[11] Jukes, T. H., these PROCEEDINGS, **48**, 1809 (1962).

[12] Wahba, A. J., C. Basilio, J. F. Speyer, P. Lengyel, R. S. Miller, and S. Ochoa, these PROCEEDINGS, **48**, 1683 (1962).

[13] Weissblum, B., S. Benzer, and R. W. Holley, these PROCEEDINGS, **48**, 1449 (1962).

A MULTIPLE RIBOSOMAL STRUCTURE IN PROTEIN SYNTHESIS

BY JONATHAN R. WARNER,* PAUL M. KNOPF,† AND ALEXANDER RICH

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

Communicated by John T. Edsall, November 3, 1962

It has been well established that the ribosomal particles are the site of protein synthesis, yet we have very little insight into the mechanism.[1] A great deal of attention has been directed toward the question of how the ribosomes contain the information necessary to effect the alignment of amino acids in a specific sequence. This problem has been resolved recently with the discovery of a rapidly metabolizing fraction of RNA, called messenger RNA, which has the ability to attach itself to the ribosomal particle and there to determine the sequence of amino acids.[2, 3] This view has been considerably reinforced by *in vitro* experiments in which naturally occurring RNA, as well as synthetic polyribonucleotides, have been shown to provide the information necessary to determine the sequence of amino acids in a polypeptide chain.[4, 5] Thus, the ribosome has a passive role in transmitting information; it can apparently polymerize a variety of proteins, depending upon the particular messenger RNA which is attached to it.

However, this state of affairs has puzzled many investigators for some time. The purely geometric aspects of the messenger RNA-ribosomal interaction leave several unresolved questions. For example, the polypeptide chains in the hemoglobin molecule each contain roughly 150 amino acids and, using a triplet code,[6] this implies a messenger of 450 nucleotides or a molecule 1,500 Å long if there is one nucleotide every 3.4 Å. How can this long polymer molecule transfer all of its sequence information to the ribosomal site at which the polypeptide chain is believed to grow? This becomes an even greater puzzle if one considers that much longer messenger RNA molecules have been found.[5] Indeed, the length is so great that it would almost be physically impossible for one ribosome 230 Å in diameter to interact with the entire messenger chain. However, Risebrough *et al.*[7] have shown that "heavy ribosomes" are seen in a sucrose gradient when labeled T2 messenger RNA is attached to *E. coli* ribosomes. More recently, several investigators[8–10] have reported that polyuridylic acid induces the formation of a rapidly sedimenting ribosomal peak when it is added *in vitro* to a cell-free bacterial extract. We have therefore been prompted to look for the possible existence of a larger multiple ribosomal structure *in vivo* which might provide insight into the detailed mechanism of protein synthesis.

In these experiments, we have used reticulocyte ribosomes because it is possible to break open the reticulocyte cell wall gently with a minimum of mechanical manipulation. This approach has been successful, and we have been able to demonstrate the existence of a multiple ribosomal structure held together by RNA. In

the reticulocyte, the predominant species contains five ribosomes. Furthermore, our experiments indicate that protein synthesis in the reticulocyte occurs only on this structure and not on a single ribosomal unit.

Materials and Methods.—Blood containing 80–90 per cent reticulocytes was collected by heart puncture from rabbits made anemic with phenylhydrazine as described by Borsook.[11] The cells were washed in a low magnesium saline (0.14 *M* NaCl, 0.005 *M* KCl, 0.0015 *M* $MgCl_2$) and then incubated at 37° in the presence of 0.2 mg/ml glucose, 0.12 mg/ml $NaHCO_3$, and 0.08 mg/ml Fe $(NH_4)_2(SO_4)_2$. After 15-min incubation, C^{14}-amino acids were added from an algal hydrolysate to a final concentration of 4 μc/ml. In one experiment, H^3 leucine was used. The reaction was stopped after 45 sec by the addition of cold saline. After centrifuging, the cells were gently lysed with two volumes of 0.0015 *M* $MgCl_2$ and then diluted with 3–5 volumes of standard buffer (0.01 *M* Tris, pH 7.4; 0.01 *M* KCl; and 0.0015 *M* $MgCl_2$). Cell walls and unbroken cells were removed by centrifugation at 10,000 × *g* for 15 min and the supernatant carefully decanted.

