# ADVANCES IN ENZYMOLOGY

*AND RELATED SUBJECTS OF BIOCHEMISTRY*

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AND RELATED SUBJECTS OF
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# ASPECTS OF THE BIOSYNTHESIS OF ENZYMES

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CONTENTS



## I. Introduction

Ten years ago very little was known about the mechanism of protein biosynthesis except that proteins form by condensation of amino acids, that this process requires an expenditure of energy, and that ribonucleic acids are somehow involved. Today, although many points are still obscure, a general theory of enzyme biosynthesis is taking shape which will soon integrate the complete process including chemical pathways, genetic control, and regulation. It is a theory which promises to be one of the major achievements of general biology in our time.

In order for an enzyme to be formed, amino acids must condense into well-defined polypeptides, the chains must fold in a specific way, and in some cases they must associate with prosthetic groups. A series of typical enzymic reactions which very plausibly describe part of the pathway followed by amino acids were discovered and analyzed by classical methods of biochemistry. On the other hand, the control of protein and enzyme structure is now understood, at least in princi-

ple, thanks to progressive studies of protein and nucleic acid structure and to the refinements of genetic analysis. However, a most challenging question remains: How do biochemical tools receive the orders given by the gene, and how do they execute these orders? Before examining this matter more closely, we shall outline in a very schematic way the present views on both the biochemical and the informational sides. Several extensive reviews on these two aspects of protein and enzyme biosynthesis have been published within the last few years (43,48,54,58,63,74,92,99,112,150,171,203,244); the reader is referred to them for discussion and detailed bibliography.

## II. The First Steps of Polypeptide Synthesis

The amino acids must first be activated, i.e., changed into high energy derivatives, in order to condense and form a peptide bond; this is an inescapable requirement of thermodynamics. As in most biochemical syntheses, the energy is funneled into the system by adenosine triphosphate (ATP). Enzymes which catalyze a reaction of ATP with L-amino acids, resulting in the liberation of pyrophosphate and the formation of amino acyl adenylates, are found in the most varied types of cells and organisms. These compounds are mixed anhydrides of the carboxyl group of the amino acid with the phosphoric residue of adenosine-5′-phosphate. They contain a high energy bond and are highly reactive chemically; they would be aminolyzed very rapidly by any free amino acid if they were not in some way protected by the enzymes which formed them and to which they stay firmly bound. All the amino acids are activated in this manner. There are probably 20 different "activation enzymes," each more or less specific for one of the natural amino acids which enter into the constitution of proteins. A few of them were obtained in a high degree of purity, or crystallized; others proved rather labile and/or sensitive to oxygen and were lost during attempts at purification. The activation of some amino acids, such as arginine, asparagine, or glutamic acid in cell extracts, is usually so weak that the very existence of enzymes similar to those which activate tryptophan, leucine, or threonine, for instance, remained a debatable matter for some time. (For detailed references and discussion, see review articles 45,48,172,250.)

The activated amino acyl moeity of the bound amino acyl adenylates is then transferred to a ribonucleic acid of a special type (114)

which was first called "soluble RNA" and is now more specifically named "transfer RNA." This RNA makes up a relatively small part of cellular RNA, perhaps 5–15% of the total. There are twenty different transfer RNA's, each able to accept one amino acid; several of them have been partly purified. Each is a single polynucleotide chain composed of some 80 nucleotides. The chain probably folds upon itself, forming loops and regions which are more or less helical in structure (148). At one end, the molecules of all the transfer RNA's terminate in the same base sequence: cytosine, cytosine, and adenine. The hydroxyls in positions 2′ and 3′ of the terminal nucleoside residue (adenosine) are free. It is precisely to one of these hydroxyls that the carboxyl of the activated amino acid is transferred, directly from the enzyme-bound amino acyl adenylate, without the participation of any other enzyme. The activation enzymes are thus able to recognize their corresponding transfer RNA and to deliver the amino acid to the right acceptor. It is not known at present whether the enzyme recognizes a certain base sequence directly, or whether it identifies the secondary structure of RNA (type of folding of the polynucleotide) which is determined by the base sequence. (For detailed references and information on transfer RNA's, cf. 112.) Recent studies of the sequence of bases next to the common C—C—A end show that differences between individual transfer RNA's already exist in this region (11). The specificity of the enzyme for the activation step is not always high, but the transfer to RNA is much more specific (13; see also 240). The bond between the carboxyl of the amino acid and the transfer RNA is a high energy bond (12). However, the integration into polypeptides of amino acid residues bound to transfer RNA's requires guanosine triphosphate (GTP), and a "transfer enzyme" which was discovered recently (14,94,118,163,164,214,215). But the exact functions of the enzyme and of GTP are not known as yet, and the nature of the interactions between transfer RNA's and the ribosome where polypeptides arise is not well understood (16,21–23,113).

The process of amino acid activation briefly outlined above was studied in many laboratories; it was found in every organism or tissue in which it was sought (45,172). It operates in the isolated nucleus as well as in the cytoplasm (2,57). *In vitro*, amino acids bound to S-RNA can be incorporated into well-defined proteins (67,111). Kinetic studies indicate that amino acyl RNA's behave as intermedi-

ates between free amino acids and polypeptides in living ascites cells (250) and in growing bacteria (143). Therefore, this process is an important pathway of protein synthesis. However, data obtained from studies on animal tissues and bacteria suggest that alternate pathways might possibly exist. Incorporation of amino acids into protein was reported to occur in fractionated bacterial extracts from which the activation enzymes had been discarded. In this system, an "incorporation enzyme" is required; this enzyme, or group of enzymes, catalyzes both the liberation of phosphate from the four nucleoside triphosphates in the presence of amino acids and the formation of various peptides (8,9,168). Liver ribosomes which have been treated with deoxycholate and are apparently devoid of activation enzymes and of transfer RNA's can incorporate amino acids into proteins in the presence of certain enzyme fractions which are different from those involved in the classical pathway (55,118,190, 195). The possible participation of lipids in amino acid activation in hen oviduct (107,108) and in bacteria (119–121) was also reported (see, however, 78).

