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A Study of Muscle Polyribosomes and the Coprecipitation of Polyribosomes with Myosin*

Stuart M. Heywood,† Robert M. Dowben, and Alexander Rich

ABSTRACT: Polyribosomes from chick skeletal muscle were prepared and found to be very active in protein synthesis. The use of high salt concentrations (0.25 M) in the buffers is necessary because myosin coprecipitates with polysomes at low salt concentrations. The coprecipitation is not limited to a particular size class of polysomes. The addition of myosin to polysomes from tissues other than muscle also results in coprecipitation. This phenomenon can be utilized to prepare purified, enriched polysomes.

Muscle is a tissue uniquely suited for the study of the biosynthesis of relatively insoluble structural proteins and their organization into an architecturally complex pattern. Muscle also lends itself for studies of the effects of hormones on protein synthesis. A number of investigators have addressed themselves to this problem by attempting to prepare cell-free polyribosomal systems from skeletal muscle capable of incorporating labeled amino acids into trichloroacetic acid precipitable material (Breuer *et al.*, 1964; Earl and Korner, 1965; Rampersad *et al.*, 1965; Strohman, 1966). In an earlier report (Heywood *et al.*, 1967), we described a cell-free polyribosomal system prepared from chick embryo skeletal muscle which was very active in incorporating labeled amino acids into proteins, including material identified as myosin. In that report it was noted that skeletal muscle polysomes tended to coprecipitate with myosin. In this communication, we report further studies characterizing the myosin-synthesizing system and describe in detail the characteristics of the interaction between myosin and polysomes.

Experimental Procedure

Leg muscles from 14-day-old chick embryos dissected free of skin and bone were homogenized in an equal volume of ice-cold M buffer (0.25 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris, pH 7.4) by five strokes in a loose-fitting Dounce homogenizer (clearance 0.12 mm). More vigorous homogenization or the use of a more tightly fitting homogenizer resulted in degradation of polysomes. The homogenate obtained from about 0.7 g of muscle was centrifuged at 10,000*g* for 10 min to remove mitochondria and cell debris. The cytoplasmic supernatant was then layered on 27 ml of a 15–40% (w/w) linear sucrose gradient in M buffer. After centrifugation for 2 hr at 25,000 rpm in a Spinco No. 25.1 rotor at −18°, fractions were collected from the botton of the tube for absorbance measurements at 260 mμ and assays of radioactivity.

For some preparations, polyribosomes were isolated by coprecipitation with myosin at low salt concentration. The cytoplasmic extracts of muscle and other tissues prepared as outlined above were diluted by the

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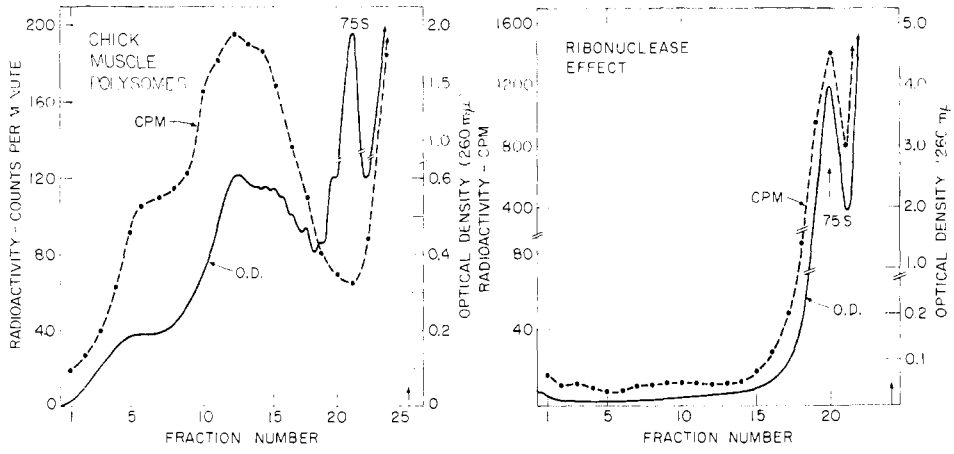

