyr. = pyrimidine nucleotides.

FIG. 25. (Belozersky, 1959).

No one knows whether the function of polynucleotidephosphorylase *in vivo* is to make RNA or to destroy it. There are reasons to believe that the synthesis of RNA follows another pathway, in which amino acids are somehow involved. In *Staphylococcus aureus* and in *Escherichia coli*, amino acid

FIG. 26. Portion of a lampbrush chromosome showing many loops of various lengths. Phase contrast. Magnification $\times 700$ (Gall, 1956).

deficiency suppresses not only protein synthesis but RNA synthesis as well (Sands and Roberts, 1952; Gale and Folkes, 1953; Borek *et al.*, 1955). In yeast, sulphur starvation prevents the synthesis of both protein and nucleic acid, although the formation of soluble nucleotides is not impaired (Schmidt *et al.*, 1956). Such observations at first suggested that RNA synthesis may depend on protein synthesis. However, chloramphenicol inhibits protein synthesis without preventing RNA formation (Gale, 1953; Wisseman *et al.*, 1954) and amino acids stimulate RNA synthesis even when protein synthesis is suppressed by the antibiotic (Gale and Folkes, 1953).

RNA synthesis in *Escherichia coli* requires the simultaneous presence of all the amino acids even when protein synthesis is 98 per cent inhibited by chloramphenicol (Gros and Gros, 1956; Pardee and Prestidge, 1956; Yčas and Brawerman, 1957). With amino acids requiring strains, under such conditions, the addition of a very small amount of the limiting amino acid will cause the synthesis of a large amount of RNA. One amino acid molecule makes possible the polycondensation of at least eight nucleotide residues (Gros and Gros, 1957). These observations can be accounted for by assuming, e.g. that the RNA precursors are amino acid-nucleotide compounds. Further developments will show whether this assumption is correct. Finally, an enzymic synthesis of RNA from ribonucleoside triphosphates has been reported (Chung *et al.*, 1960; Weiss, 1960).

The possible involvement of DNA in RNA synthesis has not been clearly observed so far in metabolic studies (see, however, Chung *et al.*, 1960). But our present knowledge on the pathways of RNA synthesis must be regarded as quite rudimentary.

Histochemical research indicates that the nucleus is an active centre of RNA synthesis, and that some RNA probably forms in the vicinity of DNA. The lampbrush chromosomes of amphibian oocytes (Fig. 26) and the giant chromosomes of insect salivary glands display protusions into the nuclear sap, which are called loops in the first case, puffs in the second. These contain RNA. Autoradiography studies, completed by ribonuclease treatment of the preparations, clearly established that the loops of lampbrush chromosomes and the puffs of giant chromosomes are the sites of a very active incorporation of radioactive precursors into this RNA (Taylor *et al.*, 1955; Ficq and Pavan, 1957; Ficq *et al.*, 1959). This probably reflects a net synthesis of RNA, since corresponding increases in basophilia were observed at similar locations (Pavan and Breuer, 1955).

In roots of *Vicia faba*, the RNA which is present in small amounts in chromatin, the DNA containing structure in resting nuclei, incorporates cytidine very rapidly (Woods, 1959; Woods and Taylor, 1959). Human amnion cells in tissue culture incorporate cytidine- ^3H in the extranucleolar

region faster than in nucleoli and much faster than in cytoplasm (Goldstein and Micou, 1959).

A nuclear element in which RNA accumulates in considerable amount is the nucleolus. Nucleoli are often observed to stay in close contact with certain regions of the chromosomes and there are many indications that the nucleoli are in some way produced by regions of the chromosome, often called nucleolar organizers (for a discussion see Brachet, 1957). During oogenesis in amphibians, the lampbrush chromosomes to which we have referred before are especially developed at the time when synthesis of yolk protein is most active, and nucleoli are also very numerous at this stage. The nucleoli might be regarded as a transitory storage place for RNA made in the chromosomes. It must be noted, however, that the nucleolar organizers are heterochromatic regions of the chromosomes, i.e. regions characterized by special staining properties, and which are believed to contain genetic controlling element involved in the quantitative expression of characters, whereas the genes of the sort we have considered so far in relation with protein structure are located in the euchromatin.

The histochemical observations on the whole support the idea that some RNA may be built up within the chromosome or in close contact with the genetic material. They are compatible with the hypothesis that RNA receives genetic information from DNA, although they cannot be taken as evidence for it.

The next task that the genetic messenger should accomplish is getting out of the nucleus and reaching the centres of protein synthesis in the cytoplasm. What is the evidence for the transfer of RNA from nucleus to cytoplasm?

Precursors of RNA are usually incorporated more rapidly into nuclear RNA than into the average cytoplasmic RNA (Jeener and Szafarz, 1950; Smellie *et al.*, 1953, 1955; Barnum *et al.*, 1953). From the kinetics of the incorporation, it was concluded that the bulk of RNA in liver cytoplasm does not derive from the nucleus, but that the possibility exists that some cytoplasmic RNA might be produced in the nucleus (Smellie *et al.*, 1953; Barnum *et al.*, 1953).

This conclusion rests on several assumptions which are very difficult to check and which now appear as oversimplifications, since it is known how heterogeneous both nuclear and cytoplasmic RNA are.

Enucleation experiments were designed to establish whether RNA can be formed at all in the absence of the nucleus. In *Acetabularia*, or in sea urchin eggs, enucleation has no effect on incorporation of labelled orotic acid or glycine into RNA (Malkin, 1954; Brachet and Szafarz, 1956; Brachet *et al.*, 1955). Precursors are also incorporated into the RNA of rabbit reticulocytes (Kruh and Borsook, 1955). Incorporation of precursors into total cytoplasmic RNA cannot, however, be equated to RNA synthesis,

it might possibly be due, e.g. to chain end metabolism (see p. 66). Direct determination of the RNA content of batches of nucleate and enucleate parts of *Acetabularia* indicated a net RNA synthesis of short duration in enucleate fragments (Brachet *et al.*, 1955). Other groups of workers observed an immediate cessation of RNA synthesis after enucleation (Richter, 1957; Noara *et al.*, 1959), or even a loss of RNA. It is probable therefore that the RNA found in the cytoplasm comes largely from the nucleus. On the other hand, cytoplasmic RNA in *Acetabularia* is not inert, it seems to undergo a turnover process (degradation and resynthesis), which is independent of the nucleus. When this cytoplasmic RNA metabolism is interfered with by ultraviolet irradiation, net loss is observed. This is, however, followed by recovery and a net synthesis of RNA is then observed in enucleate fragments (Richter, 1959). Conflicting results obtained by different groups are probably due to differences in physiological conditions of the algae under study in the individual cases. Different adjustment of the cytoplasmic steady state of RNA may result in net synthesis (Brachet *et al.*, 1955), absence of variation (Richter, 1957) or net decrease of RNA content of the cytoplasm (Naora *et al.*, 1959). Chloroplastic RNA forms in enucleate cytoplasm, apparently at the expense of other cytoplasmic RNAs (Naora *et al.*, 1960). The cytoplasmic RNA turnover observed by Richter (1959) also explains that incorporation of various precursors into cytoplasmic RNA is largely independent of the nucleus. All the data on net synthesis of RNA in *Acetabularia* nevertheless make it clear that this process is closely dependent on the presence of the nucleus, and much more so than protein synthesis which continues undisturbed after enucleation. Recent results by Richter (1959) are especially convincing: if a nucleate fragment of *Acetabularia* is grafted on an enucleate part in which net RNA synthesis had stopped, the amount of RNA again starts to increase in the old cytoplasmic fragment.

This is all very suggestive of a nuclear production of RNA which is secreted into the cytoplasm. The important point would be to know whether this special RNA fraction is bringing with it information for protein synthesis. Although this question cannot be answered yet, interesting results relevant to this question were reported by Stich and Plaut (1958). Ribonuclease inhibits protein synthesis both in nucleate and enucleate fragments of *Acetabularia*. When the fragments are transferred to normal medium after a few days of ribonuclease action, protein synthesis is restored in nucleate but not in enucleate moieties. This shows that a substance which is inactivated by ribonuclease and which is involved in protein synthesis requires the presence of the nucleus for its formation. It is exactly what would be expected if the 'central dogma' was true.

In *Amoeba*, the importance of the nucleus for the synthesis of cytoplasmic RNA is even more striking than in the case of *Acetabularia*. Thus

the RNA content of enucleate fragments of *Amoeba proteus* decreases quickly and strongly during starvation whereas it is maintained in nucleate halves (Brachet, 1955). If living *Amoebae* are transferred into a medium containing ribonuclease, the enzyme enters the cells and destroys a large part of the RNA both in the cytoplasm and in the nucleus. When the *Amoebae* are later returned to normal medium or better to a medium containing yeast RNA, nucleic acid is reformed in many *Amoebae*; under such conditions, RNA appears first in the nucleoli and later in the cytoplasm, and enucleate fragments are unable to restore their nucleic acids (Brachet, 1955 b, c).

More direct evidence for the passage of nuclear RNA into the cytoplasm in *Amoeba proteus* was obtained by Goldstein and Plaut (1955). *Amoebae* were fed with micro-organism which had been grown in a medium containing $^{32}\text{PO}_4$. The nuclei of labelled *Amoebae* were grafted into non-labelled *Amoebae*. Autoradiography studies and ribonuclease test indicated that radioactive phosphorus contained in the nuclear RNA at the beginning of the experiment was lost by the nucleus and later found in cytoplasmic RNA. Although in these experiments it is actually the fate of phosphorus atoms which was studied, not that of RNA molecules, these data strongly suggested that part of the cytoplasmic RNA originates in the nucleus.

Incorporation experiments using radioactive adenine and uracil in enucleate cytoplasm of *Amoeba* invariably showed that enucleation drastically reduces the incorporation into cytoplasmic RNA (Plaut and Rustad, 1956; Prescott, 1957). Conflicting results have been reported as to whether cytoplasm can make some RNA in the absence of the nucleus or whether all cytoplasmic RNA comes from the nucleus. The latter opinion was advocated by Prescott (1957, 1959) who believes that the low residual incorporation observed in enucleate *Amoeba proteus* is due to the activity of micro-organisms recently phagocyted by the *Amoeba*. Indeed with an *Acanthamoeba*, which can be grown in sterile medium, no detectable incorporation of adenine, orotic acid or uracil takes place in enucleate fragments (Prescott, 1960). An uncertainty which remains is whether the precursors enter the enucleate parts at all, for absorption of various substances can be affected by enucleation (see Mazia and Prescott, 1955).

Recent kinetic studies on RNA synthesis in populations of animal cells also provide evidence that some RNA of nuclear origin can be secreted into the cytoplasm. Thus cells of the human amnion grown in tissue culture were exposed for a short time to cytidine- ^3H , and then replaced in a medium containing non-labelled cytidine. Autoradiography showed a progressive movement of the label from nucleus to cytoplasm. All the label that was in the RNA of the nuclei at the time of transfer into 'cold' medium was found in cytoplasmic RNA 24 hr later (Goldstein and Micou, 1959).

Perry and Errera (1960) showed that in Hela cells irradiation of the nucleolus with a microbeam of ultraviolet light ($257\text{ m}\mu$) inhibits the incorporation of tritiated cytidine into both cytoplasmic and nuclear (extranucleolar) RNA. Kinetic studies are interpreted as indicating that RNA synthesis takes place independently both in the nucleoli and in the nucleus (outside the nucleoli), and that both fractions pass into the cytoplasm. According to the same studies, less than 10 per cent of the cytoplasmic RNA can be formed in the cytoplasm of Hela cells (Perry, 1960). Working with a similar material Feinendegen *et al.* (1960) came to the conclusion that RNA synthesis begins in the chromatin portion of the nucleus, and that RNA later passes into the cytoplasm. Amano and Leblond (1960) also concluded that, in liver, nucleolar and other nuclear RNAs are made independently and that they behave as precursors of cytoplasmic RNA.

Further support to the hypothesis of a nuclear origin of cytoplasmic RNA will be found in studies on the labelling of RNA of *Drosophila* larvae by radioactive phosphate (Herbert, 1954) or tritiated uridine (Zalokar, 1960).

Clear evidence in the same direction was obtained by Zalokar (1959, 1960b) in experiments with hyphae of *Neurospora crassa* the content of which had been stratified by high speed centrifugation. In short exposures to tritiated uridine followed by dilution of the isotopic precursors with non-labelled uridine, the nuclear RNA is labelled first, tritium-marked compounds later appear in the cytoplasm and the labelling of ergastoplasm increases with time at the expense of nuclear label. Again there remains the possibility that the labelled molecules which pass from the nucleus to the cytoplasm are not intact RNA molecules, but products from which cytoplasmic RNA is built up. But the possibility that RNA does pass is very good.

The works reviewed above dealt with several types of animal and plant cells and made use of various experimental approaches. Their results are not always in entire agreement with one another; however, a general conclusion can be safely drawn, namely that the nucleus is a very important centre of ribosenucleic acid synthesis; moreover, it is likely that a large part of the cytoplasmic RNA is made within the nucleus in the vicinity of chromatin. Clearly, these are very good reasons to retain the hypothesis that the genetic messenger which carries genetic information from DNA to the cytoplasmic centres of protein formation is probably made of RNA.

(c) *Where is the genetic messenger?* It should not be forgotten, however, that the very existence of a messenger carrying the genetic information has not been established by direct experiments, it is postulated only because most proteins arise at a distance from DNA or can be made in the absence of DNA. The experiments which come closest to establish directly the existence of genetic information outside DNA, and to provide some indications

about the nature or the state of the compound which carries it, are unfortunately not in perfect agreement with each other. Schweet *et al.* (1958) isolated microsomes from reticulocytes and showed that the non-sedimentable fraction was required together with the microsomes for amino acids to be incorporated into Hb. Liver supernatant can be substituted for reticulocytes supernatant, but liver microsomes even in the presence of reticulocytes supernatant do not catalyse incorporation into Hb. This indicates that part at least of the specific information must lay in the microsomes (see also Allen and Schweet, 1960; Allen *et al.*, 1960). On the other hand, Wainwright (1960) working with extracts of *Neurospora* reached a different conclusion. The synthesis of tryptophane synthetase could be observed in cell-free extracts of conidia. Mixed system containing soluble and sedimentable fractions prepared from wild strain or from a mutant lacking tryptophane synthetase were used. When a mutant 'particle' fraction was supplemented with wild type soluble fraction, considerable tryptophane synthetase was produced. Conversely, a mixture of wild type 'particles' with mutant 'soluble' fraction failed to develop any detectable activity. The defective component of the mutant extract has thus been located in the soluble, non-sedimentable fraction.

It is probable that in such systems, and with the progress of techniques for isolation of biologically active RNA, it will soon be possible to check whether the carrier of genetic information is an RNA, or else to discover its nature.

Reports from several laboratories have indicated that RNA extracted from micro-organisms might cause the production of certain specific proteins or might confer specific characters to homologous cells (Minagawa *et al.*, 1951; Minagawa, 1955; Reiner and Goodman, 1955; Kramer and Straub, 1956; Hunter and Butler, 1956; Hrubešova *et al.*, 1959; Kessler, 1957). It must be admitted that the results obtained in these interesting works are not always sufficiently clear and reproducible. At present, it is very difficult to evaluate them. If these observations prove to be correct, they will acquire a fundamental significance comparable only to bacterial transformation by DNA and to transmission of virus diseases by isolated RNA.

5. Concluding Remarks

The participation of RNA in protein synthesis has been known for twenty years. So far, a function has been clearly attributed to only one class of RNA — the 'transfer RNAs' which are specific carriers of activated amino acids (see p. 102). But these represent only a small fraction of total RNA. The exact function of ribosomal RNA is not yet clarified. The evidence for the participation of this RNA in protein synthesis remains the correlation between their amount and the intensity of protein synthesis,

and the fact that proteins arise in contact with the ribosomes. The nature of the evidence has not changed for twenty years; it has simply become more and more convincing as more numerous and more refined experiments were made. The exact function of ribosomal RNA in protein synthesis is still an enigma.

CHAPTER IV

Chemical Pathways of Protein Biosynthesis

A. ENERGY REQUIREMENT

For many years, it was assumed that the synthesis of protein is brought about by proteolytic enzymes. An enzyme, acting as a true catalyst, should be able to promote a reaction in both directions. Since a mixture of proteolytic enzymes split protein into smaller peptides and finally into amino acids *in vitro*, it seemed reasonable to assume that the same agents are responsible for amino acid condensation into proteins *in vivo*. The conditions within the living cell were supposed to be such that the hydrolytic process was reversed. Plastein formation in concentrated protein hydrolysate and the formation of relatively insoluble peptides under the action of proteolytic enzymes, provided some support for this theory. However, it was untenable on thermodynamical grounds. The standard free energy of hydrolysis of a dipeptide is about -3 kcal. The figure becomes less negative when the peptide chain becomes longer but it is still about -1 kcal for a soluble polypeptide of 'infinite length' (Borsook and Huffman, 1945; Linderström-Lang, 1949, 1952). The concentration of the amino acids in the cell is low; the amount of a dipeptide in equilibrium with the corresponding pair of amino acids must be very small indeed (Linderström-Lang, 1949; Borsook, 1954), and for each further condensation the equilibrium concentration will rapidly decrease and become vanishingly small. These considerations led to the prediction that the condensation of amino acids into elementary peptides and into polypeptides will not proceed to any appreciable extent unless it is driven by some process making energy available for the reaction (Lipmann, 1941; Linderström-Lang, 1949, 1952).

Borsook and Dunoff (1940) were the first to check this prediction experimentally in the case of hippuric acid formation; they determined the equilibrium constant of hippuric acid hydrolysis, and were able to show that the amount of hippuric acid actually synthesized *in vitro* by kidney or liver slices from various animals is 60–75 times greater than the amount corresponding to the thermodynamic equilibrium. Coupling with an energy-yielding process was also indicated by the fact that inhibition of respiration either by cyanide or by oxygen deprivation completely inhibited the synthesis. Lipmann (1945) later showed that the acetylation of

amines which also results in the formation of a peptide-like linkage, is blocked by dinitrophenol, indicating that the energy required for peptide bond formation is provided by oxidative phosphorylation. With a system of soluble enzymes from pigeon liver, the requirement for ATP was established, and it was found that one mole of ATP is split for each mole of amine acetylated. Similar conclusions were rapidly extended to the synthesis of other small peptides: hippuric acid (Borsook and Dubnoff, 1947), *p*-aminohippuric acid (Cohen and McGilvery, 1946, 1947) and glutathione (Johnston and Bloch, 1951; Webster, 1953). Evidence that the synthesis of enzymes in bacteria and in yeast depends on phosphorylation had meanwhile been obtained (Monod, 1944; Spiegelman, 1946).

Peptidic bond formation in small peptides was thus regarded as a model of possible mechanisms of polypeptide biosynthesis and these studies had indeed a fundamental influence on the discovery of what is known at present about the pathways of protein formation. The advent of ^{14}C -labelled amino acids was the other element which made possible the rapid progress of the last decade in this field.

As soon as the requirement for energy coupling was discovered for small peptidic compounds, it was readily established that the incorporation of labelled amino acids into protein of tissue slices or of homogenates also depends on respiration (Frantz *et al.*, 1947, 1948; Winnick *et al.*, 1947; Melchior *et al.*, 1948; Peterson and Greenberg, 1952). Amino acid incorporation, like the synthesis of small peptides, is driven by oxidative phosphorylation since the inhibition of the incorporation process by dinitrophenol, runs parallel to the inhibition of phosphorylation (Frantz *et al.*, 1948). Finally, it was clearly established that ATP or systems able to regenerate it are required for protein synthesis in homogenates (Peterson and Greenberg, 1952; Siekevitz, 1952; Zamecnik and Keller, 1954). It was probable therefore that the condensation of amino acids into polypeptides involved — at one stage at least — a coupling with ATP splitting. A great many works have now confirmed these data and extended them to many various tissues and organisms. Coupling with ATP utilization is a quite general requirement for amino acid incorporation into protein.

Most workers in the field were long reluctant to equate amino acid incorporation to *de novo* protein synthesis from amino acids. The possibility that incorporation corresponds to replacement of an old amino acid unit by a new one within polypeptides has been a constant source of worry. This question had already been raised by Schoenheimer *et al.* (1939) who were the first to observe amino acid incorporation into protein in living animals, and it became more acute in works with tissue slices or homogenates where incorporation is always very low and where no measurable net increase in protein was observed. Exchange incorporation found apparent support in certain facts. For instance the amino acid 'analogue'

p-fluorophenylalanine suppresses the synthesis of enzymes and depresses the incorporation of phenylalanine; but it does not affect the incorporation of the other amino acids (Halvorson and Spiegelman, 1952; Rabinovitz *et al.*, 1954). The first interpretation of these facts was that the amino acid analogue blocks phenylalanine utilization and net protein synthesis, and that the unaffected incorporation of the other amino acids must therefore be due to an exchange process. This interpretation later proved incorrect; actually, *p*-fluorophenylalanine does not prevent protein synthesis, it competes with phenylalanine and it is incorporated into proteins instead of the normal amino acid (Baker *et al.*, 1954; Munier and Cohen, 1956, 1959; Kerridge, 1959; Vaughan and Steinberg, 1960; Richmond, 1960). The incorporation of all the other amino acids in the presence of the analogue is due to net synthesis of abnormal proteins in which a large part of the phenylalanine is replaced by the analogue. Many of the abnormal enzymes made in the presence of fluorophenylalanine are inactive, hence the observation that the synthesis of enzymes (as measured by the increase of enzymic activity) is inhibited. Other amino acid analogues act in the same way (Sharon and Lipmann, 1957; Pardee *et al.*, 1957; Brawerman and Yčas, 1957; Gross and Tarver, 1955; Vaughan and Steinberg, 1959; Munier and Cohen, 1959; Cowie and Cohen, 1957; Cohen *et al.*, 1958; Rabinovitz *et al.*, 1955). Clearly, there is no reason to call upon exchange processes for explaining the experimental results.