Linear sucrose gradients were made in Spinco SW 25 tubes using either 15 and 30 per cent w/w sucrose or 5 and 20 per cent w/w sucrose solutions containing the standard buffer. 1 ml of the lysate was layered on the sucrose gradient and centrifuged at 25,000 rpm (55,000 × *g*), for a 2-hr period at 5°C. At the end of the centrifugation, the bottom of the tube was punctured and the solution withdrawn by use of a finger pump and collected in 35–55 fractions. The optical density of the fractions was read at 260 mμ. After the addition of carrier serum albumin (0.1 mg/ml), the fractions were precipitated in trichloroacetic acid (5 per cent final concentration) and collected on Millipore filters. C^{14} radioactivity was counted in a Nuclear-Chicago low-background gas flow counter and tritium in a Packard Tricarb Scintillation Counter. C^{14} algal hydrolysate (1.6 mc/mg) and H_3 leucine (3.6 curies/millimole) were obtained from the New England Nuclear Corporation. Ribonuclease (RNAase, 3 × crystallized) and deoxyribonuclease (DNAase, 1 × crystallized) were obtained from Worthington Biochemical Company.

Results.—When the cell contents were isolated by gentle osmotic lysis, a characteristic sucrose density gradient pattern was produced, as in Figure 1(*a*). In addition to the large amount of hemoglobin that remained at the top of the tube, there are two optical density peaks, one sharp and the other heavier and broader. However, it is the heavy peak which contains all of the radioactivity that has sedimented appreciably from the meniscus. Occasional preparations which were handled less carefully produced sucrose gradients that showed some irregularities of the type illustrated in Figure 1(*b*). These will be discussed below. Similar results were

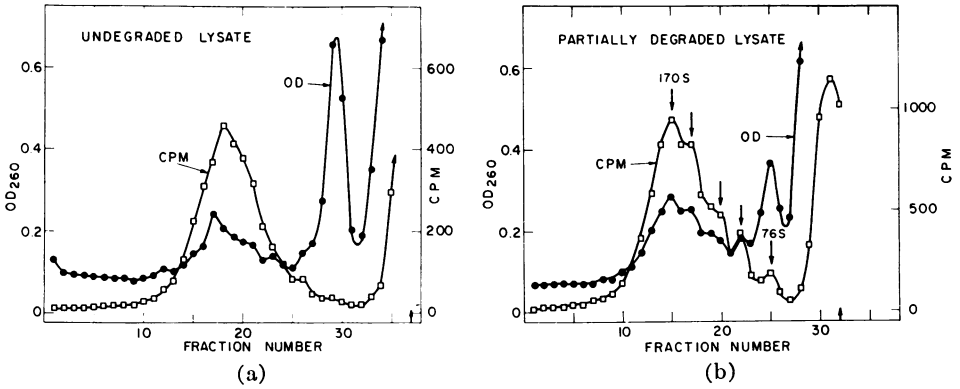

FIG. 1.—Sucrose gradients of lysed reticulocytes after a short incubation with C^{14} amino acids. 1 ml of lysate was layered on 25 ml of a 15–30 per cent sucrose gradient and then centrifuged (55,000 × *g*, 2 hr, 25° C). The short vertical arrow on the base line indicates the last fraction collected. The vertical arrows in the figure indicate peaks. (*a*) Undegraded lysate. (*b*) Partially degraded lysate.

obtained from experiments with a longer incubation period extending up to 20 min. Although there is some variation from one experiment to another, the light peak includes about 70 per cent of the area under the optical density curve, and yet it is totally inactive in the incorporation of amino acids. Since the light peak is so sharp, we conclude that the breadth of the heavy peak represents some heterogeneity, rather than spreading due to diffusion. The sedimentation constants of these peaks were determined in a Spinco Model E analytical ultracentrifuge with UV absorption optics. They were 76S for the small peak and 170S for the heavier peak. The light peak therefore corresponds to 78S ribosomes which have been isolated from reticulocytes by more vigorous methods.[1]

Ribosomes have been found attached to membranes[1] which sediment more rapidly than single ribosomes. Even though there is no endoplasmic reticulum in reticulocytes, experiments were carried out in which the lysate was incubated for 15 min with 0.5 per cent sodium deoxycholate, or 0.5 *M* NaCl. These have been shown to remove ribosomes from membranes,[1] but this treatment did not alter the 170S peak. In addition, no membranes were observed in the electron micrographs.