Thus, one should keep an open mind concerning the possible existence of another pathway to protein synthesis in addition to the one which involves the amino acid-activating enzymes, transfer RNA's, GTP, and transfer enzymes. It is possible that not all proteins are made according to the classical pathway and that a special category of proteins is formed by a completely different process. The ribosomal proteins, for instance, which are part of the protein-making machinery, might possibly be made in a different way.

## III. The Centers of Enzyme Formation

Enzymes, and proteins in general, are often formed within the subcellular structure or the organelle in which they will be integrated; nuclei, mitochondria, the cytoplasmic ground substance, chloroplasts, membranes, and myofibrils all seem to make their own proteins. The biosynthesis of enzymes is not restricted to one specialized region of the cell; it occurs almost everywhere, and there are many centers of enzyme synthesis. One essential constituent common to all of them is a ribonucleoprotein structure, the ribosome, which can be isolated from cell extracts by high speed centrifugation. Ribosomes appear as roughly spherical particles of uniform size on sections prepared for electron microscopy.

In the cytoplasm of animal and plant tissues (185a), the ribosomes are either free or associated with an elaborate structure which extends through the cytoplasm and which consists of a network of double lamellae and elongated vacuoles. In osmic acid-fixed preparations the membranes are lined on one side by Palade granules, which are ribosomes. When the cells are disrupted and their content dispersed into an adequate medium—a concentrated sucrose solution, for instance—the debris of this structure appear as small vesicles, rich in lipoproteins, to which ribosomes are attached; many ribosomes are also free in the suspension. The vesicles with attached ribosomes make up the largest part of the microsome fraction which is obtained by high speed centrifugation of a tissue homogenate as a characteristic jelly-like, transparent pellet. Treatment of the microsomes with deoxycholate dissolves the lipoprotein membranes and liberates the ribosomes which can then be sedimented by prolonged high speed centrifugation. Ribosomes can also be isolated from the nuclei of animal cells (2,76,228,229).

In kinetic incorporation studies, the ribosomes are the cell constituents in which labeled amino acids are first found in protein material. *In vivo*, the radioactivity of the proteins bound to ribosomes increases rapidly after the injection of the amino acid into the animal, and then reaches a constant value; on the contrary, soluble proteins are slowly labeled, but the incorporation continues regularly for a very long time. The labeled proteins on the ribosomes behave as precursors of soluble proteins. Newly formed enzyme molecules are indeed found in association with the ribosomes.

Mitochondria (128,129,189,194) and myofibrils (235) also contain centers of protein synthesis, and nucleoproteins also appear to be the agents of the formation of these proteins; they have, however, not yet been characterized as well as those in the other materials (56).

It has been known for a long time that the ribonucleoprotein particles are the centers of protein synthesis in animal and plant cells, but the case of bacteria has been clarified only recently. The bacterial membrane was first considered to be the protein-forming site; actually, the "membrane fraction," isolated from disrupted protoplasts, for instance (36,145,167,205), contains ribonucleoprotein particles which are the real agent of the observed synthesis (37). Ribonucleoprotein particles had been found in yeast and bacteria a long time ago, but protein and enzyme synthesis in growing bacteria occurs so rapidly

(17) that detailed kinetic studies on the incorporation of labeled 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 labeling between ribosome-bound and soluble protein. Finally, it was established (18,156,157) that in exponentially growing *Escherichia coli* the radioactivity of the ribosomes is built up to saturation within 5 seconds and that the label disappears very rapidly when the tracer amino acid is diluted out by the addition of nonlabeled compound. The radioactivity thus lost by the ribosomes appears in soluble protein. The substance which is rapidly labeled in the ribosome has the properties of a polypeptide, and it behaves like a precursor of soluble protein which is continuously chased by new nascent molecules (156,157).

In bacteria, as well as in animal tissues, newly formed enzyme molecules have been found in association with ribosomes; it is even possible to precipitate part of the ribosome population by means of an antiserum prepared against the enzyme which it manufactures (56).

Ribosomes can now be regarded as the macromolecular structures upon which amino acids assemble into proteins, in all types of living cells. Since most of the RNA of the cell, even in bacteria, is contained in ribosomes, the identification of these structures as the centers of all protein synthesis establishes the correctness of the views expressed 20 years ago as a result of histochemical studies by Brachet (24) and Caspersson (40). The correlation between the amount of RNA in a cell and the cell's capacity for making protein also applies to bacteria. Recent studies show that during periods of changes in the rate of bacterial growth owing, e.g., to transfer from one medium to another, the amount of protein made remains strictly proportional to the amount of ribosomal RNA present (135,137,165). The amount of ribosomal RNA seems to be the factor which limits the total capacity of protein synthesis of the cell, notwithstanding the operation of systems which regulate the production of individual enzymes.

The ribosomes isolated from bacteria, yeast, plant, and animal cells have much in common (102,183,184,192). They contain about equal amounts of RNA and slightly basic protein. Each ribosome might contain two RNA molecules with molecular weights of about 1.3 and $0.6 \times 10^6$, respectively (4,97; see, however, 176). Fractionation of the basic protein of ribosomes from bacteria or plant tissues revealed that it contains many different proteins (201,227), suggesting that

ribosomes are complex structures or that they make up a heterogeneous population. Ribosomes undergo processes of aggregation and dissociation depending on the cationic composition of the medium. The concentration in magnesium ions and in polyamines is especially critical. In the cell, their state of aggregation probably corresponds to that of the particles which, in extracts, have a sedimentation constant of 70–80 Svedberg units (183). These particles are also the particles on which polypeptides form in bacterial extracts (156,157, 219). In media poor in magnesium ions, these particles dissociate into several classes of smaller particles which can be separated by ultracentrifugation (20,102,183,184,192,219,220). At present, the significance of these ribosomal components for protein synthesis is not clear, and the exact function of any of their constituents is not understood.

The ribosome is the machine in which the amino acids line up in the correct sequence and condense into the genetically controlled enzyme structure. Before considering what little is known about this process, let us turn to the origin of the information which directs it and the transfer of this information to the ribosome.

## IV. Genetic Control of Enzyme Structure

It is quite certain at present that details of the structure of enzyme proteins are controlled by the nuclear genetic material. The reader is referred for detailed information, discussion and bibliography to recent reviews of the field (48,63,74,106). It will suffice here to summarize briefly a few essential points.

