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Time Factors in Protein Biosynthesis

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In spite of the voluminous literature rapidly accumulating on protein biosynthesis, no evidence is yet available concerning the time required for biosynthesis of a single molecule of any protein. This article is intended to draw attention to the mechanistic significance, apparently often unrecognized, of this unknown factor, and to advance what it is hoped may be a useful analogy.

It is generally considered that there are three likely mechanisms for incorporation of amino acids into protein, which are (i) by assembly on a "template" (usually considered to be ribonucleic acid), (ii) by exchange of free amino acids with corresponding residues in existing protein, and (iii) by transpeptidation or synthesis, or both, in free solution. The specificity of the amino acid sequence of at least large regions of proteins can only reasonably be accounted for in terms of a template hypothesis, which is now becoming widely accepted, and for which plausible detailed mechanisms can now be suggested (*1, 2*).

The absence of detectable intermediates between amino acids and proteins was at first assumed to indicate that amino acids arrived on the template surface, formed peptide bonds, and separated as a complete protein all as one effectively instantaneous process. If one considers even a small protein molecule of, say, 100 residues, it is obvious that the chance of the correct 100 amino acids colliding with the correct 100 positions on the template at effectively the same time is infinitesimal, and that even if this happened it is extremely unlikely that the protein could separate instantaneously as a unit from the template (*3, 4*). The much more reasonable hypothesis was then advanced that the protein chain peeled off from the template as the amino acid residues combined (*1*). Such a process would imply a finite time for biosynthesis of a single molecule. One of us has proposed (*3*) an extension of this mechanism involving simultaneous attachment to a template of several growing peptide chains in varying stages of growth.

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One or More Growing Chains on a Template?

Suppose A_1, A_2, A_3 . . . A_{100} represent the amino acid residues of our hypothetical protein. Let us assume for this discussion that the peptide-bound residue just leaving the template surface is adjacent to the peptide bond being formed. A situation might then obtain which is represented diagrammatically in Fig. 1. Here T_1, T_2, and so forth represent the regions of the template with which residues A_1, A_2, and so forth become associated. As bond A_2—A_3 is being formed, residue A_1 is leaving the template surface. We can picture residue A_4 as already in position on the template ready for formation of bond A_3—A_4, while residues A_5 and A_6 may still be in the medium approaching the template surface. Similarly, while residues A_9 and A_{10} are combining, residue A_8 would be leaving the template, carrying the partially formed protein molecule represented by residues A_1 to A_8. This is only a diagrammatic representation; the distance between two growing peptide chains might be very different from that shown in the seven residues pictured in Fig. 1, but the argument would not thereby be affected.

The alternative hypothesis, that a template carries only one growing chain at a time, is often assumed (for example, *4*) but appears to us to be unlikely. In the case of our hypothetical protein of 100 amino acid residues, it would imply either (i) that residues A_1, A_2, and so forth are unable to approach the template until a completed protein chain has left the surface or (ii) that residues A_1, A_2, and so forth attach themselves to the template but do not combine until information is received from region of T_{99} and T_{100} of the template that synthesis of a protein chain has been completed. A plausible mechanism for the second alternative has not been suggested. The steric hindrance implied by the first alternative seems to us improbable and becomes more improbable the greater the length of the protein chain being formed. Steric hindrance would be further reduced if there were any tendency of the growing peptide chain to adopt a helical configuration, which would result in a considerable diminution of over-all length of the peptide relative to the corresponding portion of the template from which it has separated. (This spiralization of the growing chain can be readily visualized by anyone who has watched metal turnings coming off a lathe.) In our view, it is likely that vacant positions on a template will be filled with appropriate amino acids as soon as that region of the template becomes accessible and that peptide-bond formation and "peeling off" of the chain will occur as soon as the appropriate amino acids are in position on the template.

An Analogy

If a template carries more than one growing chain at a time, the time of biosynthesis of a protein molecule ceases to be the same as the apparent time of synthesis per molecule, and it becomes all the more important to determine this true time for biosynthesis before drawing mechanistic conclusions.

