THE SYNTHESIS OF RIBOSOMES IN *E. COLI*

IV. The Synthesis of Ribosomal Protein and the Assembly of Ribosomes

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ABSTRACT The incorporation of C^{14} leucine into the protein moiety of ribosomes has been studied as a sequel to the studies of ribosomal RNA synthesis. In contrast to the latter studies, labeled leucine is incorporated directly into 50S and 30S ribosomes without measurable delay by precursor stages. There is, however, evidence of some transfer of radioactivity from the 43S group of particles to the 50S. The inhibition of protein synthesis by chloramphenicol results in the accumulation of material similar to the eosome—the primary precursor in ribosome synthesis. There is also evidence for the synthesis of some neosome. The results of the studies of ribosomal RNA and protein synthesis are combined into a model of ribosome synthesis. Finally, consideration is made of the significance of these studies of ribosome synthesis for general problems of protein synthesis and information transfer.

A. INTRODUCTION

The previous paper reports studies of the synthesis of ribosomal RNA. These studies indicated several states of organization of ribosomal RNA during the assembly of the complete 50S and 30S ribosomes. The two precursor stages of ribosome synthesis apparently differed from complete ribosomes in having a considerably lower content of protein. Study of the synthesis of ribosomal protein was therefore undertaken to elucidate the details of the process of assembly of RNA and protein to make completed ribosomes.

Results are presented showing the kinetics of incorporation of leucine into ribosomal protein. The radioactivity due to structural ribosomal protein has been separated from that of nascent protein synthesized on the ribosomes (McQuillen, Roberts, and Britten, 1959) by means of chase experiments. Finally, the general features of ribosome synthesis are summarized together with a discussion of the role of ribosomal RNA and other RNA fractions in protein synthesis.

B. MATERIALS AND METHODS

Methods of growing cells, estimating radioactivity, and fractionating cell extracts have already been described in Paper III.

The C^{14}-leucine used as a protein label was purchased from the New England Nuclear Corporation and had a specific radioactivity of 5.2 mc/mM. A P^{32} steady-state label was used as a measure of total RNA as before.

RNA synthesized during inhibition with chloramphenicol was studied with the aid of 2-C^{14}-uracil as previously described.

C. RESULTS

1. *Analysis of Pulse Labeled Extracts by Sedimentation.* Most previous sedimentation analyses have been made in higher concentrations of magnesium (10^{-2} M) in which 70S and larger ribosomes survive (Roberts, 1960). The results were only qualitative but led to the conclusion that the 50S and 30S ribosomes present in the cell extract were the precursors of the 70S. Since the present experiments were designed to complement those in which C^{14}-labeled uracil was used, the techniques and methods of analysis were completely analogous, including the use of a steady-state P^{32} label as a measure of total ribosomes and the breakage of the cells in 10^{-4} M magnesium.

Fig. 1 shows the sedimentation analysis of five total cell extracts prepared from cells given exposures to C^{14}-leucine varying from 30 seconds to 8 minutes. The P^{32} profile shows the usual three major peaks of 50S ribosomes, 30S ribosomes, and DNA and S-RNA. The C^{14} radioactivity associated with the 50S and 30S peaks is already high at 30 seconds suggesting that much of it represents nascent protein (McQuillen, Roberts, and Britten, 1959) rather than ribosomal protein. At 30 seconds about one-third of the leucine radioactivity is associated with ribosomes, indicating that the amount of nascent protein found in association is equivalent to some 10 seconds' total supply, *i.e.* 0.2 per cent. This figure is higher than that previously reported by McQuillen *et al.* This could be a result of the different method of breakage preserving more of the protein-ribosome association.

At later times the radioactivity of the nascent protein is less prominent and there is a continued rise in the amount of C^{14} radioactivity in the 50S and 30S ribosomes. In addition, the peak of high specific radioactivity (C^{14}/P^{32} ratio) between the main 50S and 30S ribosomes visible in Fig. 1(*b*) continues to increase. This radioactivity may be attributed to an object of about 40-45S presumably the same as the 43S neosome previously described (Paper III).

