The Second Symposium of
THE SOCIETY FOR
GENERAL MICROBIOLOGY

edited by

SIR PAUL FILDES & W. E. VAN HEYNINGEN

THE NATURE OF VIRUS MULTIPLICATION

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EDITORS' PREFACE

Members of the Symposium will no doubt notice that the account of the discussions of papers in this volume is uneven.

Many questions and criticisms went unrecorded on the forms supplied, or did not reach the Editors. Further, some of those which came in had been polished and made more cogent. The effect of this was that the replies already on hand were either irrelevant or ineffective.

Under these circumstances it was clear that the Editors were unable to provide a factual report of the proceedings, and they decided to base an account on ideal conditions in which the questions and answers could be well considered. In this way, they thought, the scientific, rather than the personal aspects of the discussion, might be registered more satisfactorily. As the ideal questions or criticisms were received, they were forwarded to the author of the paper discussed. He thus had the advantage of the last word. This procedure also allowed acceptance of questions which, so far as the Editors could remember, had never been asked at all.

It was found possible to collect and circulate all these documents and to recover corrected proofs of the main papers and all replies to discussions within four weeks of the termination of the Symposium.

In a subject in which movement in discovery is so rapid, four weeks is, of course, a long time and this led to such alterations in proof as normally could not be allowed. It also permitted at least one author to carry out further experimental work to clarify a matter which the Symposium had left obscure. The Editors decided that even in this case a postdated 'stop press' note came within the intentions of the Symposium.

This volume thus contains the final versions of the main contributions and, attached to each, the 'discussion' in an idealized form.

Sir William Dunn School of Pathology
University of Oxford
17 *June* 1952

PAUL FILDES
W. E. VAN HEYNINGEN

THE ORGANIZATION OF THE SYMPOSIUM

The Committee of the Society for General Microbiology having selected for a Symposium a controversial subject, it became necessary to organize it in such a way as to ensure that an even balance was maintained between opposing factions, and that their respective views should be exchanged and conveyed to the prospective audience in advance of the meeting so that the discussion should be conducted as effectively as possible. About eight months before the event the Society invited a representative group of virus experts to write a full account of their views. These were set up in type, and about one thousand sets of galley proofs were distributed to all members of the Society three weeks before the opening of the Symposium. The meeting, which took place at Oxford University on the 16th and 17th of April, 1952, was attended by some 400 persons. The galley proofs were taken as read and, after brief introductions by the authors, the papers were thrown open to discussion by invited speakers and the audience generally. Each speaker was provided with a printed form on which his remarks, whether questions or answers, were to be recorded and handed in for publication.

The organization of the Symposium on these lines entailed considerable expenditure. A large proportion of the cost of printing the galley proofs was met by a contribution of £250 from the Nuffield Foundation. Further printing costs and travelling expenses of visitors invited from abroad were met by subscriptions amounting to £710 10s. 0d. from the following organizations: Allen & Hanbury's Ltd, Boots Pure Drug Co. Ltd, The Distillers Co. (Biochemicals) Ltd, Evans Medical Supplies Ltd, Glaxo Laboratories Ltd, May & Baker Ltd, Pest Control Ltd, The Wellcome Foundation. The Society is glad to record here its gratitude for this generous support, without which it would not have been possible to run a successful Symposium.

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PROBLEMS OF PROTEIN SYNTHESIS

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A FEW FUNDAMENTAL DATA

Two proteins having the same physiological function in two different species of animals are often different enough to be easily distinguished by chemical analysis, solubility measurements or other relatively crude physical or chemical tests. Closely related strains of the same micro-organisms can be identified by small differences in their metabolism, i.e. in their enzymatic equipment, and the study of biochemical mutants in micro-organisms (Beadle, 1945; Bonner, 1946) or in higher organisms (Perutz *et al.*, 1951) has shown, in many instances, that mutations correspond to the loss or to the modification of one type of protein. The formation of a given protein in a cell thus depends on the genetic characteristics of that cell.

On the other hand, the discovery of the acquisition of new enzymes by micro-organisms grown in a new medium and the very careful analysis of this phenomenon, has shown that the actual formation of an adaptive enzyme depends on both genetic characteristics and external conditions (Spiegelman, 1946, 1949; Monod, 1948, 1950; Ephrussi & Hottinguer, 1950). A given adaptive enzyme can form only in cells possessing a suitable genotype, and its actual formation requires the presence of the corresponding substrate. Another example of the action of external factors on the production of specific proteins is the formation of antibodies specific for the antigens which caused their production.