Mutation of a Mendelian gene can manifest itself by a deficiency in an enzyme: either complete absence of the enzyme or production, instead of the normal enzyme, of a poorly active enzyme or of an abnormal protein closely related to the enzyme, e.g., serologically, but devoid of enzymic activity. In a few cases, it was possible to compare the abnormal protein of the mutant to the normal enzyme, and it was found that they differ by the replacement of one amino acid by another at one specific spot in the protein. Different mutants may show individual amino acid replacements at different places. Therefore, the nature and the location of several amino acids in the enzyme molecule are genetically controlled. For the sake of unity and simplicity, it is generally assumed that the position of every amino acid in

an enzyme protein is controlled by the genetic material, although the data available at present do not prove this completely.

In certain organisms it is possible to locate mutation spots within the genetic material with great accuracy. The mutations corresponding to the loss or modifications of a given enzyme are always clustered in one short piece of genome, the locus of the enzyme. This locus extends over a particular length of genetic material. In certain cases, the mutation spots corresponding to different mutants deficient in the same enzyme can be located with sufficient accuracy for their arrangement within the locus to be established. The distances between the mutation spots and other topological relations (10) indicate that the spots are arranged in a linear order. The primary structure of an enzyme is thus controlled by a unique and limited segment of genetic material which has a linear structure.

When two different mutants of the same locus are crossed, the progeny contains rare recombinants which make the normal enzyme. Thus, a normal piece of genetic material is reconstituted by recombination occurring between the two mutation spots within the locus of the enzyme. If the genetic materials of the two mutants are introduced into the same cell under such conditions that no recombinations occur, as in heterocaryons, for instance, in most cases the normal enzyme does not form, although the complete genetic information for making the enzyme is obviously present in the cell. After recombination the genetic information for the enzyme is all in one continuous piece of genetic material (*cis*), but in the heterocaryon, part of the information is in one piece of genome and part in another one (*trans*). Two mutants which can restore the normal type after recombination, but which fail to do so when their genomes are simply put together within the same cell, are said to be located within the same cistron. The information contained in a cistron must be used in one block; it is not expressed when it is divided. In many of the cases studied so far, the locus of an enzyme consists of a single cistron or a very small number of different cistrons. This indicates that the structural information for enzyme synthesis must be provided to the enzyme-making machine in one single piece or in a very small number of functionally indivisible pieces. On the other hand, it is known that enzymes are composed of one or of a very small number of different polypeptide chains. It appears therefore that the smallest part of an enzyme which can be made independently

must be the size of a polypeptide chain. It is probable that all the information which directs the sequence of the amino acids in a polypeptide chain is contained in one cistron.

The genetic material of higher organisms, bacteria, and most known bacteriophages is deoxyribonucleic acid (DNA). The backbone of DNA is a linear polymer in which deoxyribose and phosphate residues alternate in a perfectly regular way; this backbone is identical in all DNA's. Each deoxyribose residue carries a purine (adenine or guanine) or a pyrimidine (usually thymine or cytosine). DNA's of different origin all have the same general structure; they differ only in the arrangement and proportions of the bases. Whatever information DNA may carry must therefore reside in the base sequence.

In ribonucleoprotein viruses and in certain phages (149) the genetic material is RNA. The structure of an RNA chain is essentially the same as that of a DNA chain. Again, RNA's of different viruses differ by their base composition and base sequence.

If the sequence of amino acids in polypeptides is controlled by nucleic acids, and if the genetic information that these acids carry consists of a certain arrangement of bases along the polynucleotide chains, it seems most probable that a given amino acid sequence in the protein must correspond to a certain base sequence in the genetic nucleic acid. This is the colinearity hypothesis.

The complete sequence of the amino acids in a polypeptide can be established experimentally, but there are at present no ways of isolating the DNA corresponding to a given cistron and no method for determining nucleotide sequences in DNA more than a few nucleotides long. However, by means of genetic analysis, a very accurate map of mutation sites within a cistron can be constructed. It is therefore already within our reach to test the colinearity hypothesis, to a first approximation, by checking whether the positions of the mutation points within the cistron are correlated to the positions of the amino acid substitutions in the polypeptide. Comparison of the various abnormal enzymes corresponding to different mutations of the locus of tryptophan synthetase in *Neurospora* (19) indicated that different regions of the gene control the synthesis of different regions of the enzyme (see also 117). The alkaline phosphatase of *E. coli* contains some 380 amino acids in its polypeptide chain, and the complete sequence is not yet known, but partial trypsin hydrolysis followed by paper electrophoresis of the digest gives a pattern which is

characteristic of the protein. Such "fingerprints" of the abnormal phosphatases of two mutants corresponding to two different but closely located mutation points showed modifications in the same peptide (81a). These results are not yet sufficient to establish the validity of the colinearity hypothesis, but they are exactly what one would expect if the hypothesis was true.

Assuming that each amino acid in an enzyme is genetically determined and that a point to point correspondence exists between this amino acid sequence and the arrangement of the bases in the gene, one wonders what kind of correlation exists between the two sets of monomers. Since there are 20 amino acids in proteins (including glutamine and asparagine, which behave as independent amino acids), and essentially 4 nucleotides in the nucleic acid, the simplest hypothesis is that each amino acid is coded by a short sequence of nucleotides. Comparison of the already known sequences in proteins indicates that almost any possible arrangement of two contiguous amino acids exists and that the coding units for the individual amino acids must be largely independent. This makes overlapping codes unlikely; for if certain nucleotides in the nucleic acid would code for two contiguous amino acids, severe constraints would be noticed in the amino acid sequences, and this does not seem to be the case. Among the coding principles suggested so far, Crick's "code without commas" (58,59) is still the most attractive one. It is assumed that a sequence of, e.g., three nucleotides in the DNA chain codes for one amino acid and that the coding units are contiguous but do not overlap. Precise correlations between nucleotide groups and amino acids have been computed on the basis of the frequencies of the individual nucleotides and amino acids in the RNA and in the protein coat of several viruses (82,237,238,245,246). As a result of such calculations, the idea was proposed (246) that the coding ratio might be equal to one, i.e., that there might be just as many nucleotides in the genetic nucleic acid as there are in the corresponding protein. If such was the case, the nucleic acid would not carry enough information to specify the protein completely. Each nucleotide would specify a choice of five or six amino acids among which the right one in each specific case would be selected by a second information-carrying system. There would be two steps in the selection process. But it seems that the frequencies are also compatible with a triplet code (238). It must also be realized that all these computations rest

on the assumption that the RNA of a virus codes only for the protein of the virus coat, yet this is in no way established; it is known, for instance, that bacteriophage DNA contains the information for a large group of enzymes, besides the information which controls the synthesis of the phage particle itself. Comparison of the modifications of a single protein in several mutants (147,221,236) might be a safer way of deciphering the code, especially when the mode of action of the mutagen used is known (223).*

In most coding systems proposed so far, it was assumed that each of the four usual bases was a coding digit. There are reasons to believe at present (204) that the code might be a two-digit system in which the two meaningful alternatives would be the presence of either a keto group or an amino group at the 6-position of the purines and at the corresponding position of pyrimidines (6 according to the biochemical usage, 4 according to *Chemical Abstracts*).

