The Structure and Function of Polyribosomes

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INTRODUCTION

It has been known for many years that ribosomes are the site of protein synthesis. However, it was only comparatively recently that we gained any insight into the nature of the process. Perhaps the most significant advance was recognition of the fact that the information necessary for the manufacture of a protein is not an integral part of the ribosome itself, but is in the form of an additional molecule, messenger RNA, the base sequence of which is related to the sequence of amino acids in the protein molecule (Brenner, Jacob, and Meselson, 1961; Nirenberg and Matthaei, 1961). It has been shown that three nucleotide bases in the messenger RNA strand are probably responsible for the coding of a single amino acid (Crick, Barnett, Brenner, and Watts-Tobin, 1961). Thus it is possible to think about the problem of protein synthesis in a quantitative manner. For a molecule such as hemoglobin, which has approximately 150 amino acids in each of its polypeptide chains, we anticipate that its messenger strand would contain about 450 nucleotides. If adjacent nucleotides are separated by 3.4 A, this would imply that the messenger RNA strand was about 1,500 A long. However, the inter-nucleotide spacing can be greater than this, and the messenger strand would be correspondingly longer. This immediately poses a problem, since we would like to know how this long strand interacts with the globular ribosomal unit which has a diameter of approximately 220 to 230 A. If the messenger RNA exists in an unfolded configuration, then it is evident that there is room for more than one ribosome on each messenger RNA strand. This is the physical basis of the polyribosomal structure.

Polyribosomes or polysomes are clusters of ribosomes which are held together by RNA (Warner, Rich, and Hall, 1962). As might be anticipated, the polyribosomal structure is somewhat fragile, since the ribosomal components are held together solely by an RNA strand. They were initially seen in preparations from reticulocytes (Warner et al., 1962; Warner, Knopf, and Rich, 1963; Gierer, 1963). With this cell it is possible to carry out a gentle osmotic lysis which ruptures the cell membrane and releases the cell contents without subjecting them to any grinding or centrifuging operation. The lysate can then be layered on a sucrose density gradient, centrifuged, and by puncturing the bottom of the centrifuge tube, fractions can be collected and examined. This process results in the separation of the constituents on the basis of sedimentation constants. To carry out such an experiment, intact reticulocyte cells are first briefly incubated with C^{14}-amino acids in order to load the ribosomes with labeled nascent protein. Following this, the cells are chilled, lysed osmotically, and the material placed on a sucrose gradient. The results of the experiment are illustrated in Fig. 1, in which the direction of sedimentation is to the left (Warner et al., 1962). It can be seen that there are two peaks in optical density, one sedimenting at 76S and another approximately two and

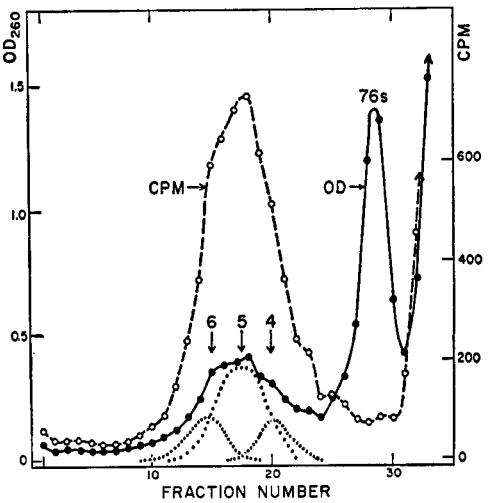


FIGURE 1. One milliliter of packed rabbit cells containing over 90% reticulocytes was incubated at 37°C in a final volume of 1.5 ml of saline containing 1 mg $NaHCO_3$, 2 mg glucose and 0.8 mg $Fe(NH_4)_2SO_4$. After 15 min incubation, 4 μc of C^{14}-amino acids from an algal hydrolysate were added, and 45 sec later the mixture was chilled by adding 25 ml of cold saline and the cells pelleted by low speed centrifugation. Osmotic lysis was carried out by adding 9 ml of standard buffer to the cells, and the cell walls were removed by centrifugation at $10,000 \times g$ for 15 min. One ml of lysate was layered on a sucrose gradient and centrifuged for 2 hr at 25,000 rpm in a Spinco Model L. The protein was precipitated by adding carrier serum albumin and trichloracetic acid. It was collected on Millipore filters and counted in an end window Geiger counter. The arrows labeled 4, 5, and 6 represented the sedimenting positions of ribosomal tetramers, pentamers, and hexamers. The dotted lines schematically represent their distribution in the gradient. Counts per minute are shown by the dashed line and OD_{260} by the solid line.

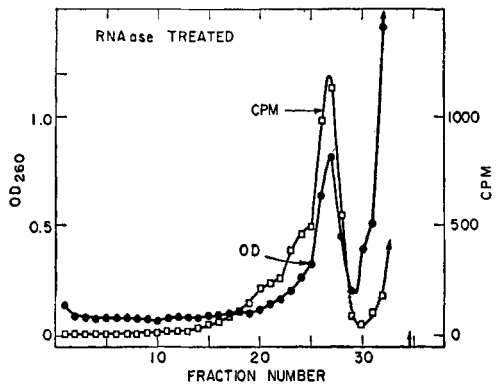


FIG. 2. Sucrose gradient of reticulocyte lysate as in Fig. 1 but incubated at 4°C for 1 hr with 0.25γ/ml RNase.

one-half times faster. The 76S peak represents the single ribosomes and the more rapidly sedimenting peak the polyribosomes. It can be seen that the radioactivity is associated predominantly with the rapidly sedimenting peak, showing that it is the site of protein synthesis. Electron microscopic examination of this material shows that the center of the main peak consists predominantly of pentamers of ribosomes as shown schematically by the dotted lines in Fig. 1. In addition, the distributions of tetramers and hexamers are also illustrated, and their centers are shown by the numbers. The polyribosomal structure is extremely sensitive to small concentrations of ribonuclease. On treating the reticulocyte lysate with 0.25γ/ml of ribonuclease (RNase) in the cold, the sucrose gradient distribution changes dramatically, as shown in Fig. 2 (Warner et al., 1963). It can be seen that the more rapidly sedimenting peaks have disappeared, and only the 76S single ribosomal peak remains. The nascent protein radioactivity has been quantitatively transferred from the polysomal peak into the single ribosomal peak. Such a result would be anticipated if the ribosomes are held together by RNA. By contrast, deoxyribonuclease has no effect on this distribution.

Thus, polyribosomes are most readily identified as a rapidly sedimenting peak with a sedimentation constant considerably greater than that seen for single ribosomes. The nascent protein migrates with the polysome peak, and the entire structure can be degraded into single ribosomal units by small amounts of ribonuclease.

THE STRUCTURE OF POLYRIBOSOMES

The polyribosomes from reticulocytes and HeLa cells have been studied in the electron microscope. While both of these are mammalian cells, they differ in a fundamental property, namely, the reticulocyte is specialized and is manufacturing a single protein, while the HeLa cell grows in tissue culture and manufactures the entire complement of proteins necessary for independent cellular life. To prepare polyribosome specimens for electron microscopy, a cell lysate or cytoplasmic extract is placed on a sucrose gradient and the polysomal material is isolated by centrifugation. Fractions from the gradient can be deposited directly on an electron microscope grid, washed, air dried, and shadowed with platinum. Micrographs obtained in this manner are shown in Fig. 3. A typical field of reticulocyte polysomes is seen in Fig. 3A (Warner et al., 1962). This was taken from the optical density peak in the polysomes, and it can be seen that the pentamer is the predominant species. A survey through the polysome region produces the distribution illustrated in Fig. 1.

However, the HeLa cell is not specialized and has a correspondingly broader distribution of polysomes (Penman, Scherrer, Becker, and Darnell, 1963; Goodman and Rich, 1963). Figure 3B shows a shadowed electron micrograph taken from a region with a sedimentation constant near 300 (Penman, Rich, Becker, Darnell, and Hall, 1963). These polysomes are much larger than those seen in the reticulocyte. Both of the specimens shown in Fig. 3 illustrate a type of characteristic clumping which is seen in air dried, shadowed preparations. It is likely that these configurations are determined largely by the surface tension forces associated with air drying. Nonetheless, in these photographs it is possible to measure the distance between adjacent ribosomal centers. This distance is approximately 300–350 A, which is considerably larger than the diameter of a single ribosome (220 A). This suggests that the ribosomes are not in direct contact with each other but are separated by a gap which is not shown clearly in this type of preparation.

However, reticulocyte polyribosomes have been studied more extensively by negative and positive staining techniques, and examples of these are shown in Fig. 4 (Slayter, Warner, Rich, and Hall, 1963). Figure 4A shows a typical field which consists of negatively-stained clusters of ribosomes. These are often separated, and in some cases, it is possible to see a thin, negative-staining thread bridging the gap between two adjacent ribosomes. Figures 4B and 4C show a field of reticulocyte polyribosomes stained positively with uranyl acetate. Under some conditions uranyl acetate is known to stain nucleic acids (Huxley and Zubay, 1960), and in these pictures we can see a thin 10–15 A diameter thread linking the various ribosomes. The positively-staining thread is seen to pass occasionally through the center, but often eccentrically, through the ribosome. Its size and staining properties are consistent with a single strand of RNA.