Another group of observations which were regarded as direct evidence for exchange processes was made by Gale (1957) on disrupted *Staphylococci*. The time curve of the incorporation of glutamic acid into acid insoluble compounds is not the same when glutamic acid is added alone or together with all the other amino acids. When a mixture of amino acids is provided, the incorporation is linear and largely irreversible, for the addition of non-labelled glutamic acid causes no loss of incorporated ^{14}C . To the contrary when the system is presented with glutamic acid alone, incorporation is fast but it rapidly stops; the time curve resembles a saturation or adsorption curve. Moreover, non-labelled glutamic acid causes a release of part of the previously incorporated labelled compound, indicating a replacement by mere exchange (Gale, 1957). These facts, no doubt, were well observed but their interpretation at present must be re-examined; at the time they were made, the existence of soluble RNA and its capacity of binding amino acids reversibly was not known; it is possible that part of the exchangeable glutamic acid was bound in this way or at the end of polypeptide (Webster, 1959). It was not known either that in bacterial preparations amino acids can be incorporated into peptide constituents of cell wall material (Mandelstam and Roger, 1958; Chantrenne and Devreux, 1958, 1960; Hancock and Park, 1958; Richmond, 1959; Roodyn and Mandel, 1960) and it would seem that part of the glycine or glutamic acid

incorporated into acid-insoluble material in the absence of other amino acids, is actually incorporated into cell wall material rather than into protein (Gale and Folkes, 1958; Mandelstam and Rogers, 1959). Thus, the apparent evidence provided by the quoted experiments in favour of direct exchange of free amino acids with amino acid residue in a protein cannot any more be regarded as valid.

It has been most clearly shown that in exponentially growing bacteria amino acid incorporation into protein is essentially irreversible (Rotman and Spiegelman, 1954; Hogness *et al.*, 1955). In animal or plant tissue, in yeast and in resting bacteria, a protein turnover does occur (Halvorson, 1958; Mandelstam, 1957, 1958), but there is no reason to assume that it is due to an amino acid exchange; protein turnover probably reflects the continued formation of new molecules from amino acids released by destruction of other protein molecules. Exchange process can still be called upon for explaining unequal labelling of proteins in various positions, but it is only one of several possible interpretations of this phenomenon which is still completely obscure (see p. 115).

In conclusion, it may be assumed that amino acid incorporation within polypeptide chains is due to net synthesis of protein material; the only restriction being that it may not always correspond to the formation of complete or perfect protein molecules.

ATP requirement for amino acid incorporation confirmed that protein synthesis is driven by energy yielding reactions, and indicated ATP as the essential energy distributor in this process as well as in many other syntheses. The mechanism of the energy coupling has been the object of many studies. An early working hypothesis assumed that ATP was used in the making of small peptides, and that polypeptides resulted from rearrangements of amino acids between these peptides by a series of transpeptidation reactions (Fruton, 1950, 1952, 1957; Hanes *et al.*, 1950; Waelsch, 1952; Linderstrom-Lang, 1949, 1952; Virtanen, 1950). The justification of this hypothesis was that it accounted for the energy coupling and ATP requirement on one hand, and that it implicated on the other hand many well observed transpeptidation processes catalysed by proteolytic enzymes. Transpeptidations involving esters or amides of amino acids or peptides can indeed result in the formation of rather long peptides *in vitro* (Fruton *et al.*, 1951, 1953; Jones *et al.*, 1952; Durell and Fruton, 1954; Waley and Watson, 1954; Blau and Waley, 1954; Schweet *et al.*, 1948; Brenner *et al.*, 1950; Tauber, 1952; Kaganova and Orekhovich, 1954; Watson, 1956; Neumann *et al.*, 1959). Glutamine and glutathione received special attention as possible initiators of protein formation, because they can transfer their glutamyl residue to a variety of amino acids and peptides (Hanes *et al.*, 1950, 1952, 1953; Waelsch, 1952; Hird and Springell, 1954) and it was conceivable that a series of transfers of carboxyl or amino moieties of small peptides could

result in polypeptide formation. However attractive this hypothesis may have looked for some time, no convincing evidence for the operation of such a mechanism has been provided, and direct attempts to test the idea of the participation of glutathione for instance as an initiator of amino acid incorporation have led to a negative conclusion (Hendler and Greenberg, 1954; Flavin and Anfinsen, 1954; Askonas *et al.*, 1955; Barry, 1956). Besides, glutamyl transpeptidases are found only in a very few tissues (Revel and Ball, 1959). If polypeptides were formed by a series of transpeptidations at the energy level of the peptide bond, the concentration of free intermediary peptides would be rather high, for the equilibrium constant of such transpeptidations should be close to unity. And no free peptides have been found to accumulate in living cell to any considerable extent. There is at present no positive reason to think that proteolytic enzymes play an important part in protein synthesis, neither that they play any part in the process. It is not excluded, however, that they might be involved in the finishing steps of protein formation (Horowitz and Haurowitz, 1959); limited proteolysis is clearly involved in zymogen activation (Davie and Neurath, 1955; Desnuelle and Fabre, 1955).

An alternative to the hypothesis considered above for explaining how energy is funnelled into protein synthesis, is to assume that the amino acids, or at least some of them, are activated directly in some process involving ATP, and that the activated forms of the amino acids then condense into polypeptides.

B. THE ACTIVATION OF AMINO ACIDS

1. *Amino Acid Activation Enzymes*

Analysis of the enzymic acetylation of aromatic amines by Lipmann's group led to the basic discovery that ATP, which is used up stoichiometrically in the process, is not split in the 'usual' way into ADP and phosphate; instead, it is broken into AMP and inorganic pyrophosphate (Lipmann, 1952; Jones *et al.*, 1953). This was the first observation of the direct utilization of the second energy rich bond of ATP. Another example of this new type of ATP cleavage was soon found by Maas and Novelli (1953) in pantothenic acid synthesis from pantoic acid and β -alanine. Experiments with purified preparations of the enzyme provided evidence that the formation of pantothenic acid involves the production of an enzyme-bound pantoyl adenylate as an intermediate. The evidence was as follows: the purified preparation catalyses an exchange of the two terminal phosphates of ATP with inorganic pyrophosphate, but this exchange requires the presence of pantoate specifically. In the presence of concentrated

hydroxylamine, pantoylhydroxamic acid and inorganic pyrophosphate are formed in stoichiometric amounts, but this reaction is much slower than the exchange. These experimental facts can be explained by the following scheme (Maas, 1955) —

The formation of a bond between AMP and pantoate was confirmed by the observation of a transfer of ^{18}O between the carboxyl group of pantoic acid and AMP.

It was soon found that a relatively slow pyrophosphate-ATP exchange, which takes place in soluble extracts of rat liver, is strongly stimulated by a mixture of the natural amino acids, and that hydroxamates are formed in the presence of amino acids and high concentration of hydroxylamine; leucylhydroxamate was identified among them (Hoagland, 1955). Completely similar observations were made by De Moss and Novelli (1955) with extracts of many bacterial strains as well as yeast and moulds. That the reactions involved are quite similar to those of pantoate activation was soon confirmed. Leucyladenylate prepared by chemical synthesis when incubated with these bacterial extracts and inorganic pyrophosphate forms ATP, and free leucine appears (De Moss *et al.*, 1956). Heavy oxygen is exchanged between the carboxyl of an amino acid and the AMP moiety of ATP (Hoagland *et al.*, 1957; Bernlohr and Webster, 1958) showing that at some time during the process the carboxyl group of the amino acid shares one oxygen atom with AMP, not with the pyrophosphate residue.

These experiments clearly established the existence of a direct activation of amino acids. Amino acid activation enzymes were then sought and found in all kinds of animal or plant tissues and in micro-organisms (see reviews by Novelli and De Moss, 1957, and by Chantrenne, 1960).

The discoverers of the amino acid activation enzymes noticed that if the total concentration of amino acids is kept constant, the ATP-pyrophosphate exchange is promoted as the number of individual amino acids is increased. This strongly suggested that the different amino acids did not compete for a common activation enzyme, and that there were probably several enzymes with different specificities. When activation was studied separately for each individual amino acid, great differences in efficiency in promoting the pyrophosphate exchange were observed between them (De Moss and Novelli, 1955, 1956; Hoagland *et al.*, 1956; Berg, 1956). Some of the natural amino acids had so little effect on the exchange that for a time it was believed that a few of the natural amino acids only can be activated in such reactions. Later on, activation of all amino acids was observed in bacteria (Nisman *et al.*, 1957, 1958; Nisman, 1959) in animal and plant tissues (Lipmann, 1958; Webster, 1959). But it remains that the reaction

of some amino acids, like proline, histidine, threonine, tryptophan, leucine is usually strong, whereas that of arginine, glutamic acid or asparagine is often so weak as to be difficult to show (Lipmann, 1958). This may be due, in part at least, to differences in stability of the individual activation enzymes in tissue extracts, as well as to exacting requirements of some of them (Schweet *et al.*, 1957; Nisman, 1959; Webster, 1959). For instance a purified alanine-activation enzyme from rat liver is very labile, it loses its activity by freezing and thawing, and it is rapidly inactivated by oxygen (Holley and Goldstein, 1959).

Davie *et al.* (1956) were able to isolate from beef pancreas an almost pure enzyme which is highly specific for L-tryptophan; the only other amino acids which react with this enzyme are certain tryptophan analogues (Sharon and Lipmann, 1957). Enzymes which activate other amino acids specifically have now been isolated or purified to various degrees: the enzyme for methionine activation was purified from yeast (Berg, 1956), tryptophan activation enzyme was separated from the enzymes for threonine and serine in an extract of pig pancreas (Cole *et al.*, 1957). Tyrosine activation enzymes were also partly purified from the same tissue (Schweet *et al.*, 1957) and from yeast (Van de Ven *et al.*, 1958). Serine activation enzyme was purified from beef pancreas (Webster and Davie, 1959) and a threonine specific enzyme from calf liver (Lipmann *et al.*, 1959). Recently, activation of the dipeptides L-leucyl-L-tyrosine and glycyl-L-leucine has been reported (Tuboi and Huzino, 1960; Brown, 1960).

Little is known at present about the structure of these activation enzymes and their mode of action. Their active centre probably contains a —SH group, for *p*-chloromercuribenzoate or oxygen inhibit the tryptophan enzyme from pancreas (Davie *et al.*, 1956) and the alanine enzyme from rat liver (Holley and Goldstein, 1959). The activity of several activation enzymes is protected by reduced glutathione (Allen *et al.*, 1960). The participation of —SH groups in activation enzymes reminds one of the case of triosephosphate dehydrogenase. This enzyme also contains —SH groups (Rapkine, 1938) to which the activated carboxyl of phosphoglyceric acid is transitorily bound as a thioester (Krimsky and Racker, 1952). Activated carboxyls are also carried by coenzyme A as thioesters. One may therefore suspect the participation of a thioester of amino acids at some stage of the activation process.

Some activation enzymes cause a rapid formation of hydroxamates although they catalyse the pyrophosphate exchange very poorly (Novelli and De Moss, 1957). Further analysis of this apparent discrepancy might throw light on the reaction mechanism.

An interesting fact is that the aminoacyl adenylates are very strongly bound to the enzymes. During the pyrophosphate-ATP exchange catalysed by the enzyme in the presence of the corresponding amino acid, no net

change of ATP concentration is observed (De Moss *et al.*, 1956), and no free aminoacyl adenylate could ever be detected (Hoagland *et al.*, 1956). Using large amounts of pure tryptophan activation enzyme, Kingdon *et al.* (1958) and Karasek *et al.* (1958) were able to liberate tryptophanyl adenylate by denaturing the enzyme. The amount of anhydride obtained is compatible with the existence of one molecule of tryptophanyl adenylate per molecule of enzyme. The aminoacyl adenylate might therefore be described as a prosthetic group of the enzyme, rather than as a substrate or reaction product. Actually, free aminoacyl adenylates would rapidly disappear in the cell and be wasted in all kinds of reactions; it is quite certain that their being bound to the activation enzyme protects them from reacting at random (Askonas *et al.*, 1957; Moldave *et al.*, 1959). They are anhydrides of a carboxylic acid with adenylic acid, and belong therefore to the class of mixed anhydrides of a carboxylic acid with a phosphoric group which carries a substituent. Substances of this type have been shown to be rather

FIG. 27. Amino acyl adenylate.

stable in water, and much more so than the corresponding compound with unsubstituted phosphoric group like acetylphosphate (Chantrenne, 1948). On the other hand, they react very rapidly with amino acids to form a peptide bond, even in 10^{-3} M solution at pH 7.4 and this is a purely non-enzymic reaction (Chantrenne, 1947, 1948, 1949, 1950; Katchalski and Paecht, 1954; Avison, 1955; Moldave *et al.*, 1959). Free aminoacyl adenylates in neutral or slightly alkaline medium would rapidly form polypeptides at random or produce a large variety of peptides by reacting with any amino acid present. The formation of well-defined proteins from such substances is possible only if they are in some way prevented from reacting at random (Chantrenne, 1950). That their binding to the enzyme protects the aminoacyl adenylates from reacting with amines is clearly shown by their behaviour in the presence of hydroxylamine. Whereas in the free state they would react extremely readily with neutral 10^{-3} M hydroxylamine, it is necessary to use at least 0.3 M, sometimes 3 M hydroxylamine to trap these 'activated' carboxyl groups (Hoagland, 1955; De Moss and Novelli, 1955; Davie *et al.*,

1956; Berg, 1956). But at such high concentrations, hydroxylamine reacts even with common esters (Chantrenne, 1948; Raacke, 1958). The reactivity towards amines of enzyme bound aminoacyl adenylates is thus much lower than that of the free form. Incidentally, the high reactivity of aminoacyl adenylates raised doubts as to the meaning of amino acid incorporation into proteins in the presence of activation enzymes which produce amino acid adenylates: could not the observed incorporation be explained by a non-enzymic reaction of the mixed anhydride with —NH_2 groups of pre-existent proteins (Zioudrou *et al.*, 1958; Zioudrou and Fruton, 1959)? The low reactivity of the enzyme bound anhydride with common amines permits us to discard this objection; but it would be a fundamental mistake to provide an experimental system with ready made free aminoacyl adenylates, for these — at least the fraction in excess to what can be bound by the corresponding enzyme — would immediately react at random with most available —NH_2 groups (Wong *et al.*, 1959).

Aminoacyl adenylates are anhydrides of the amino acids, but they cannot leave the enzyme, and they are not very reactive in this bound form even with as eager an acceptor as hydroxylamine. One may wonder then whether such an activated form can be an intermediate in protein synthesis, and what its normal acceptor is.

These two questions are actually linked. Let us consider first the fate of the activated amino acid.

In amine acetylation (Lipmann, 1950), in the synthesis of hippuric acid (Chantrenne, 1951; Schachter and Taggart, 1954), the active acyl residue of the enzyme-bound acyladenylate is picked by coenzyme A, a typical acyl carrier which conveys it to the condensing enzyme where the acyl radical will meet the acceptor amine and condense with it in a practically irreversible reaction. But aminoacyl residues are not transferred to coenzyme A (Jencks and Lipmann, 1957).

The discovery of the amino acid acceptor was a major achievement of work on tissue homogenates and disrupted cells, which will now be considered briefly. The first experiments designed with the hope of observing incorporation of labelled amino acids into homogenates gave depressingly low incorporation indeed. It was established, however, that the incorporation was real and not due to contamination, and that it depended on respiration (Winnick *et al.*, 1948) or more precisely on oxidative phosphorylation (Frantz *et al.*, 1948; Borsook *et al.*, 1949). The amino acids were shown to be incorporated within polypeptide chains (Winnick, 1949, 1950) and the incorporation of one labelled amino acid was stimulated by a mixture of the others. The recognition of a few pitfalls (Borsook *et al.*, 1949; Winnick, 1950; Peterson and Greenberg, 1952) and their elimination, and progress in the methods of preparation of homogenates (Schneider and Hogeboom, 1951; Bucher, 1953) provided extracts in which amino acid

incorporation was better and continued at a linear rate for about 15 min (Siekevitz, 1952; Zamecnik and Keller, 1954); it was possible then to obtain reproducible and reliable results. The factors required for incorporation and the interactions between several components of the system could be studied more easily and with more confidence than before.

It is in the microsomal fraction that the highest rate of incorporation was observed, in homogenates of liver as well as in the liver of the living animal. The microsomes alone were almost inactive, but they would incorporate readily in the presence of both mitochondria and the soluble fraction (Siekevitz, 1952). The function of the mitochondria in such a system is actually limited to the regeneration of ATP, for they can be dispensed with, provided substances able to furnish phosphate-bond-energy by anaerobic processes are available (e.g. hexosediphosphate, phosphocreatin, phosphoenolpyruvate). Dialysis of the soluble fraction suppressed the incorporation, but this could be restored to a large extent by a supplement of ATP and adequate metabolizable substrate to regenerate it (Zamecnik and Keller, 1954).

In later studies, Keller and Zamecnik (1956) observed that the fraction of this supernatant which plays a part in amino acid incorporation is precipitated when the pH of the dialysed supernatant is adjusted to 5. This fraction, often called 'pH 5 enzymes', is of course a very crude mixture of many components which precipitate together at that pH. The incorporation system could then be reconstituted from the microsomal fraction, the redissolved pH 5 precipitate, ATP and an ATP regenerating system. Reprecipitation or treatment of the pH 5 fraction by the anion exchanger Dowex-1 inhibited the system almost completely. Reactivation was achieved by ATP plus a small amount of guanosinetriphosphate. This is one more compound required for amino acid incorporation (Keller and Zamecnik, 1956; Littlefield and Zamecnik, 1957). The pH 5 fraction then received special attention. Hoagland (1955) had found that the supernatant fraction of a liver homogenate catalyses the exchange of pyrophosphate with ATP in the presence of several amino acids, and the formation of hydroxamates of the amino acids in the presence of hydroxylamine, thus indicating the presence of amino acid activation enzymes in this supernatant. Association of these activities with the pH 5 precipitate obtained from the crude supernatant (Hoagland *et al.*, 1956), strongly suggested that these activation enzymes are the constituents of the pH 5 precipitate which are required for amino acid incorporation.

The system at this stage seemed to consist of an energy source capable of regenerating ATP, the activation enzymes which will cause the condensation of the amino acids with ATP, the microsomal particles which are the place where newly-formed polypeptides are first observed, and guanosinetriphosphate. In between the activation enzymes and the microsomal

particles there was a big gap. Since the amino acid adenylates are strongly bound to the activation enzymes, the problem was to find out how activated amino acids were transferred to the microsomal particles.

Hultin (1956) obtained evidence for the accumulation in homogenates of some form of activated amino acids which can serve as precursors of proteins in the absence of ATP. The addition of a one thousand-fold excess of non-labelled leucine to a homogenate in which radioactive leucine is being incorporated causes a sudden and radical drop of specific radioactivity of the free leucine, but incorporation is not stopped immediately: it goes on undisturbed for a few minutes, before being completely inhibited. This indicates that some intermediate between free amino acids and precipitable protein piles up in the homogenate. The intermediate is in the soluble fraction, and it is in a form which does not equilibrate rapidly with free amino acids as the amino acid adenylates would do (Hultin and Beskow, 1956).

Holley (1957) obtained indications on the probable polyribonucleotidic nature of the acceptor by observing that ribonuclease suppresses a AMP-ATP exchange which is catalysed by the pH 5 fraction and which depends on alanine. Hoagland *et al.* (1957) found that the 'pH 5 enzymes' preparation from rat liver contains about 5 per cent ribonucleic acid, and that when this preparation is incubated with ATP and labelled leucine, the RNA subsequently isolated from this fraction is labelled. Leucine is thus bound to the RNA of the pH 5 fraction. The bond is relatively stable in acid medium, and alkali labile. Bound leucine does not exchange with non-labelled free leucine. When the leucine-labelled RNA is incubated with concentrated hydroxylamine, leucylhydroxamic acid is formed, indicating that leucine is bound to RNA through the carboxyl in such a way that this group is moderately reactive. When leucine-labelled RNA, isolated by the phenol method (Kirby, 1956) is added to a microsomal suspension, leucine leaves the RNA and it is transferred to microsomal protein material, provided guanosinetriphosphate is present (Hoagland *et al.*, 1957, 1958; Zamecnik *et al.*, 1958).

Thus some ribonucleic acid which is contained in the soluble fraction, and in the pH 5 precipitate obtained therefrom, can act as a transitory carrier of activated amino acids in between the activation step catalysed by the activation enzymes, and the microsomal particles where polypeptides first appear.

2. Transfer RNA

The discovery of amino acid binding by soluble RNA from rat liver was soon confirmed, and RNAs with similar acceptor properties were found in other animal tissues, e.g. pancreas (Weiss *et al.*, 1958), mammary gland (Fraser and Gutfreund, 1958), in a protozoan (Mager and Lipmann,

1958), in bacteria (Berg, 1958) and in yeast (Osawa, 1960; Otaka and Osawa, 1960; Monier *et al.*, 1960). Although no systematic survey has been reported, it seems probable that RNAs able to bind activated amino acids will be found everywhere. It is important to realize that activated amino acids cannot be transferred to all kinds of RNA. Ribosomal nucleic acids, which make up around 85 per cent of total cellular RNA, do not accept activated amino acids. It is only from the soluble fraction that adequate acceptor RNAs have been isolated.

The very first studies on soluble RNA (Hoagland *et al.*, 1957, 1958) already indicated that crude soluble RNA from rat liver can bind several individual amino acids, and that there is no competition between these. When the RNA had been saturated first with a given amino acid, it could still bind all the other amino acids. This suggested that crude soluble RNA contained independent specific acceptor sites for each kind of amino acid. Similar observations were made for soluble RNA from *E. coli* (Berg and Ofengand, 1958; Nisman, 1959) and from reticulocytes (Schweet *et al.*, 1958). A similar system was found in plant cells (Webster, 1959). An important question was to find out whether there was one molecular species of RNA able to bind the individual amino acids at specific sites distributed along its molecule, or whether there were several molecular species of RNA, each specific for one single type of amino acids. Fractionation of nucleic acids is not an easy matter. However, Goldthwait (1958) using an Ecteola column, Smith *et al.* (1959) with a cation starch exchanger, Holley *et al.* (1959) by means of counter-current distribution, Lipmann, *et al.* (1959) with column electrophoresis, all achieved some degree of resolution of soluble RNA. When the fractionation was applied to a crude soluble RNA, which had been loaded with various labelled amino acids, a definite although only partial separation of fractions binding different amino acids was obtained. Thus Holley *et al.* (1959) quite clearly separated a RNA fraction labelled with ^{14}C alanine from another which was loaded with ^{14}C leucine. The acceptor RNA for leucine was also partly separated from the acceptor for threonine (Lipmann *et al.*, 1959) and from that for tyrosine (Smith *et al.*, 1959). Brown (1960) has succeeded in separating almost completely the acceptor RNAs for histidine and tyrosine from all the others, by coupling chemically to polydiazostyrene the histidine and tyrosine bound to RNA. The other amino acids do not react with the resin under the conditions used; consequently, only histidine and tyrosine with their attached specific RNA are retained with the polymer. Mild alkali treatment splits the amino acid-RNA bond and releases the specific acceptor RNAs.