## V. Transmission of Structural Information from the Gene to the Enzyme-Forming System

### A. DNA IS NOT DIRECTLY INVOLVED IN ENZYME SYNTHESIS

Since DNA ultimately controls the primary structure of the individual enzyme, one may wonder whether the amino acids are arranged in the correct sequence under the immediate action of DNA itself, i.e., whether the gene is the template upon which enzymes are built.

In intact cells, newly formed polypeptides are first found in association with ribosomes, but the genetic material is present in the cell during polypeptide formation. Disrupted cell preparations are poorly active, and the systems which produce some enzyme synthesis *in vitro* are very crude and are not free of DNA. Thus, it is difficult to decide, on the basis of experiments of this type, whether DNA is specifically involved in making perfect enzymes.

For higher organisms, a clear answer to this problem was afforded by enucleation experiments (27,29a–32,41,48). Clear-cut results were first obtained with the unicellular alga *Acetabularia*. For most

* A direct way of solving the code has now been discovered: see Nirenberg, M. W. and Matthaei, J. H., *Proc. Natl. Acad. Sci. U.S., 47*, 1588 (1961). Interesting new data on the nature of the code will be found in papers by Wittmann, H. G., *Z. Naturforsch., 48b*, 729 (1961) and by Crick, F. H., Barnett, L., Brenner, S. and Watts-Tobin, R. J., *Nature, 192*, 1227 (1961).

of its life cycle, this organism consists of a single cell which contains only one nucleus which is located at one easily recognizable end of the cell. Large, enucleate fragments of *Acetabularia* can be separated easily. Such fragments continue to incorporate labeled amino acids into their proteins at the normal rate for about two weeks after enucleation (30,32,174). Enzymes such as enolase (6), phosphorylase, and invertase (53) are produced in normal amounts during this period. The cytoplasmic fragments of *Acetabularia* thus contain a perfect and complete system for making specific proteins in the absence of the nucleus. Clearly, the nuclear genetic material is not directly involved in the process. The same conclusion must be valid for animal cells as well; Enucleate pieces of human amnion cells in tissue cultures incorporate L-amino acids into their proteins at a normal rate for 10–30 hours after enucleation (87). Enucleate fragments of amoeba (25–27), sea urchin eggs (158), or newt eggs (217) retain at least part of their ability to make proteins. Mammal reticulocytes, which are devoid of nucleus, produce hemoglobin (151).

Some of the essential conclusions of the experiments with enucleate cells are: genetic information can be retained in a cytoplasm for a long time; there must exist cytoplasmic copies of the nuclear genetic information, and the information the cells keep can eventually be used for controlling protein synthesis.

Whereas it has been known for almost ten years that DNA itself is not directly involved in the synthesis of cytoplasmic proteins in higher organisms, it was long regarded by many as the probable template for protein synthesis in microorganisms. Mechanical elimination of DNA has never been achieved; indirect or incomplete evidence, often contradictory, was derived from various types of observations, and the matter remained controversial for a long time. Yeast cells which had received very high doses of X-rays continued to make enzymes at a normal rate, although their DNA was damaged to the point that it could no longer be precipitated by acid (42,49). In disrupted protoplasts of *Bacillus megaterium* destruction of most of the DNA by deoxyribonuclease did not impair enzyme synthesis (145,205). These results were evidence that DNA is not the template upon which proteins are built in microorganisms. On the other hand, in disrupted *Staphylococcus aureus*, extraction of DNA with salt solutions depressed enzyme synthesis, and the activity could be partly restored by specific DNA (80,81). Comparable results were briefly

reported recently with better-defined bacterial preparations, in which DNA stimulated enzyme formation in a specific way (130,168,169).

Decay of $P^{32}$ previously incorporated into DNA causes breakages in the DNA backbone; this type of DNA breakdown is accompanied by a parallel drop in the capacity to produce enzymes (155). Later developments of this research showed, however, that the observed, effect was quite indirect and irrelevant to the present problem (153, 154) and therefore did not prove an absolute requirement for DNA integrity.

The kinetics of enzyme formation after the introduction of the corresponding gene into a bacterium at first seemed to indicate direct involvement of DNA in the synthesis of proteins. Experiments showed that when the structural gene for $\beta$-galactosidase is introduced by bacterial conjugation into a mutant which lacks this gene, the synthesis of the enzyme begins not more than 2 minutes after the introduction of the gene, and it proceeds immediately at maximal speed. The amount of enzyme in the population of zygotes is proportional to the square of time. Since the number of zygotes formed is known to be directly proportional to time, the quadratic function indicates that the number of enzyme molecules formed is also proportional to the time elapsed after zygote formation (178). This is exactly what one would expect if the gene itself acted directly as a template upon which proteins are assembled (178). However, these experiments simply prove that the gene does not continuously produce, at a constant rate, any stable catalyst which in turn makes the enzyme, also at a constant rate; the possibility remains that a stable intermediate carrier of information is made within 2 minutes in a small number of samples, or that a short-lived information carrier is continuously produced under the action of DNA and that it rapidly reaches a steady-state concentration (178,191).

