The Ribosomal Subunit–Polyribosome Cycle in Protein Synthesis of Embryonic Skeletal Muscle*

David Kabat† and Alexander Rich

ABSTRACT: Thigh muscle from 14-day-old chicken embryos consists predominantly of young multinucleated myofibers having a broad and characteristic distribution of polyribosomes which are not attached to membranes. Autoradiographic experiments suggest that the fibers are the major site of muscle ribosome synthesis. Accordingly, large polyribosomes synthesizing fiber-specific proteins become highly labeled with radioactive ribosomal ribonucleic acid after embryos are incubated with isotopic ribonucleic acid precursors. Pulse labeling of polysomal nascent peptide chains with radioactive amino acids showed that the largest polysomes synthesize much larger proteins than those made on small polysomes. Furthermore, the time required to synthesize a complete protein increases linearly with the size of polypeptide chain. Following intravenous injection of embryos with [^{32}P]phosphate, analysis was made of the flow of radioactive ribosomal ribonucleic acid into polysomes of various sizes, into single ribosomes, and into subribosomal particles. The results suggest that equilibration of active subribosomal particles with even the largest polyribosomes is so rapid that their specific radioactivities remain nearly equal throughout the labeling. In contrast with its rapid cycling through polysomes, radioactive ribosomal ribonucleic acid enters into the pool of single ribosomes at a very slow rate. It is concluded that at least 90% of the embryonic muscle single ribosomes, and possibly all of them, do not participate in the ribosomal subunit–polyribosome cycle which accompanies protein synthesis.

It is generally believed that single ribosomes are formed after polysomal ribosomes release their newly synthesized nascent polypeptide chains and fall off the mRNA (Colombo *et al.*, 1968; Kohler *et al.*, 1968; Maden, 1968; Rich *et al.*, 1963). Single ribosomes have been found in the extracts of all cells examined, and pulse-labeling studies with radioactive amino acids suggest that they do not carry nascent polypeptide chains. However, there has not been any critical positive evidence in bacteria or in higher organisms which would suggest that single ribosomes are a normal stage in the cycling of ribosomes during protein synthesis. On the contrary, they may be an inert storage form of ribosomes. In fact it has been proposed recently that single ribosomes may not even exist *in vivo* in bacteria (Mangiarotti and Schlessinger, 1966; Cundliffe, 1968). Although other recent work has questioned these findings and has suggested that single ribosomes are present within bacteria in substantial numbers (Kohler *et al.*, 1968), the controversy illustrates the lack of definite information available concerning single ribosomes.

There is now considerable evidence that bacterial ribosomes dissociate into their two subunits between successive cycles of protein synthesis. The 30S subunits combine with $tRNA^{fMet}$ at the initiator site of mRNA; 50S subunits then associate and protein synthesis is initiated (Nomura and Lowry, 1967). Physical and metabolic studies have also demonstrated that the subunits of bacterial ribosomes exchange between ribosomes during protein synthesis (Kaempfer *et al.*, 1968; Kaempfer, 1968; Mangiarotti and Schlessinger, 1967). It is quite likely that a similar cyclic dissociation of ribosomes into subunits occurs between rounds of polypeptide chain initiation in eukaryotic cells. In HeLa and in Landshutz ascites cells, for example, it appears that radioactive subunits are able to associate with mRNA without first mixing with the intracellular pool of single ribosomes (Girard *et al.*, 1965; Joklik and Becker, 1965; Hogan and Korner, 1968). Furthermore, initiation of new globin chains in a cell-free system from reticulocytes is markedly enhanced by the addition of ribosomal subunits (Bishop, 1966). Additional evidence for such cyclic ribosome dissociation in eukaryotic cells has recently been obtained (Vaughan *et al.*, 1967; Colombo *et al.*, 1968; Hogan and Korner, 1968). Although these experiments suggest that a cycling of ribosomal subunits occurs in eukaryotic cells, it is of interest to examine in more detail the participation in this cycling of the subunits in polyribosomes and those present in single ribosomes.

We have begun a study of polysomal RNA metabolism in embryonic chicken skeletal muscle. The polysomes of 14-day-old embryo thigh muscle are unattached to an endoplasmic reticulum and thus are easily isolated. They contain a broad and unique distribution of polysome sizes, including large clusters of 50–60 ribosomes which are engaged in myosin synthesis and smaller clusters active in actin and tropomyosin synthesis (Heywood and Rich, 1968). This tissue is therefore well suited for studies of the flow of newly synthesized ribosomal subunits into polysomes of different sizes and into single ribosomes. Our results show that the newly synthesized ribosomal subunits cycle repeatedly through all sizes of polysomes, and suggest that equilibration of polysomal ribosomes with the subunit pool is rapidly established. On the contrary, these new subunits with radioactive rRNA enter into the pool of single ribosomes very slowly. Although high levels of single

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† Present address: Department of Biochemistry, University of Oregon Medical School, Portland, Ore.

ribosomes are present in embryonic muscle, at least the vast majority of them are not participating in the protein synthetic cycle.