FIGURE 1: Analyses of chick skeletal muscle. Left: a sucrose gradient analysis of the cytoplasmic supernatant of 14-day-old chick skeletal muscle homogenized in 0.25 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.4). Embryos were injected with 5 μCi: [^{14}C]-amino acid mixture intramuscularly 5 min before sacrifice. Total radioactivity in the gradient 7616 cpm; radioactivity at the top of the gradient 5382 cpm. Right: an aliquot of same cytoplasmic supernatant was treated with 2 μg/ml of ribonuclease for 15 min at 2° prior to sedimentation. This results in the disappearance of absorbance and radioactivity in the polysome region and their appearance in the ribosomal monomer and dimer region. Total radioactivity in the gradient, 8160 cpm; radioactivity at the top of the gradient, 5407 cpm. The sucrose gradients were 15–40% (w/w). The direction of sedimentation is to the left. The arrow at the lower right shows the position of the last fraction. The single ribosome peak is labeled 75 S.

addition of ten volumes of D buffer (0.01 M $MgCl_2$ and 0.01 M Tris, pH 7.4). The precipitates were collected after centrifugation at 3000*g* for 10 min and resuspended in a small volume of 1 M buffer for sedimentation in sucrose gradients or in a small volume of B buffer (0.15 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris, pH 7.4) for *in vitro* amino acid incorporation studies. Polyribosomes obtained in this way had a 260/280 mμ absorbance ratio of 1.6–1.7.

The cell-free amino acid incorporation system, which was described previously (Heywood *et al.*, 1967), contained 0.15 M KCl, 0.01 M $MgCl_2$, 6 mM β-mercaptoethanol, 0.01 M Tris (pH 7.4), 2 mM ATP,[1] 0.5 mM GTP, 10 mM phosphoenolpyruvate, 0.25–0.5 μM for each of 20 amino acids which contained 0.5 μCi of a uniformly labeled [^{14}C]amino acid mixture, 50 μg of pyruvate kinase, 0.2 mg of chicken liver tRNA, and 0.4 mg of pH 5 enzyme fraction. In some experiments, instead of the pH 5 enzyme fraction, ribosome-free high-speed supernatant fraction was used which was prepared by homogenizing tissue in M buffer, centrifuging at 150,000*g* for 1 hr, and dialyzing the supernatant fraction against several changes of incubation buffer.

RNA was extracted from the cytoplasmic supernatant of skeletal muscle homogenized in 0.5 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.4). After centrifugation at 10,000*g* for 10 min, EDTA and sodium dodecyl sulfate were added to the supernatant fraction to final concentrations of 0.015 M and 0.5%, respectively. Polysomes coprecipitated with myosin were resuspended in 0.5 M NaCl, 0.015 M EDTA, 0.01 M Tris, and 0.5% sodium dodecyl sulfate (pH 7.4). An equal volume of phenol was added to either type of suspension, and the mixture was extracted three times with 4-ml portions of chloroform containing 1% isoamyl alcohol at 55° to float the protein at the interface. After the final extraction, the aqueous phase was added to three volumes of 95% ethanol and allowed to stand overnight at −20°. The precipitate of RNA was collected and dissolved in a small volume of buffer containing 0.04 M Tris, 0.02 M sodium acetate, and 0.002 M EDTA (pH 7.6). Approximately 0.15 mg of RNA was layered on a 17-ml 5–23% (w/w) linear sucrose gradient containing 0.5% sodium dodecyl sulfate in the Tris–acetate–EDTA buffer. The gradient was then centrifuged in a Spinco No. 25.3 rotor at 23,000 rpm for 13 hr.

Protein was determined by the method of Lowry *et al.* (1951). RNA was measured by its ultraviolet absorption (E_{260} 20 cm/mg). To assay radioactive proteins, a precipitate was formed by adding trichloroacetic acid to a final concentration of 5%. The trichloroacetic acid mixture was heated to solubilize any RNA from the precipitate. The protein precipitate was then collected, dissolved in 1 M NaOH, and reprecipitated in 5% trichloroacetic acid. This precipitate was dissolved in 1 ml of 4 M NH_4OH and then added to Bray's solution and counted in a Nuclear-Chicago scintillation counter.

Merck (reagent grade) or Mann (ribonuclease-free) sucrose was used. Tris, ATP, GTP, and phosphoenolpyruvate were purchased from Sigma Chemical Co. The pyruvate kinase was purchased from Boehringer-Mannheim. The [^{14}C]amino acids were prepared by New England Nuclear Corp. All other chemicals were commercial reagent grade.

Results

Properties of Polyribosomes from Muscle. A typical polyribosome distribution from the skeletal muscle cytoplasmic supernatant of 14-day-old chick embryos is shown in Figure 1. The large peak of single ribosomes labeled 75 S has a shoulder representing dimers. The oscillations in the more rapidly sedimenting region of the optical density trace represent successively higher members of the polysome series. The profile shows two peaks,

[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).