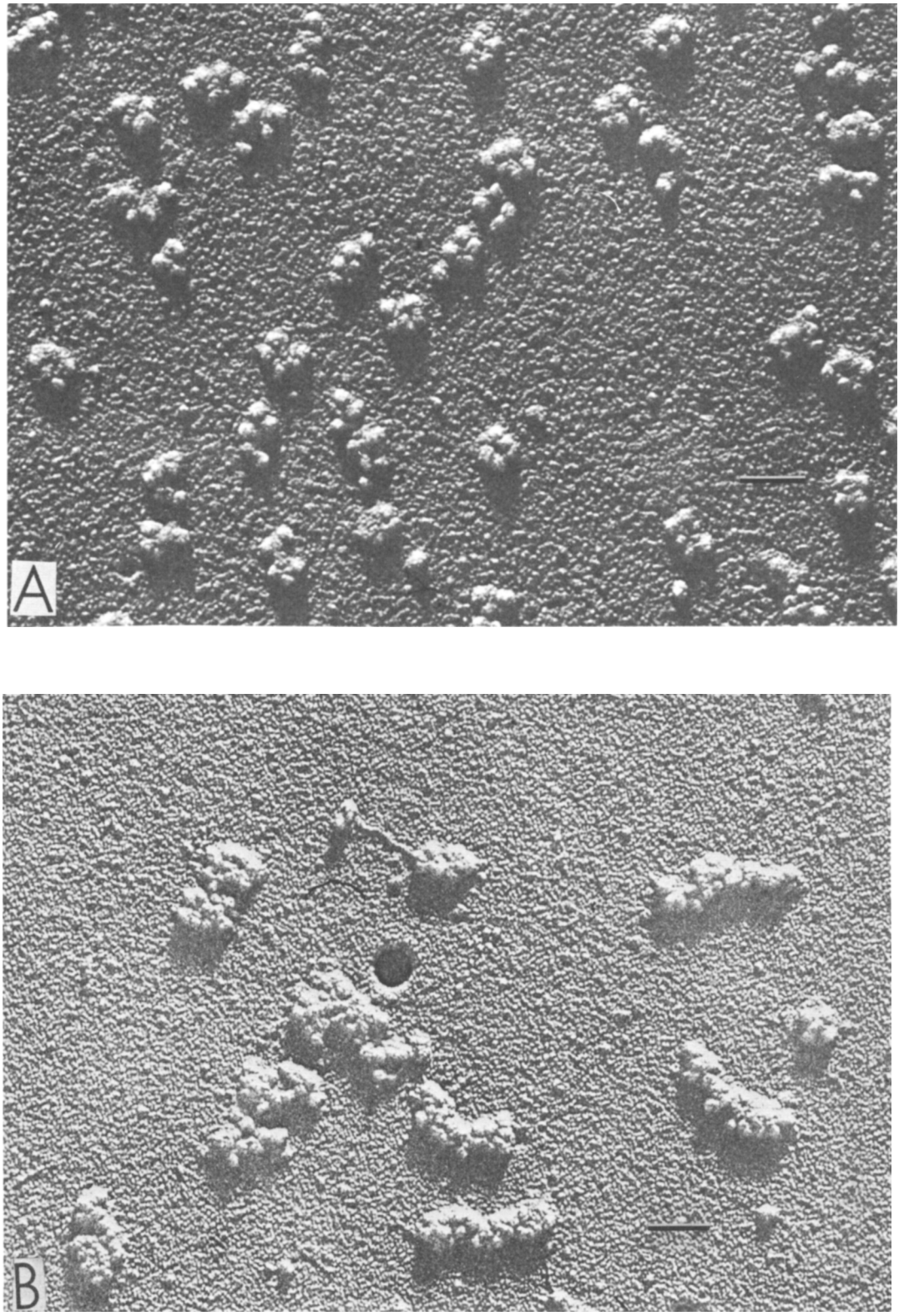
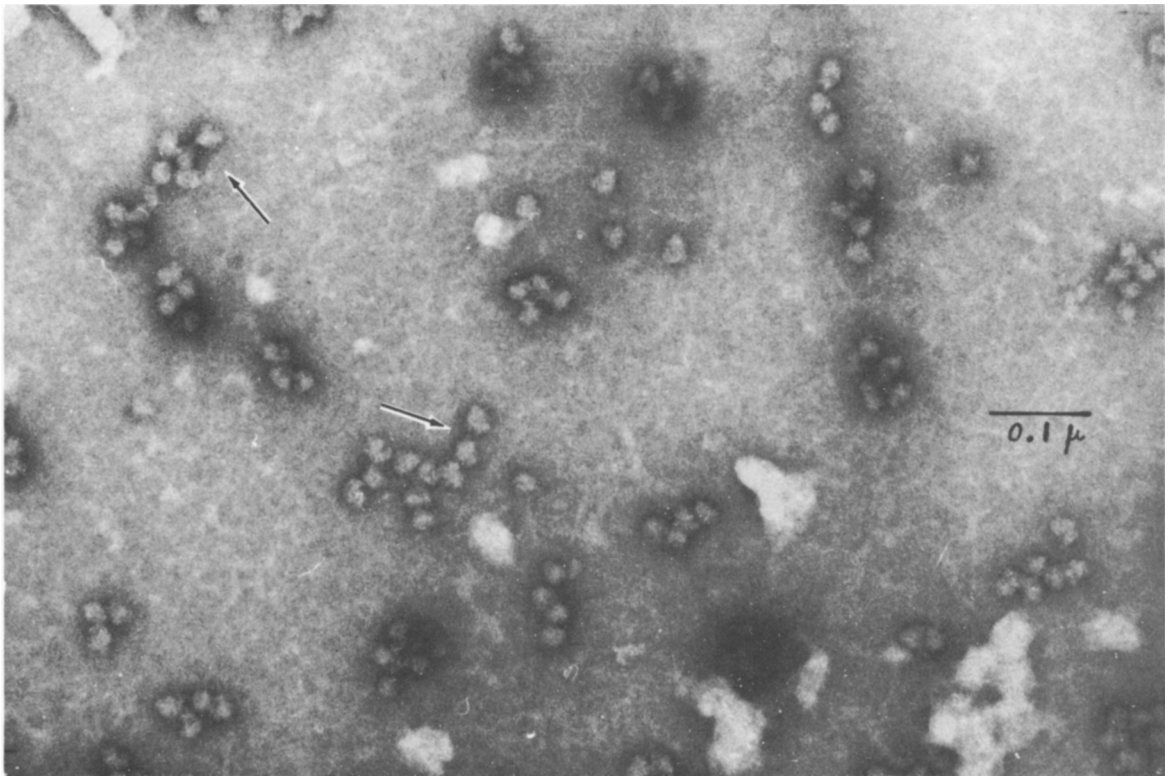


FIGURE 3. Electron micrographs of air-dried and platinum-shadowed polysomes. The horizontal mark in the lower right is 0.1 μ.

FIGURE 3A. A sample from the optical density peak of reticulocyte polysomes, as in Fig. 1. It can be seen that many of the clusters contain five ribosomes.

FIGURE 3B. A sample obtained from a HeLa polysome sucrose gradient with a sedimentation constant of approximately 300S. The polysomes are tightly clustered, which is probably a drying artifact. Although individual ribosomes are not clearly outlined, it can be seen that there are 15–20 ribosomes in some of the groups.



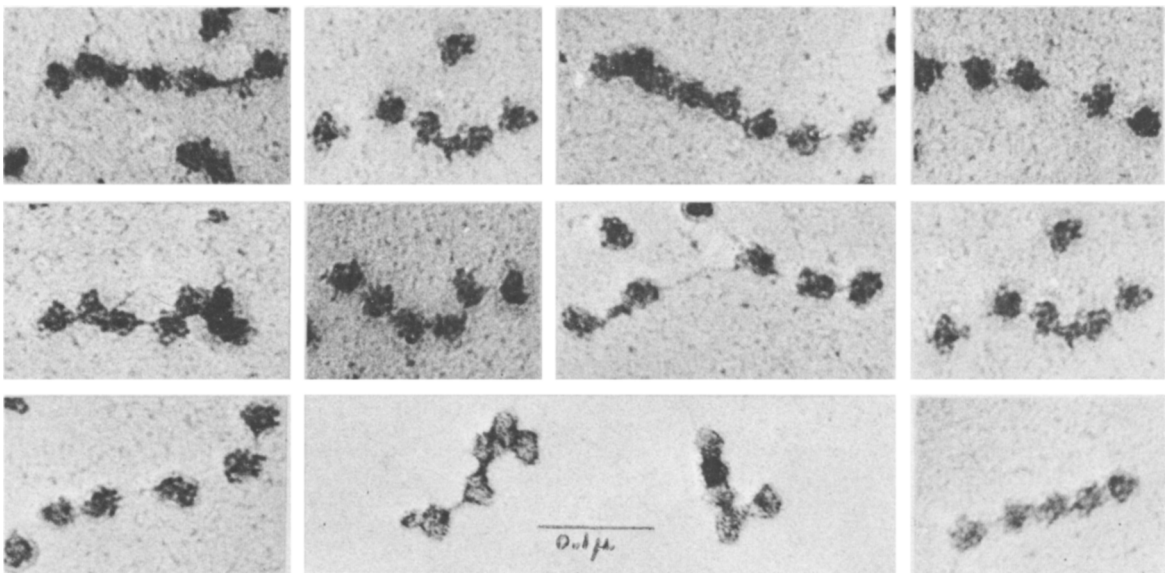


FIGURE 4B

FIGURE 4. Electron micrographs of reticulocyte polysomes. All samples are from the optical density maximum in the polysome region.

FIGURE 4A. Negatively-stained polysomes. Occasionally light strands can be seen bridging between adjacent ribosomes, as shown by the arrows.

FIGURE 4b. Positively-stained polysomes. Several examples are shown here to illustrate the extended configuration seen predominantly with this technique.