Materials and Methods

Chicken embryos were injected intravenously with 10 μCi of a mixture of [^{14}C]amino acids (New England Nuclear), with varying amounts of [^{32}P]disodium phosphate (2 Ci/mmole) (New England Nuclear), or with 100 μCi of [^{3}H]uridine (20 Ci/mmole) (Schwarz) for various times at 38° before removing and chilling the thigh muscles in ice-cold MSB buffer (0.25 M KCl–0.01 M $MgCl_2$–0.01 M Tris-HCl, pH 7.4). The veins of the chorioallantoic membrane were located for injection by candling the egg. An overlying piece of shell was gently removed and the shell membrane was rendered transparent by applying mineral oil. The exposed vessel was then injected with a maximum of 0.1-ml solution using a beveled glass capillary needle.

Polysomes from the thigh muscles of 14-day-old chicken embryos were extracted with high salt MSB buffer using the method of Heywood *et al.* (1967). They were analyzed in 27 ml of 15–40% sucrose–MSB gradients in an SW25.1 Spinco rotor. When the polysomes were to be coprecipitated with myosin, one volume of tissue was extracted with only one-half volume of MSB. Precipitation by myosin was accomplished by diluting these extracts with ten volumes of low ionic strength buffer (0.01 M $MgCl_2$–0.01 M Tris-HCl, pH 7.4) as described previously (Heywood *et al.*, 1968). Unfractionated polysomes were pelleted by layering extracts over 2 ml of 15% sucrose in MSB and centrifuging at 50,000 rpm for 50 min in a Spinco 65 rotor. Polysomes from sucrose gradient fractions were pelleted by centrifuging in the Spinco 30 rotor at 30,000 rpm for 10 hr. The single ribosomes and subribosomal particles of sucrose gradients were pelleted thoroughly by centrifugation in the Spinco 65 rotor at 50,000 rpm for 10 hr.

Radioactive fractions from sucrose gradients were precipitated with 10% trichloroacetic acid in the presence of 50 μg/ml of carrier bovine serum albumin. Precipitated fractions that were labeled with [^{14}C]amino acids were heated at 80° for 20 min and then chilled for 30 min. The radioactive precipitates were filtered onto Millipore membranes and radioactivity was measured in a Nuclear-Chicago low-background gas-flow counter. Membranes with [^{3}H]uridine were suspended in Bray's solution (Bray, 1960) and measured in a liquid scintillation spectrometer.

RNA was extracted by a modification of the TNS[1] method of Kirby (1965). The pelleted ribosomal material was suspended in 0.25 M sucrose, 0.05 M KCl, 0.001 M $MgCl_2$, and 0.035 Tris-HCl (pH 7.4) containing 5% TNS and 7% *t*-butyl alcohol. The butyl alcohol prevents detergent precipitation at 2°, the temperature used for the extractions. The solution was then shaken with one volume of water-saturated redistilled phenol which contained 10% vacuum, redistilled *m*-cresol and 0.1% 8-hydroxyquinoline. After centrifugation at 10,000*g* for 10 min, the aqueous phase was adjusted to 0.5 M NaCl and the extraction with phenol–cresol was repeated. After centrifugation, two volumes of ethanol was added to the aqueous phase and RNA precipitation occurred overnight at −20°. The precipitated RNA was washed once with 80% ethanol to remove phenol and other ultraviolet-absorbing impurities. This method gave high yields of unaggregated rRNA. Aggregation of a small fraction of the rRNA was regularly found to occur during extractions with SDS or with TNS at higher temperatures in a manner similar to that reported by Wagner *et al.* (1967). A simple method was used to test for aggregation of RNA or for hidden breaks. The RNA samples were incubated in 70% dimethyl sulfoxide in the electrophoresis buffer for 15 min at 37°. The RNA samples were then layered onto polyacrylamide gels for 30 min at room temperature before applying the current. The present extraction method gave quantitative yields of rRNA whose electrophoretic mobility was unaffected by the dimethyl sulfoxide treatment. The only change observed was the quantitative appearance of the 7S rRNA which normally associates with 28S rRNA as described by Pene *et al.* (1968).

Polyacrylamide gels (2.4%) were prepared for RNA electrophoresis as described by Loening (1967). The electrophoresis buffer was 0.036 M Tris–0.03 M NaH_2PO_4–0.001 M EDTA, containing 0.2% SDS as described by Loening and Ingle (1967). After electrophoresis, the gels were scanned for ultraviolet absorbance and then sectioned into 1-mm slices as described by Weinberg *et al.* (1967). Adjacent slices were generally placed together onto aluminum planchets and radioactivity was measured in a low-background gas-flow counter with a background level of 1.7 cpm. Samples with a low radioactivity level were routinely counted for 30 min.

For autoradiography the muscle from embryos labeled with 100 μCi of [^{3}H]uridine was fixed and washed in Bouin's fixative, followed by three washes of 0.2% perchloric acid, and then with water; 5-μ sections were made on a microtome and coated with liquid emulsion film (Ilford L.4). The developed autoradiograms were stained with hematoxylin–eosin.

Results

rRNA Synthesis in Young Multinucleated Myofibers. During differentiation of striated muscle, mononucleated myoblasts cease mitosis and subsequently fuse to form long multinucleated myofibers. Synthesis of many muscle enzymes and of the major myofibrillar proteins actin and myosin become appreciable only after cell fusion (Baril and Hermann, 1967; Fishman, 1967; De La Haba *et al.*, 1968; Coleman, 1968). Although the nuclear morphology of young myofibers is initially similar to that of myoblasts, eventually the myofiber nuclei become highly elongated and condensed as they are pressed against the peripheral sarcolemmal membrane by the central mass of contractile proteins.