As far as the entry of C^{14} radioactivity into the 50S and 30S ribsosomes is concerned there is little sign of any delays brought about by a precursor through which all the ribosomal protein must pass. The rising specific radioactivity from the very earliest times shows that most of the ribosomal protein enters the 50S and 30S directly.

Since a high proportion of the C^{14}-leucine radioactivity enters 50S and 30S ribosomes directly, it is clear that short labeling periods are necessary to demonstrate any more detailed features of ribosomal protein synthesis. In view of the contribution of nascent protein to the total ribosomal protein at early times, a short pulse of C^{14}-leucine followed by a chase with C^{12}-leucine is useful to reveal the transfer of ribosomal protein. Cells were labeled with P^{32} for three generations and then with C^{14}-leucine for one minute when a 100-fold excess of C^{12}-leucine was added. Samples were taken after 10 seconds and 1 minute, 2 minutes and 20 seconds, 4

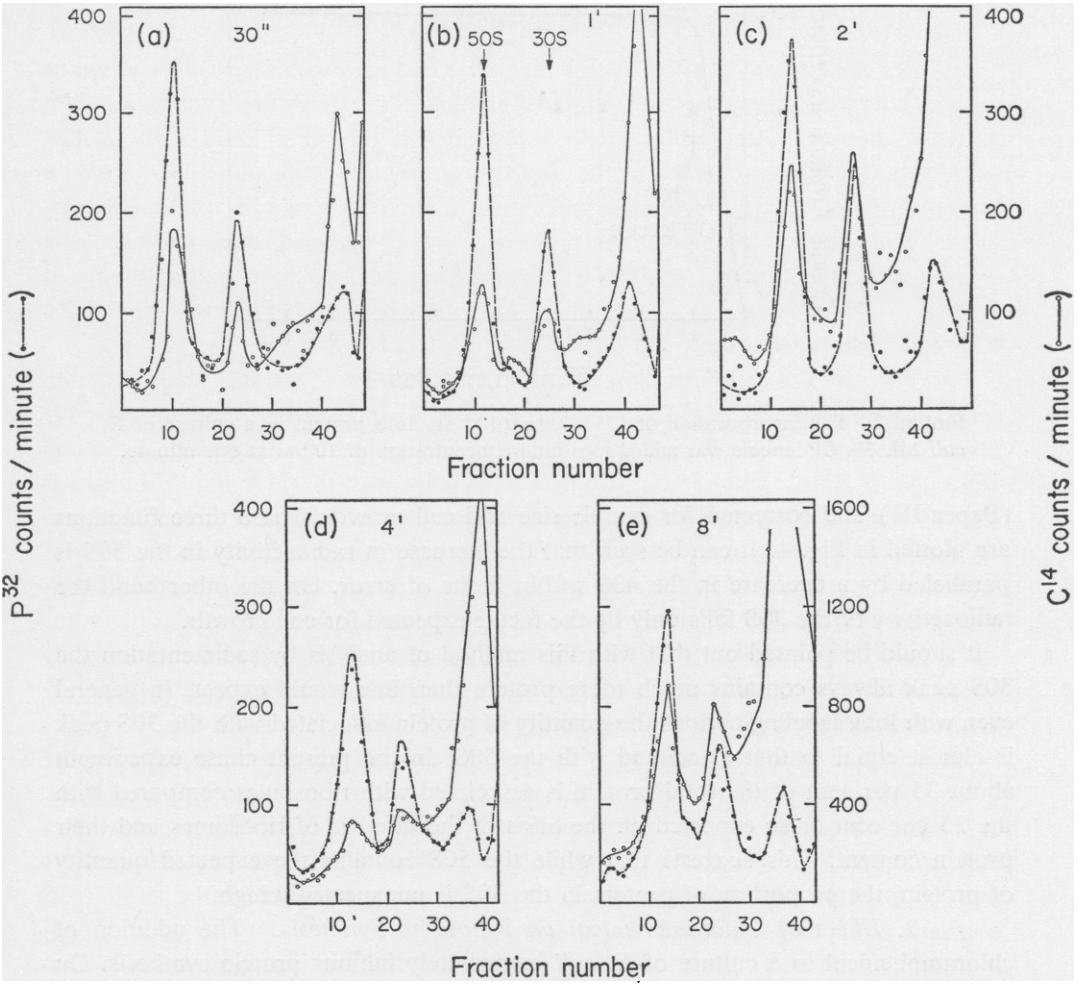

FIGURE 1 Sedimentation analysis of five total cell extracts from cells labeled with P^{32} for four generations and given (*a*) 30 seconds, (*b*) 1 minute, (*c*) 2 minutes, (*d*) 4 minutes, (*e*) 8 minutes exposure to C^{14}-leucine. Cells washed and extracts prepared in tris-HCl 0.01 M pH 7.4 $MgCl_2$ 10^{-4} M. Centrifugation 160 minutes at 37,000 RPM 4°C.

minutes, and 7 minutes and 45 seconds. Fig. 2 shows the efficiency of the chase in stopping further incorporation of radioactivity into protein.

Extracts were prepared in 10^{-4} M magnesium in the usual way and analyzed by sedimentation. Three of the analyses are shown in Fig. 3. Preliminary examination of the radiactivity profiles reveals that the 50S peak gains radioactivity relative to the 30S, and that the label in the 43S decreases as a function of time. The C^{14} present in the 30S, 43S, and 50S peaks was computed for all five analyses in the usual way