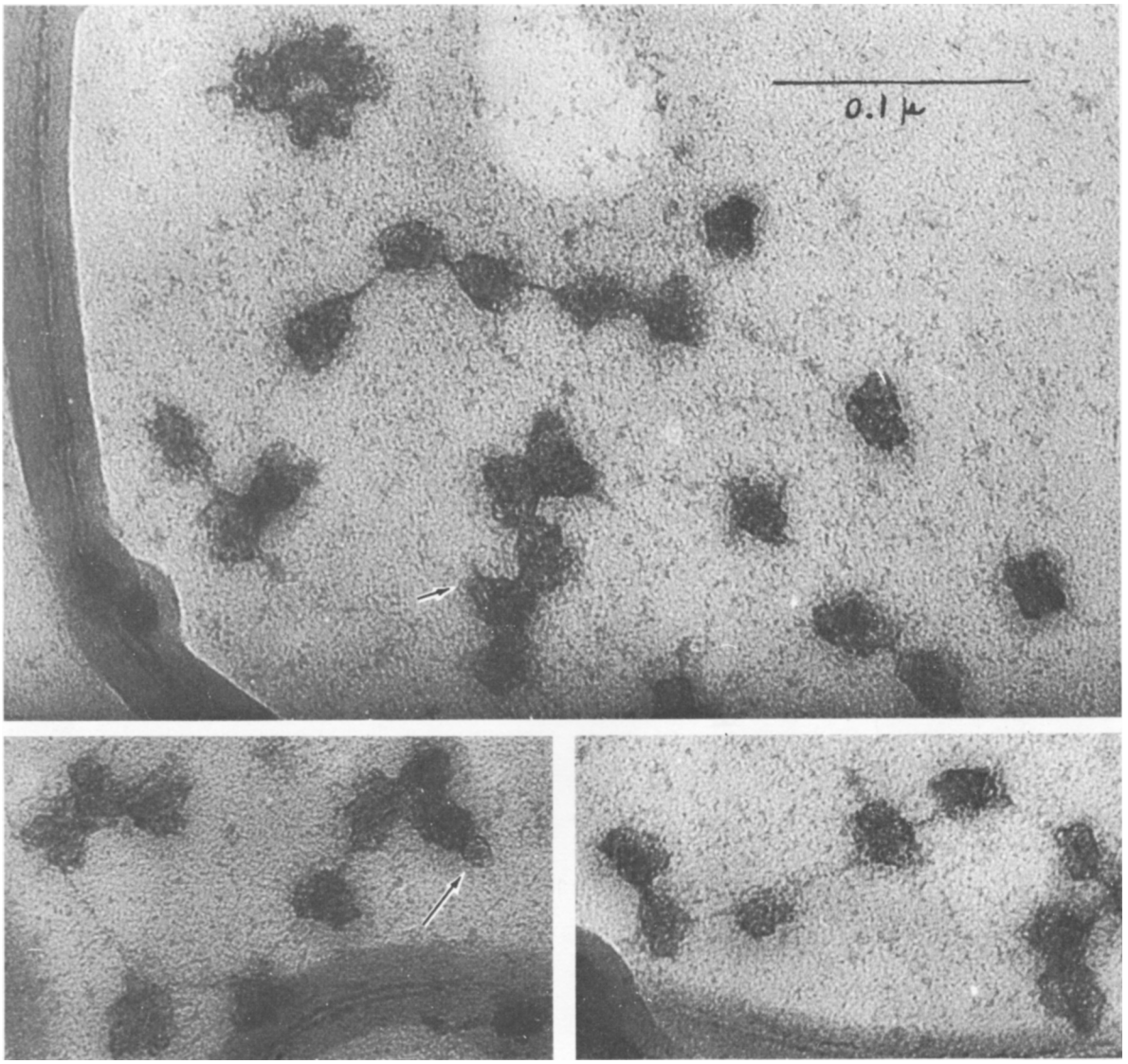


FIGURE 4C. Positively stained polysomes at higher magnification. Thin strands 10–15 Å in diameter can be seen stretching between ribosomes. Features of ribosomal substructure are shown by arrows.

Thus, in summary, the major features observed in the electron microscopy of polyribosomes are clusters of ribosomes which are separated from each other by gaps which range from 50–150 A. These are seen as linear structures in the positively-stained preparations, and they have a variety of sizes depending upon the particular cell from which the material is obtained.

THE FUNCTION OF POLYRIBOSOMES

When polyribosomes were discovered in reticulocytes, it was assumed that the RNA holding the ribosomes together was the messenger RNA. Electron microscope studies of polyribosomes from reticulocytes were consistent with this interpretation, since the overall length of the structure is in the vicinity of the 1,500 A which one would expect for a messenger RNA strand coding for a hemoglobin peptide chain (Warner et al., 1962). This assumption was also a reasonable one, since it is known that the synthetic messenger polyuridylic acid has the property of forming aggregates with bacterial ribosomes (Spyrides and Lipmann, 1962; Barondes and Nirenberg, 1962; Gilbert, 1963). Further evidence has accumulated which strongly suggests that messenger RNA holds the polysomes together. Actinomycin D is known to decrease the

production of messenger RNA, and it also decreases the number of polysomes (Penman et al., 1963; Staehelin, Wettstein, and Noll, 1963). Furthermore, pulse labeled RNA isolated from HeLa polysomes has sedimentation characteristics and base ratios compatible with messenger RNA (Penman et al., 1963).

Experiments such as those described in Fig. 1 showed that there was a natural distribution of polyribosome size in the reticulocyte which is manufacturing a single protein. Although the pentamer is the predominant species, the tetramer and hexamer are present to a lesser extent as well, and are equally as active in amino acid incorporation. It has been shown that proteins are synthesized by the sequential addition of amino acids starting from the N-terminal end (Dintzis, 1961). Thus, this natural polysome distribution was interpreted in terms of a mechanism whereby ribosomes attach themselves to one end of the polysomal cluster, and then gradually move along the messenger strand as the polypeptide chain increases in length by sequential addition of amino acids starting from the N-terminal end (Warner et al., 1962; Warner et al., 1963). At the end of the messenger chain, the ribosomes are believed to detach and the polypeptide chain is released. This process has also been discussed by Gierer (1963), Watson (1963), and Wettstein, Staehelin, and Noll (1963). Such a mechanism provides a ready explanation for the naturally occurring distribution of polysomes in reticulocytes. That is, random attachment at one end and random detachment at the other end of the messenger strand would produce a distribution of the type shown in Fig. 1. Furthermore, the fact that the ribosomes are separated from each other by gaps of 50 to 150 A means that it is unlikely that a polypeptide chain could start growing on one ribosome and then be transferred to an adjoining ribosome. In this interpretation the individual ribosome continues to be the site of protein synthesis, but the actual mechanism involves a large number of ribosomes working on the same messenger strand at the same time.

A schematic model of this concept of polysome function is shown in Fig. 5. An oncoming ribosome (diagonal shading) is shown without a polypeptide chain. This ribosome attaches at one end of the messenger RNA strand and begins to initiate the sequential addition of amino acids. The ribosome gradually makes its way along the messenger strand until all of the amino acids have been added. At the end of the messenger strand the ribosome and the completed polypeptide chain are released. This diagram shows five ribosomes which would be found in the case of a messenger RNA long enough to code for a 150 amino acid polypeptide chain, as

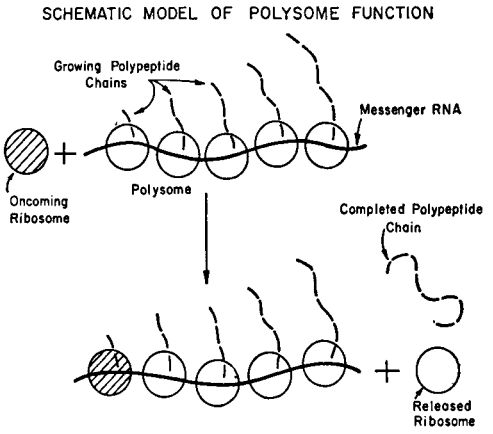


FIGURE 5. A schematic model of polysome function.

in hemoglobin. However, similar diagrams could be drawn, involving longer messenger RNA chains with more ribosomes which would be active in the synthesis of longer polypeptide chains. This would be the case in cells manufacturing a wide variety of proteins such as the HeLa cell.

It is possible to test a model of this type experimentally. In particular, one can make a number of predictions about the properties and behavior of this system under various experimental conditions. In the course of this paper we will outline a series of experimental observations which will relate to this concept of polysome function. These may be listed as follows:

A. The model predicts that the size of the polysome should be related to the size of messenger RNA coding for the protein being synthesized. In particular, the polysome size distribution in a cell synthesizing a single protein should be considerably different from that which is found in a cell synthesizing a large variety of proteins.

B. According to this mechanism polysomes contain a messenger RNA, while the single ribosomes do not. Accordingly, only the polysomes are active in protein synthesis in vivo. However, if we add a synthetic messenger RNA in vitro, we anticipate that only the single ribosomes would be able to accept the synthetic messenger.

C. The mechanism predicts that under protein-synthesizing conditions, single ribosomes should be able to attach themselves to polysomes. Furthermore, there should be a quantitative relation between the number of attached ribosomes and the number of polysome ends.

D. If we incubate polysomes under protein synthesizing conditions, the model predicts that there should be a gradual release of polypeptide chains, and the process should be energy dependent.

E. The model also predicts that single ribosomes should be released from polysomes as protein

synthesis occurs. This process should likewise be energy dependent.

F. Finally, there should be a quantitative relation between the number of growing polypeptide chains and the number of ribosomes in the polysome. Figure 5 shows one nascent chain per ribosome.

A series of experiments have been carried out using both reticulocyte cells which manufacture a single protein, and HeLa cells which manufacture a wide variety of proteins, to test these various predictions. The results of these experiments are described in the following sections.

