PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY

15

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PREFACE

Volume 15 again contains a varied diet. Dr. P. N. Campbell continues the story of the unravelling of protein synthesis, last discussed by Dr. McQuillen in Volume 12. Dr. V. P. Whittaker gives a comprehensive account of the subcellular fractionation of brain tissues, with particular emphasis on the work of his own laboratory. Prof. Brachet gives a brief account of his recent work and ideas on morphogenesis, especially concerning the role of ribonucleic acids, providing an introduction to a field which is likely to attract much attention in future years. Dr. B. B. Hyde discusses the somewhat meagre information available on the structure of chromosomes. An important biophysical subject, namely the structure and development of animal viruses, is discussed by Dr. D. S. Bocciarelli and finally Dr. D. R. Davies gives an account of recent progress in the study of polypeptide conformations by X-ray diffraction.

We should perhaps comment on the fact that the present volume is somewhat smaller than those of recent years. This is due partly to the average length of the articles being less, which will perhaps not be entirely unwelcome to our readers, but also to the non-appearance of certain articles which had been arranged for, because the authors could not finish them in time. It is hoped to include these in later volumes. While we have every sympathy with authors in their efforts to satisfy the numerous calls made upon them, we would appeal to them to make every effort to complete articles undertaken by the agreed time, as when withdrawals occur it is often too late to arrange for suitable substitutes.

J. A. V. B.
H. E. H.

15 December 1964

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1
THE BIOSYNTHESIS OF PROTEINS

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ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ATP	adenosine triphosphoric acid
poly U	polyuridylic acid
GTP	guanosine triphosphate
RNP	ribonucleoprotein
m-RNA	messenger RNA

I. INTRODUCTION

The development of the idea that the genetic information in the cell is carried from the DNA in the nucleus to the ribosomes in the cytoplasm by means of a special kind of RNA, called messenger-RNA, triggered off a surge of activity in the field of protein synthesis. Although some of the earlier experiments are not considered conclusive (see Hendler, 1963) supporting evidence for this hypothesis has rapidly accumulated. The idea received its strongest support from the experiments of Nirenberg and Matthaei (1961) who demonstrated that a polynucleotide could direct the synthesis of a polypeptide in a system containing ribosomes from *E. coli*. After a phase of feverish activity the impression now is one of consolidation involving the performance of painstaking experiments rather than the execution of a few brilliantly conceived ones. There have arisen only two serious critics of the general hypothesis (Hendler, 1963; Harris, 1963a). Such critics perform a most useful function for there is a danger in an exciting subject of selecting the experiments that support the hypothesis of the day and ignoring those which might produce results which are contrary to the current views.

There are so many excellent reviews on protein synthesis that it is difficult to avoid being repetitive. The one aspect that seems to have received least attention and which is probably of interest to biologists in general is that which concerns the correlation of structure and function of the various organelles. The tendency to concentrate attention on the genetic code may have given a distorted picture to the non-specialist for whom this review is intended. For completeness it is necessary to start with a brief description of the blueprint with which the experimental results will be compared.

II. THE GENERAL SCHEME OF PROTEIN SYNTHESIS

The first step in the synthesis of protein from amino acids is still thought to be the activation of the carboxyl group. The amino acid reacts with ATP to form the amino acid adenylate, the reaction being catalysed by the appropriate amino acid activating enzyme. In the last few years this step has come to be regarded as established and there is now relatively little work done on this aspect of the field. The various objections such as the difficulty of detecting enzymes in one biological source which would lead to activation of all twenty amino acids remain. (For a detailed discussion see Fruton, 1963.) Such matters are passed over for at present there is no reasonable alternative to the activation step.

After conversion of the amino acid to its adenylate derivative it is transferred to the terminal nucleotide of the transfer* RNA. This reaction is shown in Fig. 1. It was for long not certain whether the site of attachment on the ribose