The proteins in a non-growing cell have been considered for a long time to be stable and inert; but the pioneer work of Schoenheimer (1942) on the incorporation of labelled amino acids into the proteins has completely changed that picture, by showing clearly that the substance of the proteins in the living cell is in a dynamic state, being continuously replaced by that of small molecules of the environment. Innumerable works have confirmed this for many various types of cells. This is thus a fundamental fact that must be kept in mind in any consideration of the mechanism of protein synthesis.

The study of viruses has established two basic facts, among many others. First, that the introduction of the virus into a receptive cell causes this to produce a group of proteins that the non-infected cell

would never have produced, as if the presence of a virus particle had caused the cell mechanisms to reduplicate that particle manyfold. Second, virus studies have shown that these 'self-duplicating' particles contain a large amount of nucleic acid, be it ribosenucleic or desoxyribosenucleic. Other known self-duplicating particles have also been shown to contain nucleic acids. Thus chromosomes contain both types, chloroplasts contain RNA, and there are reasons to think that the self-duplicating particles responsible for enzyme production also contain ribosenucleic acid (Spiegelman, 1949).

RNA is most probably involved not only in the self-duplicating processes, but also in protein synthesis in general. The histological and chemical studies on the distribution of RNA in different tissues by Brachet (1941, 1947) and the microspectrophotometric researches of Caspersson *et al.* (1941) have led to the recognition of a constant coincidence in space and time of a high content of RNA and rapid protein formation. At the time when, and in the place where, proteins are being rapidly formed, the amount of RNA present is relatively high. This relation is particularly striking when the same tissue is considered under two different physiological conditions (Jeener, 1951). For instance when protein formation or growth is taking place the RNA content is high, but when the same cells are not producing proteins, there is less RNA. The number of facts all pointing in the same direction is very impressive, and the great number of experiments reported since that relation was first discovered have largely confirmed the existence of a correlation between RNA and protein synthesis (Brachet, 1950). Moreover, there is evidence that ribosenucleic acid accumulation occurs before protein synthesis (Thorell, 1947; Brachet, 1950; Davidson *et al.*, 1949).

The transformation of one type of bacterium into another by purified DNA (Avery *et al.*, 1944; Boivin *et al.*, 1945; Hotchkiss, 1948) also suggests an effect of DNA on the structure of proteins synthesized, since we are accustomed to consider a change in metabolism or constitution as the reflection of a difference in enzyme equipment. The formation of galactozymase in adapted yeasts can be inhibited by ultraviolet irradiation, and it is very impressive that the action spectrum for this phenomenon closely resembles a nucleic acid absorption spectrum (Swensson, 1950). It has also been shown, that the turnover of RNA phosphorus in yeast is several times higher when the cell is producing proteins, and that several factors inhibiting protein formation also inhibit the replacement of the RNA phosphorus (Spiegelman & Kamen, 1947). Quite recently, Hultin (1950) working on the liver of chick embryo, Keller (1951) and Lee *et al.* (1951) on rat liver, and Szafarz (1951) on a flagellate, have

observed that the rate of incorporation of amino acids into proteins is very different for various particulate fractions that can be isolated by centrifugation of the homogenized tissues. The highest speed of amino acid incorporation was found in the proteins of the 'microsomes' and especially in the smallest of those particles which are known to have the highest RNA content of all the cell particles. The larger particles containing the respiratory enzymes and the centres of high energy phosphate bond production were much less active in this respect. This is true also for the incorporation in homogenates (Borsook, 1950; Siekevitz & Zamecnik, 1951). These results extend to the cell particles the previously recognized coincidence of high nucleic acid content and protein synthesis, and may be taken as good evidence that the microsomes are a site of protein formation (Brachet, 1947).