The situation can conveniently be envisaged by analogy with an automobile factory. Here component parts (compared with amino acids) enter, are assembled in appropriate order on a pro-

duction line, and finally leave as a completed automobile (compared with protein) without any free intermediate state (compared with peptides) that is obvious to an extramural observer. If it were observed that one automobile leaves the production line of such a factory every 30 seconds, it would be unjustifiable to assume that all the components were put together effectively simultaneously and therefore to deduce that the time of assembly of an automobile is 30 seconds. If it were known that n cars in various stages of construction were on the production line at any one moment, and the more reasonable assumption were made that the component parts were assembled consecutively rather than simultaneously, it would be possible to deduce that the time required for assembly of an automobile is $30 \times n$ seconds.

A value analogous to n would be difficult to derive in the case of protein biosynthesis. The time required for automobile assembly could, however, be determined by feeding in labeled components and determining the time before emergence of the first car containing these components. Such an approach can be applied in the case of protein biosynthesis if labeled amino acid is added to an active protein-synthesizing system and the time before the appearance in the system of free labeled protein is determined.

A further complicating factor arises in the existence of "pools" not equilibrating directly with added exogenous amino acids. For example, Cowie and Walton (*5*) have shown that, in the food yeast *Torulopsis utilis,* added amino acids pass through such a pool; once they are included in this "bound" pool, their further reactions are little affected by changes in exogenous amino acids, even though they are not yet incorporated into protein.

In terms of our analogy, such a bound pool can conveniently be interpreted as a reserve of components for the automobile. Components can be imagined as being delivered onto a moving belt for transport to the production line. During the period they are on this belt, the components are neither being incorporated into the automobile, nor are they interchangeable with further similar components being delivered. The time they spend on the moving belt may be an appreciable proportion of the time these components spend in the factory, either free or as part of an automobile, yet this time is not part of the true time of construction of the automobile.

Similarly, a completed protein may not be immediately detectable as a free molecule in solution but may pass through bound "pools," just as a completed automobile may not be immediately detectable on the roads until it has passed through, for example, a distributor's pool.

Possible Experimental Approaches

If protein biosynthesis occurs by a template mechanism involving *de novo* synthesis from free amino acids, without occurrence of exchange reactions, time relationships should exist which are capable of experimental verification. The situation is illustrated in Fig. 2. The observed time T before the first appearance of free labeled protein, will be made up of two parts, a time t resulting from the synthetic process itself, and a time t_d required for such factors as diffusion of amino acids into cells, diffusion of newly synthesized protein out of cells, and time spent in bound pools.

If the specific activity of the free amino acid pool is constant, the total protein-bound activity should ultimately increase linearly. The protein-bound activity may not, however, increase linearly in the initial stages. Thus, if it is assumed that only a small part of the protein chain (presumably the "growing point") is attached to the template at any moment, it follows that for protein synthesis to occur it is not necessary for every amino acid residue in the protein to be present on the template at the same instant. If, then, one has formation on a template of a protein containing, for example, four glycine residues, and three of these residues have already been incorporated before labeled glycine is added to the system, the time of first appearance of free labeled protein, which will contain a label only in the fourth glycine residue, will not indicate the time for biosynthesis of a complete protein molecule. The time which it is necessary to observe is the time of first appearance of a labeled protein of maximum specific activity, indicated by T_1 in Fig. 2.

The increase in protein-bound activity will ultimately cease to be linear because of protein degradation. The latter will not appreciably affect the linearity while the number of labeled protein molecules in the system is still small compared with the number of unlabeled molecules. These degradation reactions are those involved in the more usually measured turnover time of the protein, which is an expression of the average life of the whole molecule and bears no necessary relationship to the time of biosynthesis. In terms of our analogy, the expectation of life of an automobile on the roads (compared with turnover time) is not a necessary function of the time required to construct the automobile.