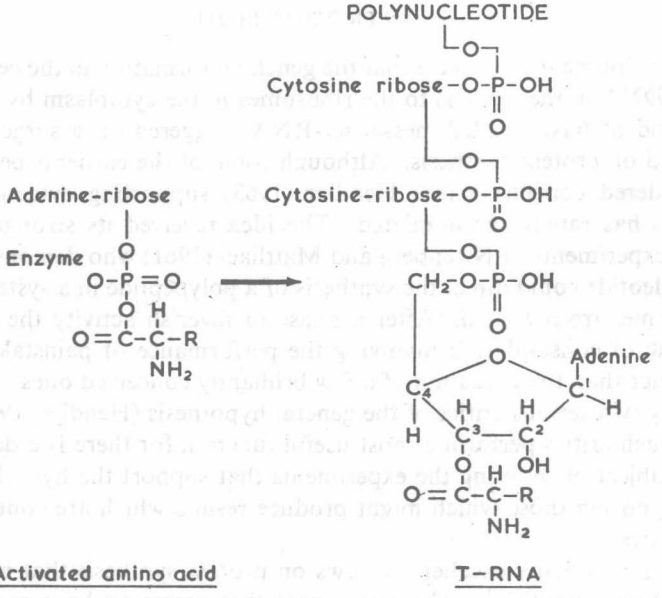

FIG. 1. Structural aspects of the transfer of amino acid from the amino acid adenylate to transfer RNA.

was the 2′ or 3′ position but this has now been clarified by Sonnenbichler, Feldmann and Zachau (1963) and the 3′ is preferred. It is clear that there is at least one specific transfer RNA for each of the twenty species of amino acid found in a protein. The attachment of the amino acid to transfer RNA is

* This kind of RNA was originally termed soluble or S-RNA, others have called it acceptor-RNA. The terms are all synonymous.

catalysed by the same enzyme that is responsible for the activation of the amino acid. This is a most interesting phenomenon for it means that the enzyme not only possesses a recognition site for the amino acid but also one for the correct transfer RNA. The separation of the different species of transfer RNA by counter-current distribution has been so successful (Weisblum, Benzer and Holley, 1962) that more than one transfer RNA has been discovered for each of the amino acids, leucine and valine. In such cases two possibilities exist, either there are multiple forms of activating enzyme for the same amino acid or the one enzyme has recognition sites for a group of transfer RNA molecules. One wonders whether the concept of allosterism could not explain this phenomenon (Monod, Changeux and Jacob, 1963).

These authors introduced the concept of allosteric proteins to explain a wide variety of cellular control systems including gene repression. The basic idea is that a substance (*effector*) becomes attached to a site on the protein (the *allosteric site*) and as a result the properties of the site that bind the substrate (the *active site*) are changed (*allosteric transition*). Thus an effector such as lactose can become attached to a protein which is so altered that it is no longer able to impair the function of part of the DNA of a cell and thereby allows an enzyme, in this case β-galactosidase, to be synthesized. In the case of the amino acid activating enzymes the idea would be that all amino acid activating enzymes have the same primary structure. They differ one from another by the attachment of one of the specific transfer RNA molecules. The presence of the RNA donates to the protein a specificity for the activation of the amino acid and its subsequent transfer to the transfer RNA. Presumably once the amino acid has been attached to the RNA the complex dissociates from the protein which is then ready to receive another molecule of transfer RNA. Such an idea has been invoked by Hele (1964) although she has not demonstrated the role of transfer RNA itself in the reaction. Davie, Koningsberger and Lipmann (1956) in their work on the isolation of the tryptophan enzyme were never able to free it completely of RNA and it remains possible that the RNA was in fact transfer RNA specific for tryptophan.

It will be obvious that a central problem is the difference in structure between the different species of transfer RNA. A recent review by Brown (1963) is an excellent account of this aspect of the field. This matter will be considered more fully at a later stage.

The next step in the synthesis of polypeptide takes place on the ribosomal particles and is depicted in Fig. 2. The information which is to control the sequence of amino acids in the polypeptide chain synthesized on the ribosome is, of course, contained in the base sequence of the DNA. The messenger RNA is assembled using the DNA as a template. Hayashi, Hayashi and Spiegelman (1963), using DNA from the *E. coli* virus ϕX 174 which is single stranded but which in its replicating form is duplex and contains both the original strand and complement, have shown that only one strand of the DNA is used for the formation of m-RNA. A similar conclusion has been reached by those working with a virus from *B. megaterium* and a phage of *B. subtilis*. In the replication

of viruses which contain RNA the RNA of the virus appears to be used as the m-RNA without the intermediary of DNA (see Montagnier and Sanders, 1963, and Cline, Eason and Smellie, 1963). The addition of synthetic polynucleotides, such as poly U, to the isolated ribosomes also circumvents the action of natural m-RNA as previously mentioned (Nirenberg and Matthaei, 1961). A great deal of evidence has been accumulated relating the composition of the polynucleotide to the amino acid incorporated, and hence the polypeptide synthesized This is summarized in Fig. 3.

