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Unsettled Questions in the Field of Protein Synthesis

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Studies on protein synthesis carried out during the past 15 years since ^{14}C became available have passed through several technical phases, which may be identified as follows: an initial disruptive period, in which the goal was to find a cell-free system in which protein synthesis occurred; a reassembly phase, in which various components of the crude homogenate were tested for essentiality in the incorporation process; and the present mechanistic or macromolecular phase, in which details of reaction mechanisms and of spatial configurations of macromolecular participants are being considered. Although a rather unitary picture, applicable to a wide variety of living organisms, may be drawn for steps involved in protein synthesis, a number of areas of uncertainty and disagreement exist, and these form the theme of the present lecture.

To begin with, there is the problem of how a transfer-RNA molecule 'recognizes' the particular activating enzyme with which it must associate in order to become esterified. Next, in the esterification reaction arises the question whether the 2'- or 3'-ribosyl hydroxyl position is the esterification site. In the subsequent sequence-ordering step just before peptide polymerization, no generally accepted picture can be drawn to indicate the geometry of the condensation reaction. How the growing peptide chain is attached to the ribosomal surface is still a matter of speculation. An actively investigated point is whether the ribosomal template on which the ordering of aminoacyl-RNA molecules occurs is a passive catalytic surface or an active template that acts only once before degradation and replacement by a new ribonucleic acid message. Finally, the coding step itself poses the problem of how the identity of each amino acid becomes translated into a sequence of nucleotide bases. Other important queries can be framed, but for the present discussion the above list will suffice, and I shall touch on these points in the order mentioned.

Recognition of the activating enzyme. The transfer-RNA molecule appears to carry out two separate functions: recognition of a particular activating enzyme, and coding with template RNA in a way that results in correct positioning of the amino acid for incorporation into the growing peptide chain.

Physical properties of this molecule, particularly as reported by Brown & Zubay (1960), suggested that the molecule doubled back on itself near the middle to form a double helical structure. The near-parity of purines and pyrimidines, even in purified Val-RNA (cf. Table 1), was consonant with this view, as was the large hyperchromic effect on heating (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959). The recent brilliant X-ray-diffraction study on crystalline transfer RNA by Spencer, Fuller, Wilkins & Brown (1962) leaves no doubt of the correctness of this prediction, and provides a picture of a uniform double helical structure (cf. Watson & Crick, 1953) of approximately 20 by 100 Å for all members of the transfer-RNA family. The present discussion has therefore been modified in details since its oral presentation, in the light of these new data on the structure of transfer RNA.

One is now in a better position to ask where on this molecule the two types of recognition function are located, and whether they can be carried out by the same group of bases on the RNA molecule. If this molecule is heated to 90° for an hour and then cooled rapidly, a treatment which disorganizes the secondary structure somewhat, its ability to accept amino acids has been reported to be virtually unimpaired (Brown & Zubay, 1960). Whether it can still transfer amino acids, however, remains to be determined. These results indicate that the secondary structure is not involved in the recognition of the activating enzyme, and that this latter recognition site is close to the amino acid-esterification position. There are at least three unpaired bases located at the hairpin bend of the transfer-RNA molecule, which could, as suggested by Spencer *et al.* (1962), serve as a coding area. The uniformity of structure of the transfer-RNA molecules, however, intensifies the puzzle of the function of the minor base constituents. The high content of 5-ribosyluracil in Val-RNA (cf. Table 1), and its apparent low content in certain other transfer RNA's, are suggestive of a special role in the coding or recognition operations. As compared with uracil, 5-ribosyluracil has of course an extra hydrogen-bonding site. On the other hand, the methylated minor bases (Dunn, 1959; Monier, Stephenson & Zamecnik, 1960) would not form

hydrogen bonds well in a double helix, and their presence would be expected to favour the formation of weak spots or unbonded loops in the helical structure in solution, not necessarily evident in the highly ordered crystalline structure. Thus on purified transfer-RNA molecules it will be interesting to determine whether the content of methylated bases is unique for each aminoacyl-RNA species (as one would expect it to be), and whether these bases are located near the known loop area in the transfer-RNA molecule. The results obtained by K. S. McCully & G. L. Cantoni (personal communication) suggest this latter to be the case.