A. The Relation Between Polyribosome Size and the Type of Protein Synthesized

It is instructive to compare the distribution of polyribosome size in two different mammalian cells. Figure 6 shows a superposition of two sucrose density gradients, one from the reticulocyte cell which is manufacturing hemoglobin, and the other from the HeLa cell which is manufacturing the wide variety of proteins that are found in a mammalian cell. The distribution of polyribosomes in the reticulocyte is similar to that shown in Fig. 1 with a predominance of pentamers. The optical density maximum in HeLa polysomes also occurs approximately at the pentamer. However, the distribution is not as narrow as in the reticulocyte but continues to extend out to considerably larger polysomes. The leading edge of the distribution sediments at approximately 350 S and contains polysomal clusters which have between 30 or 40 ribosomes in one group (Penman et al., 1963). It is difficult to determine the upper limit with great accuracy, since these very large polysomes are extremely fragile and break readily. Nonetheless, it is clear that the distribution in the HeLa cell ranges from a substantial number of di- and tri-ribosomal units up to polysomes which have 30 or 40 ribosomes connected together. This is in sharp contrast to the narrow distribution around the pentamers in the reticulocytes. The difference in distribution undoubtedly reflects differences in the proteins which are being manufactured and provides some indirect information which supports the idea that the length of the polysome is related to the length of the polypeptide chain being manufactured. However, it is clear that more direct experiments will be necessary to establish this point.

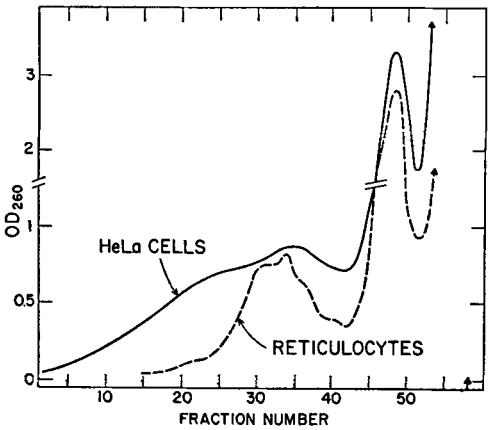


FIGURE 6. A superposition of the optical density curves obtained from 15–30% sucrose gradients of reticulocyte lysate and HeLa cytoplasmic extract. The large peak at Fraction 48 represents the sedimenting single ribosomes. The vertical arrow on the abscissa represents the last fraction.

B. The Activity of Single Ribosomes in the Presence of Added Synthetic Messenger RNA

Polyuridylic acid is the synthetic messenger for phenylalanine (Nirenberg and Matthaei, 1961). The object of the experiment described here was to determine the effect of added polyuridylic acid on the incorporation of C^{14}-phenylalanine in a reticulocyte lysate. Figure 7 (upper) shows the incorporation of this amino acid into hemoglobin in the absence of added polyuridylic acid. It can be seen that the bulk of the counts are incorporated in the polysome region in a manner similar to that seen in Fig. 1 where a variety of labeled amino acids were used. Figure 7 (lower) shows the effect of adding polyuridylic acid to this system. It can be seen that there has been a large stimulation of phenylalanine incorporation, all of which is associated with the single ribosomes.

In this experiment, the concentration of ribosomes and polyribosomes in the lysate is approximately one-fifth that which is found in the intact cell. We see that single ribosomes have taken up polyuridylic acid and started to synthesize polyphenylalanine, but the polysomes cannot add this synthetic messenger. However, because of the dilution of ribosomes, as well as the relatively large amount of polyuridylic acid which is added, the system has not been able to form polyuridylic acid polysomes. In short, the conditions of this experiment were chosen to emphasize the fact that the single ribosomes rather than the endogenous polysomes interact with polyuridylic acid. This suggests that there is no site for an additional messenger RNA molecule on a polyribosome which already has a messenger for hemoglobin production. Results similar to these have also been reported by Gierer (1963).

C. The Attachment of Single Ribosomes to Polysomes

The object of these experiments was to ascertain whether or not polysomes have the ability to add

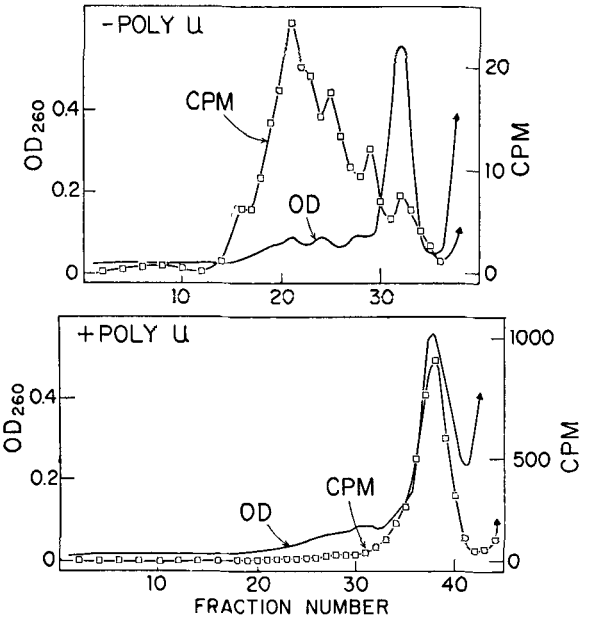


FIGURE 7. Reticulocytes were washed three times with saline and lysed with an equal volume of 0.01 M Tris-HCl (pH 7.4), 0.05 M KCl, 0.005 M $MgCl_2$. The stroma was removed by centrifugation for fifteen min at 10,000 $\times g$, and the upper portion of the supernatant dialyzed for two hr against the lysing buffer. The incubation medium contained per ml, 0.4 ml dialyzed lysate, 0.1 μmole of each amino acid excluding phenylalanine, 1 μmole ATP, 0.05 μmole GTP, 25 μmole phosphoenol-pyruvate, 40 γ pyruvate kinase, and 5 μc C^{14}-phenylalanine (60 μc/mole), with a final Mg^{++} concentration of 0.005 M. The polyuridylic acid concentration was 150 γ/ml. After seven min of incubation, the reaction mixture was diluted with one volume of 0.01 M Tris, 0.01 M KCl, chilled, layered on a 15–30% sucrose gradient containing 0.01 M Tris (pH 7.4), 0.01 M KCl, 0.0015 M $MgCl_2$ and centrifuged for 2.5 hr. Upper—no polyuridylic acid added. Lower—plus polyuridylic acid. Note the difference in radioactivity scales.

additional single ribosomes under the conditions of protein synthesis (Goodman and Rich, 1963). To carry out this experiment, cytoplasmic extract from HeLa cells was used because it contains a broad distribution of polysome sizes and thus allows us to explore quantitatively the relation between the number of attached ribosomes and the size of the polysome.

HeLa cells were grown for approximately one generation in medium supplemented with tritiated uridine. Cytoplasmic extract from these cells was placed on a sucrose gradient and single 74S ribosomes were isolated. To insure that this material was completely devoid of polysomes, a second sucrose gradient was carried out and only the center fraction from the 74S peak was used. When this preparation of tritiated single ribosomes is placed on a surcose gradient, it sediments as shown in Fig. 8 (curve with crosses). This shows that the single ribosomes have sedimented with a broad diffusion base and have a peak located in Tube 34.

For the incubation experiment tritiated ribosomes were added to an unlabeled cytoplasmic extract and incubated for a short time period (two or three min) under various conditions. Following this incubation, the mixture was chilled, centrifuged on a sucrose gradient, and analyzed for both optical density and radioactivity. The results are also shown in Fig. 8. The material is sedimenting to the left, and the single ribosome peak is at Tube 34, as shown by the solid line of optical density. The polysomes have a broad distribution of sedimentation constant which is similar to that illustrated previously in Fig. 6.

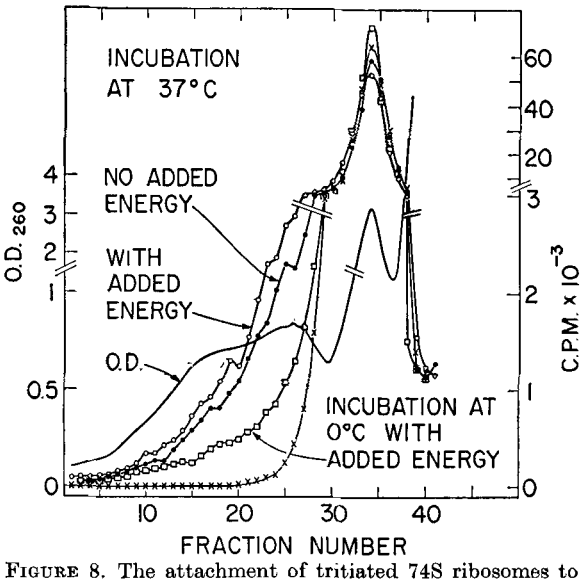


FIGURE 8. The attachment of tritiated 74S ribosomes to polysomes. 0.2 mg of 74S H^3-ribosomes were added to 1.2 ml of cytoplasmic extract and then incubated for three min. After incubation, the samples were chilled, and layered on a linear 15–30% w/w sucrose gradient (containing 0.1 M Tris pH 7.8, 0.05 M KCl and 0.005 M $MgAc_2$). The material was centrifuged for 105 min at 25,000 rpm in a Spinco SW 25 rotor. The bottom of the tube was punctured and the contents of the gradient were continuously analyzed for optical density at 260 mμ in a Gilford Spectrometer. Fractions were collected and counted in a scintillation counter. The curves are as follows: ribosomes alone (X), incubation at 37°C with no added energy (●), and incubation at 37°C with added energy (○), incubation at 0°C with added energy (□). 41 fractions were collected. The peak tube 34 is the 74S ribosomes. The components added for energy were 2.5 μM/ml ATP, 0.3 μM/ml GTP, 12.5 μM/ml phosphoenolpyruvic acid and 50 γ/ml crystalline pyruvate kinase. The H^3-ribosomes were isolated by preparing cytoplasmic extract from cells grown with H^3-uridine (2.6 c/mM; 1 mc/ml of culture medium) and then preincubating for 30 min to release single ribosomes from polysomes. The preincubation system contained 10 μM/ml Tris buffer (pH 7.8), 5 μM/ml $MgAc_2$, 5 μM/ml KCl, 6 μM/ml mercaptoethanol, the energy system above, and 0.07 μM/ml each of 20 naturally occurring amino acids. Following preincubation, the ribosomes were centrifuged (2 hr, 105,000 $\times g$), the pellet resuspended in unlabeled supernatant and then centrifuged for 325 min on a sucrose gradient. The 74S peak was collected and dialyzed overnight against standard buffer. The ribosomes were again centrifuged into a pellet and the 74S fraction was again isolated on a sucrose gradient.