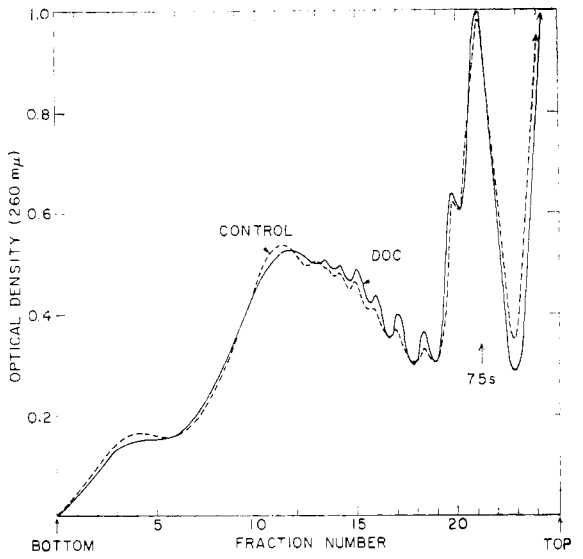

FIGURE 2: Sucrose gradient profile of cytoplasmic supernatant from chick embryo skeletal muscle with and without 0.5 % sodium deoxycholate.

one due to polysomes containing 15–25 ribosomes, and the other representing polysomes of 50–65 units, as determined by electron micrographs (Heywood *et al.*, 1967).

Deoxycholate or other detergents are frequently used in the preparation of polysomes from the cells of higher organisms in order to free them from attachment to the membranous endoplasmic reticulum. Little, if any, sarcoplasmic reticulum is present in 14-day-old-chick embryo muscle (Allen and Pepe, 1965; Fischman, 1967). It is not surprising, then, that the use of 0.5% deoxycholate in the preparation of chick embryo muscle polysomes had little effect on the polysome pattern (Figure 2). It is unlikely, therefore, that membranous material is associated with chick embryo polysomes prepared without deoxycholate.

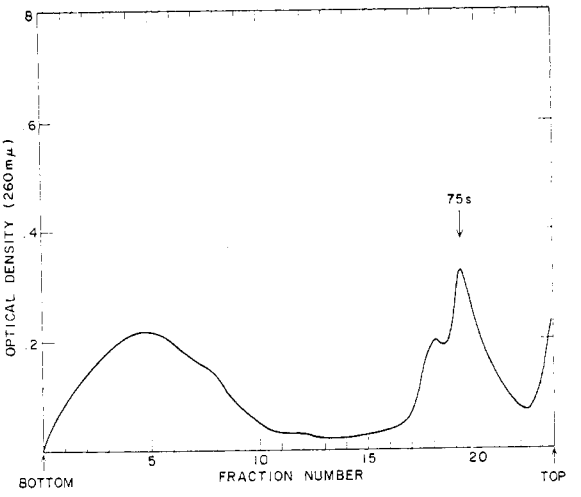

FIGURE 4: Large polysomes (50–65 units) from six sucrose gradients were pooled and pelleted by centrifugation at 150,000*g* for 3 hr. This included material from fractions 3–7 in Figure 1. The pellet was gently resuspended in M buffer and then centrifuged at 10,000*g* for 10 min. The resulting supernatant was layered on a 15–40% sucrose gradient and centrifuged at 25,000 rpm for 2 hr. A prominent peak of large polysomes was still observed.

A typical polysome profile for cardiac muscle from 14-day-old chick embryos is shown in Figure 3; it bears a general resemblance to the skeletal muscle profile. Treatment of either the skeletal muscle or cardiac muscle cytoplasmic supernatants with 1 μg/ml of ribonu-