If the coding and activating enzyme-recognition areas turn out to be separate, it is then possible that errors or changes in sequence in a protein molecule may be introduced by an aminoacyl-RNA molecule in two separate ways: (1) by an error or blurring of specificity in recognizing the correct activating enzyme; (2) by a mistake introduced into the coding area, resulting in incorrect positioning of the aminoacyl-RNA molecule on the ribosomal template. Related to these considerations in an intriguing way is the problem of where the minor base constituents are methylated.

Esterification site. Next, in the absence of knowledge of the precise site of esterification, let us

discuss whether it is more likely for this to occur on the 2'- or 3'-hydroxyl group of the terminal ribosyl moiety of the transfer-RNA molecule. Hecht, Stephenson & Zamecnik (1959) showed that the esterification reaction does not occur, even though the amino acid becomes activated as the aminoacyl-AMP mixed anhydride, unless the terminal 5'-mononucleotide is an adenylyl residue. This observation suggests that the base adenine plays a role in the esterification reaction. By means of space-filling models, it can be shown that it is possible to form a hydrogen bond between the hydrogen atom of the 2'-hydroxyl group of adenosine and N-3 of the purine ring. It is not possible to do so for the 3'-hydroxyl group. Thus the oxygen atom of the 2'-hydroxyl position would become more nucleophilic than the corresponding oxygen of the 3'-hydroxyl position, and would be favoured to initiate a nucleophilic attack on the carbonyl carbon atom of the aminoacyl-AMP, resulting in the formation of the aminoacyl ester. This point is illustrated in Fig. 1. As a possible explanation of the preference for adenine in this reaction, it may be stated that its ring nitrogen atom (N-3) is capable of acting as an internal base. Guanine is much less basic (Taylor, 1948), and the pyrimidine bases, the carbonyl oxygen atom (O-2)

Fig. 1. Postulated formation of aminoacyl-RNA.

of which is in the structurally similar position to the N-3 in adenine, must be even less basic.

This argument thus points to the 2'-hydroxyl group in the terminal adenosine residue as being the most likely initial esterification site. There is, however, the possibility or even likelihood of an aminoacyl migration from the 2'- to the 3'-ribosyl position, i.e. an ester may initially be formed on the 2'-hydroxyl group and then migrate to the 3'-hydroxyl position, actually leaving the latter site in the peptide-chain-extending reaction. A number of studies of acetylation of adenosine indicate that the 3'-acetyl derivative is the thermodynamically more stable isomer. Finding of the aminoacyl ester on the 3'-hydroxyl position in aminoacyl-RNA would therefore have limited interpretative value. It seems necessary to synthesize the as yet unknown 2'-acetyladenosine and to investigate whether it can be isomerized to the 3'-derivative before this point can be evaluated. It should be emphasized, however, that the facilitation of peptide-bond synthesis by the *cis*-vicinal hydroxyl function could occur whether the aminoacyl ester be on the 2'- or the 3'-position.

Peptide-chain-extension step. In the peptide-chain-extending reaction, a possible detailed

mechanism is shown in Fig. 2. Here the presence of a *cis*-hydroxyl group, vicinal to the aminoacyl ester, is considered to lower the activation energy of the peptide-bond-forming reaction, in which an amino group effects a displacement on the carbonyl group of the aminoacyl ester. In an analogous situation, Bruice & Fife (1961) (cf. also Henbest & Lovell, 1957; Kupchan, Johnson & Rajagopalan, 1959; Kupchan, Slade, Young & Milne, 1962), have found good evidence for assistance of the neighbouring *cis*-hydroxyl group in the alkaline hydrolysis of the ester bond of *cis*-cyclopentane-1,2-diol monoacetate. They implicate a hydrogen bond between the carbonyl carbon atom of the acetyl ester and the neighbouring hydroxyl group as increasing the likelihood of successful nucleophilic attack on the carbonyl carbon atom, by stabilization of the transition state associated therewith. The results obtained by Michelson (1961) and Michelson, Szabo & Todd (1956) also raise this possibility, and the influence of other factors in determining the reactivity of the ester linkage has also been pointed out (Zachau & Karau, 1960; Wieland, Merz & Pfeleiderer, 1960). It has of course for some years been presumed by biochemists that the presence of a *cis*-hydroxyl group increases the

Fig. 2. Postulated chain-lengthening step.

reactivity of an aminoacyl ester. With the aminoacyl-RNA, the reaction with hydroxylamine is much faster than with simple esters of amino acids.