The thigh muscle from 14-day-old chicken embryos consists predominantly of young myofibers and to a much lesser extent of nondividing myoblasts (Marchok and Hermann, 1967). In typical sections of the muscle we found that nearly all of the nuclei appear the same, slightly oblong with their long axes lying parallel to the fiber direction. We investigated the pattern of RNA synthesis in these cells by autoradiography of tissue isolated and sectioned 2 and 18 hr after the administration of [^{3}H]uridine to embryos. The vast majority of grains were rather uniformly distributed over the tissue, with no suggestion that the RNA remains localized within nuclei or that a minority cell type is responsible for the bulk of muscle

[1] Abbreviations used are: TNS, sodium triisopropylnaphthalenesulfonate; SDS, sodium dodecyl sulfate.

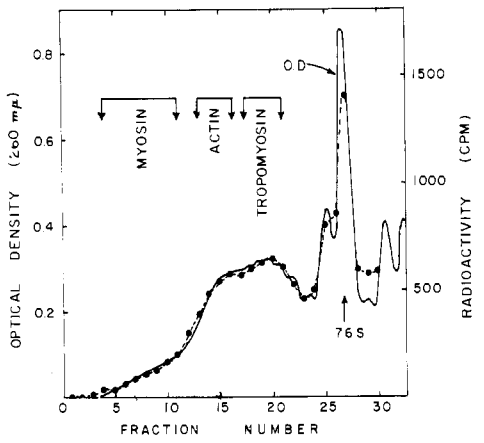

FIGURE 1: Sucrose gradient pattern of muscle polysomes 24 hr after intravenous injection of a 14-day-old embryo with 0.1 mCi of [^{32}P]-phosphate. The polysomes were quantitatively coprecipitated with myosin before redissolving and layering on the gradient. Arrows indicate the regions containing polysomes synthesizing various proteins.

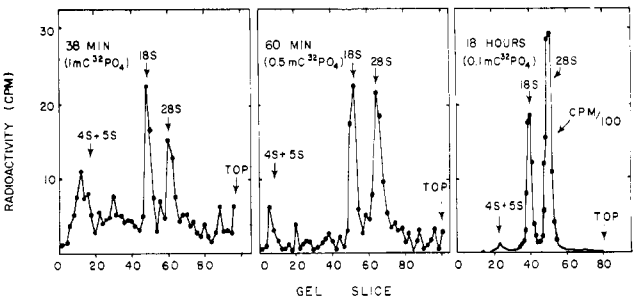

FIGURE 3: Acrylamide gel electrophoresis of RNA from muscle polysome fraction after [^{32}P]phosphate injections of embryos for various time periods. The extracts were prepared using two strokes with the Dounce homogenizer. The polysome fractions were sedimented from the extracts as described in Methods. Electrophoresis was for 130 min in the two patterns on the left and for 95 min in the right panel. The arrows indicate the positions of the absorbance peaks due to 28S, 18S, 5S, and 4S RNA.

RNA synthesis. However, a very small fraction of the tissue (about 5% of the nuclei) consists of more highly differentiated fiber bundles having very narrow nuclei. Such fiber bundles clearly showed a lowered incorporation of isotope as compared with the neighboring younger cells. In some regions of tissue, adjacent fibers were separated, and it could be clearly seen that they had accumulated labeled RNA in their cytoplasm. As will be shown later, the majority of the cytoplasmic RNA labeled after 2- or 18-hr incorporation is in 18S and in 28S rRNA.

Cytoplasmic extracts from dissected and washed 14-day-old embryo thigh muscle contain nearly all the ribosomes present in the tissue and exhibit a characteristic distribution of polyribosome sizes (Figure 1). Myosin, actin, and tropomyosin are made on different sized polysomes as shown in the figure (Heywood and Rich, 1968). After a 24-hr incorporation of [^{32}P]phosphate, nearly all of the polysome-associated label is in 18S and 28S rRNA and the distribution of these radioactive ribosomes closely follows the optical density distribution in the sucrose gradients (Figure 1). It is significant that the large polyribosomes synthesizing myosin have the same specific activity as the smaller polysomal regions. This result strongly suggests that the majority of ribosome synthesis in the thigh muscle of the 14-day-old embryo occurs in young myofibers which are active in the synthesis of proteins including myosin (Yaffe and Feldman, 1964; Yaffe and Fuchs, 1967). These contribute the bulk of polysomes to the cytoplasmic extracts. If a minority cell type with a different polysome profile was responsible for a high proportion of the ribosome synthesis, the distribution of radioactive ribosomes would differ from the absorbance profile. The data in Figure 1, together with our cytological and autoradiographic analysis, has led us to conclude that ribosome synthesis in the 14-day-old embryonic muscle occurs mainly within a large fairly homogeneous population of developing young myofibers.