In order to make sure that we were examining the bulk of the nascent protein, the location of all radioactivity precipitable with trichloroacetic acid was determined (Table 1). After this short incubation time (45 sec), the ribosomes and soluble hemoglobin are about equally labeled and less than 7 per cent of the radioactivity sediments with the cell-wall fraction. Because there are often radioactive materials at the bottom of the tube after sucrose gradient centrifugation of the clarified lysate, a short centrifugation was carried out at low speed. However, no new peaks of optical density or radioactivity appeared, even though anything sedimenting as rapidly as 4,000S would have been seen. Thus, we can conclude that more than 85 per cent of the hemoglobin is being formed in the 170S peak, and this peak is therefore the site of protein synthesis *in vivo*.

From these initial experiments, we began to suspect that the 170S peak was a multiple ribosomal structure, perhaps held together by RNA. Since it is known that very small amounts of ribonuclease have a profound effect on protein synthesis *in vitro*,[12] it seemed likely that the enzyme might similarly affect the sites of synthesis *in vivo*. Therefore, an aliquot of clarified lysate was incubated at 4° for 1 hr with 0.25 μg/ml of ribonuclease and then layered on the gradient. A similar incubation and centrifugation was carried out with deoxyribonuclease at a concentration of 10 μg/ml. These results are shown in Figure 2. The deoxyribonuclease had no effect, while the ribonuclease almost completely destroyed the 170S peak, converting it to the more familiar single ribosomes. The nascent protein, however,

TABLE 1

Cell fraction	CPM	Per cent total count
Cell walls and unlysed cells	3,980	6.6
Ribosomes	28,200	47
Soluble fraction (hemoglobin)	28,000	46.4

0.75 ml of cells were incubated with C^{14} algal hydrolysate for 45 sec, then chilled, washed, and lysed as described in *Methods*. Cell walls were centrifuged at 10,000 × *g* for 15 min and the supernatant carefully decanted. The cell-wall pellet was resuspended in 4.5 ml buffer and again centrifuged for 15 min at 10,000 × *g*, and the supernatant was added to the first supernatant. Ribosomes were collected from the supernatant by centrifuging at 35,000 rpm for 60 min in a Spinco SW 39 rotor and the supernatant (soluble fraction) decanted. Protein from each fraction was purified by a modification of the procedure of Siekevitz[20] and plated on Millipore filters, and the radioactivity was counted. All counts were corrected for self-absorption.

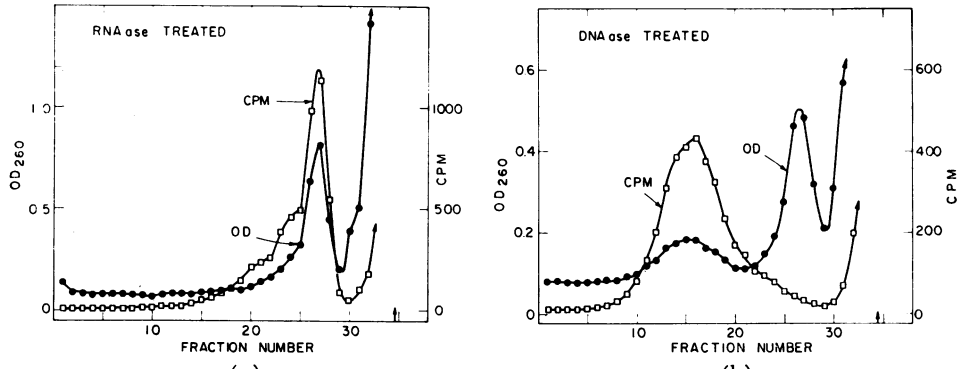

FIG. 2.—Sucrose gradients of reticulocyte lysate as in Figure 1 but incubated at 4°C for 1 hr with (*a*) 0.25 μg/ml RNAase—the 170*S* peak has disappeared and the radioactivity is now in the 76*S* peak, (*b*) 10 μg/ml DNAase—the 76*S* and 170*S* peaks are unchanged from Figure 1.

remained firmly attached to the single ribosomes. Higher concentrations of ribonuclease destroyed even the leading edge of the 78S peak. These experiments suggested that the heavy peak consisted of a multiple ribosome structure held together by RNA. We have called this a "polyribosome" or, a "polysome."