In summary, these points of view have the attractive features of relating the purine base adenine to the positioning of the aminoacyl ester as it is being formed, and of bringing into consideration the presence of the additional hydroxyl group on the sugar portion of the molecule—a distinctive feature of RNA as contrasted with DNA—in the peptide-forming step of protein synthesis.

On the above matters I have had the benefit of discussions in the University Chemical Laboratory at Cambridge with Professor Todd and with Dr C. B. Reese, Dr D. M. Brown and Dr V. M. Clark, although errors of interpretation still rest with me.

Spatial arrangements of aminoacyl-RNA and template RNA. Turning to a consideration of the spatial relationships of the participants involved in the peptide-chain polymerization step, one stumbling block to visualization of a mechanism is the disparity in size between the aminoacyl residue and the comparatively enormous transfer-RNA molecule which positions it on the ribosomal surface. A second problem is how the template RNA can have unpaired purine and pyrimidine bases available on the surface of the ribosome for the coding operation. Finally, the geometrical design of the polymerization site is a puzzle.

If one accepts indications (Brown & Zubay, 1960; Spencer *et al.* 1962) that ribosomal RNA as well as transfer RNA is folded back on itself to form a double helix, it is necessary for the ends of the double-helical template-RNA molecule (ribosomal or messenger as one chooses to designate it) to open to permit the start of the coding operation that positions the amino acids in the growing peptide chain. One might then picture a wave of hydrogen-bond opening and closing of the double helix to move along the surface of the template RNA, with at least six base pairs at a single instant being separated and rotating somewhat out of the helix (cf. Fresco, Alberts & Doty, 1960). This would permit two adjacent sequences of three (or whatever the correct number turns out to be) bases of aminoacyl-RNA molecules of the proper complementarity to pair with the template RNA at the same time. The wave of template-RNA hydrogen-bond opening would progress from the free end of the chain. The energy for the propagation would be derived from the peptide condensation reaction, the balancing of hydrogen-bond closing and opening once the wave reaction had started, and from hydrogen-bond strain induced in adjacent base pairs by thermal motion of the transfer-RNA molecules hydrogen-bonded to a single chain at a given point. The peptide chain would remain attached to that end of the transfer-RNA molecule

which projects away from the surface of the ribosomal particle, and the chain-extending reaction would occur at this point. This possibility is represented schematically in Fig. 3.

A special enzyme and a cofactor (GTP) play a role in this polypeptide polymerization step (Zamecnik, Stephenson & Hecht, 1958; Grossi & Moldave, 1960; Nathans & Lipmann, 1961). In the above-mentioned topographical scheme, since the aminoacyl ester and the peptidyl ester are both at the open ends of the double helices of the transfer-RNA molecules, it is possible that the polymerization enzyme helps to bring them into proper orientation with respect to each other, so that the peptide-condensation step can occur. Toward such a purpose, the adenylyl-cytidylyl-cytidylyl end-group common to all the transfer-RNA molecules (Hecht *et al.* 1959) might fit an appropriate site on the surface of the polymerization enzyme, which would somehow rest against the ribosome, possibly in the groove. A guanosine nucleotide-enzyme complex might also help in the geometrical orientation of the participants in this step. Such a relation

Fig. 3. Hypothetical topography of interaction of transfer RNA with template RNA.

of aminoacyl-RNA to template RNA would imply a minimal coding ratio of six template nucleotide residues per amino acid residue—three for base pairing and three unused complementary bases on the other half-chain of template RNA.