AMINO ACID CODE TRIPLETS

Amino acid	U-triplets	Non-U triplets	Shared doublets
Ala	CUG	CAG, CCG	C●G
Arg	GUC	GAA, GCC	G●C
AspN	UAA, CUA	CAA	●AA, C●A
Asp	GUA	GCA	G●A
Cys	GUU	...	...
Glu	AUG	AAG	A●G
GluN	...	AAC	...
Gly	GUC	GAC, GCG	G●G
His	AUC	ACC	A●C
Ile	UUA, AAU	...	...
Leu	UAU, UUC, UGU	...	U●U
Lys	AUA	AAA	A●A
Met	UGU	...	...
Phe	UUU	...	...
Pro	CUC	CCC, CAC	C●C
Ser	CUU	ACG	...
Thr	UCA	ACA	●CA
Try	UGG	...	...
Tyr	AUU	...	...
Val	UUG	...	...

FIG. 3. Amino acid code triplets. (From Wahba *et al.*, 1963.)

While the precise difference between the various species of transfer RNA is not yet known it is assumed that the differences concern a triplet of nucleotides. Such triplets are shown (Fig. 2) pairing with complementary triplets on the messenger RNA which is now associated with the ribosome. In this way the transfer RNA guides the appropriate amino acid to the correct position on the messenger RNA. Experimental evidence for this was provided by the work of Chapeville *et al.* (1962). They prepared transfer RNA charged with [^{14}C]-cysteine and incubated this with ribosomes from *E. coli*. They found that the transfer of the cysteine to polypeptide was stimulated by the addition to the system of GUU (see Fig. 3). If the transfer RNA charged with cysteine was first treated with Raney Nickel which converted the cysteine to alanine then the transfer of alanine was again stimulated by GUU. Reference to Fig. 3 shows that the transfer of alanine from alanine charged transfer RNA would be stimulated by CUG, CAG, or CCG but *not* GUU. They in fact found that this

was so. This proves that it is the nature of the transfer RNA and not the amino acid attached to it which determines the position of the amino acids on the messenger RNA. Additional evidence was later provided by von Ehrenstein, Weisblum and Benzer (1963) who studied the synthesis of haemoglobin by ribosomes isolated from rabbit reticulocytes. They incubated the ribosomes first with transfer RNA charged with [^{14}C]cysteine and then with the latter after treatment with Raney Nickel. In the first case normal haemoglobin peptides were synthesized with [^{14}C]cysteine in the correct position and in the second the cysteine residues in the peptide were replaced by alanine. In a rather similar experiment Chapeville, Cartouzou and Lissitzky (1963) converted tyrosine attached to transfer RNA into 3,4-dihydroxyphenylalanine (dopa) and found that the position in haemoglobin formerly occupied by tyrosine now contained dopa.

Various aspects of this scheme will now be discussed.

III. SPECIAL ASPECTS OF THE GENERAL SCHEME

(a) *Mechanism of Action of Puromycin*

Puromycin was first used by Yarmolinsky and de la Haba (1959) and shown to inhibit the incorporation of amino acid by liver microsomes. They explained the inhibition on the basis that the structure bore a sufficiently close relationship to transfer RNA charged with amino acid for it to act as an inhibitor of the

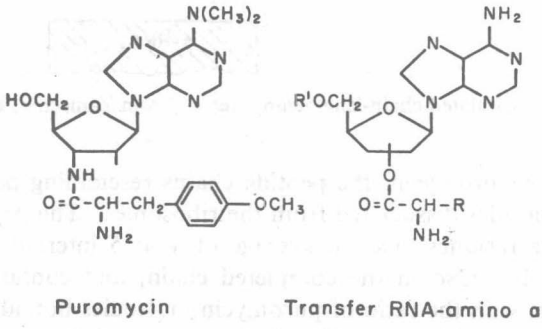

FIG. 4. Structures of puromycin and the amino acid-bearing end of transfer RNA. R represents the remainder of the amino acid residue, R^1 represents the remainder of the polynucleotide chain. (From Yarmolinsky and de la Haba, 1959.)

transfer of amino acid to the ribosome. The relationship of the two structures is shown in Fig. 4. We now know that the original idea of the authors was not quite correct but theirs has been an important contribution to the subject and it is worth considering the way in which we now believe puromycin to inhibit protein synthesis.