In general, ribosomes are very sensitive to the ionic environment and, in particular, to the concentration of Mg^{++} ions. In order to test the polysome structure for such sensitivity, a clarified lysate was dialyzed for 6 hr against three changes of a thousand-fold volume of 0.01 *M* Tris, pH 7.4, 0.01 *M* KCl, $5 \times 10^{-5} M$ $MgCl_2$. It was then layered on a 15–30 per cent sucrose gradient containing this low Mg^{++} buffer and centrifuged for 2 hr. This treatment had no effect on the centrifugal pattern and the results were the same as in Figure 1(*a*). However, when the lysate was made 0.01 *M* in ethylenediamine tetraacetate, both the polysome peak and the ribosome peak broke down to smaller units of 50S and 30S and most of the nascent protein was stripped off. Thus, in its reaction to changes in Mg^{++} concentration, the polysome resembles the "active" 70S ribosomes from *E. coli*.[4, 12]

In order to compare our results with earlier ribosome data, some experiments were done to test other techniques for preparing ribosomes. After either freeze-thawing or alumina grinding, the usual methods for preparing bacterial ribosomes, most of the optical density and nascent protein appeared in the 76S peak with no evidence of the polysomal structure. Therefore, the polysome is relatively fragile. Some indication of this fragility is suggested in Figure 1(*b*), where some fine structure begins to appear in the region between the 76S and the 170S peak. This is seen more clearly when the normal procedure is used for isolating reticulocyte ribosomes. To purify them, the ribosomes are usually centrifuged, the supernatant decanted, and the ribosomal pellet suspended free of hemoglobin. To study the effects of such treatment on the polysome structure, ribosomes from clarified lysate were pelleted and resuspended three times. The result is shown in Figure 3. Five peaks are clearly evident, both of radioactivity and of optical density. Once again, the heaviest peak has migrated about two and a half times as far as the lightest peak. Two considerations argue against this result being an artifact due to nonspecific aggregation during pellet formation. In the first place, the specific activity of the four heaviest peaks is nearly constant, while that of the lightest peak is much lower.

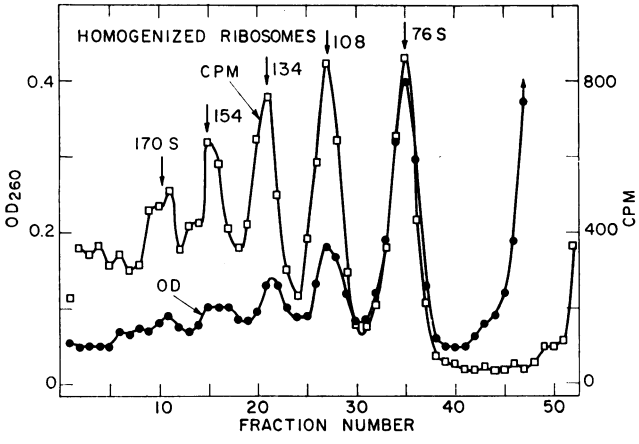

FIG. 3.—Sucrose gradient of lysed reticulocytes after incubation with H^3 leucine. After lysis and low-speed centrifugation, the ribosomes were pelleted three times at 28,000 rpm and resuspended with a homogenizer, as described in *Methods*. A 5–20 per cent sucrose gradient was used. The numbers next to the arrows represent the sedimentation constants associated with each peak.