A difficulty in this hypothetical picture is that if just one chain of the template RNA is used for coding, the entire double helix must rotate, or open completely, or else the adjacent transfer-RNA molecules have frequently to bridge over the second chain of the temporarily-opened helical structure in order to propagate the chain-lengthening reaction. One would have to postulate considerable positional flexibility of the participants to permit this. Alternatively, if only the parts of both chains disposed on the ribosomal surface were used for the coding operation, the peptide chain would be propagated by attachment of transfer-RNA molecules alternately along one and then the other of the surface arcs of the (two) coiled half-chains of template RNA that lie adjacent to each other. The second possibility would be less complicated in one sense, and yet involves the consecutive use of pieces of both chains. Even though such efforts at visualization turn out to be incorrect in detail, the consideration of a wave of hydrogen-bond opening and closing of the template RNA has great appeal, in providing a coding surface which is preserved for successive similar operations, protected until the moment of use from random hydrogen-bonding opportunities.

One is impressed by the ease with which a ribosomal particle may dissociate into subunits of its ribonucleoprotein and protein components, with lowering of the magnesium concentration of the

medium, and then may reassociate once more when the magnesium concentration is again elevated (Petermann & Hamilton, 1961). If this process of dissociation-reassociation of ribosomal components takes place within the cell, it is then possible that a competition may take place, at the instant of reconstitution, between the 'old' and newly synthesized RNA. Thus a new message (or messenger RNA) from DNA could become part of a ribosome by substituting for a previous message. This point of view has the virtue of not drawing a constitutional distinction between 'ribosomal' RNA and 'messenger' RNA; but whether it can account for all the observations on this puzzling subject is uncertain.

Mechanism of action of puromycin. The nascent peptide has been pictured above as growing step-wise (Loftfield, 1957) from the free amino end (Bishop, Leahy & Schweet, 1960; Dintzis, 1961), and being continuously attached by ester linkage as peptidyl-RNA to a transfer-RNA molecule which is hydrogen-bonded by a short sequence of bases to complementary coding bases on the template RNA. Holding this view of the polymerization step, we were much intrigued when the suggestion was made by Yarmolinsky & de la Haba (1959) that the antibiotic puromycin might inhibit protein synthesis by acting as an analogue of aminoacyl-RNA. If such were the case, we reasoned that the amino group of the *p*-methoxyphenylalanyl portion of the puromycin molecule (Fig. 4) might execute a nucleophilic attack on the carbonyl carbon atom of the peptidyl-RNA, in analogy to the mechanism postulated for the addition of the next aminoacyl-

Fig. 4. ^{14}C -labelling of puromycin.

RNA residue. The difference would be, however, that if the puromycin residue became the *C*-terminal residue of the peptide chain, the latter would separate from the ribosomal template, having no polynucleotide chain (such as the aminoacyl-RNA has), to hydrogen-bond the *C*-terminal esterified end of the peptide to the ribosomal surface. Using ^{14}C -labelled puromycin (cf. Fig. 4) and a reticulocyte ribosomal system (Schweet, Lamfrom & Allen, 1958), Dr David Allen found evidence to support this point of view (Allen & Zamecnik, 1962). In the presence of puromycin, peptide chains resembling partially formed haemoglobin molecules dissociated from the ribosomal surface (Morris & Schweet, 1961; Allen & Zamecnik, 1962; Morris, Favelukes, Arlinghaus & Schweet, 1962). These peptides bore *N*-terminal valine residues, had an average of 4 or 5 internal valine residues (rather than the 10 or so internal valine residues of the completed haemoglobin chain), and contained covalently bound puromycin, with the ratio of puromycin molecules bound to *N*-terminal valine residues being roughly 1:1. The amino group of the *p*-methoxyphenylalanyl part of the puromycin molecule incorporated into the released protein was incapable of reacting with fluorodinitrobenzene. This evidence suggests that the puromycin was bound to the peptide by means of this amino group, presumably in peptide bonding. Besides offering mechanistic details on the action of puromycin, this investigation provides indirect evidence for the concept of peptidyl-RNA chains as intermediates in protein synthesis (cf. also Koningsberger, van der Grinten & Overbeek, 1957; Hoagland, Zamecnik & Stephenson, 1959; Harris & Neal, 1961; Nathans & Lipmann, 1961) and for the postulated stepwise extension of the peptide chain from the free *N*-terminal end. It is hard to picture a successful chain-extending reaction moving in the opposite direction, since the free α -amino group of the elongating peptide chain would be too unfixed in space to execute a successful nucleophilic attack on the adjacent esterified carbonyl group of an aminoacyl-RNA molecule.