Zamecnik *et al.* (1960) have recently developed a chemical method which is, in principle, applicable to the isolation of all the individual acceptor RNAs.

The specific acceptors of the individual amino acids thus belong to

different molecular species of RNA, which can be separated by several methods.

The average molecular weight of soluble RNA has been estimated by different methods to be in the range of 10,000–50,000 (Hoagland, 1958; Zamecnik *et al.*, 1958; Goldthwait, 1958; Hoagland *et al.*, 1958). The more recent figures being 16,000 (Brown, 1960), 25,000 for *E. coli* (Tissières *et al.*, 1959) and 27,000 for yeast (Otaka and Osawa, 1960). These figures can only be rough approximations since the molecular weight determinations were made on preparations known to be mixtures of many different molecular species of RNA. They indicate that soluble RNAs are molecules containing of the order of 50–80 nucleotide residues. An end group determination gave an estimate of about 100 residues (Singer and Cantoni, 1960; Allen *et al.*, 1960).

It was shown, moreover, that the RNA acceptors of proline, leucine and valine all have that same molecular size (Klee and Cantoni, 1960). When crude soluble RNA is loaded with leucine, the maximum amount it can bind corresponds to one leucine residue for about two thousand nucleotides (Goldthwait, 1958; Hecht *et al.*, 1959). Since there are twenty different amino acids, and since the acceptor RNAs are all of the same size, assuming equivalent proportions of each, one can estimate that there is one acceptor site per one hundred or so nucleotides. Other estimates gave one amino acid residue per 70–90 nucleotides (Allen *et al.*, 1960). This being roughly the size of one soluble RNA molecule, it would seem that soluble RNAs most probably carry one single acceptor site per molecule.

Soluble nucleic acids differ from ribosomal nucleic acids on several accounts: they are soluble in molar NaCl, whereas the bulk of cellular RNA is precipitated (Smith, 1960), they contain considerable amounts of 5-ribosyl uracil nucleotide, which differs from normal uridylic acid by the position in which uracil is bound to the C₁ of ribose (Dunn, 1959, 1960; Scannell, *et al.*, 1959; Osawa and Otaka, 1959). Soluble RNA is also adsorbed by charcoal (Brown, 1960) and by *Ecteola* (Otaka and Osawa, 1960) much more readily than the bulk of RNA. Several precursors of RNA are incorporated much more rapidly into soluble RNA than into microsomal RNAs, but the results of recent investigations indicate that in homogenates adenylic or cytidylic nucleotides can be incorporated at the end of the chains of soluble RNA in a process which does not correspond to the synthesis of new RNA molecules (Heidelberger *et al.*, 1956; Paterson and Lepage, 1957; Edmonds and Abrams, 1957; Canellakis, 1957; Canellakis and Herbert, 1960; Herbert and Canellakis, 1960). It would seem that soluble RNAs are able to bind in succession two cytidylic nucleotides, followed by an adenylic residue which thus comes to occupy a terminal position in the polynucleotide chain. This last adenylic residue is bound through its 5' phosphate group to the 3' position of the last cytidylic

nucleotide, and accordingly both 2' and 3' positions of the ribose in the terminal adenylic nucleotide are free. This is established by periodate oxidizability of the terminal adenylic residue, and by the fact that when ^{14}C -labelled ATP is used as a source of terminal adenylic acid, most of the label in the alkali hydrolysate is recovered as adenosine.

FIG. 28. Terminal sequence of soluble transfer RNA with and without attached amino acid.

Prolonged incubation of soluble RNA removes the terminal nucleotides, and fixation of amino acids is possible only after the reformation of the terminal cytidyl-cytidyl adenosine (C-C-A) sequence (Hecht *et al.*, 1958, 1959). Singer and Cantoni (1960), on the other hand, provided evidence that

the other end of these RNA molecules terminate by guanosine 5' phosphate. Harbers and Heidelberger (1959) have separated RNAs having the usual terminal C-C-A sequence from yet another group in which the final sequence is uridyl-uridyl guanosine. It is not known whether the latter can bind amino acids.

Evidence discussed before indicates that the carboxyl group of the amino acids is bound to soluble RNA in such a way that it is only moderately reactive with hydroxylamine. The reactivity of this bond resembles that of an aminoacyl ester more than that of a mixed phosphoric anhydride (Raacke, 1958; Hoagland *et al.*, 1958; Lipmann *et al.*, 1959) and binding of the carboxyl of the amino acid somewhere else than on the phosphate residue is therefore indicated. A soluble RNA loaded with amino acids was hydrolysed by ribonuclease and the split products separated by paper electrophoresis. The amino acids were not released, they were found to be associated with adenosine, from which they could be separated by mild alkali. Since the adenosine amino acid compound does not reduce periodate, it must be concluded that the amino acids are bound to an —OH in position 2' or 3' of adenosine (Zachau *et al.*, 1958), for a compound with hydroxyl groups on two consecutive carbons (like 2' and 3' of adenosine) would be oxidized by periodate.

The amino acid composition of the mixed adenosine-amino acid compounds thus isolated from RNA indicated the presence in various amounts of almost all the amino acids (Lipmann *et al.*, 1959). Preiss *et al.* (1959) studying soluble RNA from *E. coli* obtained similar results. They showed that periodate treatment destroys the acceptor capacity of RNA. Moreover, if an amino acid residue is linked to the RNA prior to treatment with periodate, the acceptor site specific for the bound amino acid is protected against inactivation while the others are destroyed. This provides an elegant demonstration of the high degree of specificity of the acceptor RNA, and confirms that a free glycol structure is necessary for the fixation of all the individual amino acids.

In the present stage of our knowledge, it seems that there might exist twenty soluble RNAs, each able to accept specifically one single amino acid. This is bound by ester linkage to the —OH in position 2' or 3' of the adenosine residue of the terminal cytidyl-cytidyl adenosine sequence.

Two important questions must now be asked; how do the specific RNAs pick up the amino acids, and how do they transfer them further? The purification of several activation enzymes, and the possibility of isolating soluble RNAs by the phenol method which most probably destroys all the enzymes present, made it possible to study the requirement for the transfer of activated amino acid from the enzyme to soluble RNA. Apparently nothing else is required beside the pure activation enzyme and the RNA (Schweet *et al.*, 1958; Preiss *et al.*, 1959; Wong *et al.*, 1959). Capacity of a threonine

activation enzyme to activate the amino acid and to transfer it to RNA remained strictly proportional in the course of purification (Lipmann *et al.*, 1959). Studies by Holley (1957), Holley and Goldstein (1959) are also relevant to the mechanism of the transfer. The purified alanine activation enzyme which catalyses the pyrophosphate ATP exchange in the presence of alanine, catalyses also the exchange of ^{14}C -AMP with ATP in the presence of both alanine and soluble RNA. This last exchange is completely abolished by ribonuclease. This fact strongly suggests that the reaction between enzyme bound alanine adenylate and soluble RNA is direct. Mager and Lipmann (1958) provided evidence for the reversibility of the amino acid transfer to s-RNA in *Tetrahymena* preparations, by showing that reversion is obtained by the addition of pyrophosphate and AMP. Inhibition of the pyrophosphate exchange by s-RNA also supports a direct interaction between s-RNA and the activation enzyme (Goldstein and Holley, 1960). The aminoacyl group alone is transferred to soluble RNA, not the adenylic acid residue, which is liberated (Wong and Moldave, 1960).

One must conclude that activation of the amino acid with formation of the enzyme-bound aminoacyl adenylate, and the transfer of the aminoacyl residue to the RNA are both brought about by the activation enzyme. The donor specificity of the activation enzymes must be such that they can distinguish between the twenty different acceptor RNAs and deliver the activated acyl group to the right one only. It has been briefly reported that two activation enzymes both specific for alanine were isolated from pig liver: the one from the cytoplasm, the other from nuclei (Weber, 1960). The cytoplasmic enzyme is said to transfer alanine to cytoplasmic RNA from muscle and liver, but not to nuclear RNA or yeast RNA. There are indications that a certain degree of species specificity might also exist at this level. The activation enzymes of guinea pig will transfer to animal s-RNA, but *E. coli* RNA is a bad acceptor (Allen *et al.*, 1960).

What happens to the activated amino acids which are bound to their specific soluble RNA? They can be transferred to the ribosomes where they first appear as polypeptides. This was shown first by Hoagland *et al.* (1957, 1958) with rat liver homogenates. More recently Lacks and Gros (1959) established that the amino acids bound to soluble RNA in *E. coli* are very rapidly renewed and that any loss of labelled amino acid from the soluble RNA is accompanied by a gain of ^{14}C in the proteins.

Extremely little is known about this transfer at present, except that guanosine triphosphate (GTP) is required at this stage, possibly also ATP (Hoagland *et al.*, 1957, 1958; Webster, 1959; Ogata *et al.*, 1960). Some other factor, which is especially abundant in regenerating liver, also favours the passage of amino acids from soluble RNA to microsomal protein (Rendi, 1959; Grossi and Moldave, 1959, 1960; Nathans and

Lipmann, 1960). Evidence for the participation of the so-called S-protein (Sachs, 1957) or of some —SH containing protein at this stage was obtained by von der Decken and Hultin (1960) and by Hulsmann and Lipmann, 1960. In certain bacteria, the transfer is inhibited by chloramphenicol (Lacks and Gros, 1959), streptomycin (Erdös and Ullmann, 1959) or puromycin (Yarmolinski and de la Haba, 1959).

No doubt, the most obscure steps in protein synthesis are here, and unfortunately this is the heart of the matter. The amino acids on soluble RNA are bound in an activated state to specific carriers, but they are still isolated from one another. The next step must be their condensation into the genetically controlled sequence. Before venturing in considerations upon this still completely mysterious process, let us return to the present scheme of amino acid activation and examine how much confidence we can place in it.

There is no doubt that amino acids can follow this pathway and be incorporated into polypeptides. Most amino acids can be activated by specific 'activation enzymes' and transferred to specific ribonucleic acceptors; these in turn will loose the bound amino acids, which will eventually be recovered as polypeptides in the ribosome fraction. If an aminoacyl adenylate on the activation enzyme is labelled in the carboxyl by ^{18}O , heavy oxygen is later found in the proteins (Boyer and Stulberg, 1958). Molecules of the amino acid which had been activated by this process were therefore used in making proteins. The ubiquity of the activation enzymes indicates that they fulfil a quite general function common to all kinds of cells of animals, plants and bacteria. A more convincing indication in favour of the importance of activation enzymes for protein synthesis was obtained by Sharon and Lipmann (1957): several tryptophan analogues are known to be incorporated instead of tryptophan into the proteins of micro-organisms and animal tissues, whereas other tryptophan analogues are not incorporated. It is a striking fact that the analogues which are incorporated are those which are activated by the pure enzyme. This identical specificity of the activation enzyme and of protein synthesis as a whole, is good circumstantial evidence for the participation of this activation enzyme in protein synthesis. The mistakes made by this enzyme are reflected in the protein formed.

The exact function of soluble RNA in the process is not completely clarified. It can act as a depositary of activated amino acids. But one may wonder whether its function resembles more that of a store-house in a side street or that of a carrier of amino acids on the highway. In support of a function of carrier or obligatory intermediate, one may quote the rapid inhibition of protein synthesis by ribonuclease, which is known to degrade soluble RNA rapidly and to act more slowly on microsomal RNA (Brachet and Six, 1959). The experiments upon which the classical scheme of pro-

tein synthesis is founded were tracer experiments in which incorporation of amino acids into crude protein material was studied. But Campbell *et al.* (1960) and Ogata *et al.* (1960) obtained evidence for the validity of this scheme for labelling of a specific protein, serumalbumin, in a liver homogenate. The requirements are a source of ATP, the pH 5 fraction and GTP; ribonuclease treatment of the pH 5 fraction which contains the soluble RNA inhibits the incorporation into serumalbumin. Finally, in living *E. coli*, chasing experiments in which non-labelled amino acids were added after a period of incorporation of labelled ones showed that the amino acids bound to soluble RNA behave as if they were intermediates on the way of protein synthesis (Lacks and Gros, 1960). These results which were obtained with intact exponentially growing bacteria indicate that the pathway under consideration is indeed operative in bacteria as well as in animal tissues.

However, other observations indicate that we do not know the whole story yet, even for the activation steps perhaps. In the experiments just mentioned with living *E. coli* for instance, it would seem that the rate of renewal of the amino acids bound to soluble RNA can account only for part of the total incorporation into proteins in exponentially growing bacteria, as if an alternative pathway contributed to the incorporation. Silkworm fibroin contains 42 per cent glycine and 28 per cent alanine. However, the activation enzymes for tryptophan and for tyrosine are much more active than the one for glycine (Heller *et al.*, 1959). Although the lability of the activation enzymes makes a comparison of absolute activities difficult, and its significance questionable, these observations nevertheless again raise the question of the existence of alternative pathways.

C. OTHER FACTORS INVOLVED IN PROTEIN SYNTHESIS

(a) *The incorporation enzymes.* Beljanski and Ochoa (1958) isolated from bacteria a protein fraction which is required for the incorporation in a cell free system from *Alkaligenes foecalis*, in the absence of amino acid activation enzymes (Beljanski, 1960). This fraction 'replaces' the pH 5 fraction in the incorporation of leucine into proteins of rat liver microsomes (in this sense that it causes a very strong stimulation of incorporation, as the activation enzymes would do). This 'incorporation enzyme', however, does not catalyse the amino acid promoted exchange of pyrophosphate with ATP. Instead, this purified fraction causes the exchange of each ^{14}C -labelled nucleoside diphosphate (ADP, GDP, UDP, CDP) with the corresponding triphosphate. Differential thermal inactivation of the exchange of the four pairs of nucleotides points to the existence of four

different enzymes in the preparation, one for each type of nucleotide. It is conceivable that these enzymes catalyse the activation of amino acids by a process of the type illustrated in the synthesis of glutamine or of glutathione. The enzymes involved in the formation of these peptides have been partly purified (Elliot, 1951, 1953; Snoke and Bloch, 1952, 1955; Webster and Varner, 1954). The carboxyl group of glutamic acid must be activated in the process, for in the presence of ATP and hydroxylamine, glutamyl hydroxamic acid is formed. No free glutamic acid derivative with a reactive carboxyl could ever be isolated however, and it is assumed that the activated compound is bound to the enzyme. A bound glutamyl-phosphate intermediate is postulated (Webster and Varner, 1954; Varner and Webster, 1955; Kowalsky *et al.*, 1956), because heavy oxygen is transferred stoichiometrically to inorganic phosphate, and not at all to the ADP moiety of ATP; besides, the exchange of inorganic phosphate with the terminal phosphate of ATP requires the presence of both glutamic acid and the acceptor (e.g. ammonia in the synthesis of glutamine). The following scheme accounts for these experimental facts, although the detailed working of the enzyme cannot be considered as completely clarified.

FIG. 29.

A similar reaction scheme may be assumed for the 'incorporation enzyme'. It would account for the exchange data and for the fact that amino acids cause a net liberation of inorganic phosphate from the nucleoside triphosphates in the presence of the enzyme preparation. It would also explain the transfer of heavy oxygen from the amino acid to inorganic phosphate in bacterial preparations (Bernlohr and Webster, 1958). An interesting observation is that the individual amino acids do not cause the liberation of phosphate from all four nucleoside triphosphates equally well. Thus glycine appears to react readily with ATP, less with GTP, leucine reacts with UTP and CTP, phenylalanine with CTP only (Beljanski, 1960).

It is indeed impossible at present to decide whether these reactions play a part in protein synthesis or not. One favourable indication is that the amino acid promoted liberation of inorganic phosphate is inhibited by chloramphenicol, a typical inhibitor of protein synthesis in bacteria. The possibility that the 'incorporation enzyme' might be involved together with the activation enzymes must be kept in mind.

(b) *The S-protein.* An additional factor in amino acid incorporation in certain systems, is a protein material isolated by Sacks (1957) from the soluble fraction of liver homogenate, but which does not contain any activation enzymes. This S-protein enhances the incorporation of amino

acids into microsomal particles. Its effect is especially marked with microsomes that have been lyophilized or treated with acetone (Sachs, 1957). Microsomes treated under certain conditions by the detergent lubrol or by perfluoro-octanoate can incorporate amino acids in the absence of the amino acid activation enzymes (Cohn, 1959); in this system the S-protein is necessary. A crucial question is the dispensability of the amino acid activation enzymes. If this is well established and if the observed incorporation reflects protein synthesis, then one must conclude that there are two pathways of amino acid activation leading eventually to protein via s-RNA. This raises questions that the presently available data cannot answer. Are there two alternative pathways for the synthesis of any protein, or are certain proteins made by one process and others by the other one? Such a duality of proteins certainly does not appeal to anyone, but one should be prepared to the eventuality that e.g. the constitutive proteins of the ribosomes might be made in another way than the enzymes and the other common proteins or that certain cell organelles might not make proteins in exactly the same way as the cytoplasmic ground substance. This is supported by a few observations. Hexetidine disturbs protein synthesis in bacteria in a very strange way: in the presence of this agent the synthesis of all proteins is not uniformly affected. Incorporation of labelled precursors seems to occur in a few protein fractions only, as if the synthesis of most proteins was inhibited, leaving the formation of a few others unaffected (Halvorson and Gorman, 1959). Chloramphenicol at rather high concentration inhibits protein synthesis in isolated nuclei and mitochondria, but not into ribonucleoprotein particles (Rendi, 1959). Chloramphenicol and chlortetracyclin inhibit amino acid incorporation into large particles contained in a homogenate of *Tetrahymena* but not into the microsomal fraction (Mager, 1960).

(c) *Lipids*. Hendler (1958, 1959) presented evidence for another carrier of amino acids besides soluble RNA. A lipid fraction from hen oviduct was found to incorporate amino acids very rapidly; the amino acids seem to be held by a highly labile bond in this lipid fraction and to be rapidly renewed. Hunter *et al* (1959) also observed that a lipoprotein fraction isolated from cytoplasmic membranes of *B. megaterium* takes up labelled amino acids very rapidly. Moreover, they obtained indications that if both soluble RNA and lipids are concerned in protein synthesis, the lipids are involved at a stage subsequent to the action of soluble RNA. A lipoidic substance containing carbohydrates was shown to stimulate amino acid incorporation in homogenates (Hradec and Stroufova, 1960). These systems are still very crude, and it is not quite clear at present whether the lipids are involved in protein synthesis or in some other process, e.g. amino acid transport. Further research is certainly needed along this line.

(d) *Incorporation factors*. One further point which still awaits clarifica-

tion is the nature and functions of the amino acid 'incorporation factors' discovered by Gale. Disrupted *Staphylococcus* cells incorporate amino acids readily under a variety of conditions (Gale and Folkes, 1955; Gale, 1955, 1957). Removal of the nucleic acids by salt extraction almost completely suppresses the incorporation. The process can be restored by the addition of *staphylococcal* RNA or better of a ribonuclease digest of RNA. The activity is not due to any nucleotide or oligonucleotide fragment of the RNA as one would have anticipated. The activity is associated with several substances present in minute amounts in such digests. A purified preparation of the so-called 'glycine incorporation factor' promotes the incorporation of glycine, phenylalanine, aspartic acid, leucine, glutamic acid, arginine and lysine, and to a lesser extent that of valine, isoleucine, tyrosine and proline (Gale, 1958). The same preparation also stimulates the incorporation of adenine into nucleic acids (Gale and Folkes, 1958). The chemical nature of these factors is completely unknown. The glycine incorporation factor is reasonably stable to acid or alkali, it is devoid of charge, except at high pH values, when it becomes slightly positively charged.

According to Wagle *et al.* (1960), the glycine incorporation factor increases the (very low) incorporation which is observed in liver microsomes in the absence of the pH 5 fraction. The same laboratory has also shown that a derivative of vitamin B₁₂ stimulates very much amino acid incorporation into the proteins of homogenates of liver from vitamin B₁₂ deficient animals (Barker, 1958, 1959). The site of action of this substance has not been located exactly, but it does not seem to concern the activation steps (Mehta *et al.*, 1959; Wagle *et al.*, 1958; Szafranski *et al.*, 1960) and the observed effects might actually be indirect (Fraser and Holdsworth, 1959; Arnstein and Simkin, 1959). Bečarevic (1957) had also noted a restoration by vitamin B₁₂ of protein synthesis in ultraviolet-irradiated yeast.

Yoshikawa and Maruo (1960) have isolated an amine which is strongly bound to RNA preparations and which stimulates amylase formation by *B. subtilis*.

Many experimental facts remain to be explained and eventually integrated into a scheme of amino acid incorporation. It is possible that the presently accepted pathway involving activation enzymes, soluble RNA and microsomes is only a first approximation to a more complex reality.

D. THE SEQUENTIAL CONDENSATION OF AMINO ACIDS INTO POLYPEPTIDES

In the evolution of ideas on protein formation, two main working hypotheses which were regarded as alternative and mutually exclusive

retained the attention. One hypothesis assumed that proteins are made stepwise as the other substances, the other one that they are assembled in one stroke by a template process. The template hypothesis remained sterile for a long time because it was beyond the reach of experimentation; the current developments of genetics and molecular biology have now given so much consistency to this old daydream that it appears at present an almost certain reality.

The hypothesis of a stepwise formation of polypeptides has not been very successful; it never tried to account for the genetic control of protein synthesis. But it suggested many experiments, and gathered a number of data which will have to be integrated in the final scheme of protein synthesis.

As will be seen, the two hypotheses are incompatible only when they are both taken in their extreme form, and it is quite possible that the actual mechanism of protein synthesis presents facets in which both hypotheses can find some justification.

1. *Are there Free Peptide Intermediates?*

The question as to the existence of peptide intermediates between amino acids and proteins was the subject of much discussion a few years ago; it was thereafter disregarded, but it cannot yet be answered unequivocally at the present time. Intermediate stages must of course exist between separate amino acids and a polypeptide containing for instance thirty amino acid residues. The problem is to know whether polypeptides are made stepwise from oligopeptides or peptide derivatives having an independent existence and being handled by a series of separate catalysts, or whether all the linkages between the amino acids which make a polypeptide are formed, in more or less rapid succession, under the control of one single catalyst, the organizer molecule or template.