Under suitable conditions, it should be possible to break down the experimentally observed time T and to derive the time t that is appropriate to the synthetic process, and the time t_d that results from the sum of other factors, such as diffusion and the time spent by amino acids, or completed proteins, in bound pools. Two approaches appear promising, one of which is illustrated in Fig. 2. Suppose that the appropriate times, as defined here, in normal circumstances are T_1, t_1, and t_d and that the rate of synthesis of protein is then modified by suitable activation or inhibition, while constant conditions of temperature and amino acid concentration, and so forth are maintained, so that the nonsynthetic factor t_d remains constant. Let the observed time before first appearance of protein of maximum specific activity now be T_2, and the true time of biosynthesis under the modified conditions be t_2 (see Fig. 2). Then

$$T_1 = t_1 + t_d$$

and

$$T_2 = t_2 + t_d$$

In addition, the times required for synthesis under the different conditions should vary inversely with the rate of synthesis (to return to our analogy, in order to double the rate of production of automobiles, it is necessary to halve the time spent on the assembly line). If the rates of synthesis (that is, the slopes of the linear portions of the incorporation curves in Fig. 1) are S_1 and S_2, respectively, then, in addition:

$$S_1/S_2 = t_2/t_1$$

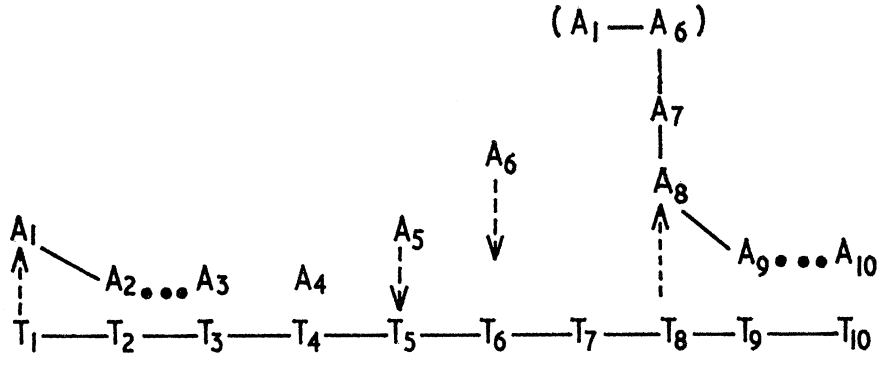

Fig. 1. Mechanism suggested for simultaneous formation of several protein chains on the same template.

As T_1, T_2, S_1, and S_2 are known, t_1, t_2 and t_d can be calculated, or alternatively, t_d may be deduced by extrapolation of the straight-line portion of the incorporation curves (Fig. 2) and determination of the intercept.

For this approach to be valid, it is essential, first, that the change in rate be due to a change in the rate of the actual synthetic process, and not, for example, to limitation of the supply of an essential amino acid. And second, there must be no simultaneous change in the amount of template material. An increase in the rate of protein synthesis is known often to be accompanied *in vivo* by an increase in ribonucleic acid, which is commonly assumed to be the template material. Results should therefore be expressed in terms of equivalent amounts of ribonucleic acid, but even this would be inadequate if an inactive (from the template point of view) form of ribonucleic acid is converted to an active form.

A second approach to separating the observed time T into its components t and t_d might be to observe the rates of formation of protein at different temperatures. In this case, both t and t_d can be expected to alter. If the variation of t_d with temperature could be predicted, it would again become possible to deduce the true time of biosynthesis, t.

If the biosynthesis of a single molecule of protein can be a lengthy process, one would therefore expect to find evidence of delays between addition of amino acids to a protein-synthesizing system and the first appearance of labeled protein. In fact, there are numerous examples of this in the literature. For example, Peters (*6*), studying synthesis of serum albumin *in vitro*, consistently found a delay of 15 to 20 minutes before the first appearance of labeled serum albumin. Similar results on synthesis of whole serum proteins *in vivo* were obtained by Green and Anker (*7*), while a comparable delay

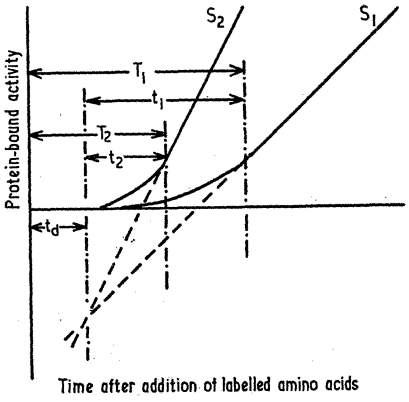

Fig. 2. Theoretical incorporation curves for the same protein-synthesizing system made to work at two different rates under otherwise constant conditions; from such curves the true time of synthesis of a single protein molecule could be deduced.