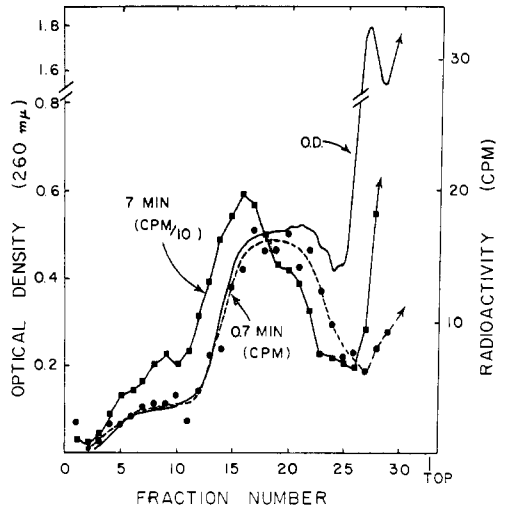

FIGURE 2: Sucrose gradient pattern of muscle polysomes from a chick embyro injected with a mixture of [^{14}C]amino acids and allowed to incubate for 0.7 and 7.0 min. After precipitation with 10% trichloroacetic acid, the fractions were heated at 80° for 20 min. The radioactivity from the 7-min injection is plotted at one-tenth scale. The optical density profile was the same at 0.7 and 7.0 min.

Linear Relationship between Size of a Polypeptide Chain and Its Synthesis Time. The muscle polysomes were analyzed at various times after injection of [^{14}C]amino acids into the embryos. When the injection lasted for 2 min or longer, the larger polysomes contained progressively more nascent chain radioactivity per ribosome than did the smaller polysomes (Figure 2). This is expected because the larger polysomes are synthesizing larger proteins. It has been shown (Heywood and Rich, 1968) that myosin chains (mol wt 220,000), actin chains (mol wt 55,000), and tropomyosin chains (mol wt 30,000) are preferentially synthesized on the polysome size classes shown in Figure 1. On the other hand, when the amino acid injection was 45 sec or less, the distribution of nascent chain radioactivity closely followed the optical density profile of the polysomes (Figure 2). The latter result would be expected after a very short pulse of radioactive amino acids if the rate of amino acid polymerization were the same on different sized polysomes. It thus appears likely that the translation times for different sizes of polysomes are approximately proportional to the size of polypeptide chain being synthesized. It perhaps

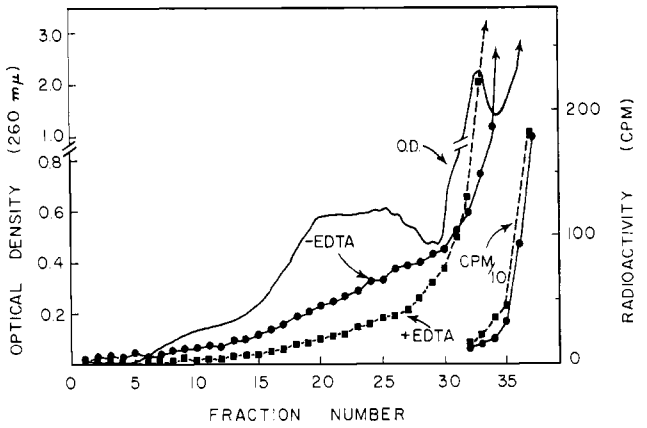

FIGURE 4: Sucrose gradient pattern of a muscle extract from an embryo pulse labeled for 40 min with 1 mCi of $^{32}PO_4$. One aliquot was adjusted to 0.05 M EDTA (pH 7.0) for 5 min at 2° prior to layering on the gradient. The optical density pattern is from the aliquot which was not treated with EDTA.

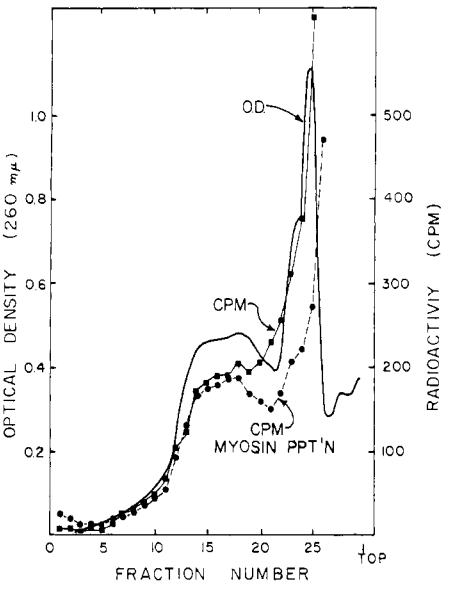

FIGURE 5: Sucrose gradient pattern of a muscle extract from an embryo labeled for 60 min with 1 mCi of [^{32}P]phosphate. One aliquot of polysomes was coprecipitated with myosin, redissolved and layered on the gradient. The optical density pattern is from this gradient. The other aliquot which was not coprecipitated had a nearly identical optical density pattern in the polysome region.

should be mentioned here that all sizes of muscle polysomes disaggregate to form single ribosomes when the tissue is treated with puromycin or with sodium fluoride. This strongly suggests that all sizes of muscle polysomes are functioning in protein synthesis. It is therefore valid to conclude from the results presented here that it requires much longer for ribosomes to cycle through larger polysomes than through smaller ones.