The direct search for oligopeptides in living cells has not been very rewarding. If common small peptides, like glutathione or carnitine, are discarded — for they do not seem to be intermediates in protein synthesis — no notable amount of free peptides have been detected in cells. Exceptions may be some plant material (Fogg, 1952; Dagley and Johnson, 1956) and resting bacteria maintained under adverse conditions (Gale and Van Halteren, 1952; Weinbaum and Malette, 1959; Šorm and Černá, 1960). Tracer experiments designed to find out whether growing bacteria could form enzymes from inactive precursors (Monod *et al.*, 1952; Halvorson and Spiegelman, 1952; Rotman and Spiegelman, 1954; Hogness *et al.*, 1955) did not detect any accumulation of complex intermediates between amino acids and proteins. These experiments of course do not prove that intermediate peptides do not exist, but they show that if they exist their concentration is so low as to escape detection by the sensitive tracer methods used, and that they are renewed very rapidly.

On the other hand, indirect and circumstantial evidence for the existence of peptide intermediates has been presented on several occasions. Peptides obtained by the action of proteolytic enzymes on proteins stimulate protein synthesis more than a mixture of amino acids in certain systems (Kihara and Snell, 1955; Rychlik and Šorm, 1956; Fox and Krampitz, 1956). The rather frequent occurrence of individual tripeptides in certain classes of proteins (Šorm, 1957, 1959, 1960) makes one wonder whether these tripeptides might not be made separately and serve as precursors for several related proteins. So far, however, all the studies on the utilization of labelled di- or tripeptides have shown that the amino acids are used more readily than the peptides and that these must actually be split into amino acids to be utilized. The amino acids are the real precursors. This was found for instance in the case of the synthesis of haemoglobin in reticulocytes (Nizet and Lambert, 1954), and of casein in the goat (Godin and Work, 1956), for amino acid incorporation in tissue slices (Hendler and Greenberg, 1954) and for bacterial growth (Meinhart and Simmonds, 1955). The stimulatory effect of peptides obtained by enzymic hydrolysis of proteins is not completely explained. It is due in certain cases to glutamine and asparagine (Rychlik and Šorm, 1957), which are contained in enzymic hydrolysates of proteins, but not in acid hydrolysates since these amides are split by acid into ammonia and glutamic or aspartic acid respectively. It is indeed established at present that glutamine and asparagine are incorporated as such into proteins (Barry, 1954, 1956; Rabinovitz *et al.*, 1956; Sansom and Barry, 1958). They should be regarded as individual amino acids which are just as different from glutamic and aspartic acids as e.g. leucine or glycine, as far as incorporation into protein is concerned. The 'complete' amino acid mixtures used in the overwhelming majority of the experiments did not contain any asparagine or glutamine, and were therefore incomplete mixtures of protein precursors.

Another possibility is that peptides might in certain cases be degraded by living cells in such a way that they would provide activated amino acids directly (Walter *et al.*, 1956). This also deserves further investigation.

A more striking phenomenon which might reflect the existence of peptide intermediates of some sort is the non-uniform labelling of proteins as observed most clearly with *in vitro* systems. Pieces of hen oviduct incorporate the carbon of $^{14}\text{CO}_2$ *in vitro* into ovalbumin and other proteins. Ovalbumin which had been labelled in this manner was split by a bacterial protease into two fragments, according to Ottesen and Wollenberger (1953). The larger fragment, named plakalbumin, can be isolated in a crystalline form, the smaller one is a hexapeptide. The specific radioactivity of the aspartic acid residues of these two fragments differed by a factor greater than four (Steinberg and Anfinsen, 1952). A similar result was later obtained for the incorporation of labelled phenylalanine and glycine into

insulin and into ribonuclease in slices of beef pancreas. This time, each protein was split into many peptides; the specific radioactivity of the same amino acid was notably different in the different peptides originating from the same protein (Vaughan and Anfinsen, 1954). More recently, non-uniform labelling was reported for collagen (Gehrman *et al.*, 1956) and procollagens (Orekhovich *et al.*, 1959), for silk fibroin (Shimura *et al.*, 1956), for amylase in *B. subtilis* (Yoshida and Tobita, 1960), for haemoglobin in the rabbit (Kruh *et al.*, 1960) and for cytochrome-*c* in isolated muscle mitochondria (Simpson, personal communication). In other cases, however, no inequalities of labelling were detected (Muir *et al.*, 1952; Heimberg and Velick, 1954; Askonas *et al.*, 1955; Simpson, 1955) but the experiments were made with intact animals and the incorporation was of relatively long duration. Non-uniform labelling is best observed for short periods of amino acid uptake and with poorly active or damaged systems.

The meaning of unequal labelling is not clear. Good discussions on the subject have been presented by Steinberg *et al.* (1956) and by Borsook (1956). The first interpretation proposed was that amino acids are first incorporated into various oligopeptides or peptide derivatives which are later combined to make a protein. According to the size of each peptide pool and to their turnover rate, a given labelled amino acid would be diluted to different degrees in each oligopeptide, and the protein formed by association of the peptides would not be uniformly labelled. The incorporation is so low in most of the *in vitro* systems considered that a pool of peptides amounting to some 2 per cent of the free amino acid pool could account for the experimental data.

Another way of explaining non-uniform labelling is to assume that in the presence of protein forming system, the peptide linkages of proteins can be labilized in such a way that an amino acid comprised within the polypeptide chain can exchange with free amino acids (Chantrenne, 1952; Gale, 1953; Borsook, 1956). Differences in exchange rate at different positions in the protein chains would explain differences in specific radioactivity. An alternative interpretation, which avoids the assumption of exchange processes for which there is no compelling evidence, is that the time required for making a protein is long in the poorly active system considered; in this sense that each individual polypeptide molecule stays on the weaving machine long enough for the specific radioactivity of an amino acid, e.g. glycine, to change considerably between two additions of this amino acid at different places along the polypeptide (Dalglish, 1953). If protein synthesis takes place by such a 'stepwise template process' (Steinberg *et al.*, 1956) one should expect to find peptide intermediates bound to some structures in the experimental systems in which unequal labelling is observed.

In the last few years, the occurrence of nucleotide-amino acid and

nucleotide-peptide compounds has been reported from several laboratories. Ascites tumour cells contain aspartic acid associated with a uridylic nucleotide (Reith, 1956), and chicken liver a compound tentatively described as adenosinediphosphoglutamic acid (Hansen and Hageman, 1956). Several compounds containing a few amino acids were isolated from micro-organisms (Hase *et al.*, 1959; Brown, 1959). The peptide linked nucleotides which were detected in yeast (Koningsberger *et al.*, 1957; Harris and Davies, 1958; Koningsberger, 1960) and in liver (Szafranski *et al.*, 1959, 1960; Steinberg *et al.*, 1960) might be of special interest, for the terminal carboxyl of the peptide moiety reacts with hydroxylamine. The compounds might thus be activated peptides acting as intermediates in some anabolic process. The chemical structure of some of the compounds from yeast has now been established (Davies and Harris, 1960). They are mixed

FIG. 30. Structure of a nucleopeptide from yeast (Davies and Harris, 1960).

anhydrides in which the terminal carboxyl of the peptide is condensed with the 5' phosphate group of the uridylic moiety of a dinucleotide (Fig. 30). Some of these compounds are extracted only by cold acid treatment, as if they were weakly bound to other cell constituents in neutral extracts. One may wonder whether the peptides found in yeast by Turba and Esser (1955) have the same origin.

The uridylic acid peptide compounds discovered by Parks (1952), and other clearly related substances (Strominger and Threnn, 1959; O'Brien and Zilliken, 1959) have not been mentioned here, because they are known to be precursors of bacterial cell wall. This serves to remind one that amino acids and peptides are found in other substances beside proteins, and that nucleotide-peptide compounds might have nothing to do with protein synthesis. However, until their function is discovered, they must

be regarded as likely intermediates, the study of which will be pursued with great interest.

2. *Are Polypeptides Made on a Template?*

In its present state of development, biochemistry offers essentially one type of explanation for each of the metabolic steps it describes, namely the presence in the cell of an adequate enzyme which promotes the reaction considered. When several possibilities exist from a purely chemical point of view, the specificity of the enzyme or of the enzyme assortment is again invoked. For instance, a great variety of fatty acids, terpenes, carotenoids, steroids can be made from acetate; the nature and assortment of the products elaborated by a cell is explained by its particular complement of enzymes.

If this type of stepwise assembly process is assumed to operate in the specific synthesis of the individual proteins, a fundamental difficulty is raised by the number of enzymes which would be specifically involved in the production of each single specific protein. This time, simply referring to the specificity of the enzymes would amount merely to displacing the problem, for the enzymes are specific proteins and they are also made in the same cell. The difficulty has been appreciated by biochemists for more than a quarter of a century, and it was suggested several times, on purely logical grounds, that the structure of the enzymes or at least of their specific part must be controlled by a model, a mould or a template, i.e. by a substance having a structure complementary to that of the enzyme synthesized.

Current developments of molecular biology (see Chapter I) give a great vitality to this old idea. Indeed it is now established that the primary structure of the proteins, i.e. the sequence of the amino acids in the polypeptide chains, is controlled by mendelian genes. The information relative to the arrangement of the amino acids is recorded on the genetic material which is made of nucleic acids. The purine and pyrimidine bases compose the ciphers in which this information is coded, since replacement of one base by another or substitution of one keto for an amino group in one base can change the information and result in the specific replacement of one amino acid by another at one specified place in the polypeptide chain. All the specific information relative to the primary structure of a protein is comprised in a unique segment of the genetic material, the locus. The size of the locus is such that it might contain enough information for controlling every single amino acid in the polypeptide. These facts almost inescapably lead to the conviction that the arrangement of the amino acids in the polypeptide correspond point to point to that of the purines and pyrimidines (or of their 6-keto and 6-amino groups) in DNA. Moreover, the information concerning a protein molecule must be transferred to the protein making system in one single package or in a very small number of

packages: the information required for the synthesis of a piece of protein of the size of a complete polypeptide chain cannot be used unless it is delivered *en bloc*. This almost certainly means that all the building blocks which make up a polypeptide are assembled under the influence of one single organizer macromolecule.

The remarkable results of the studies on the mechanism of DNA replication, in providing evidence for the operation of a rigid template process in this case (Kornberg, 1958; Lehman *et al.*, 1958), indirectly support the conviction that a mechanism of this nature operates in protein synthesis. Unveiling of the template mechanism is the task for the coming years; it is felt that our curiosity will not have to wait for a very long time to be satisfied.

Current ideas about this most important step of protein synthesis have been deeply influenced by the brilliant theory presented by Crick (1957, 1958) as a development of the coding principle called 'code without commas'. For reasons that have been discussed in Chapter I of the present book, Crick suggested that each amino acid of a protein is coded in the corresponding DNA by a sequence of three bases; there are twenty such sequences, each coding for an amino acid. The coding sequences for each amino acid are such that if they were located in a row next to one another no reading mistake could be made: the triplets corresponding to each amino acid are such that any triplet formed from contiguous parts of any two of them is nonsense, i.e. does not correspond to any amino acid. This is the coding principle.

The gene DNA which carries this coded information transmits it to some RNA by controlling the arrangement of the nucleotides in RNA by a template process of the kind discussed by Stent and by Zubay (1958). This specific RNA is the template for protein synthesis. The difficulty here is that amino acids must find their right place along a polynucleotide in order to obey the coded information. Crick suggests that specific enzymes, able to recognize amino acids and short nucleotide sequences, bind the individual amino acids to specific trinucleotides which are complementary to the coding sequences in the RNA. These serve as adaptors which will carry the amino acids and will find their right place by forming hydrogen bonds with the complementary bases on the RNA template. When the template is covered with adaptors carrying amino acids, the amino acids unite into a polypeptide chain, which has thus the primary structure specified by the gene.

If there are only two coding digits (6-keto and 6-amino) instead of four as first assumed by Crick (see p. 36), triplets should be replaced by groups of five nucleotides, but this does not change the principle of this extremely attractive template mechanism.

The experimental data reviewed before establish that amino acids are

bound individually to specific ribonucleic acids (transfer RNA) and it is very tempting to consider these as the adaptors which will carry the activated amino acids to the template and locate them at their right position according to Crick's scheme. This hypothetical mechanism (Hoagland *et al.*, 1959) is depicted on Fig. 31. The template carrying specific information is supposed to be microsomal RNA. So far, experimentation has been unable to attribute a clear function to the RNA of the ribosome, which constitutes some 80 per cent of cellular or bacterial RNA. But it has been recognized a long time ago that the intensity of protein synthesis is related

FIG. 31. A hypothetical scheme for the interaction of microsomal RNA and transfer RNAs with amino acids attached (Hoagland *et al.*, 1959).

to the amount of RNA, and essentially of ribosome RNA (Brachet, 1941). Nascent polypeptides, in liver and bacteria, are found in association with the ribosomes. The assumption that ribosome RNA is the template or is part of it, is therefore reasonable. The fact that modifications of soluble RNA can stop protein synthesis is quite compatible with the above scheme.

There is one difficulty, in this hypothetical template process, it is the size of soluble RNA. A sequence of three to five nucleotides should be sufficient to code for an amino acid. If we add to it the terminal cytidyl-cytidyl-adenosine sequence to which the amino acid is bound, this makes

eight nucleotides at most; but soluble RNA contain probably 10 times as many. Such molecules seem too bulky to operate as adaptors in the sense of Crick and it would seem that if soluble RNA is really an obligatory intermediate on the pathway of protein synthesis, the template mechanism must operate in a somewhat different way.

It may be not necessary to assume that the template is at one time covered with nucleotidic 'adaptors' each carrying its own amino acid; it is conceivable that each specific soluble RNA adds in turn its amino acid to the growing polypeptide. Such a process would of course be slower than the other one. Protein synthesis takes several minutes in animal cells (Peters, 1957; Craddock and Dalglish, 1957; Loftfield and Eigner, 1958; Dintzis *et al.*, 1958; Morris and Dickman, 1960), and only a few seconds in micro-organisms (Boeyé, 1957; McQuillen *et al.*, 1959; Cowie and McClure, 1958; Zalokar, in press). These are short times as compared to the usual duration of biochemical experiments, but exceedingly long times in terms of absolute rate of reaction. If it is assumed that soluble RNA and ribosomes must collide for the activated amino acids to be linked to the polypeptides, the collision frequency which can be estimated is sufficient to support the observed rate of peptide bond synthesis, if more than one collision out of ten thousand results in amino acid transfer (Ts'O and Lubell, 1960). Sequential addition therefore does not seem excluded. It would be compatible with many experimental data (Webster, 1959; Bishop *et al.*, 1960) including the apparent absence of intermediates in rapidly synthesizing systems like living bacteria, and the non-uniform labelling in extremely slow systems like homogenates of animal tissues.

In a template process outlined by Dalglish (1953), several peptide chains in different stages of development were attached simultaneously to the templates, each by a small region as shown in Fig. 32. The polypeptides all grow in the same direction by addition of activated amino acids, and peel off behind the assembly zone. Future developments in molecular biology will tell whether such a scheme is possible topologically.

The experimental data available at present are not sufficient for a useful discussion of a template process in terms of chemical reactions. But several of the present lines of research might soon provide information relevant to the template mechanism: further data on the relations between soluble and ribosomal RNA, a better understanding of the function of amino acids in RNA synthesis, further study on the function of peptide nucleotide compounds will be very valuable.

A metabolic relationship seems to exist between soluble and ribosomal RNA, for energy-dependent transfers of oligonucleotides in both directions have been demonstrated (Bosch *et al.*, 1959). Hultin and von der Decken (1959) and Moldave (1960) also observed a transfer of soluble RNA to the microsomes and GTP seemed to promote the transfer. Clarification of these

processes will certainly throw light upon the real function of soluble RNA and upon the mechanism of amino acid transfer to the ribosome.

It will be important also to know a little better the conditions for coaptation between polynucleotides, a promising field of studies opened by the work of Rich (1959).

A completely unexplained fact is the necessity of a complete assortment of amino acids for RNA synthesis. The fact that analogues of amino acids inhibit RNA formation is another reason to believe that the individual amino acids have a specific function in RNA synthesis. There is indeed an intriguing reciprocity between the requirement for amino acids in RNA synthesis and the requirement for precursors of RNA in protein synthesis. This led several workers to the suggestion that RNA and protein might be assembled from common precursors, visualized as some kind of nucleotide amino acid compounds (Gros and Gros, 1957; Yčas and Brawerman, 1957;

FIG. 32.

Pardee *et al.*, 1957; Spiegelman, 1957). The recently discovered interactions between amino acids and nucleoside triphosphates (Beljanski, 1960) might prove of great interest in this respect. The relation between amino acids and the synthesis of RNA might be the key to the template mechanism for the synthesis of proteins and nucleic acids. Interesting attempts at formulating in chemical terms template processes taking this idea into account have been made by Simkin and Work (1957), Kiepal-Kochanska (1958), Michelson (1958). Raacke (1958) has proposed a more elaborate process which features attachment of the carboxyl activated amino acids to the template by their $-\text{NH}_2$ groups, and which has the merit of making predictions and suggesting experiments. Future research will tell the value of these various hypotheses.

E. FORMATION OF THE PROTEIN MOLECULE

(a) *Folding*

When the amino acids have united into the genetically controlled sequence, a polypeptide is formed, not a protein molecule. The polypeptide must fold in a specific way, hydrogen bonds be established between neighbouring parts of the polypeptide, certain regions of the chain will form a helix for instance, whereas others will not. The helix is not straight, it folds upon itself, sometimes in a very intricate way, as illustrated by the structure of myoglobin (Kendrew *et al.*, 1960) (see Figs. 4 and 33). It is only when the tertiary structure is established that the protein molecule possesses its all important physiological properties: enzymic activity, serological characteristics, capacity of associating with other substances in a specific way.

It is assumed at present that once the primary structure is there, folding does not raise any further problems (Perutz *et al.*, 1960); folding is regarded as a spontaneous process which is determined by the amino acid sequence and by conditions prevailing in the cell, like pH, ionic strength, nature of the ions. Reversibility of limited denaturation indicates that the right folding of polypeptide segments can indeed re-form spontaneously. Complete uncoiling is usually irreversible, for there are too many possibilities of formation of bonds between different parts of the chain or between chains. If the polypeptides were released from the assembly line as randomly coiled molecules, their remoulding into the right configuration would be difficult. On the contrary, if the end of the polypeptide is allowed to fold spontaneously as it comes off the template, then folding might go on in a regular and unique manner as the chain grows. The tertiary structure would then be strictly determined, at each step of its formation, by the nature of the amino acid residues and by the shape and position of the already formed parts of the molecule. Folding might conceivably be influenced by other substances present in the vicinity of the template. But it must be realized that the data on the secondary and tertiary structures of individual proteins are really too scanty for deciding whether folding can always occur spontaneously or whether it requires in certain cases the active participation of special cellular mechanisms. A case like trypsinogen for instance might raise difficulties. In this molecule, part of the polypeptide is not folded in a helix; it is maintained in a somewhat extended state; the splitting of one bond in this particular region allows the polypeptide to assume a helical structure, as shown by a change in rotatory power. There is therefore a structural feature in the trypsinogen molecule which prevents it from assuming the more coiled configuration of active trypsin (Neurath, 1957). Entirely too little is known at present to realize whether the formation of a protein molecule with an inner tension, like trypsinogen, raises new problems or not.

(a)

(b)

FIG. 33. (a) Tertiary structure of myoglobin.
(b) The course of the polypeptide chain.
(Kendrew *et al.*, 1958).

(b) *Disulphide Bridges*

Most of the proteins the primary structure of which has been unravelled contain cystine. The disulphide bridges maintain a certain folding of the polypeptide upon itself as in ribonuclease or they connect together different polypeptide chains as in insulin. Disulphide bridges are essential to the stability of the protein and to its physiological properties. It is not known whether the formation of the SS bridges requires a specific enzymic oxidation, or not; the oxidoreduction state of the $-SH$ groups of proteins and its relations, in the different regions of the cell, with free $-SH$ containing substances like glutathione are very obscure, a few steps in the analysis of these interactions have only been made recently.

(c) *Association of Polypeptides*

Certain proteins are made of several polypeptide chains, either different or identical, and the physiological properties of these proteins depend on the association of the parts. Globin is a good example: each molecule is made of four polypeptides: two α chains, two β chains, assembled in an intricate structure (Perutz *et al.*, 1960). The association of the α and β chains into the finished protein occurs spontaneously *in vitro* under adequate conditions. The formation of an active enzyme by mixing extracts of two different mutants strains of *Neurospora* in the cold (see p. 18) is best explained by the spontaneous association of polypeptides into active enzyme. A related phenomenon is the association of two proteins into an active enzyme (Yanofsky and Crawford, 1959). Again the correct association arises spontaneously.

(d) *Further Transformations*

Further changes occur in certain protein molecules, which transform them into other proteins with quite different properties, as for instance the transformation of a zymogen into active enzyme. It is a matter of taste to consider these transformations as finishing stages of the synthesis of certain proteins or as modifications undergone by finished proteins. We will here adopt the latter attitude, because these further changes do not concern all protein molecules. The transformations result from the action of specific enzymes; they are of various types and each would deserve special study; a few examples will simply be quoted here. Thus transformations of pepsinogen, trypsinogen or chymotrypsinogen into the corresponding enzymes are interesting cases of limited proteolysis; so is the cascade of protein transformations involved in blood clotting. Phosphorylase-*a* results from the phosphorylation of four serine residues of phosphorylase-*b* (Fischer *et al.*, 1959), followed by dimerization (Keller and Cori, 1953; Krebs and Fischer, 1956). Collagen and certain plant proteins contain hydroxyproline. At variance with the twenty common amino acids,

ready made hydroxyproline is not used for the synthesis of these proteins (Steward and Pollack, 1958; Green and Lowther, 1959) or it is a very inefficient precursor if it is used at all (Mitoma *et al.*, 1959). Hydroxyproline exists only in bound form, it arises by a modification of proline after this has been incorporated into polypeptides (Stetten, 1949; Jackson and Smith, 1957; Wolf and Berger, 1958). A special oxidase is involved in the transformation (Van Robertson *et al.*, 1959).

Unusual observations have been reported on amylase formation in pancreas extracts. The active enzyme is released from an inactive precursor, it is not made *de novo* from amino acids in the homogenate. The appearance of amylase activity requires the presence of ATP, threonine and arginine, although these amino acids are not incorporated into the active enzyme (Straub *et al.*, 1953; Straub and Ullmann, 1957; Straub, 1958; Grabowski and Munro, 1960). An activation process of a new type might be operating here.