Aminoacyl-RNA and the coding problem. During the past year, spectacular progress made (Nirenberg & Matthaei, 1961; Lengyel, Speyer & Ochoa, 1961) offers promise of deciphering the ribonucleotide code sequences for a number of amino acids by addition of synthetic polynucleotides to the cell-free systems previously worked out (Keller & Zamecnik, 1956; Lamborg & Zamecnik, 1960; Tissières, Schlessinger & Gros, 1960; Rogers & Novelli, 1960). In order to make coding relationships firmer, it would be of great advantage to be able to put to the test ribopolynucleotides of known repeating sequence. In connexion with the use of trichloroacetic acid precipitability as a measure of

protein synthesis, it is also possible that this may give rise to a bias in apparent coding relationships, in favour of peptides with low water-solubility.

Our own interest in the coding problem has been to try to isolate a single species of aminoacyl-RNA, to which sequential degradation techniques may be applied. This has been the objective of a number of Laboratories as well, which have reported good progress toward this end with employment of such diverse techniques as countercurrent distribution (Apgar, Holley & Merrill, 1962), chromatography (Smith, Cordes & Schweet, 1959) and chemical attachment of substituents to the side chain of the aminoacyl residue of a particular aminoacyl-RNA (Brown, Brown & Gordon, 1959). Our approach is based on a combination of two *ad hoc* procedures, used in sequence. Transfer RNA prepared from yeast (Monier *et al.* 1960) is first stripped of amino acids and then labelled with a single one. Periodate is now added, and the terminal ribosyl moieties of the transfer-RNA species bearing no aminoacyl ester are oxidized to the dialdehydes. The aminoacyl ester prevents the periodate oxidation of the terminal ribosyl group to which it is attached. A hydrazide (2-hydroxy-3-naphthoic acid hydrazide) is now added and reacts with the aldehyde groups to form a hydrazono-RNA. A diazo-coupling reagent, such as tetrazotized di-*o*-anisidine, will now quickly couple to the bound hydrazone to form a bulky dye (blue in this case), firmly attached to the large fraction of the RNA molecules not bearing the aminoacyl ester. The dye-RNA molecule is less water-soluble than the non-dye-bearing RNA, and may be readily separated (Zamecnik, Stephenson & Scott, 1960). Purifications of Val-RNA, and of several other aminoacyl-RNA species, of the order of tenfold have been obtained in this way. Further improvements have also been made (Portatius, Doty & Stephenson, 1961; Zachau, Tada, Lawson & Schweiger, 1961) by the use of hydrazides bound to resins. The method is, however, based on the hope of removing the 95% or so of the transfer RNA that does not bear a single species of aminoacyl ester from the roughly 3–5% that does. Despite our best efforts, we have never been able to get rid of a small fraction (5–10%) of the treated RNA that has neither an esterified amino acid nor a dye molecule attached. In short, the periodate-hydrazone-coupling reactions fall short of 100% of completion, and the aminoacyl-RNA species reaches an upper plateau of 25–50% of purity, above which it is hard to push it by recycling or modification of conditions of the steps, including the use of resin hydrazides.

We therefore turned to other fractionating principles for further purification, and found diethylaminoethyl-dextran columns to be promising

(Stephenson & Zamecnik, 1961). Consistent purifications of Val-RNA of the order of 90 % or more have recently been obtained (Stephenson & Zamecnik, 1962). No evidence has been found for the presence of more than one species of Val-RNA, although differences might be difficult to detect. Nevertheless, with this high degree of purification, the finding of only one species of Val-RNA must be reckoned with in the hypotheses on coding.