Evidence That the Cytoplasmic Extracts Contain Heterodisperse RNA Not Associated with Polysomes. At various times after [^{32}P]phosphate was injected into embryos, muscle extracts were prepared and the polyribosomes were pelleted by centrifugation. RNA was then extracted from the sedimented polyribosome fractions. Figure 3 shows the radioactive RNA components fractionated on polyacrylamide gels. After 18 hr of exposure to label the radioactivity distribution in the gels closely follows the optical density profile (which is not shown in Figure 3). However, 38 min after injection, most of the labeled material is heterogeneous as judged by its electrophoretic mobility. By 60 min a higher proportion of the radioactivity is in the 18S and 28S rRNA species. As has been observed with other eukaryotic cells, labeled 18S rRNA appears in the cytoplasmic extracts earlier than labeled 28S rRNA. Although the results presented are typical, the quantity of heterogeneous radioactivity in the extracts was found to vary and is generally higher if the tissue is given more strokes with the Dounce homogenizer. Similar results were also observed if [^{3}H]uridine was the radioisotope or if sucrose gradients rather than polyacrylamide gels were used for the RNA separations. All of the pulse-labeled molecules were degraded to small materials by pancreatic ribonuclease. In addition, we have been unable to detect any DNA or 45S RNA in the cytoplasmic extracts, which suggests that the level of nuclear breakage may be very low.

It has been shown that a large proportion of pulse-labeled RNA in cytoplasmic extracts from HeLa cells is heterodisperse and not associated with polysomes (Attardi and Attardi, 1967; Penman *et al.*, 1968). Ribosomes and polysomes are disrupted by 0.05 M EDTA while the heterodisperse RNA, presumably attached to membrane fragments, is unaffected. In Figure 4 we show the sedimentation profile of a cytoplasmic extract from an embryo labeled for 40 min with [^{32}P]phosphate. Most of the trichloroacetic acid precipitable label sediments more slowly than the polysomes or single ribosomes. After complete disruption of ribosomes and polysomes with EDTA, only a portion of the radioactivity larger than 76S was reduced in its sedimentation rate (Figure 4). Although this experiment seems to suggest that about half of the rapidly sedimenting radioactivity was associated with EDTA-resistant structures, we cannot exclude the possibility that all of the radioactivity was only partially resistant to the treatment or that some labeled materials aggregated in EDTA.

We therefore sought another method of analyzing the polysome-associated RNA. Ribosomes and polysomes can be coprecipitated with myosin from muscle extracts by lowering the ionic strength (Heywood *et al.*, 1968). We found that the ribosome-containing structures (including the 60S and 40S ribosomal subunits) were quantitatively precipitated from concentrated muscle extracts made using one-half volume of the high ionic strength MSB buffer solution per volume of muscle tissue during homogenization. Following such coprecipitation, the precipitate was dissolved in high salt buffer and was then analyzed on a sucrose gradient (Figure 5). The polysomal material was quantitatively recovered from the coprecipitate and was almost uniformly labeled with [^{32}P]phosphate. However, a high proportion of the slowly sedimenting radioactivity was removed from the polysome preparation. We conclude that there was considerable nonpolysomal pulse-labeled RNA in the extract which was not coprecipitated with myosin. Although most of the radioactivity sediments more slowly than single ribosomes, some of this material sediments into the polysome region of the gradients. As shown in Figure 6 most of the EDTA-resistant rapidly sedimenting radioactive RNA in muscle extracts is similarly not coprecipitated with myosin.

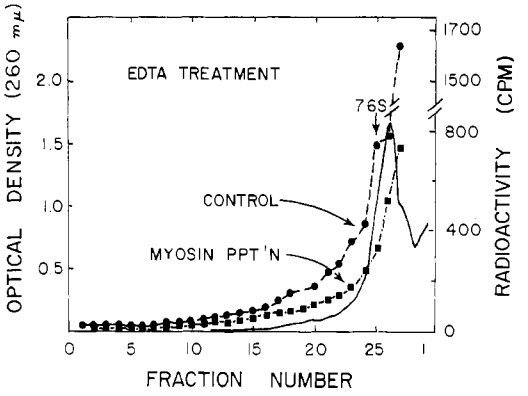

FIGURE 6: Sucrose gradient pattern of EDTA-treated muscle extract labeled for 60 min with 1 mCi of [^{32}P]phosphate. One aliquot of polysomes was coprecipitated with myosin before EDTA treatment. The other aliquot was treated directly with EDTA. The optical density pattern is from the myosin-precipitated polysomes.

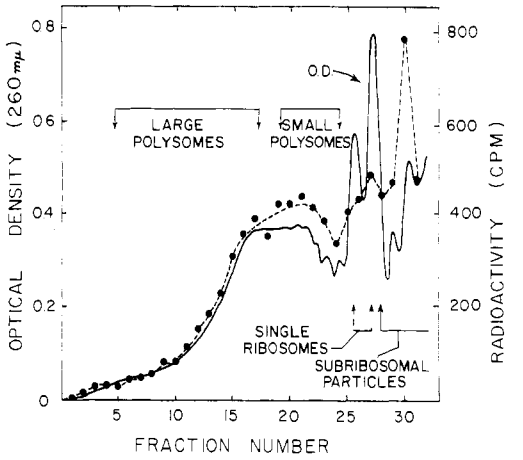

FIGURE 8: Sucrose gradient pattern of muscle polysomes and single ribosomes from an embryo labeled for 150 min with 0.5 mCi of $^{32}PO_4$. The ribosomal structures were coprecipitated with myosin before redissolving in MSB. The arrows indicate the fractions used in the experiments described in Figure 9.