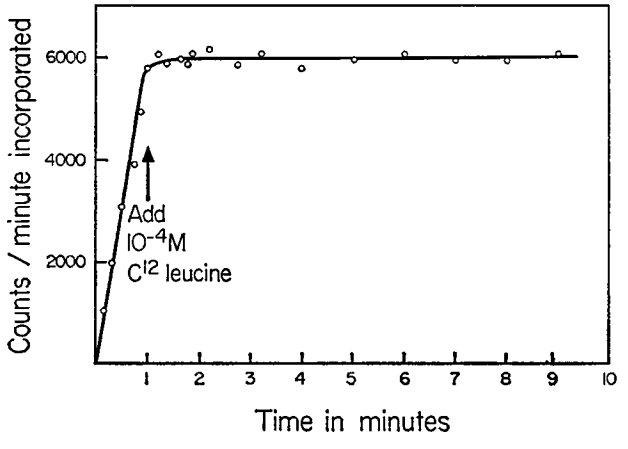

FIGURE 2 The incorporation of C^{14}-leucine (10^{-6} M) into protein in a culture of *E. coli* ML 30. C^{12}-leucine was added to a final concentration of 10^{-4} M at one minute.

(Paper III) and corrected for sample size and cell growth. These three functions are plotted in Fig. 4. It can be seen that the increase in radioactivity in the 50S is paralleled by a decrease in the 43S within limits of error. On the other hand the radioactivity of the 30S falls only by the factor expected for cell growth.

It should be pointed out that with this method of analysis by sedimentation the 30S peak always contains much more protein than one would expect. In general even with long labeling periods the quantity of protein associated with the 30S peak is almost equal to that associated with the 50S. In the present chase experiment about 35 per cent of the total protein is associated with ribosomes compared with the 25 per cent or so expected on the basis of the number of ribosomes and their protein content. This suggests that while the 50S contains the expected quantity of protein, the proportion of protein in the 30S is unexpectedly high.

2. *Effect of Chloramphenicol on Ribosome Synthesis.* The addition of chloramphenicol to a culture of *E. coli* immediately inhibits protein synthesis. On the other hand RNA and DNA synthesis continue at approximately the normal rate for the first 30 minutes or so (Gale and Folkes, 1953). During long periods of inhibition a special fraction of RNA can accumulate to a high level (Pardee, Paigen, and Prestidge, 1957) and preexisting ribosomes may be degraded (Nomura and Wat-