The question regarding the immediate participation of DNA in protein formation in microorganisms has not been completely answered. It is felt that enzyme synthesis in bacteria is not as independent of the presence of DNA as it is in higher organisms. However, in the absence of clear evidence for a direct template function of DNA, and by analogy with higher organisms in which its direct participation is excluded, it is assumed that in bacteria, too, DNA exerts only indirect control over the structure of proteins. This conviction was strengthened by the observation that in bacteria, as

well as in higher organisms, nascent proteins are found in association with ribosomes (56,136,156,157). It was also observed by radioautography that protein synthesis in bacteria is much more active in the cytoplasmic region than in the nuclear region (39).

## B. NATURE AND PROPERTIES OF THE EXTRAGENIC INFORMATION CARRIER

If DNA is not directly involved in the production of polypeptides, then the genetic information it contains must be transferred or communicated to some other substance and eventually to the ribosome where the information will determine the arrangement of the amino acids.

Direct evidence that ribosomes of animal cells contain the structural information for protein synthesis is provided by data on hemoglobin synthesis in acellular preparations from reticulocytes, or in mixed systems containing ribosomes and supernatant from different organs even from different organisms. If liver supernatant is substituted for reticulocyte supernatant, the reticulocyte ribosomes still make hemoglobin, but liver ribosomes, even in the presence of reticulocyte supernatant never make hemoglobin (199). Washed reticulocyte ribosomes can accept amino acids from a transfer RNA isolated from a bacterium, *E. coli*, and incorporate them into hemoglobin (67). Microsomes of rabbit reticulocytes make rabbit hemoglobin, even if the supernatant comes from chick reticulocytes. All this clearly demonstrates that the ribosomes or the microsomes as they are obtained in these experiments contain the information for hemoglobin synthesis. However, in systems containing rabbit reticulocytes and guinea pig supernatant, both rabbit hemoglobin and guinea pig hemoblobin appeared (142); comparable observations were made with mixed rabbit and sheep systems (144). This would indicate that the supernatant can, under certain conditions, contribute information which competes with that originally present in the ribosome and which can be expressed by the ribosome.

In *Neurospora* extracts, the soluble fraction is the one which contains the specific information; mixed systems containing soluble and sedimentable fractions were prepared from the wild type and a mutant lacking tryptophan synthetase activity. When the mutant "particle" fraction was supplemented with wild type supernatant, considerable tryptophan synthetase was produced. Conversely,

a mixture of wild type particles with mutant supernatant failed to develop any detectable activity (226). This indicates the presence in the supernatant of a soluble specific factor which can interact with the particles and cause the synthesis of the enzyme. It would seem that the carrier of genetic information can exist in different states of aggregation or that it can be more or less tightly bound to the ribosome. The chemical nature of the extrachromosomal carrier of genetic information has not been established yet, but ribonucleic acids are best suited to fulfill this function.

Virus RNA's are known to carry the information for the synthesis of the virus protein. The polynucleotide chain of RNA has essentially the same structure as that of DNA, the only difference being the presence of an extra oxygen atom in the sugar residue of the backbone and the replacement of thymine by uracil. Any information which can be recorded as a base sequence on DNA can probably also be recorded in almost the same symbols on RNA. Recent studies from several laboratories showed that cells contain an enzyme system which makes RNA from nucleoside triphosphates only in the presence of DNA and that the base composition of the RNA made under these conditions is complementary to that of the DNA present (34,52,79, 104,173,233). If RNA can be copied from DNA by some kind of a template process, it means that structural information contained in the DNA can be transcribed from DNA to RNA in this process.

There is evidence, on the other hand, that in higher organisms certain RNA's which form close to the genetic material are eventually found in the ribosomes. Thus, histological observations completed by radioautographic studies indicate that RNA is very rapidly formed in that region of the nucleus which contains chromatin (72,73,83,86, 216,241). In the lampbrush chromosomes of amphibian oocytes and in the giant chromosomes of insect salivary glands a very rapid RNA synthesis takes place in some bands as compared to others (73); this is exactly what would be expected if RNA copies of certain genes or groups of genes were being made.

Certain RNA's made in the nucleus pass into the cytoplasm; if the RNA of a living amoeba is partly destroyed by ribonuclease, and if the amoeba is then washed free of the hydrolytic enzyme, RNA rapidly reappears in the nucleus and later spreads through the cytoplasm (25–27). When a $P^{32}$-labeled nucleus of an amoeba is transferred into a nonlabeled amoeba, the labeled RNA passes into the

cytoplasm (88). In *Acetabularia*, ribonuclease can block protein synthesis; the synthesis is restored after some time in the presence of the nucleus, but not in its absence, as if the nucleus could provide a nucleic acid required for protein synthesis in the cytoplasm (209). Kinetic studies on RNA synthesis in human amnion cells show a progressive movement of cytidine-labeled RNA from nucleus to cytoplasm (86). In HeLa cells, most of the cytoplasmic RNA originates in the nucleus, and part of it in the chromatin region (71,179–181). The nuclear origin of part of cytoplasmic RNA was also indicated by experiments on *Drosophila* larvae (109), rat pancreas (3), *Neurospora* (249), and pea roots (20). Rapidly labeled nuclear RNA from a rat liver can be transferred to small ribosomes in vitro (196). Paired incorporation of radioactive phosphate into the nucleotides of RNA in animal cells indicates that an RNA fraction forms under the structural control of DNA (96,146).

It is thus reasonable to suppose that in higher organisms RNA's formed under the immediate control of DNA by a template process receive the genetic information and convey it from the gene to the executive agents of protein synthesis, the ribosomes, in which the specific RNA's are integrated or with which they become associated more or less permanently.

The effects of purine and pyrimidine analogs on enzyme synthesis in bacteria indicate that the integrity of certain RNA's is a necessary prerequisite for the formation of bacterial enzymes (42,203) and of bacteriophage proteins (125). Modifications of RNA due to incorporation of 2-thiouracil, 5-fluorouracil, or 8-azaguanine can inhibit the synthesis of certain enzymes or result in the production of abnormal enzymes (35,44,46,50,91,100,101,115,116,161). The abnormalities seem to consist of a few amino acid substitutions; it is as if the introduction of abnormal purines or pyrimidines in some RNA's could lead to mistakes in the transfer of information or in its interpretation by the protein-making system. Similar effects have also been obtained by a limited treatment of the bacteria with pancreatic ribonuclease (126).