Processes of maturation of protein systems may also be mentioned at this point. Reserve proteins of pea seeds undergo a slow evolution after they have been made: the electrophoretic pattern changes, indicating association of many protein components into a few larger units (Danielsson, 1952; Snellman and Danielsson, 1953; Raacke, 1957). These changes recall the transformations of gluten which involve oxidation of —SH groups (De Deken *et al.*, 1953). But here we may have crossed the limit of processes which are not relevant to protein biosynthesis.

The attachment of a prosthetic group may be spontaneous. This is the case of certain flavoproteins, as shown a long time ago by Theorell (1935). The porphyrin nucleus of catalase can probably find its proper place in the protein moiety since the apo-catalase made by a porphyrin-less mutant of *E. coli* can combine *in vitro* with haemin, resulting in a complex endowed with catalase activity (Beljanski, 1955). Certain *Staphylococci* also make apo-catalase when deprived of haemin, and complete the synthesis of the enzyme if haemin is added; this later step apparently involves the participation of coenzyme A (Jensen, 1957). Incidentally, these results indicate that the proper folding of the polypeptide chain did not depend on the prosthetic group since apo-catalase was made in its absence.

These processes give the finishing touch which confers to the individual proteins their typical features and physiological properties, but the biochemical problems raised are minor as compared to the making of the chains with their genetically controlled sequence of amino acids.

(e) *Integration of Proteins into Structures*

Association of proteins into structures of a higher level can also occur spontaneously. The best example of this phenomenon is the association of the protein of tobacco mosaic virus into rods resembling the virus, or better

the formation *in vitro* of the complete virulent virus by spontaneous association of some 3000 molecules of protein and one RNA chain into the well-known structure.

The association of proteins with lipids and other substances into microscopical structures like membranes, fibres, lamellae, mitochondria, etc.... will raise other problems which have practically not been touched at the molecular level so far. These organelles do not seem to arise *de novo*, they develop from similar or related pre-existing structures, as if they could only grow by accretion of new material on to structures which insure the correct arrangement of the building blocks.

The thiol-disulphide equilibrium seems to be determining in the formation of structures. Embryologists have reported on many occasions striking inhibitions of morphogenesis by sulphydryl reagents like iodoacetamide (see Brachet, 1946, 1960). Certain of these effects are due to the inhibition of some common SH enzymes, or to the inhibition of mitosis. But more subtle changes in morphogenesis caused by various thiols or disulphide containing compounds certainly reflect processes of a completely different nature. As a rule, free thiols disturb morphogenesis; on the contrary disulphide substances improve it, if anything. This was observed in widely different morphogenetic processes: development of amphibian embryos, regeneration of the tail in amphibian tadpoles, regeneration of the head of planarians, and cap formation in *Acetabularia mediterranea* (Brachet, 1959). This last-mentioned case is especially interesting, for the alga consists of a single cell (see p. 50) and the morphogenetic processes here do not depend on cell division or cell migration. Growth continues in the presence of thiols, synthesis of new material is not prevented, but the newly made material does not form the normal macroscopic structure. Instead of a well-shaped hat, the alga develops a swollen club-like structure. It would seem that the thiol-disulphide equilibrium is of great importance for the relative positions in space that the substances synthesized during growth come to occupy. This strongly suggests that when the formation of disulphide bridges is interfered with, proteins cannot associate correctly into structures, either because the folding of the structure proteins themselves is not right, or because disulphide bridges play a fundamental part in the association of constituents — proteins or otherwise — into microscopic structures.

A new field of study will soon develop here, for the progress of electron-microscopy, X-ray diffraction analysis and light microscopy will certainly make it possible to bridge the gap between biochemistry and morphology, and eventually give the first molecular interpretation of form.

CHAPTER V

Regulation of Protein Synthesis

A. ENZYMIC ADAPTATION

The rate of protein synthesis, like that of all the anabolic processes, depends on temperature, pH, energy production, availability of building blocks, etc. ... More interesting is the specific control of the rate of synthesis of the individual proteins, exerted by external or internal agents.

The assortment of proteins actually made by a cell is conditioned by the genetic material; the presence of the gene, however, confers but a potentiality, the expression of which depends on specific controlling systems. Let us ignore for a moment the problem of differentiation and consider only micro-organisms; even for these relatively simple cells, the assortment of the proteins actually produced does not depend on the genetic material alone: it can be influenced by changes of the medium composition. Such mould will start producing a protease when transferred on to a medium which contains milk. A bacterium which makes a phosphatase in given surroundings may stop to manufacture the enzyme if inorganic phosphate is added to the medium. Certain substances can thus specifically induce a cell to make certain proteins, others can specifically repress the production of an enzyme without affecting the synthesis of the other proteins. Induction and repression of enzyme synthesis have been studied mostly in bacteria and yeasts; comparable effects have been observed in plants and animals, but they are much more complex especially in animals where homeostatic mechanisms oppose changes of conditions and where superposed hormonal actions make the results extremely intricate (see p. 170).

It is not the purpose of the present chapter to discuss thoroughly the problem of enzymic adaptation and repression; many excellent reviews and discussion have been published on the subject. Reading them in the chronological order gives an extremely vivid picture of the detours of discovery in the field, with a succession of bright hypotheses, blind alleys and break through, resulting in the slow elaboration of a more and more accurate picture of the phenomenon (Wortman, 1882; Duclaux, 1900; Dienert, 1900; Karström, 1936; Stephenson and Yudkin, 1936; Yudkin, 1938; Monod, 1947, 1956, 1958, 1960; Spiegelman, 1950; Stanier, 1951; Mandelstam, 1956; Spiegelman and Campbell, 1956; Pollock and Mandelstam, 1958; Vogel, 1958; Pollock, 1956, 1959; Pardee *et al.*, 1959). Only a

few facts which throw light upon the mechanism of protein synthesis and upon its control will be considered here.

1. *Induced Synthesis of Enzymes in Micro-organisms*

Enzymic adaptation refers to the adjustment of a specific enzyme activity caused by a change of the medium under such conditions that selection of mutants can be excluded. In several cases, it has been clearly established that the adaptive increase of certain enzyme activities in micro-organisms corresponds to the complete *de novo* synthesis of the enzyme proteins (e.g. Cohn and Toriani, 1953). For instance, *E. coli* was grown in a medium containing radioactive protein precursors so that every protein present in the bacteria was strongly labelled. The bacteria were then transferred into a non-labelled medium and induced by a galactoside to make β -galactosidase. The enzyme was isolated in a state of great purity, and its radioactivity was measured. Practically no label was found in the enzyme; β -galactosidase had been synthesized completely from non-radioactive precursors, it did not pre-exist in the cell in any form before the addition of the inducer galactoside. The inducer therefore caused the complete synthesis of the enzyme from amino acids (Rotman and Spiegelman, 1954; Hogness *et al.*, 1955). The usual genetic control of enzyme synthesis remains, however, for certain mutant strains of *E. coli* are unable to make β -galactosidase even in the presence of galactosides (Lederberg, 1951); these strains result from mutations which are all located within a narrow region of the genome, called the *z* locus (Pardee *et al.*, 1959; Monod, 1960). Transfer of a small piece of genome containing the *z* locus to a *Shigella* confers to this bacterium the capacity of making a galactosidase which is identical to the *coli* enzyme (Cohn *et al.*, 1960). The *z* region of the genome must be the locus which specifically controls the primary structure of the enzyme.

What then is the function of the inducer? Does it add a little piece of structural information to the main information provided by the gene? Does it select among several possibilities the folding corresponding to the active enzyme? Does it pull the enzyme off the template? Does it control the operation of the genetic locus or that of the assembly template in some specific way?

Sometimes, a variety of substances are able to induce the formation of the same enzyme; these various inducers are structurally related to the substrate of the enzyme. Thus β -galactosidase synthesis in *E. coli* (Monod *et al.*, 1951) or in *B. megaterium* (Landman, 1957) can be induced by various β -galactosides or thiogalactosides, some of which are not split by the enzyme. A comparable situation exists in the induction of a glucosidase in yeast (Duerksen and Halvorson, 1959) and of penicillinase in *B. cereus* (Pollock, 1956). In spite of differences of structure of the inducers, the properties of the enzyme produced are always the same, although

the amount of enzyme may depend on the inducer. One can induce the synthesis of another enzyme, galactokinase, in *E. coli* in the absence of any exogenous inducer by causing the development of a temperate phage which had introduced the corresponding structural gene by transduction (Buttin *et al.*, 1960).

Mutants which normally produce the enzyme in the absence of any added inducers have been isolated in a few cases; these are 'constitutive' mutants. Penicillinase produced by a constitutive mutant of *B. cereus* was compared to the induced enzyme made by the adaptive parent strain. None of the physical, chemical, enzymological and serological tests applied could reveal any difference between the constitutive and the induced enzymes, which had both been highly purified and crystallized (Kogut *et al.*, 1956; Pollock, 1956). Similar, although less complete evidence for identity of constitutive and inducible enzyme were obtained for the penicillinase of *B. subtilis* (Manson *et al.*, 1954) and for the β -galactosidase of *E. coli* (Monod and Cohn, 1951) and of *Neurospora crassa* (Landman, 1954).

The capacity of constitutive mutants to produce perfect enzyme in the absence of added inducer raises an interesting dilemma: are these strains constitutive because they can really make the enzyme without any participation of an inducer, or is it because they themselves manufacture the inducer, whereas the adaptive strains cannot do so? The second alternative at first looked very promising: it provided a unitary view (Cohn and Monod, 1956) in which all the enzymes were regarded as inducible, the constitutive enzymes being induced by an endogeneous inducer. Moreover, the occurrence of endogeneous inducers was indicated by numerous cases of sequential induction (Stanier, 1947, 1950, 1955; Suda *et al.*, 1949).

When the synthesis of an enzyme is induced by the substrate, the inducer substrate is acted upon by the induced enzyme and it often happens that the product of this enzymic transformation induces the formation of a second enzyme. Addition of the first inducer in such a case results in the sequential synthesis of two enzymes. The second one is actually induced by an endogeneous inducer. If this process repeats itself several times, a series of enzymes can be induced in succession. The classical example is the sequential induction in *P. fluorescens* of seven enzymes which catalyse the oxidation of the indol ring of tryptophan into β -keto-adipic acid in seven successive steps. It is assumed that each intermediary metabolite acts as an inducer for the synthesis of the next enzyme. It is remarkable that in many cases the different enzymes are actually made in succession. Kinetic studies on the utilization of intermediates or on the formation of enzymes made it possible to unravel several metabolic pathways by making use of sequential induction (Stanier, 1947; Karlson and Barker, 1948; Ajl, 1950; Wiame, 1951).

If all the enzymes were induced either by an endogeneous or by an

exogenous inducer, it could be visualized that the inducer brings a complement of structural information, and that it is involved for instance in organizing the active centre of the enzyme. Should the inducer cause the polypeptide chain of the nascent enzyme to fold in a specific way, the remarkable coaptation of enzyme with substrate would receive a rational interpretation; sequential induction would even partly explain the establishment of metabolic chains of successive reactions (Pollock, 1953).

The unitary hypothesis of general induction in enzyme synthesis looked indeed very promising. It was difficult to test experimentally, however, for the failure to isolate from a constitutive mutant a substance which can induce an inducible strain does not prove much; success has actually been claimed (Kramer and Straub, 1956) in penicillinase induction, but the results proved difficult to reproduce and the nature of the active agent could not be quite clearly established. The problem has recently been approached by the methods of genetics in the exceptionally favourable case of β -galactosidase of *E. coli* K 12. As mentioned in Chapter I, the genome of a Hfr bacterium, or part of it, can be transferred during conjugation to an F^- recipient bacterium. Crosses of this type were made between bacteria which differed by the presence of normal or mutated genes either in the z locus controlling the structure of β -galactosidase, or in the i region which controls the inducible or constitutive character of the synthesis of the enzyme (Pardee *et al.*, 1959). Let us call z^+ the normal form of the z locus and z^- any mutated form of this locus which prevents the formation of active enzyme protein. In the same way, i^+ will refer to inducibility, i^- to constitutivity. Let us consider the two genetic formulae z^+i^+ and z^-i^- ; none of these strains will produce β -galactosidase in the absence of added inducer: the former because it must be induced to do so, the second because it does not possess the correct structural information for making the enzyme. Zygotes resulting from the conjugation of a donor z^-i^- and of a recipient z^+i^+ do not produce the enzyme in the absence of exogenous inducer, although they possess both the structural information z^+ and the constitutivity character i^- . On the other hand, if a z^-i^- strain which is potentially constitutive but does not possess the structural information receives the information z^+ together with i^+ , i.e. inducibility, from a donor strain, it immediately starts making the enzyme, but the synthesis stops after some time; it will then be obtained again if an inducer is added. The zygote at first behaved like a constitutive, but it became adaptive after some time. When both forms of the i gene are present in the same bacterium, the phenotype corresponding to inducibility prevails; the 'inducible' character is dominant, 'constitutive' is recessive. The constitutive bacterium, however, contains all that is necessary for making the enzyme, this is an obvious certainty. Therefore, it must be concluded that the i^+ gene which causes inducibility prevents the spontaneous production of the enzyme by

establishing some kind of specific inhibition. Since in the second type of crossing inducibility (as opposed to constitutivity) takes some time to be established, it would seem that the development or the operation of the inhibitory mechanism is rather slow. On the other hand, an exogenous inducer is able to release the inhibition rapidly.

These genetical studies do not indicate the nature of the inhibitory system. They are not incompatible with the general induction theory. The i^+ gene might be responsible for the production of an enzyme D which destroys the endogeneous inducer; since this endogeneous inducer must be present in the constitutive cytoplasm, the z^+ gene would be expressed as soon as introduced; to the contrary, the expression of the dominant i^+ gene would take the time required for the enzyme D to reduce the steady state concentration of the endogeneous inducer below the induction threshold. A completely different interpretation of the experimental results can also be considered, which rejects the generalized induction theory. It may be assumed that the i^+ gene (inducibility) controls the production of a substance which blocks specifically the synthesis of galactosidase, and the action of which is antagonized by exogeneous inducers. The delay in the establishment of the 'inducible' character in the second type of crossing is then explained by the time required for reaching the threshold concentration of the repressing agent.

Recent data by Pardee and Prestidge (1959) indicate that the i^+ gene is able to express itself under conditions which prevent protein synthesis almost completely. If this conclusion is correct, the first model can be discarded. Besides, these experiments indicate that the i^+ gene does not control the structure of any protein, and — adopting the second of the models discussed above — that the repressing agent is not a protein.

The generalized repression theory departs from the generalized induction theory in that it leaves all the structural information to the structural gene, the inducer and the repressor are mere regulators of the enzyme producing systems. The generalized repression theory unites in a single concept the phenomena of induction and of repression of enzyme synthesis, which will now be examined briefly.

2. *Repression of Enzyme Synthesis in Micro-organisms*

Galactose inhibits the synthesis of constitutive β -galactosidase in *E. coli*. Tryptophan and certain of its analogues inhibit the formation of tryptophan synthetase in the same organism (Monod and Cohen-Bazire, 1953). The specificity of these inhibitions indicated that the substances interfere with the process of induction of the enzymes (Cohn and Monod, 1953). More cases of such inhibitions have been observed and further analysed. Thus Gorini and Maas (1957) and Vogel (1957), who coined the name *repression* for this phenomenon, observed that the synthesis of each enzyme of the

anabolic chain which makes arginine starting from acetylornithine is inhibited by arginine. Valine represses the synthesis of valine transaminase (Adelberg, 1953), uracil represses the synthesis of at least three constitutive enzymes of the metabolic chain leading to orotic acid (Yates and Pardee, 1957); similar repression phenomena were found in the pathways of synthesis of the purines (Magasanik, 1957, 1958), of methionine and cysteine (Cohn *et al.*, 1953, Wyesundera and Woods, 1953, 1960; Bourgeois *et al.*, 1959, 1960) and histidine (Ames and Garry, 1959). Inorganic phosphate represses the synthesis of alkaline phosphatase in *E. coli* (Horiuchi *et al.*, 1959; Torriani, 1960).

Inhibition of the synthesis of certain enzymes by glucose had been known for a long time, especially in cases of induced enzyme synthesis (Dienert, 1900; Stephenson and Yudkin, 1936; Gale, 1943). This glucose effect is well illustrated by diauxie (Monod, 1942). When bacteria are grown in a medium containing two carbon sources, e.g. glucose and maltose, they use glucose first; growth slows down very much when most of the glucose has been used up, and it is only then that the synthesis of the enzyme which attacks maltose is permitted. The utilization of the new carbon source makes possible a restoration of growth, resulting in the familiar diauxie growth curve. The glucose effect, which had not been analysed very much because it was observed for many enzymes and therefore seemed to be unspecific, is a case of repression (Neidhardt and Magasanik, 1956, 1957; Magasanik, 1957; Magasanik *et al.*, 1959).

Repression is thus as common a phenomenon as induced synthesis of enzymes. As a rule, enzyme synthesis is induced by the substrate and repression is caused by the product of reaction or by the end product of the anabolic chain in which the enzyme is involved. Induction and repression of enzyme synthesis thus afford a remarkable adjustment system which selects within the genetic potentialities of the cell an enzyme assortment adapted to the conditions of the medium. Induction has an immediate or very rapid effect on the level of the enzyme; repression changes much less rapidly the level of enzyme: it simply stops further production of an enzyme which was being made, the level of the enzyme usually decreases as it is being diluted in the increasing cell mass of the growing bacterial population.

3. *Mechanisms of Repression and Induction*

The formal antagonism of inducer and repressor suggests that these substances might both act in opposite ways on the same controlling system; there might exist between them a relation similar to that of the substrate of an enzyme and a competitive inhibitor. Recent experiments by Gorini (1960) indeed showed that ornithine can reverse the repressive effect of arginine on the formation of ornithine transcarbamylase, and induction by

ornithine occurs only under conditions of partial repression by arginine. The effects of ornithine, the inducer, and of arginine, the repressor, seem to be competitive.

Sulphate represses tyrosinase formation in *Neurospora* (Horowitz *et al.*, 1960) and aromatic amino acids release the repression. Tyrosinase thus behaves as an adaptive or as a constitutive enzyme depending on the concentration of sulphate in the medium. Here it is difficult to visualize how sulphate and aromatic amino acids could compete for the same site; it should be realized that there is no reason to think that the exogenous inducers and repressors act as such on the protein making machinery. The necessity for the transformation of the added inducer into the actual inducing agent has been postulated in several cases of enzyme induction. Clayton (1960) provided evidence for the elaboration of an intermediary substance responsible for induction of catalase by the exogenous inducers oxygen or hydrogen peroxide. New words will be needed to distinguish between the inducers and repressors that the experimenter adds to the cell suspension and the resulting active intracellular agents which operate the regulatory mechanism. Competition probably takes place between these 'elaborated' inducers and repressors.

Since induction and repression are highly specific, inducers and repressors must exert their action on the system which controls the specificity of synthesis of the individual proteins. Three sites of action of the regulators can be envisaged at present: the structural gene, the genetic messenger which carries the information to the enzyme making system, and the template itself. Presently available data do not permit to decide. Enucleation experiments should make it possible to establish whether induced enzyme synthesis can take place in the absence of the gene. Unfortunately, the type of cells which can be easily enucleated, so far proved unfavourable to enzyme induction studies. An increase of catalase activity was caused by hydrogen peroxide in anucleate as well as in intact *Acetabularia mediterranea* (Brachet and Chantrenne, 1953), but it could not be clearly established whether catalase was synthesized in the process (Chantrenne, 1955b). Induced enzyme formation in yeast is not inhibited by very high doses of ionizing radiation, which cause such damage to the DNA of the cell that it cannot be precipitated with acid after alkaline extraction (Chantrenne and Devreux, 1959). Induced enzyme synthesis has been observed in bacterial preparations from which most of the DNA had been removed by salt extraction or destroyed by deoxyribonuclease (Spiegelman, 1956). These facts suggest that the site of action of the inducer is not the DNA; unfortunately, the results are not entirely convincing, because the nature of the damage caused to DNA by radiation is not clearly understood, and the disrupted bacterial system from which most of the DNA was extracted synthesized new DNA while making enzymes.

Since RNA is most probably the specific constituent of the template which organizes proteins, the question as to the requirement of specific RNA synthesis for enzyme induction is relevant to the problem under discussion. If the inducer or repressor operate at the level of the gene and regulate the production of cytoplasmic replicas made of RNA, one would expect enzymic induction to be accompanied by the synthesis of new specific RNA molecules, the organizers of the induced enzyme. Several years ago, some experimental data were quoted in favour of such a view. Thus Creaser (1955) observed that the induced synthesis of a β -galactosidase in *Staphylococcus aureus* is stimulated by purines and pyrimidines. The ability of resting yeast to form an induced α -glucosidase is strongly dependent upon the level of the free nucleotide pool (Spiegelman *et al.*, 1955). Several purine and pyrimidine analogues were reported to inhibit the induced synthesis of enzymes much more than the synthesis of constitutive enzymes. However, this conclusion proved unfounded (for a discussion see Chantrenne, 1958). The incorporation of labelled uracil or adenine is stimulated during enzyme induction in disrupted *Staphylococci* (Gale and Folkes, 1955) and in yeast (Chantrenne, 1956, 1958); this at first sight could be taken as indicating the synthesis of new RNA molecules. Further studies on the yeast system, however, showed that the stimulated incorporation was probably linked in an indirect way only to the induction process, and it cannot be taken as convincing evidence for the synthesis of new RNA molecules (Chantrenne, 1958; Chantrenne and Devreux, 1959). The discovery of chain end renewal in soluble RNA (see Chapter III) adds one more difficulty in evaluating the significance of increased incorporation of precursors into total cellular RNA.

Release of repression does not seem to require any RNA synthesis. The formation of ornithine transcarbamylase is repressed by arginine; it starts as soon as arginine is removed from the medium. With a uracil-less strain, it is possible to prevent RNA synthesis by uracil deprivation. Even so, removal of arginine causes immediate synthesis of the enzyme under conditions which prevent net RNA synthesis (Rogers and Novelli, 1959). Studies on diauxie also indicated that little RNA is made during the lag which corresponds to the release of repression (Magasanik *et al.*, 1959). It must be realized, however, that the synthesis of an amount of RNA representing 1 per cent of total RNA would escape observation; and the amount of RNA that one would expect to be synthesized during adaptation would not exceed that value: there are several hundred different proteins in a bacterial species, there must be as many specific RNA varieties; the appearance of one new type of template RNA among several hundred pre-existing RNAs would indeed be very difficult to detect by quantitative biochemical determinations. Moreover, the absence of net synthesis does not mean that no new molecular species of RNA can form; Barner and Cohen

(1958) indeed showed that a rapid RNA turnover takes place in uracil-less mutants during protein synthesis.