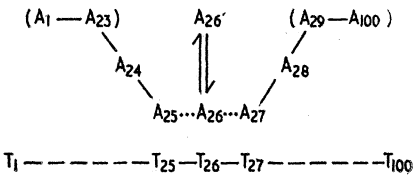

Fig. 3. The way in which the same template that controls *de novo* synthesis might also control exchange reactions.

in appearance of labeled γ-globulin *in vivo* has been found by Askonas, Humphrey, and Porter (*8*), and for Bence-Jones protein by Putnam, Meyer, and Miyake (*9*).

Green and Anker studied the delay in some detail. The delay of 20 minutes or so before first appearance of labeled protein was followed by a linear increase of protein activity for at least 50 minutes. The delay was independent of the nature or amount of labeled amino acid, but it varied with temperature, and it was considered to be specifically related to the biosynthetic process.

Junquiera, Hirsch and Rothschild (*10*) found a delay of about 50 minutes before the appearance of activity in rat pancreatic juice proteins, and they showed that this delay was not due to any appreciable extent to the time required for passage of the proteins down the pancreatic duct; they also pointed out that the existence of a long delay period is in itself evidence against the occurrence of incorporation by exchange, which involves few steps compared with synthesis of a complete peptide chain and for which no appreciable delay is therefore to be expected.

Relationship Between de novo Synthesis and Exchange

The relationship between exchange and *de novo* synthesis is another aspect of protein biosynthesis that calls for further investigation. The occurrence of exchange without *de novo* synthesis has been shown unequivocally by Gale and his coworkers (for example, *11*) to occur in bacterial systems. This exchange, like *de novo* synthesis, is an energy-requiring process. It appears often to be assumed that exchange and *de novo* synthesis involve different mechanisms, but it seems more likely to us that pure *de novo* synthesis and pure exchange are the two extremes of a common mechanism.

It has already been suggested as likely that as soon as a growing peptide chain leaves the template surface, this surface will be ready to receive appropriate amino acids. In the ordinary course of cellular events, it is to be expected that a protein chain in the medium might also collide with the template surface. If part of such a protein chain were to collide with the corresponding part of a template, it is not unreasonable to suppose that temporary attachment might ensue over a short region, with labilization of the appropriate residues and possible exchange, as represented diagrammatically in Fig. 3.

If active *de novo* synthesis were occurring, the partially formed peptide chains attached to the template would be likely to hinder the approach to the template of a free protein molecule in the medium, so that under these conditions exchange would not be likely to contribute significantly to amino acid incorporation. On the other hand, if an essential amino acid were lacking, so that protein synthesis could not proceed (as is the case in Gale's experiments where exchange occurs), the free protein molecules in solution should be able to approach the template very much more easily, with the resultant possibility of amino acid exchange.

Experiments in this laboratory on the biosynthesis of rat pancreatic ribonuclease (*12*) have suggested that the balance between the contributions to amino acid incorporation of *de novo* synthesis and exchange may be markedly altered between *in vivo* and *in vitro* systems. It thus seems desirable to determine times of biosynthesis under conditions as near as possible to those obtaining *in vivo*.

Small proteins, especially those with specific biological actions, are likely to contain a specific arrangement of all amino acid residues. Formation on a template of the complete molecule of such proteins is therefore likely. In the case of large protein molecules, however, the possibility that certain regions formed specifically on templates are combined to regions formed nonspecifically must also be considered, and the problem of determining the time of biosynthesis of a molecule formed in such a way is formidable. However formidable such problems may be, it is hoped that this discussion has demonstrated the importance for the elucidation of mechanisms of protein biosynthesis of the determination of the time required for biosynthesis of single protein molecules.

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