Figure 7 shows an acrylamide gel analysis of RNA extracted from myosin-precipitated and from control polysomes. The myosin coprecipitates contained much less heterodisperse RNA but the same level of rRNA as the original tissue extracts. These experiments demonstrate that the bulk of heterodisperse RNA in muscle extracts is not associated with ribosomal However, a portion of the nonpolysomal heterodisperse RNA is usually present in myosin precipitates so that the method cannot be used to completely purify the ribosomal components. We do not know whether the heterodisperse RNA in muscle extracts is in the nucleus or the cytoplasm in the intact cell. This RNA contributes only a small fraction of the radioactivity after long periods of labeling (Figure 3) which suggests that it may be rapidly degraded or is being constantly transferred into structures which are resistant to our extraction method.

Flow of Newly Synthesized Ribosomal Subunits into Polyribosomes. The selective purification of ribosomal structures by myosin coprecipitation allows us to analyze more easily the flow of newly synthesized rRNA through these structures. In such a preparation from an embryo labeled for 150 min with [^{32}P]phosphate, the polysomes were heavily and almost uniformly radioactive, whereas the single ribosomes were considerably less radioactive (Figure 8). Figure 5 shows that a similar result was observed when the incorporation lasted for only 60 min. These results suggested that newly synthesized ribosomes were mixing with all sizes of polysomes but entered only slowly into the pool of single ribosomes. We decided to analyze the time sequence in the distribution of newly synthesized 18S and 28S rRNA in the four regions of the sucrose gradients illustrated in Figure 8. With respect to the unfractionated muscle extracts, these regions were enriched in their contents of large polysomes, small polysomes, single ribosomes, and subribosomal particles, respectively. RNA extracted from these regions was analyzed by electrophoresis on polyacrylamide gels and the results are presented in Figure 9.

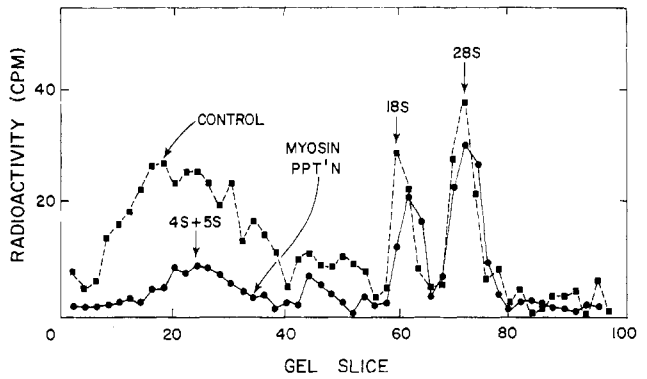

FIGURE 7: Removal of heterodisperse RNA from polyribosomes coprecipitated with myosin. The embryo was labeled for 60 min with $^{32}PO_4$ and homogenized using four strokes with the Dounce homogenizer. One aliquot of polysomes was coprecipitated with myosin and then redissolved in MSB. Both aliquots were then centrifuged to pellet the polysome fractions. The RNA was extracted and 50-μg samples were fractionated by gel electrophoresis.

Discussion

Rapid Cycling of Newly Synthesized Ribosomal Subunits through Polyribosomes. Following injection of the embryos with [^{32}P]phosphate, labeled rRNA continues to flow into the cytoplasm for many hours. It is well known that the different ribosomal subunits separately enter the cytoplasm from the nucleus and that the smaller subunit containing 18S rRNA emerges before the larger 28S rRNA-containing subunit. We can refer to the pathway followed by subunits during one round of protein synthesis as the subunit cycle. The results in Figure 9 illustrate four features of this cycle.

(i) The labeling of single ribosomes remains lower than labeling of polysomes. Therefore, single ribosomes are not a precursor of polysomal ribosomes. On the other hand, the labeling of subribosomal particles is fully consistent with the idea that they are the precursors. Perhaps the subunits associate on the initiator site of mRNA as has been demonstrated for bacteria (Nomura and Lowry, 1967). These findings are in agreement with those of previous workers using other types of eukaryotic cells (Girard *et al.*, 1965; Joklik and Becker, 1965; Hogan and Korner, 1968).