The question as to whether enzyme induction requires the synthesis of new specific RNA molecules is not solved. Several data which were taken as circumstantial evidence for a necessary RNA synthesis now appear irrelevant, and no positive evidence has been afforded so far. On the other hand, the pieces of evidence against the synthesis of specific RNA are not completely convincing either. The problem therefore is still open.

A more direct approach has been the search for specific RNA in adapted cells. More or less crude preparations containing RNA were extracted from induced bacteria and added to suspensions of non-adapted cells. In a few cases, it was reported that these extracts induced the synthesis of the enzyme that was looked for, although there was no regular inducer in the preparations used (Minagawa, 1955; Reiner and Goodman, 1955; Hunter and Butler, 1956; Nomura and Yoshikawa, 1959). Kramer and Straub (1956, 1957) reported that an extract, obtained by treating a penicillinase constitutive mutant of *B. cereus* with M NaCl at 100° C, causes the inducible strain to produce penicillinase, provided the recipient bacteria have been pre-treated with ribonuclease. Penicillinase production lasts but for 20–30 min, and it is inhibited by chloramphenicol. Ribonuclease abolishes the activity of the sodium chloride extract. Further analysis of this interesting phenomenon is needed in order to establish whether the effect is specific and whether it corresponds to an actual synthesis of enzyme (Pollock, 1959). It is difficult to evaluate the significance of these observations; the fact that they are difficult to duplicate and that they involved crude preparations is no reason to disregard them.

Lack of clear and positive evidence for the formation of new specific RNA organizers during enzyme induction encouraged the idea that induction and repression of enzyme synthesis rest on the control of pre-existing protein forming machines (Monod, 1958; Vogel, 1957; Magasanik *et al.*, 1959). The inducers and repressors could for instance facilitate or prevent the separation of the nascent protein from the assembly template (Vogel, 1957, 1958; Monod, 1958), or activate it in any other way.

More recent data on the genetic control of β -galactosidase synthesis in *E. coli* indicate that the inducer and repressor (or more precisely the real controlling agents derived from them) might actually operate a switch of some sort which blocks or releases the operation of the protein weaving machine. Jacob *et al.* (1960) showed that the regulatory genetic determinant *i* which controls the induced versus constitutive nature of β -galactosidase synthesis controls simultaneously the expression of several structural genes which are closely linked on the genetic map. A similar situation was observed in the pathways of tryptophan formation (Cohen and Jacob, 1959) and of histidine synthesis: histidine represses the synthesis of the

four enzymes involved in the conversion of imidazolglycerolphosphate into histidine (Ames and Garry, 1959). The use of a choice of mutants makes it clear that the effect cannot be explained by the prevention of sequential inductions. The four genes corresponding to the four enzymes are contiguous (Hartman, 1956). It looks as if a group of closely linked genes could be put into operation or blocked simultaneously. The existence of a lock or 'operator' is supported by direct genetical studies in the case of β -galactosidase (Jacob *et al.*, 1960) where mutants have been isolated which have the properties one would predict if the mutation had occurred within the operator. It was also shown that in heterozygotes the operator controls the expression of the genes when they are in *cis* position with the operator, and does not act on the genes which are in a piece of genetic material separated from the homologous one which contains the operator. These facts can be tentatively interpreted in the following way (Jacob *et al.*, 1960): besides the locus which contains the structural information for the synthesis of an enzyme, one should distinguish a new genetical unit, the *operon*, made of an operator and a group of closely linked loci, the expression of which is controlled by the operator. The operator is acted upon by a repressor produced under the action of the controlling gene (for instance, in the case of galactosidase). The repressor blocks the operon; the locked operon can be released by an inducer.

This fascinating scheme contains implications which are amenable to experimental test; future research will tell whether it is a good picture of reality. It provides an interesting interpretation for the clustering of genes controlling the enzymes of a single metabolic pathway (Hartman, 1956). At first sight, the operator hypothesis might be taken to mean that the repressor acts at the level of the genome. Actually, the available data are just as compatible with an action of the repressor on cytoplasmic replicas of the operator. This would, however, imply that the operon is copied as a unit and that the cytoplasmic templates corresponding to the several genetic loci of an operon work in a co-ordinate manner. It will be interesting to compare the size of the operon with those of the DNA molecule and of ribosomal RNA. The DNA molecule is large enough to accommodate several loci, and the RNA molecule of the ribosome large enough to serve as a template for several proteins. One may wonder whether the new physiological unit of expression, the operon, will be superposable to the chemical unit of structure, the molecule, and to the RNA quantum of the ribosomes.

How can one visualize at the molecular level the process of repression and induction in this perspective? The keys which lock or release the system must be of such a nature that they can interact with the lock. The operator may be assumed to be a region of a DNA molecule or of its cytoplasmic RNA replica. The key should be able to interact in a specific way with a

nucleotide sequence. In view of the present knowledge of the interactions between polynucleotides a sensible hypothesis is that the active repressing agent contains a nucleotide sequence complementary to that of the operator or to its cytoplasmic replica. The polynucleotidic repressing agent could be made by the controlling gene (i^+ for galactosidase) which indeed does not seem to control the structure of any protein (Pardee and Prestidge, 1959). A more difficult matter is to fit the exogenous, or endogenous, inducers like lactose into the picture; do they cause or prevent the production of the repressor at the level of the gene, do they become associated with a specific nucleotide sequence (Szilard, 1960), but then by what enzyme action? Where does the competition take place? Thus we are confronted with the same kind of questions as before. But because they are asked in new terms, these questions will certainly suggest new experiments which will throw new light upon the mechanism of induction and repression of enzyme synthesis.

Torriani (1956) and Halvorson and Jackson (1956) observed that the early phase of induction is exceptionally sensitive to ultraviolet irradiation. During the lag period of maltase induction by maltose in resting yeast, a fraction of RNA becomes acid soluble without being degraded to free nucleotides, and this fraction is reincorporated into acid insoluble RNA when the synthesis of the enzyme begins (Gobert — see Chantrenne, 1958). This small RNA fraction is released from the ribosomes (Gobert, unpubl.). One may wonder whether these observations have some bearing on the transitory participation of fragile nucleotidic compounds in the process which triggers enzyme synthesis.

Finally, it may be remarked that if induction and repression of the synthesis of enzymes is a process in which the specific templates are put to operation or switched off, the rate of production of an enzyme must be determined by the number of templates which are in operation. In a constitutive strain, the rate of production of an enzyme must depend on the total number of templates corresponding to the enzyme. Almost nothing is known about the number of identical templates in the cell, neither is it known when the templates are produced: are they being continuously produced or only at one stage of cell division (Mitchinson, 1958; Plaut, personal communication)? Do all the structural genes produce the same number of cytoplasmic replica, or is another regulation mechanism operating on the production of templates? In haemoglobin synthesis in man, the templates depending on two homologous genes present in a heterozygote produce their specific protein at about the same rate, since the amounts of normal and sickle cell haemoglobins produced are comparable.

It will be noticed that a large part of the present picture of induction and repression of enzyme synthesis is derived from studies on a few micro-organisms and a few enzymes. By far the most thoroughly analysed case is β -galactosidase synthesis in *E. coli*; it has been the object of exceptionally

far-reaching genetical studies. The uniformity of fundamental biochemical mechanisms in all kinds of living cells allows one to assume that the mechanisms involved in the control of β -galactosidase synthesis are probably of wide significance. It would, however, be misleading to suppose that these solve all the problems of regulation and that no other mechanisms can be operating. Other types of processes may be superimposed upon those described above. For instance, evidence that enzyme transformation occurs in certain cases has been presented recently.

4. *Induced Transformation of an Enzyme*

In anaerobically grown yeast, oxygen induces the formation of the enzymes and electron carriers of the respiratory chain (Ephrussi and Slonimski, 1950; Slonimski, 1953, 1954, 1955) and of several peroxidases (Chantrenne, 1954, 1955; Chantrenne and Courtois, 1954). Complete synthesis of the cytochrome-*c* protein during this adaptation has been demonstrated (Yčas and Drabkin, 1955), but more complex processes also occur. Changes in the absorption spectrum of the yeast cells suggest the replacement of certain haemoproteins by others (Slonimski, 1953; Yčas, 1956). This might be due to protein turnover, for at variance with bacteria, yeast readily makes new enzymes in the absence of any external source of nitrogen, at the expense of the intracellular pool of free amino acids and of proteins which can be degraded to amino acids (Halvorson, 1958). However, the formation of lactic dehydrogenase during respiratory adaptation seems to involve a direct transformation of enzymes.

In anaerobically grown yeast, there is a lactic acid dehydrogenase which catalyses the dehydrogenation of D-lactic acid only; on the contrary, after respiratory adaptation, an enzyme can be isolated from yeast which oxidizes the L-isomer of lactic acid specifically. Meanwhile, the anaerobic enzyme almost completely disappears (Slonimski and Tysarowski, 1958; Labeyrie *et al.*, 1959; Nygaard, 1959, 1960). This replacement of an enzyme by another related enzyme is not due to the destruction of one protein while the other is being made; there is evidence that the anaerobic D-lactic acid dehydrogenase is transformed into the L-enzyme normally present in aerobic cells (Kattermann and Slonimski, 1960). Amino acid analogues preclude the formation of active catalase, cytochrome oxidase, cytochrome peroxidase, presumably by being incorporated into the proteins; but these analogues do not interfere with the replacement of the anaerobic lactic dehydrogenase by the aerobic type of enzyme. Stereospecificity is not the only difference between the two enzymes: the aerobic type reduces cytochrome-*c*, the anaerobic does not. Thus the transformation involves the acquisition of cytochrome reductase activity beside the change in stereospecificity. An intermediary stage in the transformation was detected: the anaerobic enzyme which is specific for D-lactic acid first acquires the

property of transferring electrons to cytochrome, and it is later changed into the L-specific enzyme. The complete transformation takes more time than the synthesis of cytochrome oxidase or catalase, which seem to behave according to the classical scheme of induced enzyme synthesis (Nygaard, 1960).

5. *Permeases and Enzyme Synthesis*

Certain exogenous inducers induce enzyme synthesis only if they can enter the bacterium and if their concentration at their site of action is sufficient. Small molecular weight substances sometimes can enter a cell by diffusion. More complex processes are involved in most cases; the 'permeability' of cells is very specific and cannot in any way be compared to the old misleading model of the semipermeable membrane which retains certain molecular species and allows water and other substances to diffuse freely. Some cells absorb and concentrate proteins readily but are completely impermeable to glycine or succinic acid for instance. The proteins are taken up by a complex process of pinocytosis. Moreover, all types of cells are able to concentrate various substances, like amino acids or sugars, from an outer dilute solution. Thus *E. coli* grown on lactose is able to absorb and concentrate several β -galactosides (Monod, 1956; Cohen and Monod, 1957); the concentration inside the bacterium can be a hundred times the outer concentration, and its maintenance requires a continuous expenditure of energy. Azide, which prevents phosphorylations, inhibits the concentration mechanism. The accumulated galactosides seem to be free within the bacterium and this considerably increases the inner osmotic pressure; if the cell wall is damaged by enzyme action, its mechanical strength is reduced, and the internal osmotic pressure resulting from the accumulation of the galactosides can be high enough to cause the rupture of the bacteria (Rickenberg *et al.*, 1956; Siström, 1958). This permeation system, which was called 'permease', is not present in bacteria which have not been in contact with galactosides; it is an inducible system which responds to the same inducers as β -galactosidase.

When a non-adapted bacterium is confronted with an adequate galactoside, some galactoside enters the cell by diffusion, and there is a certain probability that the formation of one molecule of permease is induced. Once this molecule is formed, the internal concentration of galactoside increases, and this results in the induction of more permease and of β -galactosidase at the same time (Novick and Weiner, 1957). The permease — the chemical nature of which is unknown — thus influences the induced formation of the enzyme β -galactosidase in an indirect way, by concentrating the inducer of the enzyme within the cell. The kinetics of β -galactosidase formation actually reflects that of the formation of permease.

Rather complex consequences of this situation can be observed. If

non-induced cells are grown in a medium containing glucose and a little β -galactoside, the permease and the enzyme are not produced, due to repression by glucose. However, if the bacteria are first grown on lactose, and later transferred into the medium which contains glucose and a galactoside, they will continue to make both the permease and β -galactosidase, because the permease they contain to start with is able to concentrate the galactoside enough to release the glucose repression. Thus two populations of identical genotype in an identical environment can be made to differ in their assortment of enzymes. This situation, once it has been established, is very stable; it could be maintained for more than 150 generations (Cohn, 1958).

If unadapted bacteria are given a threshold concentration of inducer for a short time, the probability of acquiring the first molecule of permease is not great; therefore in a population of bacteria certain individual cells will adapt, and others will not. By increasing then the glucose to inducer ratio in the medium adequately, it is possible to maintain in the same population individual cells which do make the enzyme and others which do not. The formation or non-formation of the enzyme is passed on clonally by the cells to their descendants, as if it were a regular hereditary character (Cohn and Horibata, 1959). Maintenance in the adapted cells is of course conditioned by the presence of the inducer in the medium.

This illustrates well the very complex consequences of the occurrence of permeases for the regulation of enzyme synthesis. Comparable permeation systems have been found for amino acids and for various sugars and derivatives.

6. *Enzymic Adaptation in Animal Tissues*

A few cases of enzymic adaptation in animals have been reported. The tryptophan peroxidase activity of rat liver increases when tryptophan is injected into the animal (Knox and Mehler, 1950, 1951); the same enzyme can be evoked in dissociated embryonic cells (Stearns and Kostellow, 1958). Tyrosine transaminase is increased by injections of tyrosine (Lin and Knox, 1957). A strong increase of xanthinoxidase activity in the liver of mice is observed about a week after they have received injections of xanthine (Dietrich, 1954). Formation of adenosine deaminase in chick embryos after administration of adenosine was also reported (Gordon and Roder, 1953); however, systematic studies by Solomon (1960) did not confirm this observation. A strong decrease in kidney glutaminase is caused by glutamate injection; this might be an example of repression (Goldstein, 1959).

Enzymic adaptation in animals does not show the schematic simplicity of the process found in micro-organisms. A review by Knox *et al.* (1956) shows very well how intricate and complex is the regulation of the level of

enzyme activity in animals. The system which has best been studied from the biochemical point of view is the stimulation of tryptophan peroxidase activity of rat liver. It was soon observed (Knox, 1951) that an increase of this enzyme activity can be obtained not only by tryptophan, but also by the injection of histidine or of adrenaline. Adrenalectomy reduces the normal basic level of the enzyme. Due to this and other hormonal actions, involved controlling effects are observed. Cortisone and hydroxycortisone stimulate the production of the enzyme (Knox and Auerbach, 1955). Adrenocorticotrophic hormone (ACTH) stimulates peroxidase activity, and hypophysectomy depresses it. This effect of ACTH is probably mediated through the steroid hormones since ACTH causes the synthesis of corticosteroids (Koritz *et al.*, 1957). Reserpin (Canal *et al.*, 1959) and insulin (Schor and Frieden, 1958) also increase the level of the enzyme. The stimulating effects of tryptophan and of insulin or hydroxycortisone are additive, as if these agents were acting in different ways (Civen and Knox, 1959; Schor and Frieden, 1958).

Increase in tryptophan peroxidase activity was obtained in isolated perfused liver (Price and Dietrich, 1957) and in liver slices (Ephimotchkina, 1954). Such systems make it possible to avoid the hormonal effects. Even in cell-free systems, an increase of tryptophan peroxidase was caused by tryptophan (Clouet and Gordon, 1959), but the increment of enzyme activity was correlated with changes in permeability of the mitochondria, which strongly suggests that the increase in activity might reflect changes in the accessibility of the enzyme rather than an actual *de novo* synthesis (Gordon and Rydzziel, 1959). There were indications that a net synthesis of tryptophan peroxidase occurs during *in vivo* induction by tryptophan (Gros *et al.*, 1956). On the other hand, convincing evidence was recently obtained that substrate induction of this enzyme activity may be due — at least in part — to an intracellular translocation of the enzyme or of an activator (Greengaard and Feigelson, 1960); this reminds one of certain observations by Lee and Williams (1953) pointing in the same direction. It is therefore doubtful whether tryptophan peroxidase 'induction' has anything in common with the induced synthesis of enzymes as observed in micro-organisms; it might be a case of adjustment of the activity of pre-existing enzyme systems. This may explain the rapid disappearance of the enzyme activity during deadaptation (Feigelson *et al.*, 1959).

The activity of tyrosine α -ketoglutarate transaminase can also be increased by injecting tyrosine or hydroxycortisone (Lin and Knox, 1958). It would seem at present that the increase rests on a process of activation or release and not on protein synthesis, as shown by experiments combining the use of labelled amino acids and the serological isolation of the enzyme (Kenney, 1960).

One should therefore be very careful in assimilating these effects with

the induced synthesis of proteins. Enzymic adaptation in animals — at least for the presently analysed cases — may be irrelevant to the control of enzyme synthesis.

B. NON-MENDELIAN HEREDITARY FACTORS IN ENZYME SYNTHESIS

According to the presently accepted theory of enzyme synthesis, the nuclear gene contains information relative to the arrangement of the amino acids in the polypeptides. The information is transferred to some cytoplasmic constituent, most probably a specific RNA, and the amino acids condense in the genetically determined sequence on the surface of the ribosome. The resulting polypeptide is released and it folds in the correct way, thus giving rise to the active enzyme.

At some point along this sequence of events, controlling agents intervene, either to trigger or to inhibit the formation of individual proteins or of restricted groups of proteins.

A few important experimental facts are not clearly integrated into this scheme. For instance, the ability of yeast and moulds to make several enzymes of aerobic metabolism is known to be carried over from parent to progeny in a manner which does not obey mendelian laws of heredity. Let us summarize first some of the observations made by Ephrussi and his group on yeast.

When a culture of *Saccharomyces cerevisiae* is plated on a solid medium containing glucose, most of the colonies which develop are of nearly identical size; however, a few colonies of distinctly smaller diameter are always observed. This is not due to contamination or to heterogeneity of the yeast strain used, for the same result is obtained when the culture is initiated with one single cell isolated from a large colony. Such cells constantly produce in their progeny a majority of cells identical to themselves, and a few cells of a different type, recognizable by the small size of the colonies they form. To the contrary, cells from small colonies give rise to small colonies exclusively; they never recover the capacity of forming large colonies (Ephrussi, 1949, 1953). The change from large colony to small colony type is hereditary, and it is irreversible as far as the present data indicate; the difference in size of the colonies is due to a difference in rate of multiplication of the cells. Actually, this difference is observed only when the cells are grown in the presence of air; in a nitrogen atmosphere, normal yeast does not grow faster than the mutant. It was indeed clearly established that the small colonies mutant is deprived of a functional respiratory system, and that it lacks several important enzymes of the respiratory chain, for instance cytochrome oxidase, cytochrome-*a* and cytochrome-*b*

(Tavlitzki, 1949; Slonimski and Ephrussi, 1949; Ephrussi and Slonimski, 1950; Slonimski, 1949, 1953).

The mutation responsible for the appearance of respiration deficiency differs from normal mutations in several respects. It occurs spontaneously with a frequency of about $3 \cdot 10^{-3}$ per cell per generation, whereas usual genetic mutations occur one thousand times less frequently. The rate of mutation is as high in diploid as in haploid cells. It can be increased enormously by various treatments: effect of acridine dyes (Ephrussi, 1949, 1953; Ephrussi and Hottinguer, 1950), triphenyltetrazolium chloride (Laskowsky, 1954), manganous ions (Nagai and Nagai, 1958), an elevated temperature (Yčas 1956; Sherman, 1959), ultraviolet light (Raut, 1954) and by various other agents (Lindegren, 1959). In the presence of euflavine, under proper conditions, practically every bud formed is a respiration deficient mutant. The mutation affects simultaneously the formation of several enzymes. However, when the yeast cells are treated by a threshold concentration of acriflavine, some of the freshly arisen small colony cells can still give rise to both normal and respiration deficient cells; it looks as if they remained for a few generations in an unstable state from which they can still revert to the normal type or become irreversibly respiratory deficient (Ephrussi and Hottinguer, 1951). All these features indicate that this mutation is indeed of an exceptional type. It is exceptional also in its hereditary transmission, as we shall see presently.

Haploid *Saccharomyces cerevisiae* of opposing mating types can be crossed; the diploid zygotes multiply by budding for unlimited periods, remaining diploid. They can, however, be induced to sporulate by being transferred onto adequate medium. The asci contain four haploid spores. The spores, if isolated, give rise to haploid cultures which will remain indefinitely haploid unless crossed with a haploid of the opposite mating type.

When two haploid yeasts differing by one single mendelian character (A' versus A) are crossed, and the resulting diploid is caused to sporulate, two spores in each ascus contain the allele A , the other two contain the allele A' . This is a regular mendelian segregation of the character. When a normal haploid yeast is crossed with a respiration deficient mutant obtained as indicated above, different results can be obtained, depending on the particular mutant used. Two extreme types have been encountered: the so-called neutral and the suppressive mutants. Crosses of normal yeast with neutral small colony mutant give normally respiring zygotes, and the four spores in each ascus derived from such zygotes are all of the normal respiratory type. Respiration deficiency is not transmitted to any of the spores, no mendelian segregation of the character is observed, the character simply disappears, although in the very same asci typical mendelian markers were shown to segregate in the regular manner. These and other

FIG. 34. Colonies formed on normal solid medium by yeast grown in normal medium (A), and in media containing $1/100,000$ (B) or $1/10,000$ (C) of acriflavine (Ephrussi, 1950).

genetic experiments established that the mutation to respiration deficiency does not concern a chromosomal gene. It must consist in the loss or irreversible inactivation of an extrachromosomal factor which is necessary for the synthesis of respiratory enzymes (Chen *et al.*, 1950). This extra-chromosomal factor is perpetuated during cell proliferation, since it is transmitted indefinitely through generations in normal yeast.

Crosses involving the 'suppressive' type of small colony mutant give a slightly different picture. Zygotes formed by fusion of a normal with a suppressive are able to respire and they can be made to sporulate.