This is what would be expected if the polysomes were being degraded by steps to monomers. In addition, we have already seen indications of these intermediate peaks in preparations that were never centrifuged into a pellet (Figure 1 (*b*)). By calibrating the sucrose gradient with the 76S and 170S peaks, we could assign S values to the intermediate peaks (108S, 134S, 154S). It is of interest that three earlier papers have presented evidence for these additional peaks without realizing their origin.[13–15]

The regularity of the increments in sedimentation constant clearly suggested that we were observing a sequence of quantized steps in the disintegration of a multiple ribosomal structure, starting from the 170S peak and terminating in the 76S ribosome. The simplest hypothesis is that each new peak is created by the elimination of one ribosomal unit from the polysomal structure. By simple enumeration, we anticipated that the 170S peak should be a pentamer, and this was readily confirmed by studies in the electron microscope. Electron micrographs of shadowed preparations are shown in Figure 4(*a–c*). Figure 4(*a*) shows a typical field of 76S ribosomes, which have a diameter of approximately 230 A. Figure 4(*b*) shows a field taken from the trimer peak (134S), and it can be seen that most of the ribosomes are present as triads. Figure 4(*c*) shows a typical polysomal field from the 170S peak. Here, most of the clusters contain five ribosomal units, although occasional tetramers or hexamers are seen. A common configuration seen in the air-dried preparations is a tightly clumped group. In the air-dried preparation, the center-to-center distance between the ribosomal subunits is usually 300 to 350 Å, although frequently it is somewhat longer. In some cases, it is possible to see a thin fiber of diameter 10–20 Å connecting two adjacent ribosomes. Staining with uranyl acetate resulted in positive staining; occasionally, a thin thread of high electron density could be seen running between two ribosomal units in the polysome structure. The separation between the ribosomes was usually more clearly resolved in the preparations that were stained with uranyl acetate or phosphotungstic acid. A full description of these results will be published elsewhere.[16]

Discussion.—The evidence presented above clearly points to the polysomal structure as the site of protein synthesis. Earlier experiments in which the ribosomal monomers were isolated by more vigorous procedures, including the formation of

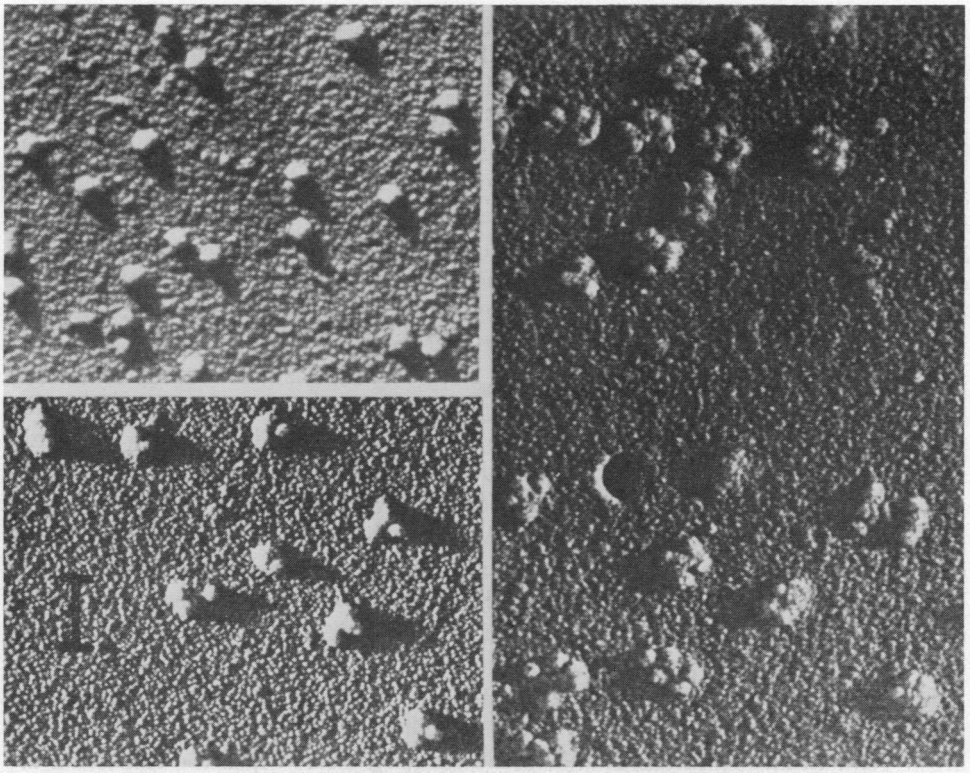

FIG. 4.—Electron micrographs of reticulocyte ribosomes. The ribosomes were deposited on the grid as a droplet from the sucrose gradient, rinsed with buffer, and air-dried. The Pt shadowing was at a 5:1 angle. The vertical mark indicates 0.1μ. (*a*) Upper left—droplet from the 76*S* peak. (*b*) Lower left–droplet from the 134*S* peak showing triplets of ribosomes. (*c*) Right—droplet from the 170*S* peak showing several ribosome pentamers. (Photographs courtesy of C. E. Hall.)