Recent comparative studies on the activity of the cytoplasm in the presence or absence of the nucleus, have confirmed the conclusion that protein synthesis is essentially a cytoplasmic function. *Acetabularia mediterranea* is a large mono-cellular alga that can be cut into two halves one of which contains the nucleus, while the other is free. Both halves will survive for several weeks. It has been observed (Brachet & Chantrenne, 1951) that the rate of incorporation of $^{14}CO_2$ into the proteins of nucleated and unnucleated halves of the algae remains the same for two weeks after division. After thirty-eight days, the rate of incorporation into the proteins of the unnucleated halves still amounted to 70% of that in the nucleated halves. This indicates that the complete system for amino acid incorporation into proteins is cytoplasmic, and that the action of the nucleus on this process is indirect and remote.

The same conclusion has been reached in an interesting study (Jeener & Jeener, 1952) on the behaviour of a thermobacterium grown in the absence of either uracil, which limits RNA synthesis, or of desoxyribonucleosides, which limit DNA formation. Growth appears to be related directly to the formation of RNA, but much more remotedly to the formation of the DNA of the nucleoids.

The fascinating work on enzymatic adaptation, and on mutations caused by acriflavine in yeast (Ephrussi) indicates that adaptive enzymes and even such a fundamental enzyme as cytochrome oxidase, are indeed formed in the cytoplasm, where they are produced by self-duplicating particles. In turn the duplication of these particles is controlled by genetic factors segregating in accordance with Mendelian laws. (Chen *et al.*, 1950; Spiegelman, 1946; Monod, 1948).

Direct biochemical studies on protein formation have been difficult so far. Net protein synthesis *in vitro* has been obtained in a few cases.

Peters & Anfinsen (1950) have obtained serum albumin formation by slices of chicken liver. They found that the respiration of the slice, and the integrity of its mechanisms for producing high energy phosphate substances were necessary for albumin synthesis. Hokin (1951*a*) observed the *in vitro* formation of amylase by pancreas slices; in this tissue also respiration producing high energy phosphate bonds was required. Again Hokin (1951*b*) made the very important observation that the addition of the essential amino acids to the incubation medium enhances the amylase production. Methionine however has no effect. This is an interesting fact indeed, since amylase does not contain any methionine. Thus, the net formation of a protein responds very rapidly to the presence of its constituent amino acids.

Tracer work on the incorporation of the amino acids into the proteins of slices or of homogenates of various animal tissues (review by Borsook, 1950) also emphasizes the necessity of high energy phosphate bonds for the incorporation of the amino acids, with the possible exception of lysine. When several amino acids are incorporated simultaneously by a given system, there seems to be no mutual interference, and the amino acids are incorporated independently (Borsook *et al.*, 1950). This last result is apparently at variance with the requirement for all the amino acids in a net synthesis as observed by Hokin (1951*b*) and especially apparent from feeding experiments in which it has been conclusively shown that the essential amino acids are not able to sustain growth or even nitrogen balance in an animal when they are fed at intervals of, e.g. two hours, whereas they are effective when fed simultaneously (Geiger, 1950).

This brief review of some major experimental facts shows that in spite of a fairly large accumulation of data, we are still far from understanding the mechanism of protein production. In the rest of this paper, we are going to examine a few of the main questions that are still to be solved, and discuss the present state and perspectives of different aspects of the problem of the mechanism of protein biosynthesis.

PEPTIDE BOND FORMATION

There is very little information about possible intermediate polypeptides or lower peptides between the free amino acids and the proteins, and so far it has not been possible to study separately the formation of a part of a protein. Most of the information now available on the condensation of amino acids is derived by analogy with somewhat comparable and simpler systems in which peptidic linkages are formed.

For instance, the formation of the CONH bond in acetylamines, hip-

puric acid, glutamine and glutathione have been extensively studied. There is one common feature of all these reactions, the synthesis depends on high energy phosphate compounds. The case of acetylamines has been elucidated by the investigations of Lipmann and his coworkers. In pigeon liver, acetylsulfonamide is formed in the following way: adenosinetriphosphate (ATP) and acetate react enzymatically with coenzyme A, with the formation of acetyl coenzyme A, phosphate and ADP. The acetyl coenzyme A then reacts with the amine in the presence of another enzyme; this results in the formation of the acetylamine, with regeneration of coenzyme A. The mechanism of this peptide bond formation thus essentially consists in the activation of the carboxyl group at the expense of high energy phosphate, followed by the transfer of the acetyl radical via coenzyme A to the amino group, which does not undergo, as it seems, any preliminary transformation, except its fixation on the enzyme.