But in this case, the four spores in each ascus all produce respiratory

FIG. 35. Result of a cross between a normal strain (G) and a cytoplasmic mutant (P) of yeast. The capacity to form respiratory enzymes is indicated by stippling (Ephrussi, 1953).

deficient mutants which are of the suppressive type. Here again the respiration capacity fails to segregate as mendelian markers do, indicating that it depends for its transmission on an extrachromosomal factor. This time, the small colony character prevails; it would seem that the suppressive mutant not only fails to perpetuate the normal extrachromosomal object, but that it also prevents its reproduction in the cytoplasm of the hybrid.

Comparable observations were made on *Neurospora crassa*, for the synthesis of the same group of respiratory enzymes. Several respiration deficient mutants were isolated by Mitchell and Mitchell (1952). Some of

them are maternally inherited; this was well established for the mutant 'poky' and for a few others designated by the symbol *mi* (for maternally inherited). The character again does not segregate as a mendelian marker. In these mutants, the respiratory system shows various abnormalities in the cytochromes system (Mitchell *et al.*, 1953; Tissières *et al.*, 1953; Tissières and Mitchell, 1954).

In *Neurospora* as well as in yeast, the formation of several enzymes of the respiratory chain thus depends on some hereditary non-mendelian factor. In *Neurospora*, just as in yeast, it looks as if an extrachromosomal element were required, as if this object could ensure its own continuity through generations of cells, and as if it were in certain cases able to replace the corresponding factor of the wild type (Pittenger, 1956).

To complete the picture, it must be emphasized that although the synthesis of the respiratory enzymes in yeast and mould depends on the presence of an extrachromosomal factor, it also depends nevertheless on typical mendelian genes, just like any other enzyme synthesis. This last point is established by the existence of still another type of respiration deficient mutants, in which the character segregates in a perfectly mendelian manner. The mutation, this time, concerns the synthesis of a single protein (Chen *et al.*, 1950; Mitchell *et al.*, 1953). For instance, the mendelian mutants C-115 and C-117 of *Neurospora* lack cytochrome-*a* and cytochrome-*b* respectively. The synthesis of these proteins, like that of any other enzyme, is therefore controlled by regular mendelian genes; the extrachromosomal factor is a further requirement for the formation of the respiratory enzymes, which has not been observed for the common enzymes.

Another group of enzymes which depend on cytoplasmic hereditary factors are those of the chloroplast in green plants. Certain green flagellates possess one single chloroplast per cell; this chloroplast normally divides before cell division and each daughter cell receives one chloroplast. Occasionally, the plast division lags behind cell division, and the daughter cells separate at a time when the division of the chloroplast is not completed; under such circumstances, one of the daughter cells is deprived of chloroplast. The striking fact is that cells which have thus lost the chloroplast, as a result of a mechanical accident, never recover the capacity of producing a new chloroplast. The progeny of such cells is for ever devoid of photosynthesis apparatus (Lwoff, 1949).

Because of these facts and of observations made on mosaics in higher plants, chloroplasts are commonly described as self-duplicating organelles. This is a mere expression of direct microscopical observation.

It is possible to influence by various means the relative rate of division of the cell and of the chloroplasts in protozoans like *Euglena*, by changing the medium or the temperature or by growing the cells in the dark. The num-

ber of chloroplasts per cell can thus be considerably reduced, and some cells may eventually lose their last plast, thus giving rise to a clone of permanently white cells. Ultraviolet irradiation also causes the appearance of many white cells. A mass transformation of green *Euglena* into white cells can even be brought about by growth in the presence of streptomycin (Provasoli *et al.*, 1951). Loss of the photosynthesis apparatus under these conditions is irreversible as far as one can judge at present. However, this permanent loss is preceded by a reversible lesion. When *Euglena* are kept in streptomycin for 2 hr (1/10 of the mean generation time) and then transferred to normal medium, many of the descendants of the treated cells are white and at each generation some green cells give rise to new white cells (De Deken-Grenson and Messin, 1958).

Certain of the white cells are apparently changed irreversibly, for their progeny remains white for ever. Others, however, can produce a normal green progeny after more than ten white generations. A certain unstable state can thus result from a short streptomycin treatment, just as yeasts which have been kept in low concentrations of acridine remain during a few generations in an unstable state in which respiratory deficient cells can produce either respiratory deficient or normal buds (Ephrussi and Hottinguer, 1951).

The hereditary loss of chloroplasts in *Euglena* has indeed much in common with the hereditary loss of the respiratory system in yeast (De Deken-Grenson, 1960). Both appear as a mutation, the frequency of which can be increased enormously and in a specific way by the action of chemicals, or by unfavourable conditions.

No genetical studies comparable to those made on yeast or *Neurospora* could be applied to *Euglena*, because of the absence of sexual reproduction. But the importance of an extrachromosomal factor is shown by the fact that the mechanical loss of the last chloroplast results in permanently white cells; this indicates that the factor is the chloroplast itself or one of its constituents. On the other hand, it is almost certain that the formation of at least certain of the chloroplastic enzymes is under nuclear genetic control, since several typical mutants blocked at various stages of chlorophyll synthesis have been isolated from *Chlorella* (Granick, 1950; Bogorad and Granick, 1953).

Although the lack of direct genetic analysis makes any conclusion uncertain, it is most probable that the formation of the photosynthesis apparatus, like that of the respiratory system of yeast, depends on both nuclear genes and extrachromosomal factors (chloroplastic in the green plants).

What are the respective functions of the mendelian gene and of the extrachromosomal factor in the formation of the respiratory enzymes in yeast and mould or of chloroplastic proteins in *Euglena*?

When the experimental facts led to the conclusion that an extra-chromosomal factor was involved, that this factor was perpetuated through generations, that it could eventually be lost irreversibly, it was perfectly normal to visualize this factor as a kind of free lance gene. It was indeed commonly referred to as plasmagene, self-dividing particle, self-duplicating particle, extranuclear autoreproducing particulate factor, etc. ... In this perspective it was assumed that acridines, high temperature, tetrazolium salts, etc., interfered with the multiplication of this particle 'endowed with genetic continuity'. The nuclear gene was regarded as controlling the multiplication of the self-reproducing cytoplasmic particle or its function. This was very satisfactory especially in view of the fact that several other biological phenomena appeared to fit into a very similar picture (see Symposium sur les Unités biologiques douées de continuité génétique, 1948).

In the present perspective, however, this interpretation looks less convincing than it was only a few years ago. The function of the nuclear gene is better understood at present: it is known that the gene controls the sequence of amino acids in the polypeptide chains (see Chapter I). As a result, questions about the nature and function of the extrachromosomal factor necessary for the synthesis of respiratory enzymes can now be asked in a different manner. One wonders especially whether the object contains an extra piece of *information* about the arrangement of amino acids in cytochrome-*a*, *b* and cytochrome oxidase, or whether it is involved in some regulation process.

The irreversibility of the non-chromosomal mutation, the existence of two types of cytoplasmic respiratory deficient mutants in yeast, the fact that the induction of the mutation is subordinated to cell multiplication, no doubt suggest that it is due to a change in a particulate object which carries with itself some features necessary to its own perpetuation. But these facts in no way imply that the extrachromosomal factor carries any piece of specific *structural* information required for its own duplication. There is no positive evidence either that the extrachromosomal factor carries any piece of *structural* information for the synthesis of the respiratory enzymes.

It has been mentioned that the progeny of certain white *Euglenae* which form after a short streptomycin treatment can recover the capacity of making chloroplasts, although they had no visible chloroplast left. In the same way, some respiratory deficient yeasts in the unstable state which follows a moderate acriflavin treatment can give rise to normal respiring cells. When they are in this unstable state, it is quite certain that yeasts or *Euglenae* have not lost any part of the structural information required for the synthesis of the respiratory enzymes or of the chloroplastic constituents; it is only the *expression* of this information that was blocked for several generations (De Deken-Grenson, 1960).

When the capacity of making respiratory enzymes or chloroplasts is lost permanently, it is not possible so far to decide whether this is due to the eventual loss of some structural information or to the permanent jamming of the mechanism of its expression, but the latter possibility looks very probable in view of the facts just mentioned.

In the case of the 'poky' mutant of *Neurospora crassa*, there is clear evidence that no structural information is lost: young cultures of this maternally inherited mutant are devoid of 'succinoxidase' and cytochrome oxidase, but older organisms actually make these enzyme systems in amounts which exceed 50 per cent of the titre of the normal wild strain (Haskins *et al.*, 1953). Therefore, the complete structural information for these proteins is contained in 'poky'; the abnormality is that, in spite of the presence of the structural information, the synthesis of the enzymes failed to occur before the fifth or sixth day of growth. This strongly suggests that the extrachromosomal factor in 'poky' in some way *regulates* or *conditions* the biochemical process of synthesis of the respiratory enzymes.

If this is so, the extrachromosomal hereditary factor need not be regarded any more as a wandering gene-like particle or plasmagene or self-duplicating entity; it should rather be considered as a piece of a controlling mechanism. One may even wonder whether the experimental facts could not be explained by the existence of alternative and mutually exclusive steady states maintained by some kind of feed back system; the non-chromosomal mutation might be an irreversible switch from one of these states to the other. It has indeed been long realized that alternative stable steady states could mimic heredity (Wright, 1945; Delbruck, 1949; Pollock, 1953; De Deken-Grenson, 1957; Beale, 1958).

In the case of *Euglena*, the rate of cytoplasmic mutation depends entirely on the physiological state of the cell at the time the mutagenic agent is applied (De Deken-Grenson, 1960). A steady state system would account for these observations more easily than a plasmagene model.

In recent discussions on cytoplasmic inheritance (Ephrussi, 1958; Lederberg, 1958; Nannay, 1958; Catcheside, 1958; De Deken-Grenson, 1960) the respective merits of the plasmagene versus steady states were compared; efforts were made in most cases to advocate one of the models or to decide into which picture a given phenomenon can best be fitted. But it is more and more clearly apparent from these discussions that each type of explanation has valuable features, although none accounts satisfactorily for all the aspects of cytoplasmic heredity. In the author's opinion, a satisfactory model should have the sharpness of the plasmagene theory and the flexibility of the steady state principle.

A unifying theory could possibly be derived from the concept of 'auto-catalytic particle' which was proposed (Campbell, 1960) as an interpretation of long term adaptation of yeast to galactose (Winge and Roberts,

1948; Spiegelman *et al.*, 1951; Campbell and Spiegelman, 1956). A clear distinction is made here between *autoduplicating* particles like genes or viruses, which carry structural information for their own multiplication, and *autocatalytic* objects which are required, albeit remotely, for the formation of identical objects because they fulfil a certain biochemical or biophysical function. In this broad sense, all the 'indispensable' enzymes of a living cell are autocatalytic objects, and so are many other cell constituents, e.g. the phospholipids of the membranes. For if the *activity* of the enzyme is blocked or if the phospholipid is destroyed, the cell dies and stops making the enzyme and the phospholipid.

If the physiological activity of an autocatalytic object X was required for the synthesis of the respiratory enzymes, but completely unnecessary for the life of the cell and for its multiplication, that autocatalytic object would indeed have many properties in common with the 'extrachromosomal hereditary factor' of yeast. Accidental loss or destruction would obviously deprive the cell of the capacity of making the respiratory enzymes and of perpetuating the factor.

But it must be clearly realized that the loss of the object X *per se* is not the essential thing; what is all important is the disappearance of the biochemical or biophysical *function* that it normally fulfilled. This special feature of the autocatalytic particle has two fundamental consequences. The first is that any inhibitor of the *activity* or *function* of X is a potential mutagen. If the biochemical *activity* or the biophysical *function* of the object X is interrupted, the synthesis of more X is blocked, together with that of the respiratory enzymes. In resting cells, the only observable fact will be the inhibition of the synthesis of the respiratory chain. But if the cells do multiply, while X cannot, X will be lost by dilution for part of the progeny, and mutants will appear. This is exactly what happens in the presence of euflavine in yeast. Euflavine first inhibits the synthesis of the respiratory enzymes, and respiratory mutants appear a few hours later (Slonimski, 1953; Marcovich, 1958). Among the acridines, only those which specifically interfere with the synthesis of cytochrome oxidase are mutagenic (Slonimski, 1953). A fairly good correlation between the conditions which depress chlorophyll synthesis and those which favour mutation in *Euglena* was also observed by De Deken-Grenson (1960) who suggested that the hereditary transformation might be a consequence of the primary phenotypic inhibition of chlorophyll synthesis.

A second consequence of the properties of an autocatalytic object like X is that its loss is not *irreversible-by-nature* as would be that of a structural gene. It will be reversible or irreversible depending on whether or not the *function* normally accomplished by X can be temporarily fulfilled by an action of the experimentator, or due to a change in conditions. This will indeed restore the synthesis of X which will then take over its normal

function. A beautiful illustration of such a phenomenon is the loss and recovery of galactosidase synthesis in *E. coli*. The factor X here is obviously the permease. It is an autocatalytic object, since its function is to pump galactosides into the bacterium, and since the achievement of a certain concentration of galactoside within the cell is required for the induction of permease formation and β -galactosidase synthesis. Loss of the permease occurs during growth under conditions which abolish the function of the permease, e.g. when there is no galactoside available to be pumped into the cell. Loss of the permease is reversible because when a sufficient concentration of galactoside is applied from the outside, free diffusion can compensate momentarily for the function of the permease, and thus induce its formation, together with β -galactosidase synthesis. The adapted state can then be transmitted clonally as long as the conditions allow the permease to function. Long-term adaptation of yeast to galactose, for which the model of autocatalytic particle was first proposed (Campbell, 1960) is another example of reversible loss of the particle.

In other cases, of course, the loss will be irreversible for all practical purpose simply because it will be impossible to compensate for the biochemical or biophysical *function* of the autocatalytic factor long enough to allow the synthesis of it to be restored. For instance, if the function of the autocatalytic factor is to be an essential piece of some subcellular structure it might be altogether impossible to restore this function from the outside.

The concept of an autocatalytic object which does not carry any genetic information but simply accomplishes a certain physiological function necessary for its own formation and eventually for other anabolic processes as well, thus leads to a general theory which accommodates cytoplasmic heredity and other phenomena which mimic heredity. Besides, it leads to predict puzzling and somewhat conflicting features of cytoplasmic heredity: the loss of the cytoplasmic factor as if it occurred by dilution of particles, the mutagenic effect of inhibitors of certain enzyme synthesis. The unstable state in which yeast or *Euglenae* can exist after a threshold treatment by the mutagen might be a state in which the activity of X is just sufficient to insure its own synthesis but not that of the enzymes; slight changes of the medium can tilt the equilibrium either towards recovery or towards irreversible loss.

Any theory can of course be stretched to accommodate any experimental facts and it would be without interest for the present discussion to elaborate further the concept of autocatalytic element. This model is presented in an attempt at making a unifying synthesis. The only merit that it claims is of placing old facts in a new perspective and, in so doing, of suggesting new experiments.

Whatever the ultimate nature of the non-mendelian factors in enzyme synthesis may be, it is becoming more and more probable that these factors

are 'epigenetic' or 'epinucleic', to take the same words as Nanney (1958) and Lederberg (1958), i.e. 'metabolic' (De Deken-Grenson, 1960), rather than of the nature of wandering genes or virus-like elements. That is the justification for considering non-mendelian heredity in the chapter of the present book which deals with the regulation of protein synthesis.

C. CHANGES IN PROTEIN SYNTHESIS DURING DIFFERENTIATION

The unique cell out of which a metazoan grows, the fertilized egg, contains all the information for making all the specific proteins of the complete organism. The egg does not grow and multiply like a bacterium which gives rise to an offspring of identical bacteria. The egg first divides a few times without growing. This 'cleavage' results in a group of cells which are not identical to each other and which are disposed in space according to a certain pattern. Some of these cells, or blastomeres, contain more mitochondria, others contain more storage material; some are larger, some are smaller; droplets of lipids may be found mostly in one region of the segmented egg, grains of pigment in others. Enzymes also are differently distributed among the blastomeres: certain regions of the blastula reduce oxidized dyes much more actively than others, the oxidation enzymes, sulphhydryl rich proteins, ribonucleoproteins, phosphatases, are distributed in the cells according to certain gradients which in some cases coincide with the morphogenetic 'fields' defined by the embryologists to describe and explain development. During the segmentation period, little protein synthesis, if any, takes place. But after the egg material has been fragmented and distributed into so many cells, with their special position in space, growth begins and with it — as a part of it — protein synthesis. Certain regions of the segmented egg will grow faster than others, sheets of cells will move under or above other groups of cells; interactions between these will cause visible changes in the morphology and arrangements of the individual cells, and thus the embryo will begin to take shape. Specialized structures, tissues and organs appear; differences in shape and properties of the various regions of the embryo become clearer and clearer as development progresses.

Thus one fertilized egg gives rise to a very heterogeneous offspring of cells, the properties of which diverge more and more during development, until they are fully 'differentiated' in the adult organism. It is barely necessary to emphasize the differences in the assortment of enzymes and proteins between liver, brain, muscle and pancreas. Clearly, these cells make a completely different set of proteins. When and how do such differences in the specificity of protein synthesis arise during development?

Consider haemoglobin synthesis in the embryo. The cells of the gastrula do not make haemoglobin; it is certain that the cells from which the blood islets originate contained the gene which specifies the sequence of amino acids in haemoglobin; synthesis of this protein, however, did not take place to any appreciable extent, it was prevented in some way. Controlling or regulatory processes therefore certainly play a part in the differentiation of protein synthesis.

Interesting studies were made on the appearance of a few highly specialized or characteristic proteins during differentiation. Thus lens proteins are not detected by a serological precipitation method before the appearance of the lens itself (Flickinger *et al.*, 1955; ten Cate and van Doorenmaalen, 1950). Even in the head ectoderm wherefrom the lens is formed, or in the eye vesicles, which induce the transformation of head ectoderm into lens, no lens antigen is found by the precipitation method (Woerdeman, 1955). With the Coons method for localizing antigen on histological sections which uses a fluorescent antiserum as a specific stain, a characterized localization was found only when the lens was formed; before this, some lens antiserum was bound everywhere. It may be therefore that typical lens proteins were made before, but in small amounts, by all the cells of the embryo, and that their formation was later restricted to a few territories.

Ebert (1954, 1958, 1959) did a similar study on the appearance of heart myosin in the chick embryo. At the end of gastrulation, a protein which reacts with anticardiac myosin serum arises everywhere in the embryo; later this protein is restricted to the heart-forming area. Actin, on the contrary, is not detected except in this area, and only when myosin localization is already restricted to it.

The appearance of skeletal muscle myosin and of collagen have also been studied by similar and other methods; and a wealth of data on the changes in many enzymic activities during development can be found in the literature of chemical embryology (see Brachet, 1944, 1960).

In a way, all these data can be taken as more elements to be added to the morphological observations; they are an extension of morphology down to the chemical level. As a rule, specific proteins appear indeed at the same time as the corresponding organ. It may be argued that the question as to which comes first, specific proteins or morphological differentiation, is not very consistent: in so intricate a process as development, where each step may influence the steps to come in very indirect ways, it is certainly not easy to distinguish causes from effects. But proteins are the most immediate expression of the genetic characters, and the enzymes do control all the biochemical operations by which cell material is made. Proteins therefore must occupy a key position in differentiation. As the mechanism of protein synthesis and its control are becoming better and better understood, the

study of changes of protein synthesis during development is certainly a promising approach to some of the basic problems of embryology.

A fundamental question is whether two differentiated cells of the same organism produce two different sets of proteins because they have a different complement of mendelian genes. This is, in newer words and in a more restrictive sense, the problem of the equipotentiality of the nuclei which retained the attention of Driesch (1894) and of Morgan (1934).

The amount of DNA in a diploid somatic cell is just double that of the spermatozoon. The number of chromosomes and their general shape

FIG. 36. Distribution of actin and myosin in the early chick embryo at successive stages of development, as shown by Coon's method (Ebert, 1954).

usually do not change during development. In insects, certain tissues contain enormously enlarged chromosomes, and a correlation has been established between the visible bands of the chromosomes and the genetic map computed from recombination frequencies. The same pattern of bands is found in the chromosomes of the salivary gland and of the midgut of *Drosophila* (Berger, 1940) or in the rectum, the Malpighian tubes, the midgut and the salivary glands of *Sciara* (Beermann, 1952); there is no evidence of any change in structure of these chromosomes during differentiation. These are indications that not many genes are lost on the way; but subtle changes in the state of the genes might escape these crude

observations. The actual presence of a gene can be shown only by its function. But when a character fails to show up, unless the character reappears in the offspring of the cell, it is impossible to tell whether the gene had disappeared or whether its expression was blocked. It is not known whether irreversibly differentiated cells have lost certain genetic determinants or whether the expression of the corresponding character was made irreversibly impossible. What we need to answer such a question is a way of testing the activity of the genetic material in a non-differentiated cell or in a cell which underwent another type of differentiation.

It has not been feasible so far to exchange the DNA or the chromosomes of two differentiated cells to see what happens. But nuclear transfer comes closest to it. Spemann (1928) and Seidel (1932) had found that during early cleavage, the nuclei are equipotential: the nucleus of a dorsal blastomere can be replaced by a nucleus from the ventral part without affecting embryonic development. Up to a time when the embryo contains about one hundred cells, the nuclei remain equipotential, they are not irreversibly differentiated. But it should be realized that growth and synthesis (except for nuclear material) had not started yet at that time. Briggs and King (1953, 1959) succeeded in replacing the nucleus of frog eggs by nuclei taken from frog embryos at more advanced stages of development. When the nucleus was taken from a blastula or even from an early gastrula, a stage at which protein synthesis just begins, it was able to replace the nucleus of the egg and ensure a complete development of the embryo into a tadpole. Clearly, the nuclei at those stages had retained all their potentialities, they possessed the same genetic information as the nucleus of a fertilized egg. If the nucleus was taken at a slightly later stage, segmentation took place normally but development was blocked during gastrulation, as if this nucleus had lost some of its capacities. More striking still are the serial transplantations in which the nuclei were taken at an early stage from embryos which had themselves originated from nuclear transfer, and whose development had stopped at an early stage. These never permit full development (King and Briggs, 1956, 1957).

Similar experiments were made on another amphibian, *Xenopus*, by Fischberg *et al.* (1959) who succeeded in obtaining complete development after transferring nuclei taken from somites, i.e. from a region of the embryo which is already clearly differentiated and which will soon form skeletal muscle; some of the nuclei were still totipotent 9 hr before the somites became contractile. It would seem therefore that these cells which were well advanced on the way of differentiation still possessed all the genes and that their nuclei were still able to accomplish all the functions of an egg nucleus, when returned to the cytoplasm of an egg. At later stages, however, they became unable to ensure development.