hard pellets and their resuspension, resulted in degradation which suggested that the nascent protein was associated with the 76S particle.[13] Directly this leads us to ask whether such polysomal structures exist in other cells as well. We are inclined to believe that this is the case, even though complete evidence is not available as yet. Palade observed long strings of ribosomes in thin slices made from a ribosomal pellet of guinea pig pancreas.[17] In addition, Risebrough *et al.*[7] have studied the interaction of T_2 virus messenger RNA and *E. coli* ribosomes. Their sucrose density gradient patterns show evidence for the existence of ribosomal dimers, trimers, and even small amounts of tetramers. It is quite likely that the adoption of more gentle preparative procedures will reveal even larger units in bacteria. Langridge and Holmes[18] have shown that ribosomes can apparently form linear aggregates in concentrated gels. It remains to be seen if this is related in some way to polysome formation.

Four lines of evidence suggest that the ribosomal units are held together by RNA, possibly a single strand. Mild treatment with ribonuclease results in dissociation to the ribosomal units; the mechanical fragility of the polysome suggests that the structural link is not large; the 10–20 Å threads seen in the electron micrographs are consistent with a single RNA strand; finally, these threads stain posi-

tively with uranyl acetate, which is known to react with nucleic acids. Somewhat less direct evidence suggests that the ribosomes are held together by messenger RNA. As mentioned above, the attachment of labeled T_2 messenger RNA to *E. coli* ribosomes leads to the formation of ribosomal aggregates with high *S* values.[7] Ribonuclease also breaks up that polysomal structure. In addition, the attachment of synthetic messenger RNA, polyuridylic acid, results in the formation of aggregates with large *S* values.[8-10] The simplest interpretation for all of these experiments is that the long thread of messenger RNA is attached to several ribosomes, five being the predominant number for hemoglobin synthesis. By comparing the center-to-center distance between ribosomes with their diameter, we note that there is a 50–100 Å spacing between ribosomes. Five ribosomal diameters plus these spacings have an overall length near the 1,500 Å which is the anticipated length for a messenger RNA strand coding for approximately 150 amino acids. However, further work will be needed to define the exact length of the hemoglobin polysome.

The width of the 170S peak suggests that it may include other species than the pentamer. A qualitative survey of electron micrographs confirms this, since tetramers as well as hexamers are seen. Air drying is known to produce an artificial clumping; accordingly, we cannot say much about the polysome configuration. We believe it to be a linear structure with considerable flexibility because the ribosomes are separated by short stretches of flexible RNA. However, we cannot exclude a circular arrangement.

Let us assume for the moment that a strip of messenger RNA has five ribosomal units attached to it. It has been shown by Dintzis[19] that the polypeptide chain grows sequentially from the amino to the carboxyl end. Since the ribosomes are slightly separated, it is most reasonable to assume that the growing polypeptide chain remains attached to an individual ribosome rather than skipping from one ribosome to the next. Since the ribosome must use the sequence information in the messenger RNA to govern the assembly of amino acids, it follows that the messenger RNA strand must move in some fashion relative to the ribosome. The possibility of such a ribosomal movement was first raised by Gilbert[8] in connection with his experiments on polyphenylalanine synthesis in an *E. coli* cell-free extract. A tentative view which we can adopt is that ribosomal particles begin protein synthesis by attaching to one end of a messenger RNA strand and then move along it as the polypeptide chain lengthens. If the messenger strip is very long, a correspondingly greater number of ribosomes will be attached at any one time. When the ribosomes reach the end of the strand, they release the polypeptide chain as well as the RNA. Thus, the released ribosome would contribute to the pool of inactive 76S monomers which we observe.