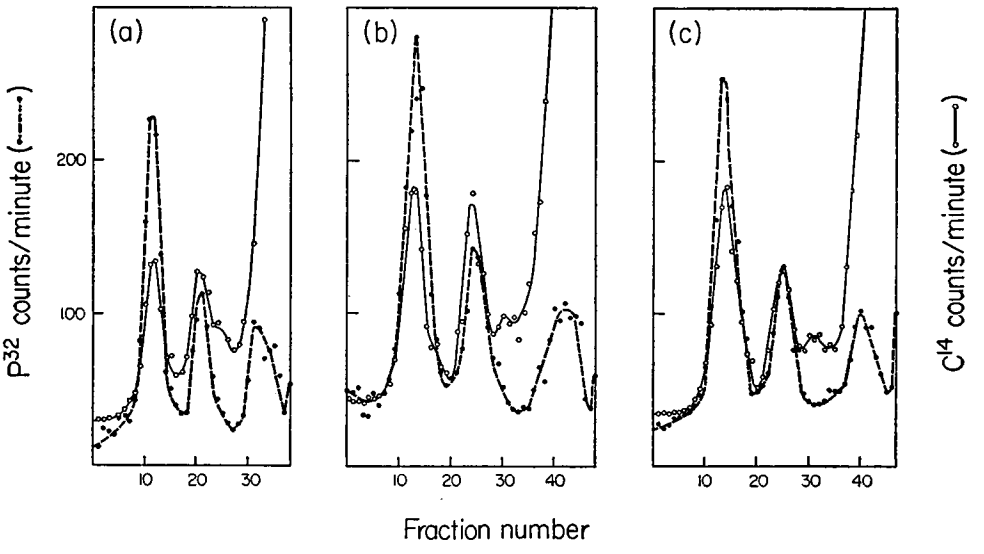

FIGURE 3 Sedimentation analysis of three total cell extracts prepared from cells labeled with P^{32} for four generations and C^{14}-leucine for one minute followed by (*a*) 10 seconds, (*b*) 2 minutes and 45 seconds, (*c*) 7 minutes and 45 seconds in C^{12}-leucine (See Fig. 2). Cells washed and extracts prepared in tris-HCl 0.01 M pH 7.4 containing $MgCl_2$ 10^{-4} M. Centrifugation 165 minutes at 37,000 RPM 4°C. The numerical scale refers to both P^{32} and C^{14} counts/minute.

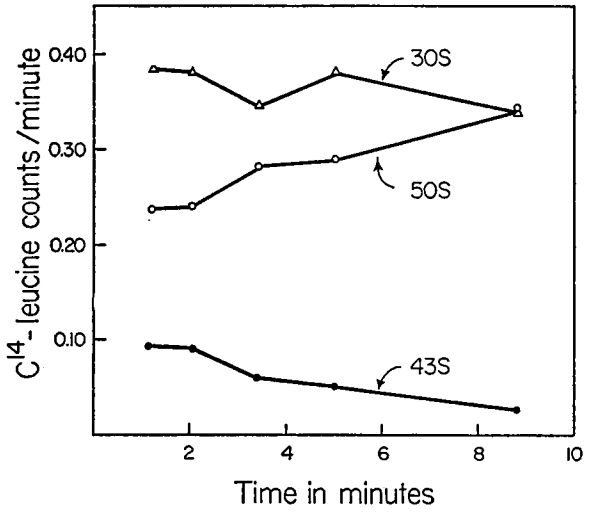

FIGURE 4 The C^{14}-leucine radioactivity in the 30S, 43S and 50S components as a function of time after a one minute pulse and chase. Data from Fig. 3.

son, 1959). Finally, removal of chloramphenicol results in the degradation and loss of much of the chloramphenicol RNA (Neidhart and Gros, 1957).

The overall base composition of RNA synthesized in the presence of chloramphenicol is identical to normal bacterial RNA (Pardee and Prestidge, 1956). Fractionation of the RNA produced on a column of DEAE cellulose gives two components, one having the base composition of soluble RNA and the other that of ribosomal RNA (Bolton, 1959). It was therefore of considerable interest to examine the relationship of the RNA formed to the normal stages of ribosome synthesis.