In the cases of 8-azaguanine and of 5-fluorouracil, the kinetics of the action of the analogs indicate that the essential effects are due to a modification of a small fraction of RNA with a relatively high turnover (42,162). A secondary specific effect concerning RNA, with a longer life was also observed in the case of 8-azaguanine (47,48,50).

An obvious possibility is that the base analogs damage RNA's which carry specific information for enzyme synthesis.

Evidence for the existence of RNA with the base composition of DNA (except for the substitution of uracil for thymine) was first obtained in studies on bacteriophage multiplication. When *E. coli* is infected with bacteriophage $T_2$, the synthesis of bacterial RNA immediately stops, but a small amount of a new type of RNA very rapidly forms (110). Damage to this type of RNA might explain the inhibition of phage protein synthesis by ribonuclease (125). The base composition of this special RNA is very different from that of the average bacterial RNA, and it resembles that of bacteriophage DNA (224,225). This special RNA, which looks as if it was a copy of bacteriophage DNA, sediments at a slower rate than ribosomal RNA does (170). A fraction with similar properties was also detected in yeast (247) and in normal growing bacteria (93). Its low sedimentation constant and its high turnover rate distinguish it from ribosomal RNA which is metabolically much more stable (60). It represents only a small per cent of total bacterial RNA; its base composition resembles that of DNA. When it is heated together with DNA of the same bacterium species and then allowed to cool slowly, a complex containing DNA and high-turnover RNA forms; but no such complex forms with DNA of other bacterial species. This suggests that the base sequence of the high-turnover RNA is such that it can form a paired structure with DNA (98). The high-turnover RNA behaves as if its base sequence was complementary to that of DNA. Estimations of the molecular weight of this special RNA are not very accurate at the moment, but they indicate values in the range of $5 \times 10^5$. Such a molecule would contain about 1700 nucleotides. It might be the copy of a piece of genetic material able to code for 300–600 amino acids, if one assumes a coding ratio of 3 or 6. This corresponds to a protein molecular weight of about 60,000.

Obviously, the high-turnover RNA has many features suitable to an intermediary carrier of genetic information; it might be an "informational RNA."

The metabolic lability of this RNA and the fact that it represents only a very small fraction of total bacterial RNA, perhaps 1%, makes its study and its isolation difficult. Two ways have been recently found to accumulate it in bacteria. In the presence of 8-azaguanine, it would seem that *Bacillus cereus* produces increased amounts of an

RNA which has about the same base composition as DNA, with part of the guanine replaced by azaguanine (47,51). When bacteria grown in a rich medium are transferred into a medium in which they grow more slowly, it seems that for some time after the transfer they produce informational RNA almost exclusively (206).

Studies of the relations, metabolic and physical, between the rapidly labeled RNA fraction and ribosomal RNA are of great interest for understanding how the information eventually reaches the ribosome where it is deciphered and interpreted.

Incorporation of radioactive phosphate or of $C^{14}$-uracil followed by sedimentation analysis of the nucleic acids contained in the bacterial extracts prepared from the labeled bacteria shows that the only fraction which is appreciably labeled in 20 seconds is the fraction which is suspected to be "informational RNA." Chasing experiments in which the bacteria are transferred to a nonlabeled medium after a short time of labeling indicate that this RNA fraction rapidly loses its radioactivity, which is now found in ribosomal fractions (4,93,170). As mentioned before, the sedimentation pattern of ribosomes depends very much on the magnesium ion concentration; when the magnesium concentration is high enough, *E. coli* ribosomes are distributed essentially in two peaks with sedimentation constants in the ranges of 70 and 100 s. At very low magnesium concentration the ribosomes are distributed in peaks corresponding to about 20, 30, and 50 s. In chasing experiments, radioactivity is transferred from the rapidly labeled RNA to all these fractions, which seem to acquire radioactivity in the order of increasing sedimentation constant (4, 93,152,193). The radioactivity from rapidly labeled RNA may thus become irreversibly incorporated into the "active" 70 s ribosomes which are those which make proteins *in vitro* and *in vivo* (156,157,218). On the other hand, by adequately changing the magnesium concentration, rapidly labeled RNA can be made to associate with 70 s ribosomes in a reversible way *in vitro* (93).

Two different interpretations were advanced for these experimental facts. According to one interpretation, the rapidly labeled RNA is a precursor of ribosomal RNA (152,177,188,193). The fact that its base composition differs radically from that of ribosomal RNA is not a very serious objection to this interpretation, for it is quite conceivable that only part of the rapidly labeled RNA is integrated into the ribosome; this part might even carry structural information (207).

According to the other intrepretation, the rapidly labeled RNA is a short-lived RNA which becomes temporarily associated with pre-existing ribosomes; it brings the information to the ribosome which interprets it, much the same way as a magnetic tape brings the information which causes the magnetophone to play a certain tune. After directing the formation of one or a few samples of polypeptide chain, the tape RNA, or "messenger RNA," is destroyed. This interpretation is an essential element of a fascinating theory of the mechanism of genetic regulation of protein synthesis (124) in which it is assumed that inducers and repressors of enzyme synthesis act on the genes and control the production or the release of "messenger RNA."

The messenger hypothesis received support from a study on the synthesis of phage protein (33). It was shown that during a period extending from the second to the seventh minute after infection, no new ribosomes are formed; the rapidly labeled RNA is bound to pre-existing ribosomes which make proteins. These are probably phage proteins because the synthesis of bacterial protein is blocked by infection. It would seem that phage "messenger RNA" indeed uses ribosomes and gives them the genetic information for making phage proteins, in conformity with the messenger hypothesis. This theory accommodates many experimental data on the kinetics of enzyme synthesis and of induction and repression; it accounts for the requirement for RNA precursors for protein synthesis and for the action of base analogs; and it explains why DNA may be required in certain bacterial preparations without being directly involved. The idea that ribosomes are devoid of specificity should probably not be pushed too far; at the present time, it is merely the simplest assumption. Bacteriophage might indeed be a special case; their parasitism must establish at one or another biochemical level. Phage uses certain ribosomes of *E. coli* for making its own proteins, but it is not known whether all the ribosomes of *E. coli* are interchangeable.