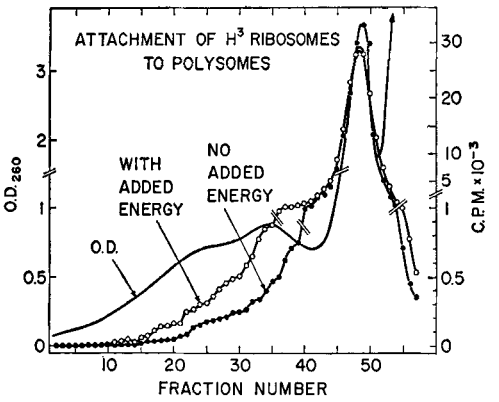


FIGURE 9. The experimental procedure is identical to that described in Figure 8, except that the incubation period was two min rather than three.

It can be seen (Fig. 8) that some of the tritiated ribosomes now sediment with the polysomes when they have been added to the cytoplasmic extract. Furthermore, there is even some displacement of tritiated ribosomes down the gradient after incubation at 0° C. However, incubation at 37° in the cytoplasmic extract results in a considerable increase in the number of ribosomes which are attached to the polysomes. The addition of more energy at 37° results in a further increase of attached ribosomes. This increase is not very large in the experiment shown in Fig. 8; however, it is considerably larger in the experiment illustrated in Fig. 9. There, approximately a 100% increase is seen in the number of tritiated ribosomes attached to the polysomes when additional energy is supplied to the system.

We have interpreted these experiments as indicating that it is possible to attach single ribosomes to polysomes during a short period of incubation. The experiments in Fig. 8 show that it is possible to obtain some attachment to a polysome at 0°C, even though there is no concomitant synthesis of a polypeptide chain at that temperature. It has also been shown that *E. coli* ribosomes will attach themselves to polyuridylic acid at 0°C (Barondes and Nirenberg, 1962). We believe that this attachment to HeLa polysomes is specific rather than nonspecific in character, since raising the temperature results in the synthesis of a polypeptide chain and the attachment of even more ribosomes. Furthermore, the addition of added energy to the cytoplasmic extract substantially increases the number of labeled ribosomes attached during the same time period. The attachment of ribosomes to polysomes in the cold suggests that the protein synthetic mechanism may be divided in two parts, ribosomal attachment to polysomes and the actual polymerization of the polypeptide chain. The former step does not have the same kinetic temperature coefficient as the enzymatic reactions which are necessary for protein synthesis to occur. It is of interest that reactions between polynucleotides have a much lower temperature coefficient than those associated with enzymatic reactions, and it is possible that the initial attachment of ribosomes to polysomes at 0°C may be a reaction of this type.

Experiments of the type shown in Figs. 8 and 9 can be interpreted quantitatively. In particular, we can ask what is the relation between the number of attached labeled ribosomes and the number of ends of messenger RNA? This can be calculated in the following way. Knowing the specific activity of the tritiated ribosomes, we can readily calculate the number of tritiated ribosomes which are found at any point along the gradient. Furthermore, by measuring the optical density, we can determine the number of ribosomes which are found in any fraction along the polysomal peak. We have only to determine the size of the polysomes as a function of the fraction number along the polysomal distribution in order to calculate the number of polyribosomal or messenger RNA ends. It is clear that the number of ends will be inversely related to the number of ribosomes in the polysomal unit and directly related to the number of ribosomes in that fraction.

We have been able to use three methods to deduce the number of ribosomes in the polysomal cluster as a function of fraction number. In some gradients it is possible to see fine structure or contours in the optical density profile which go out to as many as ten or twelve ribosomal units. Each of these contours represents an additional polysomal species. This has been shown most clearly in the experiments with hemoglobin polyribosomes where it has been possible to separate these peaks and examine them individually in the electron microscope (Warner et al., 1963). In this way we can determine the position along the gradient of species containing up to 10 or 12 ribosomes. In addition, it is possible to calculate an approximate sedimentation constant for polyribosomal clusters by assuming they exist in the form of an ellipsoid of revolution (Gierer, 1963). Finally, as mentioned above, the number of ribosomes have been determined in HeLa gradients directly by electron microscope studies (Penman et al., 1963).

Once we know the number of ribosomes in a particular fraction and the length of the polysomal chain in that fraction, it is possible to plot a curve as shown in Fig. 10 (solid curve) which shows the number of polysome ends as a function of the number of ribosomes in the polysome chain. This curve is shown, together with a curve showing the number of ribosomes found in the various fractions

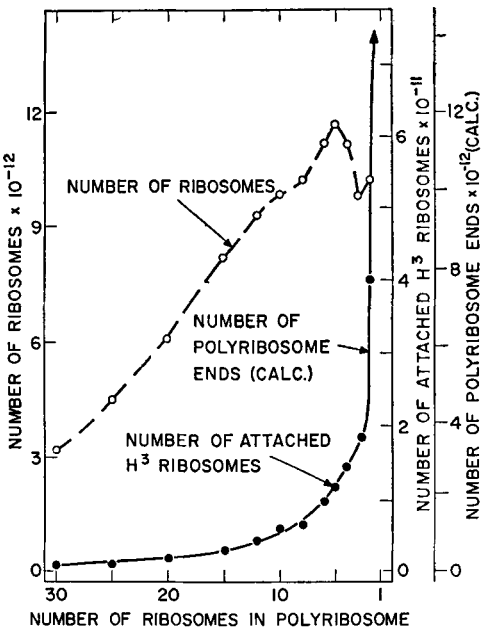


FIGURE 10. The relation between the number of polyribosome or messenger RNA ends and the number of attached tritiated 74S ribosomes after two min of incubation at 37°C in the cytoplasmic extract with added energy. The dashed line shows the number of ribosomes in various fractions as a function of the size of the polyribosome. The solid line is the calculated number of ends and the solid circles are the experimental points obtained from the data in Fig. 9.

(dashed curve). In addition to this data, the solid circles shown in Fig. 10 represent the number of labeled ribosomes which are attached to the various sized polysomal clusters from the experiments shown in Fig. 9. With a suitable adjustment of ordinate, it can be seen that these points fall almost precisely on the calculated solid curve showing the distribution of polysome ends. In the experiments shown in Fig. 9, approximately 5% of the polysome ends have a labeled ribosome attached to them after two min of incubation at 37°C in the presence of added energy. The number of labeled single ribosomes in this preparation was approximately one-third the number of unlabeled ribosomes in the cytoplasmic extract. Thus, it can be estimated that approximately 20% of the polysomes have added a ribosome during this short time period. However, it should be noted that the concentration of both ribosomes and polysomes in the cytoplasmic extract is less than one-tenth that which is found inside the cells in vivo. Therefore, it is quite likely that this amount of attachment represents only a small fraction of what is actually attaching in vivo during the same time period.

These experiments show that polysomes are able to have single ribosomes attached to them, and furthermore, the number attached is proportional to the number of polysomes but independent of its length. This suggests that all of the polysomes in the HeLa cytoplasmic extract have the same number of attachment sites, since the number of attached ribosomes is proportional to the number of ends. Since a messenger RNA strand is a polar molecule, we interpret this to indicate that there is a single attachment site on the polysome as is shown in the schematic diagram in Fig. 5.

D. The Detachment of Polypeptide Chains from Polysomes

Another type of experiment was devised to demonstrate the detachment of polypeptide chains during incubation for protein synthesis (Goodman and Rich, 1963). Polyribosomes were labeled in vivo by exposing HeLa cells to a 1.5 min pulse of C^{14}-amino acids. Following this, a cytoplasmic extract was prepared and the ribosomes and polysomes were then centrifuged into a pellet and the supernatant decanted. This pellet with the attached labeled nascent protein was resuspended in an unlabeled supernatant at the original volume. Thus the system was reconstituted with unlabeled amino acids and S-RNA, and the only radioactivity present in the system was that which had been pelleted with the ribosomes.