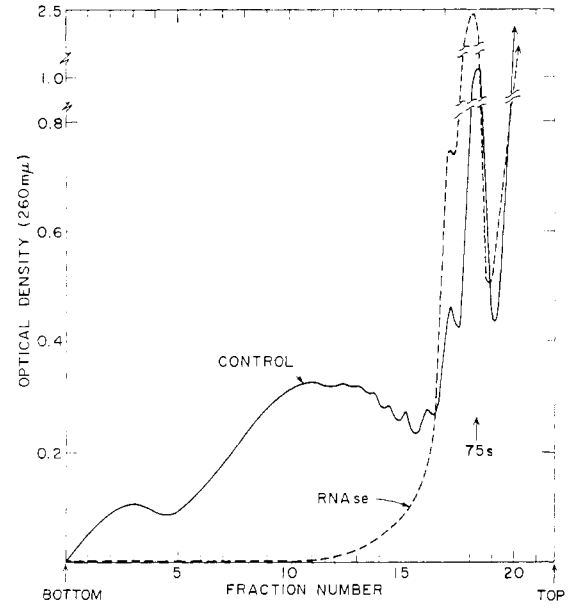

FIGURE 3: Sucrose gradient analysis of the cytoplasmic supernatant of 14-day-old chick embryo heart prepared in the same manner as that from skeletal muscle. An aliquot of the cytoplasmic supernatant was treated with 2 μg/ml of ribonuclease for 15 min at 2° prior to sedimentation.

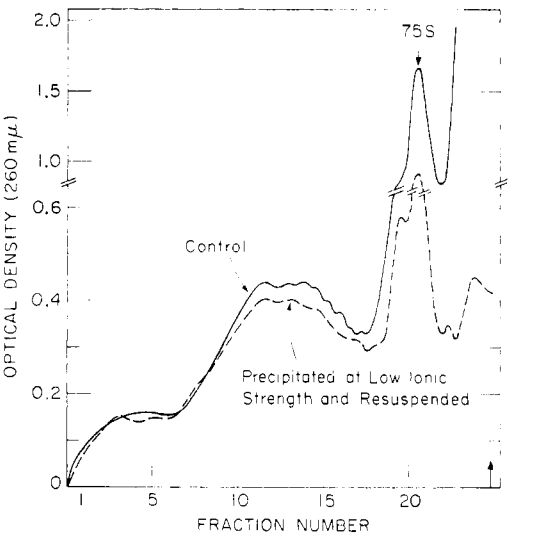

FIGURE 5: The KCl concentration of a cytoplasmic extract of chick embryo skeletal muscle was lowered to 0.025 M. The precipitate was harvested and resuspended in buffer containing 0.25 M KCl. The profile of the sucrose gradient of resuspended material is compared with that of an unprecipitated control prepared as in Figure 1. Virtually all of the polysomes and about 60% of the single ribosomes were recovered.

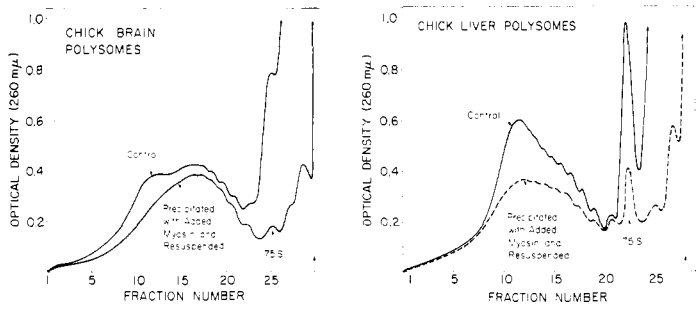

FIGURE 6: Brain or liver (0.7 g) was homogenized in 0.25 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.4) and 0.5 % deoxycholate was added to the cytoplasmic extracts. Chicken myosin (2 mg/ml) was added and the KCl concentration was lowered to 0.025 M by dilution. The precipitates were harvested and resuspended in buffer containing 0.25 M KCl. The profile of the sucrose gradient is shown together with a control sucrose gradient obtained directly from the cytoplasmic extracts. Virtually all of the polysomes was recovered from the myosin coprecipitate but only a fraction of single ribosomes was recovered.

TABLE I: Effect of Salt Concentration upon Recovery of Polysomes from Cytoplasmic Extract of Chick Embryo Skeletal Muscle.[a]

KCl Concn (M)	Polysomes Recovd (mg)
0.05	0.2
0.1	0.5
0.25	0.8
0.3	0.8
0.5	0.8

[a] Chick embryo skeletal muscle (0.7 g) was homogenized in 0.01 M $MgCl_2$ and 0.01 M Tris (pH 7.4) plus various amounts of KCl. The absorbance at 260 mμ was measured in pooled polysome fractions after sedimentation of the cytoplasmic supernatant in a 15–40% sucrose gradient.

clease at 2° for 15 min resulted in complete degradation of the polyribosomes and their conversion into single ribosomes (Figures 1 and 3).