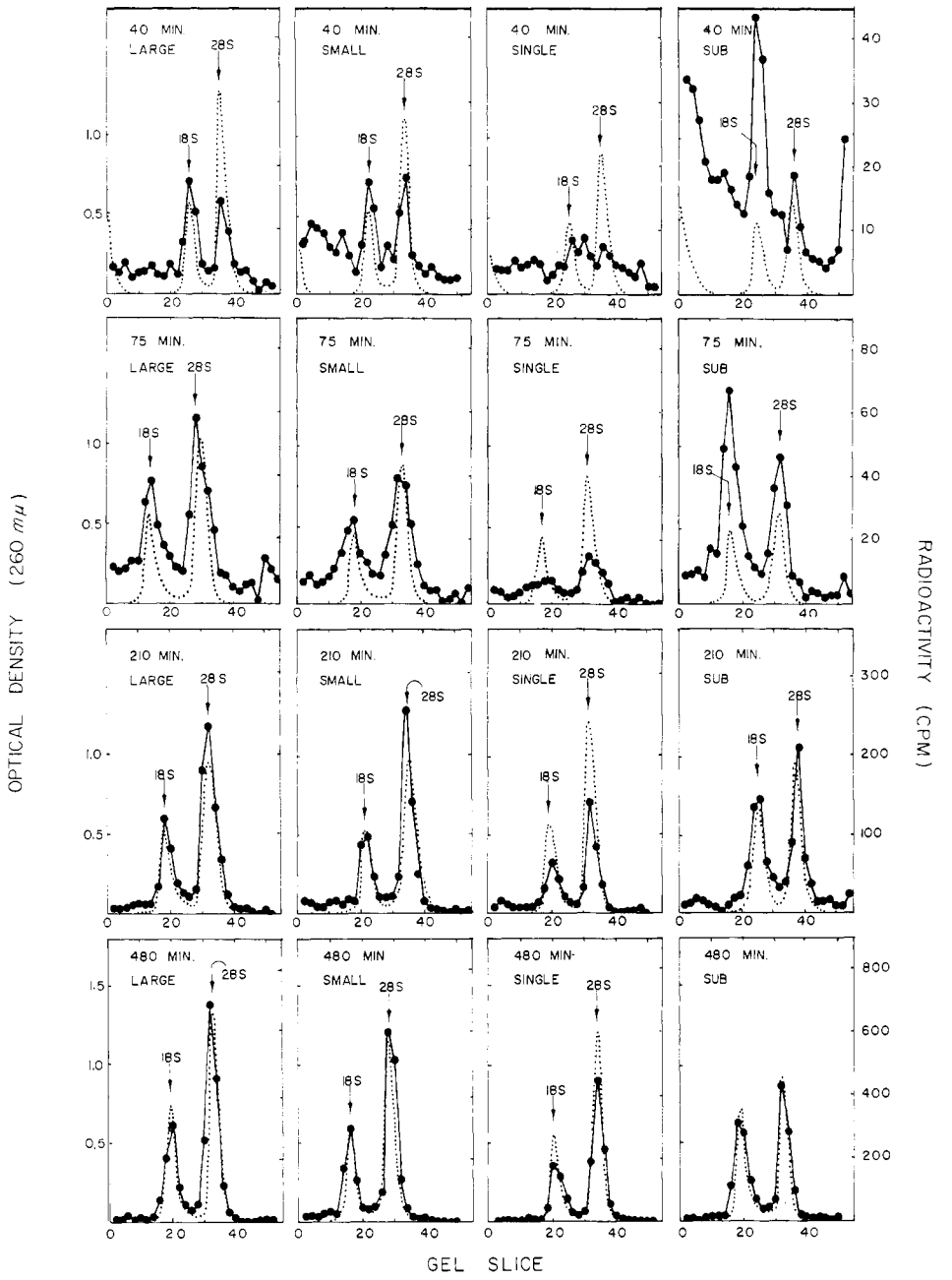

FIGURE 9: Acrylamide gel electrophoresis of $^{32}PO_4$-labeled ribosomal materials pooled from different regions of sucrose gradients as indicated in Figure 8. Large and small refer to polysome sizes; sub refers to ribosomal subunits. The material from three embryos was pooled for each time point. Embryos were injected with $^{32}PO_4$ and were harvested at 40 min (1 mCi), 75 min (1 mCi), 210 min (0.1 mCi), or 480 min (0.1 mCi). The optical density is the dotted line while the radioactivity is the solid line.

(ii) Throughout the labeling the specific radioactivity (counts per minute per optical density) of rRNA in large muscle polysomes remains approximately equal to the specific radioactivity of rRNA in small polysomes (Figures 8 and 9). We have also found this to be true when four polysomal regions rather than two were used for analysis. However, the studies with radioactive amino acids suggest (see Figure 2) that it requires much longer for ribosomes to flow through the very large muscle polysomes than to flow through the small polysomes. If the specific radioactivities of the *functional* subunit pools were doubling rapidly as compared with the translation time for large polysomes, we would expect the small polysomes to have much higher specific radioactivity than the large polysomes throughout the labeling. The result observed can be explained only if we conclude that the flow of newly synthesized ribosomal subunits through even the largest muscle polysomes occurs rapidly as compared with the doubling time of specific radioactivity of the precursor subunit pools. Since about 80% of the ribosomal subunits exist in polysomes, it follows that the specific radioactivity of active subunits must be nearly equal throughout the various stages or compartments of the subunit cycle.

The conclusion that there occurs a relatively rapid flow of newly made ribosomes through polysomes is consistent with

our knowledge about the rate of protein synthesis in other systems. In rabbit reticulocytes it has been estimated that 0.25 min at 37° is required to polymerize a globin chain of 145 amino acids (Knopf and Lamfrom, 1965). At this rate it would require 3.5 min to synthesize the myosin chain of 2000 amino acids. Although the exact kinetics of radioactive ribosome flow into the cytoplasm is difficult to measure in embryonic muscle, our studies with many embryos indicate that the specific radioactivity of cytoplasmic rRNA doubles only about every 10–15 min between 38 and 75 min after [^{32}P]phosphate injections. A similar relatively slow rate of entry into the cytoplasm occurs in HeLa cells (Joklik and Becker, 1965; Girard *et al.*, 1965) and in ascites tumor cells (Hogan and Korner, 1968).

(iii) In view of the latter conclusions, the long delay observed in the labeling of single ribosomes (Figure 9) indicates that at least the majority of these particles do not participate in the subunit cycle (see further discussion below).