This material was incubated under conditions of protein synthesis as described in Fig. 11. At various times period samples were collected and sucrose gradients analyzed. At zero time (Fig. 11) it can be seen that most of the radioactivity is in the polysome distribution. The optical density curve differs slightly from that shown in Fig. 6, the difference being attributable to a small amount of breakdown of polyribosomes during the pelleting and resuspending procedure. Further evidence of this breakdown is seen in the small peak of radioactivity associated with the single 74S ribosomes at zero time. As incubation proceeds, the bulk of the radioactivity in the polysomes gradually decreases, and the amount of radioactivity at the top of the gradient increases. This material at the top of the gradient has been shown to be soluble protein. The distribution of radioactivity during this incubation process is shown in Table 1. At the starting time 75% of the counts are associated with the polysomes

TABLE 1. DISTRIBUTION OF RADIOACTIVE PROTEIN DURING IN VITRO INCUBATION

Time	Total counts	Per cent of counts in various fractions: Polyribosomes	Single ribosomes	Soluble protein
0	15,146	75	7	18
5′	14,276	58	11	31
10′	15,135	42	12	46
25′	17,158	18	14	68

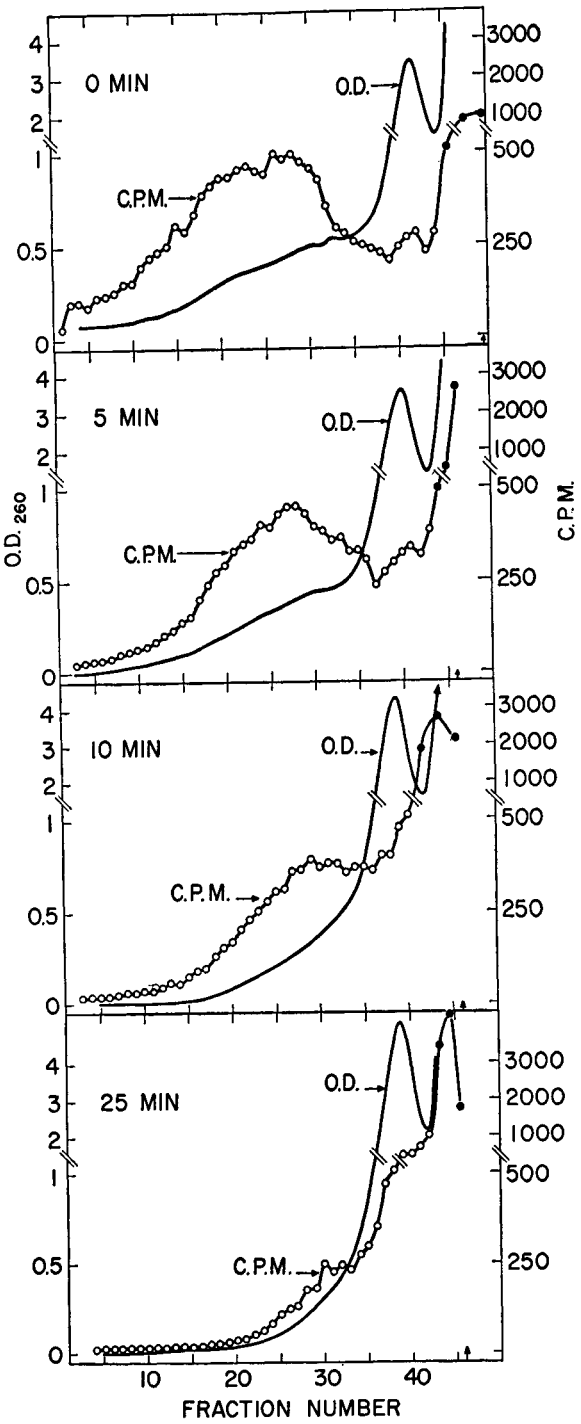


FIGURE 11. Sucrose gradients showing the results of an in vivo labeling with C^{14}-amino acids followed by an unlabeled in vitro incubation of the ribosomes. One liter of logarithmically growing HeLa cells (4×10^8 cells) were labeled for 1.5 min with 100 μc of C^{14}-algal protein hydrolyzate (1.6 mc/mg). Cytoplasmic extract made from these cells was centrifuged and the pellet was resuspended in 6 ml of unlabeled supernatant. After the addition of energy, the material was incubated at 37°C and 1.5 ml samples were removed at 0, 5, 10, and 25 min for sucrose gradient analysis. The fractions were prepared for counting by the addition of 0.5 mg of serum albumin carrier protein and trichloroacetic acid was added to a final concentration of 5%. The precipitate was plated on Millipore filters and counted. The samples in the soluble protein region (●) were prepared for counting by a modification of the method of Siekevitz (1952). The vertical arrow on the base line indicates the last fraction.

and 18% with the soluble protein, which is probably due to trace amounts of the radioactive supernatant in the pellet. After 25 min of incubation, the polysome counts now have only 18% of the total counts, while 68% are found in the soluble protein. Thus, incubation with cold amino acids under conditions which favor protein synthesis has resulted in the release of the bulk of the nascent protein radioactivity associated with the polysomes and their accumulation in soluble protein.

An important consideration is the possible effect of trace amounts of free ribonuclease. During incubation there was a small increase from 7 to 14% in the radioactivity associated with the single ribosomal peak. Earlier experiments with reticulocytes (Fig. 2) showed that small amounts of ribonuclease bring about a quantitative transfer of both polysomes and the nascent protein into the single ribosomal peak (Warner et al., 1963; Gierer, 1963). Thus it is conceivable that this increase from 7 to 14% may be associated with the ribonuclease present in this system. Separate experiments have been done to assay amounts of ribonuclease present in the cytoplasmic extract from HeLa cells. C^{14}-labeled polyuridylic acid was added to the incorporating system and its half life determined during incubation at 37°. In these experiments its half life was approximately 50 min, (Goodman and Rich, 1963) which is a marked contrast to the results of incubation in an *E. coli* cell-free extract in which the polyuridylic acid has a half life of less than one minute (Barondes and Nirenberg, 1962). These experiments therefore suggest that there is very little ribonuclease free in the HeLa cytoplasmic extract.

It is possible that the increase in counts in the single ribosomal region could have arisen by quite a different process. In cell-free incubations, the detachment of ribosomes is favored compared to their attachment. Accordingly, there is a gradual decrease in the size of the polysomal distribution during this incubation. A continuation of this process would finally lead to a single ribosomal unit which is still attached to its messenger RNA in the process of completing its polypeptide chain. The radioactivity of its nascent protein would then be found in the single ribosomal peak.

These experiments demonstrate that in vitro incubation with an amino acid incorporating system results in the liberation of radioactive polypeptide chains which were formed during an in vivo labeling. This release of polypeptide chains

is not caused by ribonuclease and occurs under conditions associated with protein synthesis.

E. The Detachment of Ribosomes from Polysomes During Protein Synthesis

In the previous section it was demonstrated that labeled nascent protein on polysomes formed in vivo is released from the polysome during in vitro incubation with unlabeled amino acids. In the present section we show that it is possible to release ribosomes from polysomes during incubation. Experiments are described here using both the reticulocyte lysate (Warner, 1963) and the HeLa cell extracts (Goodman and Rich, 1963).

A reticulocyte lysate was prepared in which the final concentration of polysomes and ribosomes was approximately one-tenth that which exists in the intact cell. When this extract is incubated with energy and labeled amino acids, the incorporation of radioactivity into both total protein and nascent protein attached to polysomes can be measured (Fig. 12). For the first five min most of the label is attached to the polyribosome, but as the incubation continues, most of the radioactivity is found in the form of free protein at the top of the sucrose gradient.

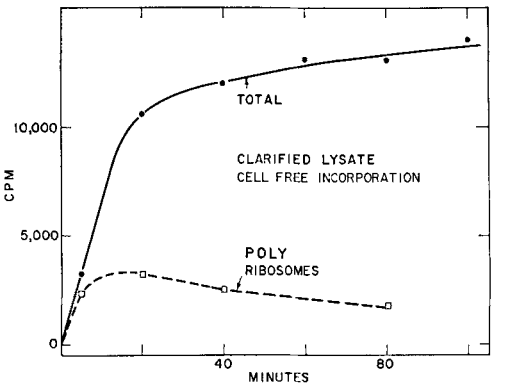


Figure 12. Washed cells were lysed by the addition of an equal volume of 0.0015 M $MgCl_2$. After removal of the stroma, the lysate was dialyzed for 16 hr against 0.01 M Tris (pH 7.4), 0.01 M KCl, 0.0015 M $MgCl_2$, and 0.005 M mercaptoethanol. The reaction mixture was as described in the caption to Fig. 7, except that the concentration of lysate was 0.2 ml/ml; 1.3 μc/ml of C^{14}-algal hydrolyzate (1.6 mc/mg) was used instead of phenylalanine and only the amino acids missing from the algal hydrolysate were added to the incubation mixture. 1.2 ml aliquots were removed at various times. 1.0 ml was analyzed on a sucrose gradient (see Fig. 1), and 0.1 ml was prepared for counting by the Siekevitz (1952) procedure. The data for the polyribosome curve in Fig. 12 was taken from the gradients in Fig. 13.