When 5 μCi of [^{14}C]amino acid mixture was injected intramuscularly into the embryos 5 min before sacrifice, most of the radioactivity in the sucrose gradient was found in the polysome fractions and relatively little was associated with the single ribosomes (Figure 1). After treatment with ribonuclease, the radioactivity was found in the single ribosome peak. The absence of appreciable amounts of radioactivity in the single polysome peak suggests that the polysome preparation is largely undegraded (Rich *et al.*, 1963).

A prominent feature of the muscle polysome pattern is the peak representing very large, 50–65-unit polysomes. To determine whether they represented large polysomes or aggregates of smaller polysomes, fractions containing these heavy polysomes were pelleted by centrifugation at 150,000*g* for 3 hr. The pellet was resuspended in a small quantity of M buffer, followed by centrifugation at 10,000*g* for 10 min to remove any material not resuspended. The supernatant was layered on a 15–40% sucrose gradient and resedimented. Even though losses were incurred in the pelleting and resuspension, and despite gentle handling, some mechanical disruption was unavoidable. Nevertheless, 60–70% of the larger (50–65) unit polysomes resedimented in the same region (Figure 4).

As noted previously (Heywood *et al.*, 1967), polyribosomes from muscle were obtained in high yield only when prepared in buffer containing a high salt concentration. The yield of muscle polysomes increased with increasing KCl concentration in the homogenization medium up to 0.25 M KCl (Table I). Further increases in KCl concentration did not improve the yield. At 0.25 M KCl, the conditions correspond approximately to those required for dissolution of the actomyosin complex (Portzehl, 1950). At low salt concentration, the polysomes coprecipitate with myosin.

Coprecipitation of Polyribosomes and Myosin. The coprecipitation phenomenon was examined in more detail. A cytoplasmic supernatant of chick embryo skeletal muscle was diluted with ten volumes of D buffer. The precipitate which formed was collected and redissolved in a small quantity of M buffer, layered on a sucrose, gradient and sedimented. A typical profile of such a gradient together with that of a standard control preparation run simultaneously are shown in Figure 5. The results indicate that the precipitates obtained by lowering the salt concentration contain virtually all of the polysomes and 20–35% of the single ribosomes in comparable control preparations. There is no indication that any size class of polysomes is selectively precipitated.

The tendency for polysomes and myosin to coprecipitate in buffers of low salt concentration is not restricted to muscle polysomes, but appears to be a general property of polysomes. A density gradient profile of polysomes isolated by coprecipitation after the addition of chicken myosin to the cytoplasmic supernatant of chick brain is shown in Figure 6a. For comparison, a density gradient profile of a comparable quantity of cytoplasmic supernatant is also shown. Figure 6b depicts a similar set of profiles using chick liver. The brain and liver polysomes were almost completely recovered from the myosin precipitate. However, only 20–25% of the single ribosomes was precipitated. Similar results were obtained using myosin coprecipitation in the preparation of rat liver, rabbit reticulocyte, and *Escherichia coli* polysomes.

The coprecipitation of RNA and myosin was studied further by means of ^{14}C-labeled synthetic polyribonucleotides and myosin from adult chicken pectoral muscles was prepared by the method of Finck (1965). The results are summarized in Table II. In 0.04 M salt solution, 92–98% of the labeled polyribonucleotides was found in the washed myosin precipitates. Of a 1:1 mixture of poly A and poly U, however, 82.1% was precipitated with myosin. On the other hand, labeled tRNA or rRNA did not coprecipitate with myosin.

TABLE II: Coprecipitation of Polyribonucleotides and Myosin.

Polyribonucleotide	% Radioactivity Recovd −Myosin	% Radioactivity Recovd +Myosin
[^{14}C]Poly A	0	97.5
[^{14}C]Poly A + [^{14}C]poly U (1:1)	0	82.1
[^{14}C]Poly (UA)	0	98.5
[^{14}C]Poly (UAC)	0	92.5
[^{3}H]tRNA	0	1.6
[^{3}H]rRNA	0	6.0

To 1 ml of buffer (0.5 M KCl, 0.01 M Tris, and 0.001 M EDTA, pH 7.0) containing 0.1 mg of radioactively labeled polyribonucleotide was added 1 ml of buffer containing 1 mg of myosin. The solution was diluted 12-fold with water, and the precipitate was collected, washed, and assayed for radioactivity. Parallel runs were made without myosin.