Base analysis on valyl-RNA. For a sample of Val-RNA of approximately 80 % purity, the base composition is shown in Table 1, as determined by Dr C.-T. Yu (unpublished work). As compared with analyses of samples of the unfractionated transfer RNA carried out by two separate techniques, it may be seen that the percentages of adenine, cytosine, guanine and uracil are in general little changed. The amount of 5-ribosyluracil has, however, risen from 3.6 to 6.6 moles per cent, which is far outside the limits of error of the methods involved. Holley, Apgar, Merrill & Zubkoff (1961) have recently reported a molar percentage composition of 4.9 % of this same minor base in a partially purified specimen of Val-RNA, and the two observations are thus in accord. Since there would be approximately 80 mononucleotide residues in a transfer-RNA molecule of molecular weight 25 500 (Tissières, 1959), a pure Val-RNA molecule would, by extrapolation of the upward trend indicated in Table 1, contain six 5-ribosyluracil residues. As suggested above, one is led to wonder whether this residue plays an important role in determining the configuration of the secondary structure of individual transfer-RNA molecules, thus helping to confer on each aminoacyl-RNA species a unique spatial conformation.

In anticipation of a species of aminoacyl-RNA of purity sufficient to justify sequential degradation,

we have developed a procedure that may be of use for this purpose (Yu & Zamecnik, 1960), based on previous methods (Brown, Fried & Todd, 1953; Whitfield & Markham, 1953) plus an observation by Barry & Mitchell (1953) and a suggestion by Hakomori (1959). The transfer-RNA molecule is treated with periodate, which oxidizes the terminal ribosyl residue to the dialdehyde, provided that the aminoacyl ester has first been removed. Cyclohexylamine is then added at neutral or acid pH, and a di-Schiff base is formed, followed by a molecular rearrangement, with cleavage of the glycosidic bond and the phosphodiester bond, the total result being removal of the terminal nucleoside residue. With samples of unfractionated transfer RNA as substrate, it has been difficult to be more precise than to say that the degradation reaction proceeded to at least 90 % of completion at a single step. Dr A. M. Michelson has, however, kindly furnished us with the trinucleotide adenylyl-(3'-5')-adenylyl(3'-5')-cytidine (ApApC), which Dr C.-T. Yu (unpublished work) has subjected to this degradative procedure, with results indicating that close to 100 % removal of a single mononucleotide residue may be obtained, and that the process may be repeated.

In summary, work of the past few years has revealed a series of identifiable steps in the pathway from free amino acid to completed protein, and exciting developments are occurring in several separate research salients, with promise in particular of clarification of the relationship between RNA and amino acid coding. A recurrent theme of the present lecture is that there appears to be a separate species of RNA esterified to each amino acid, with this uniqueness residing in the primary sequence of mononucleotide residues and possibly in features of the secondary structure that the order

Table 1. *Base composition (moles per cent) of yeast transfer RNA*

Sample 1: Alkaline hydrolysis, paper electrophoresis, isotope dilution; average of five determinations (Kaltreider & Scott, 1962).

Samples 2 and 4: Alkaline hydrolysis, paper electrophoresis, paper chromatography; average of two determinations (C.-T. Yu, unpublished results).

Sample 3: Alkaline hydrolysis, two-dimensional paper chromatography; average of two determinations (C.-T. Yu, unpublished results).

Sample 4: Approx. 30 μ m-moles of valine/mg. of RNA. E_{260} 21.4 cm.² mg.⁻¹ (Stephenson & Zamecnik, 1961).

Base	Transfer RNA (1)	Transfer RNA labelled with ¹⁴ C valine			Purified valyl-RNA (Holley <i>et al.</i> 1961)
		(2) Original	(3) 50 % pure	(4) 80 % pure	
Adenine (A)	21.3	20.6	21.1	19.6	19.1
Cytosine (C)	23.3	24.6	25.1	25.1	27.5
Guanine (G)	36.2	34.9	28.5	33.1	29.5
Uracil (U)	19.3	16.3	20.1	15.6	19.0
Uracil from 5-ribosyl- uracil (5rU)		3.6	5.3	6.6	4.9
A + G	1.35	1.25	0.99	1.11	0.95
C + U + 5rU					

of major and minor base constituents impose on this intramolecularly hydrogen-bonded macro-molecule.

In apology for the speculative queries raised, one may quote the old adage: 'He who asks no questions will have facts but no answers.'

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