The sucrose gradient analysis of this experiment is shown in Fig. 13. At five minutes the optical

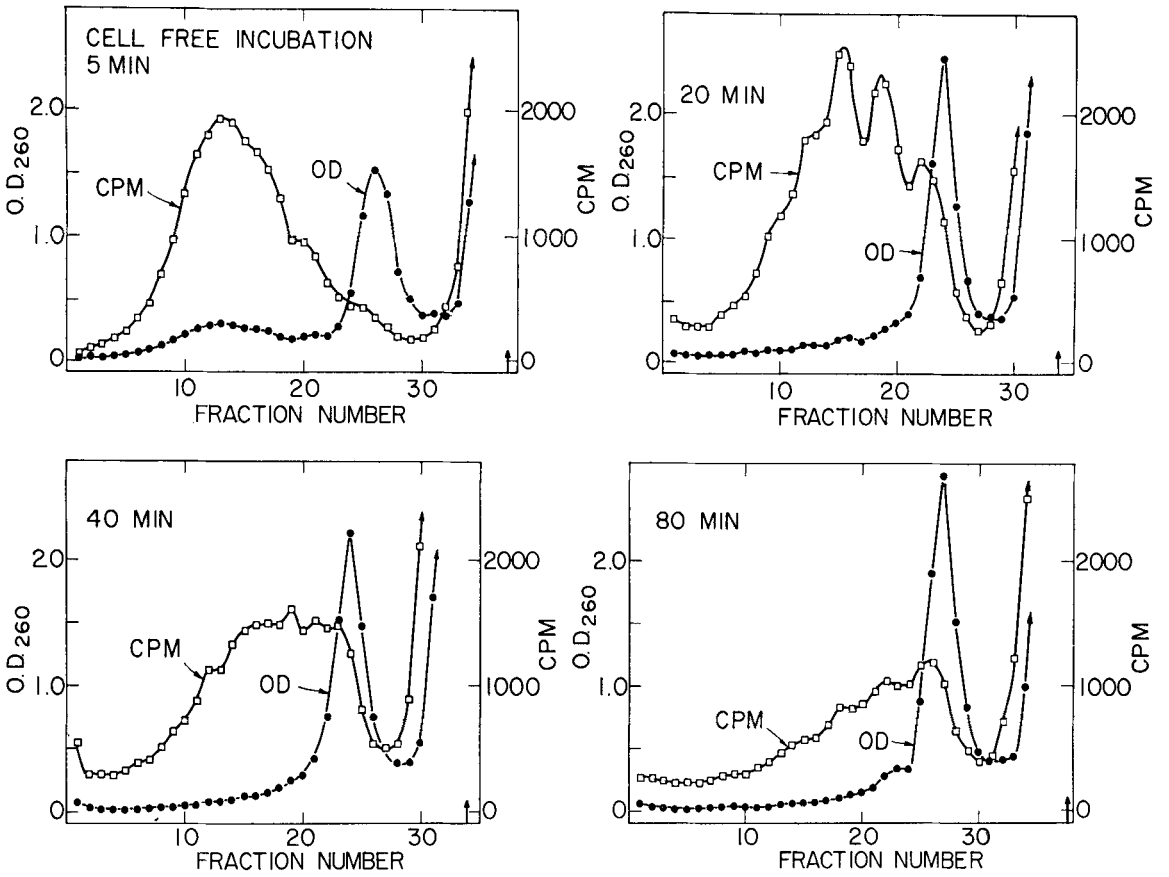


Figure 13. 1 ml portions of the incubation mixture described in Fig. 12 were analyzed on a sucrose gradient as described in Fig. 1. The centrifugation was for 2.5 hr.

density and radioactivity distribution is very similar to that seen in an in vivo incorporation. Most of the counts are found in the polysome region in a peak which has sedimented two and one-half times the distance of the single ribosomal peak and is largely in the ribosomal pentamer. By 20 min the radioactivity peak is associated with the tetramer region, and there is a corresponding decrease in the optical density of the polysome peak. By 40 min the radioactivity peak is spread through the regions of polyribosomes of length 2, 3, and 4, while at 80 min the maximum is clearly in the single ribosomal position. At the same time there is a corresponding rise in the optical density of the single ribosomal peak during this incubation. This shows a gradual decrease in polyribosomal size which accompanies cell-free incubation.

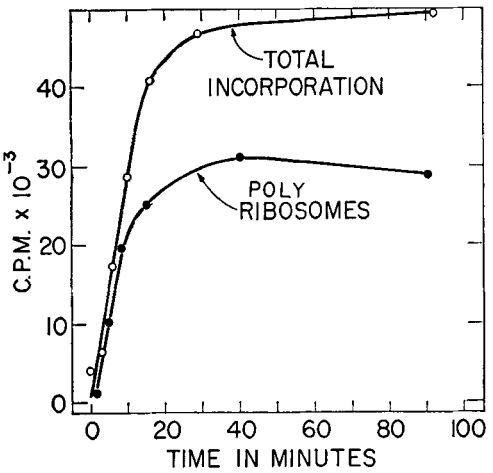


FIGURE 14. The kinetics of C^{14}-amino acid incorporation into cytoplasmic extract. The incubation conditions were as in Fig. 8, except that 8 μc/ml of C^{14}-algal protein hydrolyzate was added at zero time. 0.1 ml samples were removed at various time periods, and the data is presented for the 1.5 ml samples which were used in Fig. 15. The total incorporation was counted by the modified Siekevitz method (1952). The polyribosome curve was obtained from the sucrose gradients shown in Fig. 15a.

A similar experiment was carried out with the HeLa cytoplasmic extract, and the results are shown in Fig. 14 (Goodman and Rich, 1963). In this system a larger percentage of the counts remains attached to the polysomes compared to the reticulocyte results shown in Fig. 12. However, this system is manufacturing a variety of proteins, including some which are very large, and this may be related to the higher percentage of counts found in the polysomes. During the first 10 min or so, most of the polysomes are taking up radioactive amino acids rapidly, and there is very little release of protein. Samples were isolated for sucrose gradient analysis at various time periods, and the results are shown in Fig. 15. Figure 15a shows that the radioactivity is incorporated continuously throughout

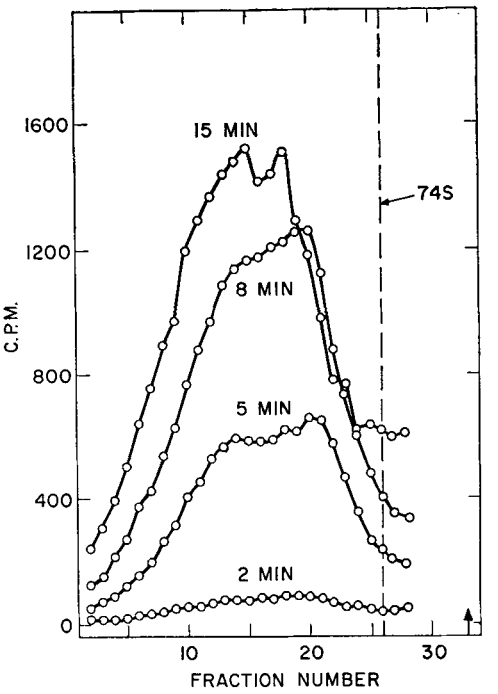


FIGURE 15. Sucrose gradient analysis of in vitro amino acid incorporation. The conditions for incorporation are as in Fig. 11. 1.5 ml samples were removed at various time periods and analyzed on the sucrose gradient as described previously.

FIGURE 15a. The radioactivity associated with the polysome peak for various time periods.

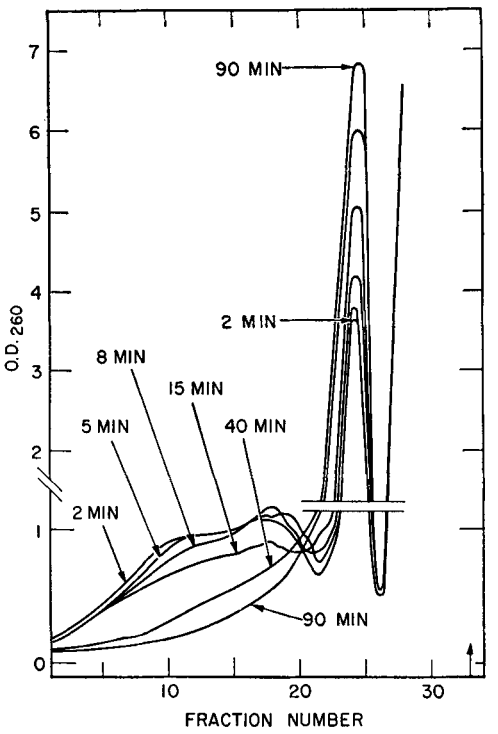


FIGURE 15b. The optical density of gradients at various time periods of in vitro incubation. The peak height in the 74S region rises steadily with time from 2 min to 90 min.

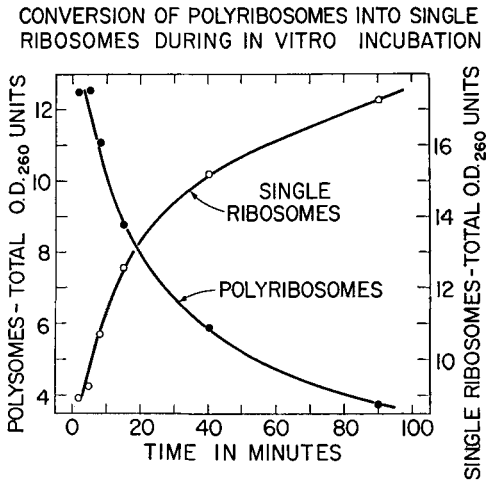


FIGURE 15c. The change in total optical density of polysomes and single ribosomes as a function of time.

the entire polysome region. All sizes of polysomes are active in protein synthesis, in agreement with the attachment experiments which show that ribosomes are attached to all the different lengths of polysomes. The total radioactivity associated with the polysomes increases during the incubation process. Somewhat similar observations have been made by Noll, Staehelin, and Wettstein (1963) in their experiments with rat liver polysomes.

The optical density profile is shown as a function of time during the incubation in Fig. 15b. It can be seen that there is a gradual decrease in the size distribution of polysomes and a corresponding increase in the optical density accumulated in the single ribosome region. This result might be anticipated in an in vitro system in which the rate of attachment of ribosomes to polysomes is much less than the rate of detachment. Thus the effect of incubation is to bring about a quantitative conversion of polyribosomes into single ribosomes, as shown in Fig. 15c.

These experiments show that in vitro incubation is accompanied by a gradual decrease in polysome size, and the liberation of ribosomes from polysomes. By carrying out appropriate controls in which dialyzed cytoplasmic extract is used, it has been demonstrated that this process is energy-dependent as is protein synthesis. With a dialyzed cytoplasmic extract, incubation at 37°C does not bring about the changes seen in Fig. 13 or 15b.