The messenger theory could easily be extended to higher organisms, provided it is realized that the time scale must be considerably expanded, and provided it is accepted that the messenger RNA's have a longer life in higher organisms and remain active longer when associated with the ribosomes. In *Acetabularia* the information for the synthesis of several enzymes survives in the cytoplasm at least 2 weeks after enucleation, and reticulocyte ribosomes retain the in-

formation for making hemoglobin at least a few days after the disappearance of the nucleus. In rapidly growing animal cells, such as HeLa cells in tissue culture, irradiation of the nucleolus immediately blocks the synthesis of an RNA fraction and its transfer to the cytoplasm; this is shown by the immediate cessation of incorporation of precursors into cytoplasmic RNA; but it is only 6 hours later that a partial inhibition of protein synthesis is observed (69).

Whatever the exact nature of the relations between rapidly labeled RNA and ribosomal RNA, it seems quite reasonable to assume as a working hypothesis that the rapidly labeled RNA contains genetic information on its way to the ribosome; whether it behaves as a short-lived messenger which disappears after having accomplished its mission, or whether it is integrated into the ribosome as a permanent constituent is an open question.

One fact may not have received enough attention so far, namely, that, in higher organisms at least, all the enzymes of a cell do not seem to be placed under equally strict or direct nuclear control. Enucleation experiments on *Amoeba* indicate that the synthesis of a phosphatase and of an esterase depends much more directly on the presence of the nucleus than the synthesis of several other enzymes does (26,27,200). In *Acetabularia*, the synthesis of enolase, phosphorylase, invertase (6,53), and most proteins (31,32) continues for 2 weeks in the absence of the nucleus, but the synthesis of acid phosphatase stops soon after enucleation (132) and resumes within 2 days if a nucleate fragment is grafted on the enucleate cytoplasm (131). The same is observed in *Acicularia*, a closely related species. One might at first think that this enzyme is made in the nucleus and simply released into the cytoplasm, but grafting experiments between *Acetabularia* and *Acicularia* indicate that the process might not be so simple. The acid phosphatase of the two species can be separated by electrophoresis. When an enucleate fragment of *Acicularia* is grafted upon a nucleate fragment of *Acetabularia*, a phosphatase of the *Acetabularia* type appears in the *Acicularia* cytoplasm after 2 days, whereas the amount of phosphatase of the *Acicularia* type decreases and disappears after 5 days. In reciprocal grafts, the *Acetabularia* phosphatase was the only one to be produced (131). It would seem that factors which are closely dependent on the nucleus cooperate, in shaping the enzyme, with other specific factors which persist for a long time in the cytoplasm.

## VI. Formation of the Polypeptide

Let us assume that in some way the genetic information has reached the ribosome; how is it deciphered, interpreted, and translated into an amino acid sequence? No answer can be provided to this question at present except in terms of hypothetical models. For instance, in Crick's model (58), it is assumed that specific enzymes bind the individual amino acids to oligonucleotide "adaptors" which contain a short sequence of bases complementary to the coding sequences on the template RNA. The adaptors thus allow the individual amino acids to find their right place on the template. In view of what is known about the first steps of protein synthesis, it is tempting to equate the transfer RNA's to the adaptors, and the activation enzymes to the enzymes which are able to bind specifically the amino acids to the adaptors. However, transfer RNA does not simply deliver the activated amino acid to the template; there are indications that part of the RNA might be integrated, at least temporarily, to the ribosome (1,21–23,113,159). Other models of templates have also been proposed, and there are not enough experimental data available at present to discuss the merits of these different models usefully. But a few very interesting data bearing on this matter have been recently reported and it may be that the template mechanisms will soon be better understood.

The formation of polypeptides is often described as being very rapid because it takes only a few seconds in microorganisms and perhaps one minute in animals. In terms of absolute rate of reaction, these are exceedingly long times. With *in vitro* systems, the process is still much slower. The progress of polypeptide formation on the template was actually observed in the case of hemoglobin synthesis. By short time labeling and location of the labeled residues within the chain, it was shown (15,65) that the polypeptide chain grows by the steady sequential addition of amino acids. Growth of the chain starts at the N-terminal end and proceeds steadily toward the carboxyl end. Very short time labeling in *E. coli* (85), as well as a study of the kinetics of inactivation by ultraviolet light of amino acid incorporation into ribosomal and soluble protein (77), led to a similar conclusion. It would seem that at each step the ribosome template is charged of incomplete chains at various stages of growth. This most probably explains the nonuniform labeling of proteins which was observed a long time ago in poorly active systems. One may also

wonder whether the nucleotide-peptide compounds found in several types of cells, and especially in yeast, are growing peptides artificially detached from the template (1,61,62,103,139,140,175,197,213).

## VII. Release of the Polypeptide from the Template and Emergence of the Protein Molecule

In *in vitro* systems, it is easy to observe incorporation of amino acids into protein material, but it is very difficult to obtain the formation of well-characterized individual enzymes or proteins. Release of the polypeptide from the ribosome does not seem to be easy. Evidence was presented for the existence of a special enzyme for releasing the newly made polypeptide (232); this enzyme seems to be especially abundant or well preserved in pea seedling extracts, and this may explain why this system is better than many others at producing enzymes (186,187,231). A release enzyme also seems to exist in reticulocyte extracts (160). Polyamines might play a part in removing the newly made polypeptide from the ribosome (202).

In order to become a protein molecule, the polypeptide must fold into a well-defined three-dimensional structure and hydrogen bonds must form between different parts of the chain; in this manner certain amino acids which are far from one another in the chain will be brought close together. Thus, the active center of the enzyme will be formed, and the perfect protein will arise with all its physical, immunological, and enzymic properties. In certain cases, several identical (or different) polypeptides must associate and prosthetic groups must be added.

At present it is generally assumed that once the primary structure—i.e., the amino acid sequence—is established, folding and stabilization by hydrogen bonds occur spontaneously as the polypeptide slowly peels away from the template. Folding is thought to be strictly determined at each step by the nature of the amino acid residues (182) and, as the synthesis proceeds, by the structure of the part of the polypeptide chain which is already formed. From studies on myoglobin and hemoglobin (134,182), it would seem indeed that folding is mostly determined by a few key amino acids. The polypeptide chain of human hemoglobin and that of whale myoglobin have almost the same tertiary structure, and the proteins have very similar functions, although they differ greatly in amino acid composition, except for a few amino acids which clearly occupy key posi-

tions responsible for the type of folding and for the attachment of the prosthetic group (230).