(iv) By 210 min after [^{32}P]phosphate injections of embryos, the specific radioactivity of rRNA in the subribosomal region has become very nearly equal with that in polysomes (Figure 9). It should be noted that the proportion of 28S rRNA as compared with 18S rRNA is lower in the subribosomal fractions compared with that in the polysomes or single ribosomes. The eventual equalization of the specific radioactivities of polysomes and subribosomal particles has been demonstrated previously and in more detail with other animal cells (Joklik and Becker, 1965; Vaughan *et al.*, 1967). This has led to the conclusion that subribosomal particles are an obligatory stage in the subunit cycle (Hogan and Korner, 1968).

On the other hand, the apparently slow attainment of this equilibration does not at first sight appear consistent with the conclusion that equilibration of subunits and polysomes is rapid. In the early stages of the labeling it appears on the contrary that the subribosomal particles contain more highly labeled rRNA than do the polysomes. The differential appears especially pronounced for the 18S rRNA as is also apparent in the results of Hogan and Korner (1968) using Landschutz ascites cells. This discrepancy can be explained, however, if we assume that the subribosomal particles which first enter into the cytoplasm are inactive in protein synthesis. Thus, the specific radioactivities of *functional* subribosomal particles might be equal with those in polysomes throughout the labeling. Indeed, Perry and Kelley (1966, 1968) have shown that the buoyant densities of subribosomal particles in cultured L cells are anomalous just after they emerge from nuclei. Some time is required before the buoyant densities become equal to those of mature subribosomal particles. Thus, we believe that our data are in agreement with the conclusion that equilibration of polysomal ribosomes with functional subribosomal particles is rapid in embryonic muscle.

Existence of Single Ribosomes Which Do Not Participate in the Subunit Cycle. The labeling characteristics of single ribosomes both with [^{32}P]phosphate (Figure 9) and with amino acids (Figure 2) suggests that they exist in the tissue and are not derived from broken polysomes or from aggregated subribosomal particles during the preparation of muscle extracts. Moreover, the very slow labeling of single ribosomes with [^{32}P]phosphate (Figure 9) indicates that *neither subunit* of the single ribosomes was participating in protein synthesis *in vivo*. A simple explanation is that the subunits were associated in the cell and were perhaps inactive by virtue of this association.

Although we cannot exclude the possibility that a small fraction (10% or fewer) of the single ribosomes participate in the subunit cycle after falling off the mRNA and releasing the newly made polypeptide chain, we do not believe that there is any evidence that they participate in normal protein synthesis. Single ribosomes are seen to accumulate in cells starved of needed hormones (Wool *et al.*, 1968), of growth factor proteins (Levinthal and Rubin, 1968; Cohen and Stastny, 1968), of amino acids (Hogan and Korner, 1968), or treated with fluoride or puromycin. However, this accumulation may reflect another process rather than a block in the normal subunit cycle. The results presented here suggest that a significant portion of single ribosomes in muscle do not have a role in the normal subunit cycle.

The eventual flow of labeled rRNA which occurs into single ribosomes (Figure 9) makes it unlikely that they are sequestered into cells which are inactive in ribosome synthesis. A slow flow of label into single ribosomes also occurs in HeLa cells (Joklik and Becker, 1965). Although it might be imagined that single ribosomes accumulate in older myofibers as they lose activity in rRNA synthesis, the older fibers remain very active in protein synthesis (Yaffe and Fuchs, 1967) and the proportion of single ribosomes does not increase during maturation of thigh muscle between 14 and 18 days of embryonic development (Heywood and Rich, 1968). Thus, although the proportion of mature fibers obviously increases in the developing muscle, no proportional rise is observed in the level of single ribosomes. We cannot exclude the possibility that the single ribosomes are present in cells which are in the process of programmed cell death. Such cells might contain a constant proportion of the total muscle ribosomes throughout muscle development. We think it most likely, however, that the inactive single ribosomes are present within all cells of the population but are participating in a process other than protein synthesis. It is possible that they are attached to mRNA which is being stored or to fragments of mRNA being degraded. As such, they would be a part of the cellular regulatory mechanism.

Previous studies of the radioactive labeling of ribosomes in eukaryotic cells (Girard *et al.*, 1965; Joklik and Becker, 1965; Hogan and Korner, 1968) are in agreement with our conclusions. However, in these studies it seemed that the ribosomes in polysomes equilibrate only slowly *both* with the subunit pool and with the pool of single ribosomes. The apparently slow equilibration of polysomal ribosomes with the subunit pool made it difficult to rigorously interpret the slow equilibration of polysomal with single ribosomes. For example, it could be argued that the newly synthesized ribosomes were particularly sluggish in protein synthesis (Girard *et al.*, 1965) so that they cycled only slowly through the protein-synthesizing machinery, or else that the subunits, the polysomes, and the single ribosomes were each in separate compartments of the cell. In the present work, by analyzing the flow of newly made ribosomes into different sizes of polysomes we have been able to conclude that the *functional* subribosomal particles equilibrate very rapidly with polysomes but not with single ribosomes. As discussed above, newly made subribosomal particles are apparently inactive in protein synthesis for some time after they emerge from the nucleus into the cytoplasm. It seems likely that in all eukaryotic cell populations the vast majority of single ribosomes are not functionally engaged in the ribosomal subunit–polysome cycle. It will be of interest to study

single ribosomes further and to learn about their functional significance.

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