Morris and Schweet (1961) studied the effect of puromycin on the synthesis of haemoglobin by ribosomes from rabbit reticulocytes (see also Morris *et al.*,

1963). In the normal system they found that there was comparatively little incorporation of valine into the N-terminal valine of the haemoglobin chain. However if the ribosomes after incubation with [^{14}C]valine were treated with puromycin the proportion of label in the N-terminal position rose. They interpreted this to mean that the puromycin stripped the partially completed peptides from the ribosomes. Allen and Zamecnik (1962) obtained evidence to support this idea when they used ^{14}C-labelled puromycin in a similar system.

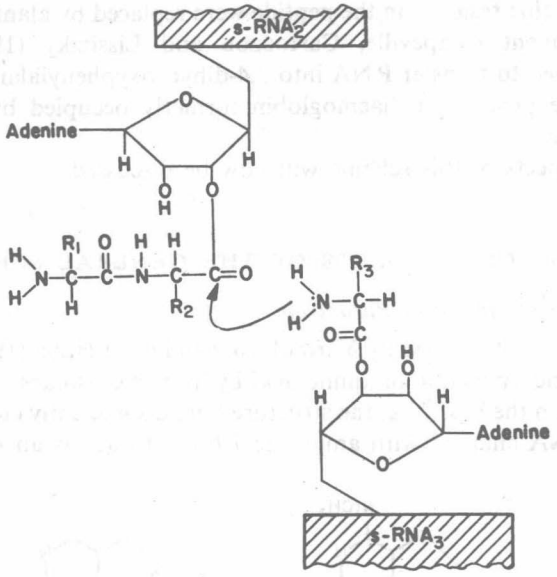

FIG 5. Postulated chain-lengthening step (From Zamecnik, 1962)

In the presence of puromycin, the peptide chains resembling partially formed haemoglobin molecules dissociated from the ribosomes. The peptides bore the N-terminal valine residues, had an average of 4 or 5 internal valine-residues (rather than the 10 or so in the completed chain) and contained covalently bound puromycin with the ratio of puromycin molecules bound to N-terminal valine residues of roughly 1 : 1. The amino group of the *p*-methoxyphenyl-alanyl part of the puromycin molecule incorporated into the released peptide was incapable of reacting with fluorodinitro-benzene. This evidence suggests that the puromycin is bound to the peptide by means of this amino group presumably in peptide bonding (see Zamecnik, 1962). The chemical events at the site of chain extension are shown in Fig. 5.

The above work provides evidence for the concept of peptidyl-RNA chains as intermediates in protein synthesis and for the postulated stepwise extension of the peptide chain from the free N-terminal end as shown in Fig. 1. It also explains the mechanism of action of puromycin. The puromycin residue becomes the C-terminal residue of the peptide chain instead of transfer RNA.

Since puromycin lacks the polynucleotide chain of transfer RNA it cannot hydrogen-bond with the m-RNA and so the peptide with the puromycin attached separates from the ribosome and the synthesis of the polypeptide is terminated prematurely.

Bishop, Leahy and Schweet (1960) first showed in their ribosome system that haemoglobin was synthesized from the N-terminal end of the chain. This was confirmed by Dintzis (1961) and Naughton and Dintzis (1962) working on the synthesis of haemoglobin by intact rabbit reticulocytes.

Gilbert (1963b) has studied the effect of puromycin in the synthesis of polyphenylalanine by ribosomes from *E. coli* incubated in the presence of poly U. His results which will be considered again were in complete agreement with those obtained with reticulocyte ribosomes. Takanami (1962a) had previously come to a similar conclusion.

(b) *Mode of Attachment of Transfer RNA to the Ribosome*

It has for long been known that ribosomes either from *E. coli* or reticulocytes dissociate into two unequal subunits when they are placed in a medium of low magnesium content. The same phenomenon is found with liver ribonucleoprotein particles isolated after the microsome fraction has been treated with deoxycholate. The two subunits of ribosomes from *E. coli* have sedimentation constants of 30S and 50S, the whole ribosome having a constant of 70S. Not only can the two components be separated in the ultracentrifuge but they may also be detected by electron microscopy as shown by Huxley and Zubay (1960). McQuillen (1961) deals with this subject extensively in a former review in this series, entitled "Ribosomes and the Synthesis of Proteins".