Direct evidence that the secondary and tertiary structures of ribonuclease are completely determined by the amino acid sequence was also presented (95). The enzyme was inactivated by reduction of its disulfide bridges; reoxidation restored the activity. Moreover, if the enzyme is first split into two pieces by subtilisin, reduced to suppress its activity, and later reoxidized, the activity reappears in spite of the fact that one peptide bond was broken (95,234). These experiments also suggest that the formation of S—S bridges in an enzyme molecule does not raise a very difficult problem and that no extra information is required for placing the polypeptide in the right position to allow the easy formation of the bridges, granted an adequate oxidation system is available.

That polypeptides can spontaneously associate into enzyme or protein molecules is an experimental fact. Each molecule of hemoglobin is comprised of four chains: two $\alpha$ and two $\beta$ chains. Their association into the finished protein molecule occurs spontaneously *in vitro* under adequate conditions. Moreover, genetic data strongly suggest that the two types of chains are made independently, that they dimerize as soon as formed, and that $\alpha,\alpha$-dimers then associate spontaneously with $\beta,\beta$-dimers, thus forming the complete globin molecule (5,122,123). Active insulin also reforms *in vitro* by combination of the separated, inactive A and B chains (66). The formation of an active enzyme in the cold in mixed extracts of two different mutants of *Neurospora* (242) is best explained by the spontaneous association of polypeptides into active enzyme. Synthesis of hybrid enzymes in heterozygotes (198) again points to the association of two polypeptides which were made separately.

Attachment of the prosthetic group may also occur spontaneously. The porphyrin of catalase can probably find its proper place in the finished protein moiety, since the apocatalase made by a porphyrineless mutant of *E. coli* can combine *in vitro* with hemin, resulting in a complex endowed with the properties of normal catalase (7). *Staphylococci* also make apocatalase when deprived of hemin, and later complete the synthesis when hemin is added; in this case, however, there is evidence that coenzyme A is involved (127), suggesting that the association of the prosthetic group with the protein is completed by an energy-requiring enzymic condensation. The proper folding

of the polypeptides of apocatalase does not depend on the prosthetic group either, since apocatalase can be made in its absence. This again supports the idea that the secondary and tertiary structures of proteins are determined by their amino acid sequence and that no extra information is needed for obtaining the correct folding which is all important for the enzymic and physiological properties of the protein. It is obvious, nevertheless, that the conditions under which folding takes place are also very important: temperature, pH, and the concentration of various ions are determinants. The presence of particular lipids might possibly play a part in certain cases at this last stage of protein formation; the formation of serum albumin molecules seems to be influenced by the lipoproteins of the liver ribosomes (38).

The fact that perfect enzymes can form by spontaneous folding and association of polypeptides does not exclude the possibility that in certain cases an active finishing process might be required. The unusual requirements for amylase formation from a precursor in pigeon pancreas extracts might find their explanation in a final transformation which is necessary for the appearance of enzyme activity; this process requires a special RNA and the presence of amino acids, although these are not incorporated into the enzyme (90,211,212). In *B. subtilis*, also, the formation of amylase involves the transformation of a precursor protein (248). Phosphorylase *a* results from the phosphorylation of four serine residues of phosphorylase *b* followed by dimerization (75,133,141). The activation of trypsinogen and chymotrypsogen results from a spontaneous change in the tertiary structure which occurs when a peptide bond is broken (166). It will be very interesting to know whether the trypsinogen structure with its inner tension acquires its tertiary structure spontaneously as it comes off the template, or whether a special mechanism is involved in such cases. Oxidation of proline into hydroxyproline in collagen (105,208, 222,239) and methylation of lysine in flagellin (210) occur after the polypeptide is formed. These are finishing steps.

Finally, the perfect enzyme with its characteristic structure and properties can associate spontaneously with other enzymes or proteins into organized complexes which catalyze a concerted sequence of reactions. The cases of tryptophan synthetase of *E. coli* (84,243) or of pyruvate dehydrogenase (89,138) are examples of this process which give a first glimpse of the organization of complex enzyme systems at

the molecular level. The next step in complexity will be the integration of enzymes into functional structures of a higher order (104a) such as mitochondria; there is already evidence that the arrangement of the enzymes in space is of great importance for their concerted activity (70).

## VIII. Concluding Remarks

According to the present concepts of enzyme biosynthesis, some aspects of which were considered above, the amino acid sequence is completely determined by the DNA gene. The structures of a higher order and the catalytic properties of the enzyme are thought to emerge from the amino acid sequence. Somewhere between the gene and the completed polypeptide, specific regulatory agents, repressors and inducers, may inhibit or stimulate the production of individual enzymes. Although these regulatory mechanisms are not completely understood yet, their study is well advanced (99,124,185).

A few important facts have not been integrated into the present schemes, among them cytoplasmic heredity (68) and differentiation of enzyme biosynthesis (29). The formation of two important groups of enzymes—namely, the respiratory chain of yeasts and molds and the photosynthetic system of green plants—is indeed controlled by hereditary factors which do not obey Mendelian laws and are therefore regarded as extrachromosomal. Their nature and exact function are not known. The enzymes which depend on extrachromosomal factors for their formation do not escape the usual nuclear genetic control, and there is every reason to believe that their structure is controlled by nuclear genes, just as are those of the other enzymes for which no cytoplasmic factors have been detected. It is not known at present whether the extrachromosomal factors also contain a piece of structural information which completes that of the gene, whether they are autocatalytic objects necessary, albeit indirectly, for their own formation and for that of the enzymes (48), or whether they are the mere representation of alternative self-sustaining steady states (64,64a) owing to special properties of regulatory processes. Although nonchromosomal hereditary factors were shown to play a part in the case of only a few enzymes, the question concerning their exact function is of great importance; its solution might provide a test for the current assumption that the specific information for protein structure is all contained in the nuclear gene.

The changes in the pattern of enzyme synthesis during differentiation raise, at the molecular level, problems familiar to embryologists. The irreversible loss of the capacity to produce certain enzymes and the accompanying increased production of others are fundamental problems which in many ways resemble those of cytoplasmic heredity. Although everyone interested in enzyme biosynthesis now has them in mind, they have not been approached very often, except in a purely descriptive manner. Their solution is a task for the next decades.

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