Hippuric acid formation from benzoate and glycine is probably dependent on a very similar process, since coenzyme A is again involved (Braunstein, 1950; Chantrenne, 1950). On the other hand, coenzyme A is not required for glutamine synthesis (Speck, 1949) and it is inhibitory for the incorporation of labelled glycine into glutathione (Bloch, 1951). In glutamine synthesis, it is clear that the γ-carboxyl group of glutamic acid is activated at the expense of ATP, and it has been suggested that the intermediate might be γ-glutamylphosphate, but this is not established. Recently Bloch (1951) found an unknown coenzyme to be involved in amino acids incorporation into glutathione, and developments in this field will be watched with great interest, for the cysteine–glycine bond in glutathione is the only true α-peptide linkage between two amino acids, the formation of which has been studied so far. It might provide the best model of peptide bond formation.

The cases of peptide bond formation we have just described can be considered, at best, as models of a mechanism of peptide synthesis, but there is no evidence that similar processes play a part in protein formation. However, the experimental fact that protein synthesis depends on phosphorylation suggests at least a possible analogy with the simpler cases of peptide linkage formation, and allows one to take as a working hypothesis that high energy phosphate compounds serve to activate the carboxyl groups of amino acids prior to condensation (Lipmann, 1941).

This is not the only possibility though, and it is quite conceivable that the individual amino acids are not activated directly at the expense of high energy phosphate. It is possible that a few types of peptide bonds only, e.g. those of glutathione or glutamine, are made at the ex-

pense of phosphorylation mechanisms and then 'transferred' to other peptides by transpeptidation (Linderstrøm-Lang, 1949; Fruton, 1950, 1951; Virtanen, 1950).

Enzymes catalysing transpeptidation reactions have been found in various tissues, but little information has been obtained so far concerning their significance for polypeptide and protein formation. It is our opinion that two types of transpeptidation reactions may be distinguished among the data of the literature: transpeptidations catalyzed by proteolytic enzymes and transpeptidations catalyzed by enzymes that might be called 'non-hydrolyzing transpeptidases'. It is clearly established that pure proteolytic enzymes like chymotrypsin or papain catalyze the direct replacement of one of the members of a peptide by another molecule (Behrens & Bergmann, 1939; Johnston *et al.*, 1950*b*; Fruton, 1951) but at the same time the same enzymes catalyze the hydrolysis of all the peptides present, including the newly-formed peptides. There is a competition between water and the amine for the carboxyl group at the level of the enzyme, and this competition is bound to be decided in favour of water, since the hydrolysis equilibrium for all the peptides is far to the side of complete hydrolysis. It seems to us that such transpeptidations have little chance of playing an important part in polypeptide synthesis in the living cell unless, for some reason, water could not enter the competition, i.e. unless the functional enzymes *in vivo* were not hydrolyzing. The same remarks apply to the transfer of the carboxyl group of an ester onto the amino group of an amino acid, that has been shown to be catalyzed by proteolytic enzymes (Brenner *et al.*, 1950, 1951).

The other type of transpeptidation is exemplified by the glutamyltransferase of Stumpf & Loomis (1950) and possibly also of Hanes *et al.* (1950) and Grossowicz *et al.* (1950). An enzyme isolated from fruit seeds catalyzes the replacement of the γ-NH_2 group of glutamine by -NHOH when the enzyme is incubated with glutamine, hydroxylamine, Mn ions and a catalytic amount of ATP. The process is very different from the other type of transpeptidations. In the present case there is *no hydrolytic cleavage* of glutamine or of the hydroxamic acid formed and the initial γ-glutamyl group is transferred, without loss, in a stoichiometric reaction (Stumpf, 1950). This type of reaction might of course operate with a high yield and should be considered as a model of reactions that might be involved in polypeptide formation. The transglutamylations observed by Hanes and co-workers (1950) between glutathione and various amino acids might be of a similar type. It seems probable that the enzyme splitting the γ-glutamyl bond of glutathione is not a hydrolytic enzyme but a transglutamylase that will transfer the

glutamyl group from the NH_2 group of cysteinylglycine to that of various amino acids. A point that may be very important, and that as far as we know has not been stressed, is that a second enzyme associated with the first (Olson & Binkley, 1950), and present in Hanes's system, hydrolyzes cysteinylglycine very rapidly, although it will not touch glutathione. The result of this is that glutathione completely disappears, one of its peptide bonds being transferred to a new peptide (e.g. γ-glutamylvaline) and the other hydrolyzed. If we consider the equilibrium of the two reactions we can see that the splitting of cysteinylglycine shifts the equilibrium of the first reaction towards the right, and that the two reactions are coupled.