Cannon, Krug and Gilbert (1963) have made an extensive study of the point of attachment of the transfer RNA to the *E. coli* ribosome in high concentrations of magnesium ions. They found that the binding occurred in the cold, was not catalysed by supernatant enzymes and did not require energy. Once bound the transfer RNA could be displaced by adding more transfer RNA or by lowering the magnesium ion concentration. The binding was not affected by puromycin and it was immaterial whether the transfer RNA had amino acids attached. The binding required the integrity of the terminal sequence of nucleotides in the transfer RNA and the point of binding was therefore near the site to which the amino acid would be attached. The 50S unit of the ribosome binds the transfer RNA. In a previous paper Gilbert (1963a) had shown that for protein synthesis the intact 70S ribosome was required, i.e. both the 30S and 50S subunits in combination. He found that after protein synthesis in a cell free extract of *E. coli* a small fraction of the transfer RNA that is bound to the ribosomes in high concentrations of magnesium ions was resistant to removal when the magnesium ion concentration was lowered. This tightly bound transfer RNA appears on the 50S subunit of the 70S ribosomes which carry the polypeptide chain and which do not dissociate in low magnesium. It is postulated that the transfer RNA is more tightly bound to the 50S unit when it has a

growing peptide chain attached to it. Hence it is likely that there is only one site for polypeptide synthesis on the 70S ribosome.

The main conclusions of this work are depicted in the diagram in Fig. 6. One point that arises concerns the role of GTP. For long it has been reported that GTP is involved in the transfer of amino acid from transfer RNA to polypeptide. Gilbert finds that it is not required for the attachment of transfer RNA to the ribosome. This is in agreement with Takanami (1962b). Hence the role

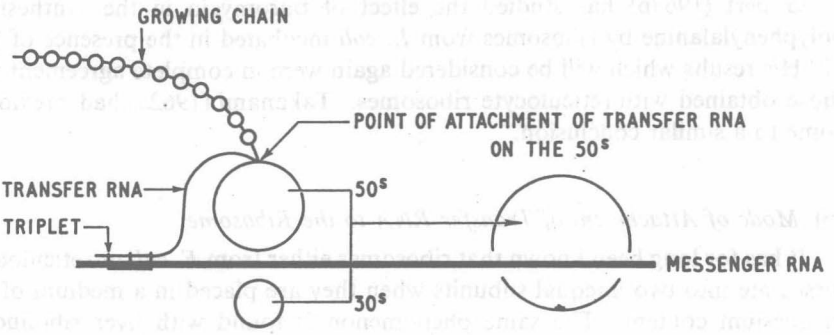

FIG. 6. Diagrammatic representation of the relationship between a ribosome, messenger RNA, transfer RNA and the polypeptide chain. Messenger RNA is attached to the 30S subunit of the ribosome. The transfer RNA is attached at one point to the 50S subunit of the ribosome. This point of attachment is at the same site as that of the growing polypeptide chain. It is postulated that the transfer RNA contains a region that base-pairs with the messenger RNA, as indicated in Fig. 2. This scheme is based on the work of Gilbert (1963a).

of GTP must concern the formation of the peptide bond. Another point concerns the finding that transfer RNA can bind to the 50S subunit even if it is not charged with amino acid. It has always been recognized that if this were to occur during protein synthesis the process would be stopped. Gilbert envisages a situation where there are two equivalent sites for attachment of transfer RNA in the 50S subunit. Either site alone can bind the transfer RNA tightly but if both are full both transfer RNA molecules are bound loosely. If the approaching transfer RNA has no amino acid attached then no reaction takes place and it will diffuse away to be replaced by another molecule with amino acid so that the chain can be lengthened.

(c) *The Significance of the Degenerate Code*

The basic problem of the code between nucleic acid and protein has been of course the way in which the four letter alphabet represented by the bases of the nucleic acids could code for the twenty different amino acids that occur in the proteins. Crick (1963) has explained all the arguments in his recent article on

this subject. The present evidence certainly suggests that the code consists of a triplet of bases for each amino acid. However, four bases would give a total of 64 different triplets which is considerably in excess of requirements. Thus it came as no surprise when by the use of the synthetic polynucleotides more than one triplet was found to code for one amino acid (see Fig. 3). This situation which is known as "degeneracy" has been treated so far with a feeling of relief for it is better than the alternative known as "ambiguity". In this case one triplet codes for two different amino acids. This so far has only been reported for phenylalanine and leucine in the case of poly U. This is indeed unfortunate for with a homopolymer there can be no doubt concerning the nature of the triplet involved as there may be in a heteropolymer. So far no satisfactory explanation has been provided for ambiguity.