These experiments indicate that some differentiation can take place

without irreversible change occurring in the nucleus. The significance of the loss of capacities of the transplanted nuclei taken at later stages is of course much more uncertain, for it is very difficult to establish whether the change is due to differentiation or to damages caused to the nuclei during transplantation; the operation becomes indeed more and more difficult as development progresses. It is not certain at present whether the nuclei undergo a differentiation process.

In giant chromosomes, metabolic processes have been observed which suggest that some differentiation might take place within the chromosomes themselves. Although the pattern of the bands of the giant chromosomes is the same in the different organs in which they are found, very striking metabolic processes occur at specific regions of the chromosomes. Certain typical euchromatic bands, i.e. bands which are supposed to contain mendelian genes, are changed into large bulbs (Breuer and Pavan, 1955). During this process, a very active incorporation of thymidine takes place, indicating localized DNA synthesis (Ficq and Pavan, 1957; Ficq *et al.*, 1958); RNA is also produced at the same locations. It is remarkable that these metabolic processes occur at the level of well-defined bands, and at certain stages of larval development only. This might possibly reflect a process of nuclear differentiation.

The sequence of events which result in cytoplasmic and eventually nuclear differentiation is unknown. There is clear evidence that the cytoplasm influences the state of the nucleus, and that both must in some way be adjusted to one another for proper co-operation. Hybrids between two sea urchins *Paracentrotus* and *Arbacia*, or between two frogs, *Rana pipiens* and *Rana sylvatica*, or *Rana esculenta* with *R. temporaria* are blocked more or less early during development, usually at gastrulation. In these lethal hybrids, the nuclei of many cells are abnormal, they are overloaded with RNA (Brachet, 1944, 1954; Zeller, 1956).

If the nucleus of the fertilized egg of *R. pipiens* is transferred into an enucleate unfertilized egg of *R. sylvatica*, development starts, but it is blocked at the blastula stage. If the nucleus of one of the blastomeres is now transferred back into an enucleate *R. pipiens* egg, development is blocked at the beginning of gastrulation. This suggests that the nucleus had been irreversibly changed due to its reduplication in a foreign cytoplasm (Moore, 1958, 1959).

It is therefore quite reasonable to consider that changes in the properties of the nuclei might be the consequence of differences in cytoplasmic properties. But changes in nuclear activity certainly influence cytoplasm. A cumulative effect will thus progressively be observed, and it is easy to visualize that once nucleus and cytoplasm are engaged in such a cascade of changes which determine one another, the resulting evolution of the cell line will rapidly become irreversible.

FIG. 37. Radioautograph of the distal segment of the salivary C chromosome of *Rhyncosciara angelae* at three different stages of larval development. The larvae were fixed 24 hr after receiving an injection of ^3H -Thymidine. Heavy blackening shows localized strong incorporation of thymidine (Ficq *et al.*, 1959) (courtesy Dr A. Ficq).

By what kind of nuclear and cytoplasmic changes is the specificity of protein synthesis changed during development? No answer can be provided at present to this question. If one adopts the common — but unchecked — assumption that each cell of a metazoan contains the complete collection of mendelian genes, then the changes must concern the control of protein synthesis. The difficult point is that these changes, although they may not be genic, are transmitted clonally. One is left with guesses inspired by observations on simpler phenomena which share certain features with differentiation, or on special cases of controlling mechanisms.

The interplay of inducers and repressors of protein synthesis, for instance, indicate a mechanism by which the production of specific groups of enzymes could be switched on or off. Not very complicated modalities of such systems can indeed ensure the clonal maintenance of certain metabolic states, and of specific protein synthesis (see p. 138). The known cases of cytoplasmic mutations, in yeast, *Euglena* or *Paramecium*, provide a model for clonally transmitted restrictions of the capacity to make certain specific proteins (see p. 141). For these reasons, the theories proposed for explaining cytoplasmic heredity — whether they involved plasmagenes or steady states — were often suggested as models of differentiation. The wandering controlling elements found by McClintock (1956) to inhibit certain genes in maize, or the episomic elements of bacteria (Jacob *et al.*, 1960) also suggest mechanisms by which the mathematical rigidity of mendelian control can be tempered.

Processes of differentiation are often presented as being irreversible by essence; or rather irreversibility is often taken as a criterion of differentiation, i.e. as part of its definition. Although differentiation 'properly speaking' may be irreversible, this irreversibility should not blind us, for it may be merely a usual consequence of the type of process involved in differentiation. Changes of cell constitution or cell structures which are not irreversible, might be brought about by the same fundamental processes as differentiation during development, but remain reversible because they are less extent and less intricate. It might be very informative to know more about the mechanisms of changes like sporulation, spore germination or even the changes in enzyme constitution during the growth phase of micro-organisms, changes in the mating type, i.e. in the constitution of the cilia in *Paramecium*; differentiation during growth of the slime mould (Sussman, 1958; Wright and Anderson, 1958) might provide a transition between micro-organisms and metazoans. It is true that in these simpler processes differentiation is controlled or triggered by changes of the medium, whereas in higher organisms interactions between cells (inductions) play an essential part. But a few known facts indicate that the outer agents of differentiation are not always very involved; simple chemicals can have rather specific actions in some aspects at least of

embryonic development. Differentiation of cells of the neural crest into pigment cells normally occur as a result of interactions with the mesoderm which lies below. The appearance of the pigment cells can be prevented by analogues of phenylalanine and, in explants of ventral mesoderm, differentiation is obtained when phenylalanine is added to the medium. It seems that the production of phenylalanine by the mesoderm and its utilization by the ectoderm is an essential factor in the differentiation of these cells (Wilde, 1955, 1956). Specific nutritional requirements have also been observed in cultures of organs (Wolff, 1957). Differences in requirements are simply a sign of chemical differentiation, but they also point to the possibility that certain tissues might depend on their neighbours for simple compounds. These elementary interactions may be of fundamental importance. Studies on the formation of specific proteins in such systems might be illuminating.

Pioneer work on the transfer of specificity by cell extracts and especially by microsomes must be mentioned here. When liver microsomes are spread on the chorioallantoic membrane of a developing chick embryo, a thickening of the membrane is observed and the amount of glucose-6-phosphatase, an enzyme characteristic of liver microsomes, increases considerably (Le Clerc, 1954) as if the microsomes had carried over the capacity of making this enzyme. More recently, Ebert (1959) implanted on the chorioallantoic membrane a microsomal fraction from adult cardiac muscle together with Rous sarcoma virus. In the tumour masses which developed, muscle tissue was observed to form in 27 per cent of the cases. These observations open new perspectives and a wide field of new possibilities to the experimental study of differentiation.

D. THE SYNTHESIS OF ANTIBODIES

If bacteria, foreign cells, foreign proteins or polysaccharides are injected to an adult rabbit, new γ -globulins appear in the serum of the animal a few days after the injection. These special γ -globulins are recognizable by their ability to form complexes with the injected substance or to bind specifically on the surface of the injected foreign cells. These new γ -globulins are the antibodies, the injected cells or substances the antigens. The antibodies are highly specific of the antigen which caused their appearance in the blood.

The antibodies do not arise by transformation of pre-existing γ -globulins: when ^{14}C labelled normal γ -globulins are injected into the blood of a rabbit immunized against *Pneumococcus*, the antibodies do not become labelled (Gros *et al.*, 1952; Green and Anker, 1954). On the other hand, when labelled amino acids are injected to an animal which is producing anti-

bodies, these become strongly labelled (Green and Anker, 1954; Askonas *et al.*, 1956; Taliaferro, 1957). Production of antibodies therefore rests on the synthesis of new specific proteins all the way from amino acids.

The use of a protein antigen coupled with a fluorescent dye makes it possible to locate on tissue sections the cells which contain the corresponding antibodies: the fluorescent antigen is bound or precipitated *in situ* by the antibodies (Coons and Kaplan, 1950; Coons, 1958). It was shown by this method, completed by tracer experiments (Askonas and White, 1956) that antibodies are produced by individual cells or by groups of cells of the reticuloendothelial system, the plasma cells, which are found especially in the lymph nodes and in the spleen.

Antibody production thus reflects the *de novo* synthesis of specific γ -globulins by specialized cells under the stimulus of an antigen. Superficially at least, this phenomenon is comparable to the induced synthesis of enzymes. The antigen, like the substrate of an enzyme, induces the synthesis of a protein which combines specifically with it.

Obviously antibody formation raises the same problems as induced enzyme synthesis. The most fundamental question, in the present perspective of protein synthesis, is whether the antigen brings to the antibody producing cell part of the structural information relative to the antibody, or whether the antigen simply triggers a ready-made machine to produce a protein the structure of which is completely specified by a gene.

The first theory of antibody production (Ehrlich, 1900) assumed that the antigen, through chance affinity, binds certain receptors or special outgrowths of a cell surface; the cell reacts by replacing the bound receptors by two or more new ones, part of which are cast off in the blood as antibodies. According to this view, the antibody structures pre-exist, the antigen simply stimulates their production and their release into circulation; it selects an antibody out of many, but it does not bring any structural information to the antibody producing system.

Landsteiner (1933) coupled proteins with various azaaryl groups under conditions which do not cause denaturation. He showed that such substituted proteins are good antigens, and that the antibodies that they evoke in the rabbit are quite specific of the artificial chemical groups bound to the protein. Using substituents in which tartaric acid residues had been introduced, he showed that the antibodies can even distinguish between L and D isomers. These fundamental studies established that antibodies are specific of individual chemical groups in the antigen molecule, rather than of the molecule as a whole. It was clear also that antibodies can be evoked by many kinds of chemical structures, including some which are not likely to ever come in contact with a rabbit, were it not for the deliberate intervention of an organic chemist. It seemed incredible that the animal could possess a ready-made specific antibody to cope with

each synthetic antigen. It was deemed much more probable that the antigen itself participates in the molding of the corresponding specific antibody.

Thus Breinl and Haurowitz (1930) advanced the view that the antigen interferes with the process of globulin synthesis so that globulin molecules complementarily adjusted to the shape of the specific groups of the antigen are formed. Proteins can differ by the amino acid sequence in the chains or (and) by the way these chains are folded. Pauling (1940) elaborated a model of antibody synthesis in which it was assumed that the polypeptide chains of the globulins or certain regions of them were likely to assume many different types of folding. In the absence of an antigen molecule, the newly synthesized polypeptide chain might fold up in any one of a number of ways, representing the maximum stability under the conditions then existent in the cell. If, however, an antigen molecule were present, the polypeptide chain would fold up into a configuration complementary in structure to the antigen molecule. The specific shape of the antibody molecule would thus be imprinted upon the plastic polypeptide chain of the globulin molecule by the antigen itself.

It must be noted here that the structural information brought by the antigen adds to the unchanged genetic information, and that it is of a completely different nature. The additional information controls the establishment of the secondary and tertiary structures of the polypeptide only. It is a kind of structural information which is disregarded in the present concepts of protein synthesis in which all the attention is focused on the primary structure of the protein (see p. 122). The antigen-template would act after the nucleic acid template has assembled the amino acids into a polypeptide in the genetically controlled sequence.

The direct antigen template hypothesis has implications which could, in principle, be checked experimentally. It assumes that the antigen must be present for antibody production to continue; it predicts that all antibodies, or at least many antibodies, should have the same amino acid sequence. This prediction is amenable to experimental check. Five different antibodies produced by the rabbit against ovalbumin and against four strains of *Pneumococcus* respectively, all terminate by the same amino acid sequence Ala-Leu-Val-Asp-Glu and four more antibodies which have not been studied as thoroughly also have a terminal Ala (Porter, 1950a; McFadden and Smith, 1955). Within experimental limitations, the amino acid composition is the same for all (Smith *et al.*, 1955). These facts lend support to the above hypothesis. It should not be overlooked, however, that the globulins are large molecules containing some 1500 amino acid residues and that the replacement of a few amino acids by others would not be detected easily. But the techniques for determining the amino acid sequence have advanced considerably in the last few years, and it is to be

hoped that the interesting works quoted above will be extended and that the primary structure of a large part of the antibody molecule will be unravelled. This does not appear beyond the reach of experimentation, for the large antibody molecule can be split into fragments some of which contain the specific groups which react with the antigen (Porter 1950b, 1958). Knowledge of the primary structure of these fragments would be of fundamental importance. It would provide not only a test for the direct template hypothesis but also an essential foundation of any coherent theory of immunological response.

According to the direct template hypothesis, antibody can be produced only as long as the antigen is present. It is a fact that immunity can last for several years after the last injection of antigen; unfortunately it is very difficult to find out experimentally whether the antigen does persist for years in antibody producing cells and it is impossible as well to prove that it does not persist, for in a template process a few molecules per active cell might be sufficient to ensure continued antibody production.

A theory, which derives from the preceding one but avoids the requirement for the persistence of the antigen, was proposed by Burnet and Fenner (1949). Here the antigen is assumed to modify the agents of globulin synthesis permanently and in such a way that they will produce a new globulin, the antibody, even when the antigen has been eliminated. This is the indirect antigen-template theory. The permanent modification is assumed to concern a piece of the protein making machine which is responsible for the specificity of the synthesis. For instance, a modification of the DNA of the antibody producing cell has been suggested as an obvious possibility (Schweet and Owen, 1957).

Recently, the old natural selection theory of Ehrlich was revived in a new form by Jerne (1955). Ehrlich's theory has been abandoned because many workers found it incredible that antibodies specific for strange artificial organic groups could pre-exist in the organism. But this opinion was based on the more or less implicit assumption that each antigen evokes an antibody which is different from any other globulin and which is perfectly adapted to the antigen. Actually, the response is not as perfect and the specificity of the reaction not so absolute. Many proteins are bad antigens, not all chemical groups have antigenic properties, and all the animals do not react equally well to an individual antigen. In response to an antigen, an animal often produces a heterogeneous group of antibodies (Talmadge, 1957; Lapresle, 1959). Cross reactions occasionally occur between 'unrelated' antigens (Dixon and Maurer, 1955). The blood of a non-immunized animal often contains proteins which manifest a great affinity for certain foreign substances (Jerne, 1956). It is conceivable therefore that an animal produces a broad but finite assortment of γ -globulins able to form complexes with many various kinds of chemical groups. When introduced into

an organism, a foreign substance may combine by chance affinity with those of the γ -globulins which happen to have such a configuration that they form a rather stable complex with the injected substance. Let us assume that the organism reacts by producing more of this diverted γ -globulin, the production of specific antibodies will be accounted for. This is essentially Ehrlich's theory reworded in molecular instead of morphological terms. The next question is how the organism is stimulated to produce specifically the γ -globulins which happen to combine with the exogenous substance. Jerne (1955) imagines that the γ -globulin-antigen complexes are picked up by competent cells and that they stimulate these cells to copying the particular γ -globulin contained in the complex. A very interesting feature of this theory is that it predicts an increasing fitness of the antibody produced in the course of immunization, for the globulins which have the greatest affinity for the antigen will be produced in greater amount and will eventually overwhelm all the others. The assumption that the swallowed γ -globulin brings into antibody producing cells the structural information for the synthesis of identical γ -globulin molecules is difficult to integrate into the present views on protein synthesis: information is known to flow from the nucleic acids to the proteins, but there is no evidence that proteins can ever transmit the structural information for their own reproduction to a protein making system. Whatever the value of the hypothetical mechanism proposed by Jerne, the revival of a selection theory of antibody synthesis was very stimulating. Newer hypotheses were soon developed in which the selection was assumed to occur between cell lines rather than between molecules (Burnet, 1959; Lederberg, 1959). These theories also account for several other experimental data which should now be summarized briefly.

When an antigen is injected for the first time into an animal, antibody appears in its blood after a few days; the titre of antibody then decreases gradually and it may eventually become very low, or undetectable. This is the primary response. If a few weeks or a few months later the animal receives a new injection of the same antigen, it soon produces large amounts of antibody and keeps producing it for a long period. This secondary response indicates that although antibody production ceased, the organism had kept memories of its first contact with the antigen, since it was made more responsive to a second contact. This memory effect should be accounted for. Another puzzling feature of antibody synthesis is that it is caused by foreign substances only, as if the organism was able to distinguish self from foreign. Moreover, embryos or newly-born animals do not respond to an injection of antigen by producing antibodies; nevertheless the injected substance can change the antibody producing system in such a way that the animal in its adult life will not produce antibodies against the foreign antigen to which it has been exposed during its fetal or early

life; it has acquired an 'enduring immune tolerance' towards this foreign substance (Billingham *et al.*, 1953). An unresponsive period in which an adult behaves in this respect as a newly-born animal can be observed after irradiation with a high dose of X-rays (Dixon and Maurer, 1955). Injection of a very large amount of antigen can have similar effects (for a review see Chase, 1959). The recognition of self and non-self, and the adoption as 'self' of an antigen injected during an unresponsive period raise the most challenging problems of the control of antibody synthesis. Cytological observation with fluorescent antigens shows that after the first antigen injection, thousands of cells in the lymph nodes may pick up the antigen; but antibody appears in a much smaller number of these cells, and after a few days antibody is demonstrable only in a few cells. In a secondary response, many hundreds of cells containing traces of antibody can be seen; they are scattered throughout the areas where the antigen was deposited after the first injection. These cells seem to spring up independently and to multiply, so that their descendants often coalesce to form clumps of cells (Coons, 1958).

Immunological response is thus associated with processes of cell multiplication and differentiation.

If two antigens A and B are injected into the same animal, and the antibody producing cells are located by the Coons' method using a different fluorescent marker for each antigen, it is observed that antibodies reacting with each individual antigen are often produced by different plasma cells, as if certain cells had specialized in the production of antibodies against antigen A, and others against antigen B (White, 1958). The same fact was observed by a completely different method. Tissues from an immunized animal can continue to produce antibodies *in vitro* (Fagraeus, 1948). Cells of lymph nodes of a rabbit immunized against two different bacteria were isolated in microdrops, and tested for the production of antibodies against each bacterium species. About 10 per cent of the isolated cells produced one or the other antibody, but none were found to produce both (Nossal and Lederberg, 1958). However, in similar experiments with rabbits immunized against phages T₂ and T₅, 8 per cent of the isolated cells produced antibody against one or the other phage exclusively, but about 2 per cent produced both types of antibodies (Attardi *et al.*, 1959). These results indicate that when an animal is injected with two different antigens, individual cells often form one species of antibody only, but formation of one antibody does not prevent the cell from making another antibody at the same time.

Burnet (1959) has proposed a new theory which takes in consideration the enduring immune tolerance, the fact that antibodies are not produced by all individual cells of the competent organs, and that antibody producing cells do multiply. Burnet assumes that the mesenchymal tissues are made

of a heterogeneous population of cells with various protein synthesis abilities; this heterogeneity arises in the course of development and differentiation. There are a certain number of different clones (i.e. classes of cells all derived from one single cell by mitotic divisions) which are each competent towards a certain antigenic determinant. If the relevant determinant comes in contact with such a cell, it stimulates this to proliferate into plasma cells which produce the corresponding antibodies. It is further assumed that if an antigen reacts with the cell at a certain early stage of differentiation, that cell is functionally eliminated. This would account for the elimination of cells able to produce antibodies against antigenic substances present in the host or introduced into it during early life. One should eventually obtain a residual population of clones able to react only with foreign antigens.

The fact that different cells often specialize in the production of one or the other antibody (Nossal and Lederberg, 1958) is certainly in line with a selection theory in which the selective process operates at the cellular level. The observation that certain cells can produce two antibodies simultaneously (Attardi *et al.*, 1959) conflicts with Burnet's theory which in its most extreme form indeed assumes that each antibody is produced by a different clone of cells, but it is not incompatible with the basic idea of the theory, which can easily be readjusted accordingly.

Lederberg's views (1959) are very similar to Burnet's; it is assumed that the stem line of antibody forming cells is genotypically heterogeneous owing to relatively frequent mutation in the γ -globulin gene. An antigen functions by recognizing those cells already competent to produce a globulin which reacts with the antigen. These cells are selected in the sense that they are stimulated to proliferate; on the other hand, their particular ability to produce antibody is also stimulated, or allowed to express itself. The event which is assumed to cause heterogeneity among the stem cells is mutation of a highly mutable globulin gene. Obviously, a 'cytoplasmic' mutation, or any kind of change which can be clonally transmitted would serve the same purpose (Lederberg, 1959; Schultz, 1959).

It has been mentioned earlier (p. 139) that in bacteria an adapted state, once it has been established, can be maintained clonally through many generations under conditions which could not cause adaptation (Cohn, 1957; Novick and Weiner, 1957); this is due to the special properties of a permease system. A model of antibody formation has been derived from this phenomenon by Monod (1959). In the primary response, the antigen is assumed to induce in certain cells both the antibody and a specific permease or a specific cellular antibody which captures the antigen. The cells which have acquired this specific antigen-capture system will exhibit an increased sensitivity to a further antigen injection since they are now able to concentrate it specifically. If they multiply, this increased sensitivity

will be transmitted to the clone. Heterogeneity among the population will be achieved through the channel of this inducible antigen-capture system, without change in the genetic material.

Szilard (1960) also developed a theory which rests entirely on the presently accepted views about induction and repression of enzyme synthesis and on feedback mechanisms. It gives an interpretation of all the major facts of antibody production, but this is at the cost of several assumptions.

All these theories of antibody formation are extremely attractive; they are unfortunately highly speculative, because facts are scarcer than ideas in the present state of biochemistry of antibody synthesis. Progress in cell cultivation methods and the possibility of studying antibody production *in vitro* might pave the way for a new type of experimental approach. One may hope that these brilliant working hypotheses will help to design clear experiments and will thus eventually lead to a better understanding of this intriguing process, which is not of academic interest only. A broad field lays open for experimentation.

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ERRATA

- p. 13, line 31: insert (Forro and Wertheimer, 1960).
 p. 14, line 16: insert (Chase and Doermann, 1958).
 p. 20, line 31: read Horowitz and Leupold instead of Horowitz.
 p. 25, line 40: insert (Schramm *et al.* 1955; Schuster *et al.* 1956).
 p. 26, line 25: insert (Schäfer *et al.* 1959).
 p. 32, line 11: insert (Chargaff *et al.* 1950; Elson and Chargaff 1954).
 p. 47, line 5: insert (Vanderwinkel *et al.* 1958).
 p. 109, line 3: insert (Perlmann 1953; Perlmann and Gustafson 1948).
 p. 110, line 5: insert (Speck 1947, 1949).
 p. 124, line 7: read Van Robertson instead of Robertson.
 p. 137, line 21: insert (Halvorson *et al.* 1955).
 p. 161, line 2: insert (Hašek, 1956).