$$\gamma\text{-glutamylcysteinylglycine} + \text{valine} \rightleftharpoons \gamma\text{-glutamylvaline} + \text{cysteinylglycine}$$
$$\text{cysteinylglycine} + H_2O \rightarrow \text{cysteine} + \text{glycine}$$

Glutathione in such a system might function as a very efficient γ-glutamylating agent. The net yield in new peptide bond formed is only 50% if the first enzyme is not hydrolytic, but the new glutamylpeptide is stable, for the equilibrium of the total reaction is just as much in favour of the formation of the new glutamylpeptide as it is in favour of the free amino acids when a peptide is exposed to proteolytic enzymes. This seems to be an example of coupling of hydrolysis to synthesis of peptide bond that is reminiscent of a mechanism suggested by Bergmann & Fruton (1944).

Before closing this section, let us remember that carboxyl activation of amino acids and transpeptidations are not more, at present, than suggested models of peptide formation and that the actual mechanism involved in protein formation is not known.

ARRANGEMENT OF AMINO ACIDS INTO POLYPEPTIDE CHAINS

The formation of a given polypeptide chain raises a second question, namely the mechanism of the selection of the amino acids from which the peptide bonds will be formed. At present we have no reason to believe that there exists any regularity of arrangement of the individual amino acids in the chains. With the refinements of analytical methods the idea of a periodicity of the amino acids had to be given up, and when examining the arrangement of the amino acids in a chain of insulin, as revealed by the outstanding work of Sanger (1951), one cannot indeed find any regularity or periodicity; the amino acids are arranged apparently in the most arbitrary order. However we are bound to consider that they are arranged in a definite order and not distributed at random in the chains, otherwise a research like Sanger's would have led to no

consistent result, and the constant biological and physico-chemical properties of insulin would be impossible to account for.

If the amino acids are not distributed at random in the protein molecule, then there must be some mechanism in the cell which causes a certain arrangement to be repeatedly and consistently produced. For each protein a unique arrangement is formed among the very great number of isomers or homologues one can think of. We know nothing about the actual mechanism involved, but several suggestions have been proposed; let us examine some of them and see to what extent they are supported by facts.

It is possible that amino acids are first activated at the expense of ATP and then united in polypeptides under the action of a series of enzymes that have not yet been discovered. This is evidently hypothetical and is based only on the necessity of phosphorylation for protein synthesis.

Following the discovery of the specificity of proteolytic enzymes for some arrangements of amino acids, and their ability to catalyse transpeptidation, it was suggested that a given assortment of proteolytic enzymes might organize the peptide chains by a series of transpeptidations (Behrens & Bergmann, 1939). Simple rearrangements of this type have actually been observed (Fruton, 1950; Waley & Watson, 1951). However they suffer from the unfavourable competition with water. This makes it rather doubtful that such systems might operate efficiently for protein synthesis, unless the properties of the enzymes involved *in vivo* be somewhat different.

We would like to suggest a related but different possibility for the selection of arrangements by the proteolytic enzymes. If peptides were built by transpeptidation of the Stumpf type, and if after each transpeptidation specific proteolytic enzymes would *hydrolyse* a bond of one of the peptides just formed, the specific attack on, and the splitting of the 'forbidden arrangement', would stabilize the other arrangement by making the transpeptidation complete. This system of selection is merely a generalization of the system observed in the transpeptidations with glutathione, in which the 'forbidden arrangement' is cysteinylglycine.

In any of the mechanisms just considered, many enzymes would be involved in the building up of a polypeptide chain. It should be realized, however, that the production of a protein with a definite arrangement of amino acids raises a question that does not appear in the formation of the other constituents of living systems. The specificity of any biochemical process is always explained, correctly we think, by the specificity of the enzyme or of the many enzymes involved in a chain of successive reactions. But when the formation of a protein is considered