It is worth considering what degeneracy implies in terms of the messenger RNA hypothesis. There seem to be two possibilities in terms of the incorporation of amino acid into protein, either (a) each triplet coding for the same amino acid is responsible for the incorporation of that amino acid into all the different positions it occupies in the polypeptide chain of the protein or (b) certain positions in the chain are coded for by one triplet and others by an alternative triplet.

We cannot at present choose between (a) and (b) for the results of experiments designed to make the choice have not yet been reported. If the answer is (b) then the coding hypothesis seems to be safe and we might even have an answer to the way in which chains are initiated which will be discussed later. If, however, the answer is (a) the implications are more serious. This would mean that for example in the case of alanine one would have to have a m-RNA in one molecule of which all the alanines were coded for by CUG, another for CAG, and another for CCG. In fact the situation would be even more complicated for molecules combining all these three would have to be available. In other words for each protein there would have to be a multiplicity of messenger RNA molecules. This idea cannot seriously be contemplated and so other alternatives must be considered. One is that the results of experiments with synthetic polypeptides are not good models for natural messenger RNA. This would mean that the transfer RNA for a particular amino acid has only one recognition triplet for the synthesis of a natural protein but several for the synthesis of an unnatural polypeptide. This explanation seems to have been excluded by the experiments of Weisblum, Benzer and Holley (1962) and von Ehrenstein and Dais (1963). They have shown that different molecules of transfer RNA carry the recognition sites for only one of the alternative triplet codes. Thus three transfer RNA fractions could be isolated for leucine, one recognizing poly UC, another poly U, and yet another poly UG. Moreover Bennett, Goldstein and Lipmann (1963) working with *E. coli* ribosomes and f2 phage RNA have shown that both of the leucyl-transfer RNA molecules (UC and UG) feed leucine into the phage coat protein with the same kinetics. The answer to this problem is therefore not yet apparent.

(d) *The Sub-Fractionation of Transfer RNA*

It was Zamecnik and his colleagues who first showed that the amino acid was attached to the ribose of the adenine nucleotide at the end of the polynucleotide chain of transfer RNA (Hecht *et al.*, 1958; Hecht, Stephenson and Zamecnik, 1959). As shown in Fig. 1 the next two nucleotides have cytosine as the base and it was possible to show that the so-called -pCpCpA sequence was common to the transfer RNA molecules for all 20 amino acids. It follows that the difference in the nucleotide sequence as between the different transfer RNA molecules must reside elsewhere in the chain. Berg, Lagerkvist and Dieckmann (1962) determined the sequence of nucleotides adjacent to the -pCpCpA end of isoleucine and leucine transfer RNA. They showed that the sequence for isoleucine is pGpCp(UpC)pApCpCpA, and for leucine they found two sequences pGpCpApCpCpA and pGpUpApCpCpA. It is apparent, therefore, that the sequence immediately after the -pCpCpA end differs as between the various species of transfer RNA. Ishida and Miura (1963) have reached a similar conclusion.

In the fractionation of transfer RNA two problems exist. First one wants to separate the different species of transfer RNA. There is at present no ideal method which is available for all amino acids. The method of choice must to some extent depend upon whether one wants to isolate a product which is (a) specific for one amino acid and is homogeneous in so far as there is more than one transfer RNA for the particular amino acid or (b) which is merely specific for a particular amino acid. A method designed to achieve (a) depends on the use of synthetic polynucleotides to effect the attachment of a particular amino acid to the transfer RNA. For example one can incubate ribosomes from *E. coli* in the presence of phenylalanine and poly U. A chain of polyphenylalanine is then built upon the specific transfer RNA. If the complex of transfer RNA and ribosome is disrupted with sodium dodecyl sulphate then the polyphenylalanine transfer RNA is isolated. Incubation at alkaline pH removes the polypeptide and the specific transfer RNA is recovered. Theoretically at least this kind of approach could be applied to other amino acids. An alternative is merely to fractionate the mixture of transfer RNA from which amino acids have been removed by a physical method such as counter current distribution. This has been very effectively used by Holley. An example of this method is the isolation of three transfer RNA fractions for leucine each specific with respect to a different synthetic polynucleotide (Weisblum, Benzer and Holley, 1962).

Methods to achieve (b) type fractionation have often depended on the attachment of one amino acid to the transfer RNA and then the destruction of the other species of transfer RNA by the application of periodate oxidation. This method depends on the original work of Preiss *et al.* (1959) in which they showed that the attachment of the amino acid rendered the terminal ribose insensitive to oxidation by periodate. A new intriguing method is due to Mehler and Bank (1963). In this the transfer RNA is charged with one amino acid.