F. The Number of the Polypeptide Chains per Ribosome

The reticulocyte polyribosome system is advantageous in several respects for the determination of the number of growing polypeptide chains per ribosome (Warner and Rich, in preparation). In the first place, the reticulocyte is making a single protein, hemoglobin, whose composition, as well as amino acid sequence is known (Borsook, Fischer, and Keighley, 1957; Naughton and Dintzis, 1962; Diamond and Braunitzer, 1962). Furthermore, in this system we are able to separate the inactive ribosomes from the active ones.

To obtain a reliable estimate of the number of growing chains per ribosome, it is necessary to use the intact cell, because cell-free incorporation systems operate at a considerably reduced level, and may therefore introduce artifacts. The experiment is carried out by adding a fixed amount of labeled leucine of known specific activity and measuring its incorporation in polysomes. To overcome the problem of the intracellular pool of amino acids, one can add varying amounts of the radioactive amino acid to a given volume of cells and measure the difference of incorporated radioactivity due to dilution by the nonradioactive pool. In this experiment, three quantities of C^{14}-leucine were added to an incubation mixture containing 0.2 ml of packed reticulocytes (Table 2A). After

TABLE 2
A. CALCULATION OF INTERNAL LEUCINE POOL OF RETICULOCYTES

Experiment 1 Tube	μmoles C^{14} leucine	specific activity (cpm/OD)
a	0.02	346
b	0.05	606
c	0.20	1018
Tubes compared	calculation of internal pool of leucine (mμmoles)	
a–b	50	
b–c	55	
a–c	58	
	average calculation of internal pool (mμmoles)	(μmoles/ml packed cells)
Experiment 1	54	0.27
Experiment 2	51	0.25

TABLE 2
B. CALCULATION OF NUMBER OF NASCENT POLYPEPTIDE CHAINS PER RIBOSOME

	No. leucine/ ribosome	No. growing chains/ ribosome
Experiment 1		
10 min sample	7.2	0.91
Experiment 2		
5 min sample	7.3	0.92
15 min sample	7.6	0.96

0.2 ml aliquots of reticulocytes, diluted to 0.9 ml with saline, were incubated at 37° as described in Fig. 1, with the addition of 0.2 μmole of each of the nineteen amino acids, excluding leucine. After 15 min of incubation, aliquots of C^{14} leucine (25.3 μc/μmole) were added and the incubation continued for 10 min. The cells were chilled, washed, lysed, and the lysate analyzed in sucrose gradient as described in Fig. 1. The specific activity shown in Column 2 of Table 2 A is derived from the six to eight tubes encompassing the polysome peak. The calculations are described in the text.

incubation for 10 min, the cells were chilled, lysed, and sucrose gradients analyzed as illustrated in Fig. 1. The specific activity of the polysome peak region was determined, and the internal pool calculated assuming complete mixing of intra- and extra-cellular amino acids (Table 2A). The assumption of rapid equilibrium is reasonable, since several experiments yielded approximately the same answer for the internal pool. Furthermore, data has been presented by Lingrel and Borsook, (1963), which indicates that there is a rapid equilibrium even with external leucine concentrations ten times greater than that used in this experiment.

Now, using the actual specific activity of leucine inside the cell, and the extinction coefficient and molecular weight of the ribosomes (Ts'o and Vinograd, 1961; Dintzis, Borsook, and Vinograd, 1958), it is possible to calculate the number of leucine residues per ribosome in the polysome region (Table 2B). The sequence data on rabbit hemoglobin shows that there are 17.5 leucine residues per average ($\alpha + \beta$) chain, with the leucine displaced by 10% toward the carboxyl end of the chain. Furthermore, the experiments of Naughton and Dintzis (1962) strongly indicate that the length of the growing polypeptide chain is random. Using these facts, one can calculate the number of growing chains per ribosome. As shown in Table 2B, the results lie between 0.91 and 0.96. Assuming the possibility of a 10% error in such a calculation, it seems clear that there is one growing chain per ribosome.

This figure, of course, contains the assumption that all the ribosomes in the polysomal pool are active in protein synthesis. However, on investigating this point, we have not been able to detect a polyribosomal fraction inactive in protein synthesis in this system.

DISCUSSION

Polyribosomes have now been demonstrated in a variety of different organisms. They have been found in several mammalian tissues, including reticulocytes, liver, and tissue culture cells. In chick embryos it is possible to demonstrate the existence of polysomes in cartilage, brain, muscle, skin, and eye lens. Similar investigations have been carried out in other phyla, and polysomes have been isolated and characterized from the protozoan *Tetrahymena* (W. Phillips, 1963, personal communication), primitive slime molds (Phillips, Rich, and Sussman, 1963) and bacteria, including protoplasts of *B. megatherium* and *E. coli* (P. C. Fitz-James, 1963, personal communication; Kiho, Zubkoff, and Rich, 1963, in preparation). There is also evidence for their existence in pea seedlings (Raacke, 1963, personal communication). Thus, they appear ubiquitous.

Although we have been able to answer a few questions concerning the functional activity of polysomes, we are left with a large number of unanswered questions, and it is worth listing some of them here. For example, what is the nature of the ribosomal attachment site on the polysome? Is there a specific position on the messenger strand to which ribosomes will attach and which serves as the site for initiating protein synthesis? Alternatively, is there a different mechanism which prevents ribosomes from adding onto polysomes in the middle of a cistron, i.e. in the middle of a region which is coded for a particular polypeptide chain? Are there polycistronic messenger RNAs in the polysomes of normal cells? It has been suggested that a series of cistrons under the control of a given operator gene may act in sequence at the polysome level (Warner, et al., 1963). Thus a ribosome may start at the end of a polycistronic messenger and work its way along past several cistrons, sequentially releasing the proteins. Is there also an alternative mechanism in which a polycistronic messenger operates so that the ribosome is able to initiate protein synthesis in the middle of a messenger RNA strand, between two cistrons? It is possible that a system of this type may be found in viral RNA molecules (Penman, et al., 1963), but more work must be done to identify and clarify this mechanism.

In a similar fashion one might ask whether there is a specific detachment site which couples the release of the polypeptide chain with the release of the ribosome. These two events may be coincident in time, but may also be separate. They may occur at the end of the messenger RNA strand, but this is not necessary.

We have described a relative movement between the messenger and the ribosome. Nothing is known of the nature of this movement, except that it must have a rachet-like property in that the movement is unidirectional, stepwise in nature (the steps may be 10 A long if they involve a codon of three nucleotides) and presumably must not reverse itself. Clearly a deeper understanding of this mechanism must depend upon learning more about the structure of the ribosome.

In comparing the polysomal size distribution in the HeLa cell with the reticulocyte cell (Fig. 6), we see that the most frequent species is the pentamer. This suggests that the largest number of polypeptide chains being manufactured are those with a molecular weight near that found for one of the hemoglobin subunits (17–18,000). It is of interest that similar distributions are found in polysomal

preparations from chick embryo, *Tetrahymena*, slime molds, and *B. megatherium*. We assume that this implies that the most frequent cistron that is found contains structural sequence information for a molecule in the neighborhood of 150 amino acids. However, it should be pointed out that the association of a protein with a larger polysome does not necessarily imply that its polypeptide chain is longer. Indeed, it is possible that some of the larger polysomes may be polycistronic and carry structural information for more than one protein. Further research will be necessary to resolve this point.

One additional consideration is worth mentioning. Sucrose gradient centrifugation of polysomes separates them into different sizes, since the movement depends upon the sedimentation constant. If it is established that an individual protein is associated with a characteristic size polysome in a manner analogous to that seen for hemoglobin, then it is clear that this technique can be used as a dispersion tool for separating different classes of nascent proteins. Furthermore, this may then enable us to learn something about the size of the cistron coding for the individual protein.

CONCLUSION

In this article we have described a variety of experiments which have been carried out on polyribosomes both from reticulocytes and HeLa cells. These systems have contrasting advantages, since the former manufactures a single protein and the latter a variety of proteins. It has been possible to demonstrate a number of reactions which are compatible with the general scheme of polyribosomal function outlined in Fig. 5.

ACKNOWLEDGMENTS

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DISCUSSION

SCHWEET: What evidence do you have that all the ribosomes in the reticulocyte polysomes are active in protein synthesis? This is an important point in deriving your figure of one growing polypeptide chain per ribosome.

WARNER: We have two indications that there is only one class of polysomes in reticulocytes.

(a) It is possible to fractionate reticulocytes in a serum albumin gradient, where the lighter cells are more active in protein synthesis than the heavier cells (H. Borsook, J. B. Lingrel, J. L. Searo, and R. L. Milette, 1962, Nature *162:* 347). We have examined the polysomes from three of such fractions, where the ratio of polysomes to single ribosomes ranged from 1.4 in the lightest fraction to 0.8 in the heaviest. In all three fractions the specific activity of the polysomes was the same.

(b) If one follows the breakdown of the polysomes as a result of lowering the magnesium concentration of the medium, the specific activity of the polyribosome remains constant. Thus there is no inactive fraction which is more sensitive to lowered magnesium concentration than the active polysomes. It should be noted, however, that if the ribosomes are pelleted and resuspended, many of the single ribosomes will stick to the polysomes. It is not unlikely that these would be prematurely released by changing the ionic environment.

Although these two pieces of data do not prove that all of the polyribosomes are active, they support such an idea as a reasonable working hypothesis.

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The Structure and Function of Polyribosomes

Alexander Rich, Jonathan R. Warner and Howard M. Goodman

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