With the experience already gained in studies of the incorporation of uracil into ribosomes (Paper III) a period of labeling in the presence of chloramphenicol was chosen such that in the control culture most of the label would be present as product ribosomes and yet the eosome and 43S neosome components would still be visible. A culture of *E. coli* ML 30 was randomly labeled with P^{32} for three generations and then split into three equal fractions. To two of these chloramphenicol was added at concentrations of 50 μg/ml and 200 μg/ml. After two minutes an equal quantity of C^{14}-uracil was added to each culture at 10^{-5} M. After 10 minutes each culture was harvested, washed, and extracts prepared in tris-HCl 0.01 M, pH 7.4 containing DNAase at 1 μg/ml. The two chloramphenicol-inhibited cultures incorporated 80 per cent as much C^{14}-uracil as did the control.

The sedimentation analysis of two of the cell extracts is shown in Fig. 5. As the

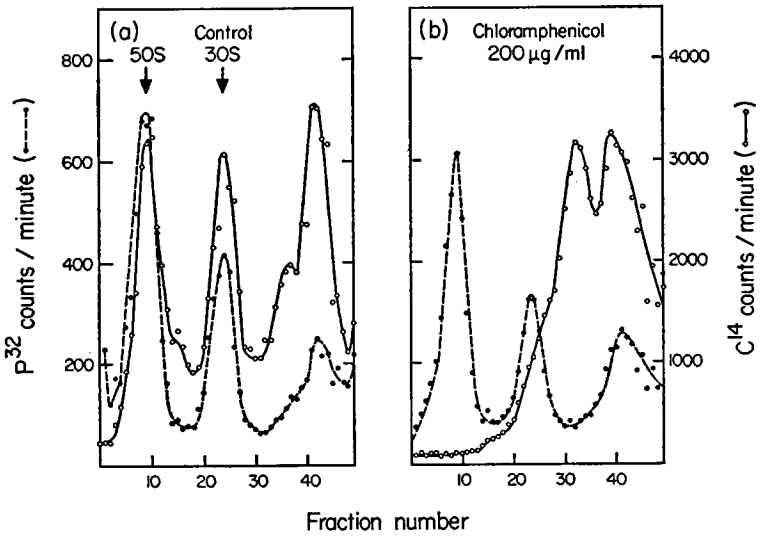

FIGURE 5 The effect of chloramphenicol on the incorporation of C^{14}-uracil into ribosomes. Cells were grown for three generations in P^{32} and given a 10 minute exposure to C^{14}-uracil. (*a*) control, (*b*) in the presence of chloramphenicol 200 μg/ml added two minutes before the uracil. Cells washed and extracts prepared in tris-HCl 0.01 M pH 7.4 $MgCl_2$ 10^{-4} M in the presence of DNAase. Centrifugation 160 minutes at 37,000 RPM 4°C.

analysis of the two chloramphenicol-inhibited cultures were identical, only one is shown. In the control culture most of the C^{14}-uracil is present in the 50S and 30S ribosome peaks and S-RNA. However shoulders of neosome at 43S and eosome at 14S are still visible. In the extract from chloramphenicol-inhibited cells most of the labeled uracil is associated with material of sedimentation coefficient less than 20. Moreover there is a separation into two peaks, one coincident with S-RNA and the other at the position occupied by eosome precursor in short pulse experiments (Paper III).

There are, however, signs of other minor labeled components. Examination of the C^{14} profile suggests that the shoulder on the leading edge of the main peak could be due to another component of about 25S. By analogy to the normal sequence of ribosome synthesis this could be identified as the neosome second stage precursor known to have a sedimentation coefficient of somewhat less than 30 (Paper III). It can also be identified as the 24S particle observed by Nomura and Watson (1959) in cell extracts after prolonged chloramphenicol inhibition.