Of course, such a model raises questions. For example, what makes the ribosome flow over the messenger RNA strand? Is it possible that there is an energy-consuming mechanism, perhaps distantly related to that which occurs in muscle contraction? However, in addition to raising questions, such a model gives rise to some suggestions. For example, this represents a very efficient use of messenger, since several ribosomes are operating on it at the same time. It is possible that the hemoglobin messenger RNA may be stabilized by its five ribosomes. A new ribosome may attach just as another ribosome falls off at the other end with its completed chain. If these two events do not occur simultaneously, a certain

number of tetramers or hexamers will form; this might account for the broad spread observed in the 170S peak. In addition, the mechanism described above raises the possibility that related groups of proteins are all manufactured at the same time. Thus, one genetic locus in the β-galactosidase system controls the production of a series of enzymes. If one long piece of messenger RNA was produced containing the information for all of these enzymes, ribosomes flowing over this strip might then produce the entire group of enzymes, one after another. Much of this is speculation at the present time, since it is based on the assumption that a messenger RNA strand passes through the five ribosomes. In any case, the demonstration that protein synthesis occurs in a polysomal structure will undoubtedly enhance our understanding of the function of the ribosome.

Summary.—It has been demonstrated that protein synthesis in the reticulocyte takes place in a multiple ribosomal structure containing five ribosomes. These ribosomes appear to be held together by an RNA strand, probably messenger RNA. The mechanism of protein synthesis is discussed in light of these observations.

We would like to thank C. E. Hall and Marsha Glynn for their assistance in obtaining electron micrographs. We acknowledge the capable technical assistance of Melissa Quinn. This research was supported by research grants from the National Institutes of Health and the National Science Foundation.

* Predoctoral Fellow of the National Science Foundation.

† Present address: MRC Laboratory of Molecular Biology, Cambridge, England.

[1] A review of ribosomal properties is published by Ts'o, P. O. P., *Ann. Rev. Plant Physiol.* **13**, 45 (1962).

[2] Gros, F., H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Risebrough, and J. D. Watson, *Nature*, **190**, 581 (1961).

[3] Brenner, S., F. Jacob, and M. Meselson, *Nature*, **190**, 576 (1961).

[4] Nirenberg, M. W., and J. H. Matthaei, these PROCEEDINGS, **47**, 1588 (1961).

[5] Tsugita, A., H. Fraenkel-Conrat, M. W. Nirenberg, and J. H. Matthaei, these PROCEEDINGS, **47**, 846 (1962).

[6] Crick, F. H. C., L. Barnett, S. Brenner, and R. J. Watts-Tobin, *Nature*, **192**, 1227 (1961).

[7] Risebrough, R. W., A. Tissières, and J. D. Watson, these PROCEEDINGS, **48**, 430 (1962).

[8] Gilbert, W., personal communication.

[9] Spyrides, G., and F. Lipmann, these PROCEEDINGS, **48**, 1977 (1962).

[10] Barondes, S. H., and M. W. Nirenberg, *Science*, **138**, 813 (1962).

[11] Borsook, H. C., L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.*, **196**, 669 (1952).

[12] Tissières, A., D. Schlessinger, and F. Gros, these PROCEEDINGS, **46**, 1450 (1960).

[13] Wallace, J. M., R. F. Squires, and P. O. P. Ts'o, *Biochim. et Biophys. Acta*, **47**, 130 (1960).

[14] Arnstein, H. R. V., *Biochem. J.*, **81**, 24 P.

[15] Lamfrom, H., and E. R. Glowacki, *J. Mol. Biol.*, **5**, 97 (1962).

[16] Warner, J. R., A. Rich, and C. E. Hall, *Science* (in press).

[17] Palade, G. E., in *Microsomal Particles and Protein Synthesis* (New York: Pergamon Press, 1958), p. 36.

[18] Langridge, R., and K. C. Holmes, *J. Mol. Biol.* (in press).

[19] Dintzis, H. M., these PROCEEDINGS, **47**, 247 (1961).

[20] Siekevitz, P., *J. Biol. Chem.*, **195**, 549 (1952).