D. DISCUSSION

1. *The Assembly of the Ribosomes.* The experiments on the synthesis of ribosomal protein do not add any fundamentally new features to the sequence of ribosome synthesis based on studies of RNA synthesis (Paper III). They do, however, confirm previous indications that some of the steps in the sequence represent the addition of protein. The fact that a high proportion of the leucine label enters 30S and 50S ribosomes directly with delays of less than one minute shows that the last stage, 43S neosome → 50S ribosome, involves the addition of protein. This is in agreement with the fact that no new RNA is incorporated directly into ribosomes and with the low protein/RNA ratio of neosomes indicated by their column behavior.

One other feature is clear from the leucine pulse and chase experiments. Since the 43S receives some label at early times, it is evident that not all the protein of the 50S is added in one step. In fact the chase experiment shows that the 50S increases some 30 per cent in specific radioactivity during an 8 minute chase period at the expense of the 43S. Rough estimates made from the two leucine experiments suggest that the 43S has a protein/RNA ratio one-quarter to one-third that of the 50S ribosome.

The final flow diagram, including both the RNA and protein moieties of ribosomes, is shown in Fig. 6. The open and shaded areas are proportional to RNA and protein contents. The eosome is shown as pure RNA since there are no measurements of its protein content and it must certainly be small. There is evidence of excess leucine radioactivity at early times (Fig. 1(*a*) and (*b*)) in the 14S region but the significance of this is not clear. The 30S neosome is shown with less than half of the protein of the 30S ribosome by analogy to the 43S neosome. However,

its protein content cannot be measured because of the lack of resolution between objects in the 30S size range. The 30S neosome which is precursor to the 43S is shown as a separate object from that leading to the 30S ribosomes, which may well be an unnecessary complication.

The 43S neosome is shown with only one-quarter of the protein of the 50S ribosome. This quantity is uncertain and it is not known what fraction of its protein enters directly in the formation of the 43S or by way of the 30S precursor to it. While these estimates of relative protein contents in neosome and ribosome are

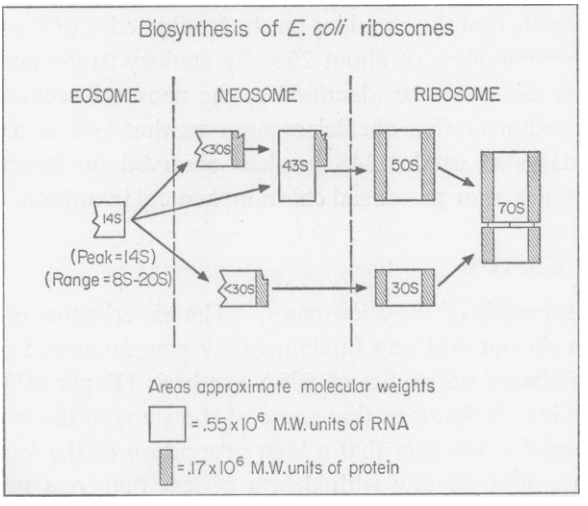

FIGURE 6 The biosynthesis of ribosomes in *E. coli*. The open and shaded areas are proportional to weights of RNA and protein respectively.

crude and preliminary, it is clear that the conversion of neosome to ribosome involves only the addition of protein and that the greater part of the ribosomal protein is added in this step.

The effect of chloramphenicol is just what one would expect from this model. The predominant effect is the accumulation of what appears to be eosome material by both sedimentation and column analysis. Moreover the nucleotide composition of this material is like that of ribosomes (Bolton, 1959). Apparently chloramphenicol inhibits the addition of protein to eosomes. The minor peak appearing at about 25S may represent small quantities of neosome produced in the presence of chloramphenicol since even at 200 μg/ml the inhibition of protein synthesis is not complete.

One feature of the general sequence of ribosome synthesis must be emphasized. The addition of RNA and protein occur in time-separated stages. This is clear from the very observation of RNA-rich intermediates.

2. *Role of Ribosome Synthesis in Replication.* Ribosomes are ubiquitous,

appear in greatest concentration where or when protein synthesis occurs, and newly synthesized protein is found in association with them. Furthermore, the rate of protein synthesis depends, not on the rate of RNA synthesis, but on the number of ribosomes present (Kjelgaard, 1961). Thus it seems likely that the steps in ribosome synthesis are relevant to the process of replication but the exact correlation with other parts of this process is difficult to discern. For example, is the synthesis of the ribosome directed by DNA or autocatalytic? Is the process of ribosome synthesis related to the transfer of information? At this time no exact answers can be given to these questions. There is, however, information which justifies speculation and suggests future experiments.

In the high concentrations of magnesium, a large fraction of the eosome is found in association with 70S and larger ribosomes. This observation is open to four interpretations. First, this association may be meaningless due to simple adsorption of eosome on the large ribosomes. Second, it may be due to the existence of large, newly formed particles which are broken down to eosomes and neosomes when magnesium concentration is reduced. Third, eosomes may actually have been synthesized in association with the 70S ribosomes. Finally, the eosomes may have been transferred from their sites of synthesis to the large ribosomes, perhaps in order to carry out a role in information transfer.

The third and fourth of these alternatives have the most theoretical interest and may well represent different views of the same process. Both these alternatives are consistent with the experiments of Brenner, Meselson, and Jacob (1961) if it is assumed, in the case of bacteriophage infection, that ribosome synthesis is effectively halted at the eosome stage.

The third alternative gains some support from the autocatalytic rate of ribosome synthesis which occurs when cells recover from magnesium starvation (McCarthy, 1962). The fourth alternative is supported by the observation of Caro and Forro (1961) that newly synthesized RNA shows the same localization as does DNA. Thus the eosomes may be formed in association with DNA and subsequently removed from the DNA by transfer to association with a ribosome. Consequently the rate of synthesis might be limited, both by the quantity of DNA acting as template, and by the number of ribosomes available to strip the template. The picture of the process is highly speculative and there is no information whatever on the location of other events such as the addition of protein.

The eosome might act as template for protein synthesis for the period before it is covered by other RNA and protein to become a finished ribosome. Its lifetime in the eosome stage does correspond to the lifetime of the enzyme-forming unit for β-galactosidase (Pardee and Prestidge, 1961; Boezi and Cowie, 1961). This correspondence, however, is between the average lifetime of all eosomes and the lifetime of one particular enzyme-forming unit. There may well be a broad distribution of eosome lifetime which would allow some protein-synthesizing units to act

for prolonged periods without replenishment of the template. Reticulocytes are able to synthesize a particular protein (hemoglobin) for long periods when DNA is apparently absent from the cell and RNA synthesis occurs at very low rates (Kruh and Borsook, 1956; Nathans *et al.*, 1961).

While it is clear from kinetic studies (Paper III) that the majority of the material in the eosome fraction must be considered ribosome precursor, it is possible that other types of RNA molecules in this size range are also present. For example it appears that in the case of bacteriophage infection, there is synthesized an RNA molecule which reflects the nucleotide composition of the infecting DNA and is able to form hybrid double strands with it (Hall and Spiegelman, 1961). In addition, the presence of RNA molecules with similar properties in uninfected cells can be demonstrated by growth under special conditions (Hayashi and Spiegelman, 1961). The kinetic study reported in Paper III, Fig. 7, suggests that there does exist a small component of very rapidly labeled RNA more closely associated with the 70S ribosome than the majority of the eosome.

In view of the complexity of the processes of RNA synthesis in the growing cell, rapid labeling cannot be considered to demonstrate turnover (in the sense of synthesis and degradation), nor does association with large ribosomes prove an object's role as informational RNA (Gros *et al.*, 1961). If indeed a distinction does exist between the eosome (considered as ribosome precursor) and messenger (considered as rapidly turning over RNA) new evidence is needed in order to assess their relative quantities and roles in the growing cell. Further attempts at fractionation, measurements of nucleotide compositions and ability to specifically hybridize with DNA may resolve the question.

In any event the eosomes, or a fraction of them, seem to be likely candidates for template material. There is as yet no binding evidence which requires the existence of an RNA other than that normally destined to be ribosomal RNA to carry out the function of information transfer from the DNA to the sites of protein synthesis.

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