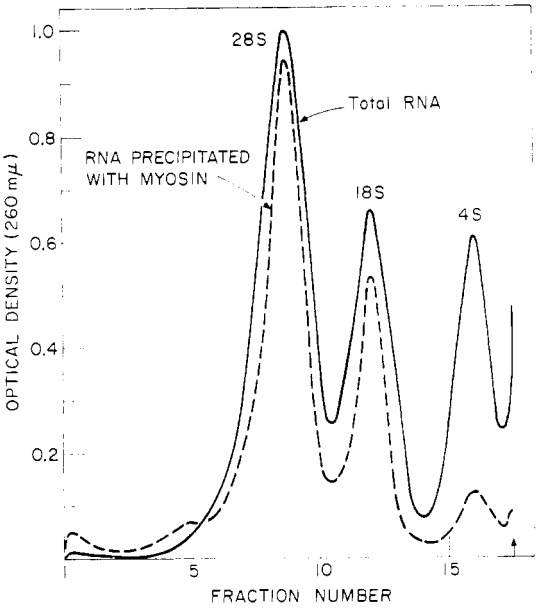

FIGURE 7: RNA was extracted from whole cytoplasmic supernatant and from myosin-preipitated polysomes of chick skeletal muscle as described in the text. Sucrose gradient analyses of the two preparations are depicted. The recoveries of 28S and 18S RNA were similar but only a small fraction of the 4S RNA was recovered from the myosin–polysome precipitate.

Experiments were carried out to analyze the RNA of the myosin–polysome precipitate. A density gradient absorption profile of RNA obtained from muscle polysomes coprecipitated with myosin at low salt concentration is shown in Figure 6. For purposes of comparison, the profile of total RNA obtained directly from an equal quantity of cytoplasmic supernatant is also shown. The amounts of 28S and 18S RNA in the myosin-precipitated polysomes and in the cytoplasmic supernatant are approximately equal. However, relatively little 4S RNA was recovered from the myosin-precipitated polysomes. The small 4S peak in the precipitated material probably represents tRNA bound to polysomes. In addition, it probably contains the 5S RNA, a type of RNA that has been isolated in small amounts from mammalian and bacterial ribosomes (Comb and Katz, 1964) which differs in its base composition and physical properties from 4S tRNA (Figure 7).

In Vitro Amino Acid Incorporation by Myosin-Precipitated Polysomes. The property of coprecipitation may be used to advantage for the rapid preparation and enrichment of polysomes from tissues other than muscle. Some purification is achieved because most of the other constituents of the cytoplasmic supernatant do not precipitate with myosin but remain suspended. Polysomes prepared in this way can be used for *in vitro* incorporation of labeled amino acids into hot trichloroacetic acid precipitable material. In addition to polysomes, ATP, GTP, and pH 5 enzyme fraction were required for *in vitro* incorporation of ^{14}C-labeled amino acids in trichloroacetic acid precipitable material.

Using chick embryo skeletal muscle polysomes prepared by precipitation at low salt concentrations, the optimal concentrations of KCl, pH 5 fraction enzymes, and polysomes were determined. The incorporation of labeled amino acids at various concentrations of KCl is shown in Figure 8; greater incorporation occurred in buffer (0.01 M $MgCl_2$ and 0.01 M Tris, pH 7.4) containing 0.15 M KCl than if the concentration was lower or higher. It may be noted that the optimal KCl concentration, 0.15 M, is higher than that commonly used in mammalian systems for *in vitro* amino acid incorporation. In Figure 9, the extent of labeled amino acid incorporation is depicted for various concentrations of polysomes using 0.4-mg/ml pH 5 fraction enzymes, and for various concentrations of pH 5 fraction enzymes using 0.5 mg/ml of polysomes.

In the cell-free system, incorporation of amino acids proceeds linearly at a more or less constant rate for 60 min when pH 5 fraction enzymes are used. After this time, the rate of incorporation diminishes (Figure 10), coming to a virtual halt. When a high-speed, ribosome-free supernatant was used in place of the pH 5 fraction enzymes, amino acid incorporation proceeded linearly for about 90 min, continuing at a gradually diminishing rate for the duration of the experiments (2 hr). Amino acid incorporation by the cell-free system ceases promptly upon the addition of ribonuclease, and this procedure can be used to terminate the reaction.

In order to evaluate the efficiency of the release of newly synthesized protein by the polysomal system, the reaction was stopped after various periods of incubation by the addition of ribonuclease (20-μg/ml final concentration) and the mixture was chilled. The KCl concentration was adjusted to 0.3 M and the mixture was centrifuged for 2 hr at 150,000*g* to pellet ribosomal material. The hot trichloroacetic acid precipitable material in the pellet and in the supernatant was assayed sep-

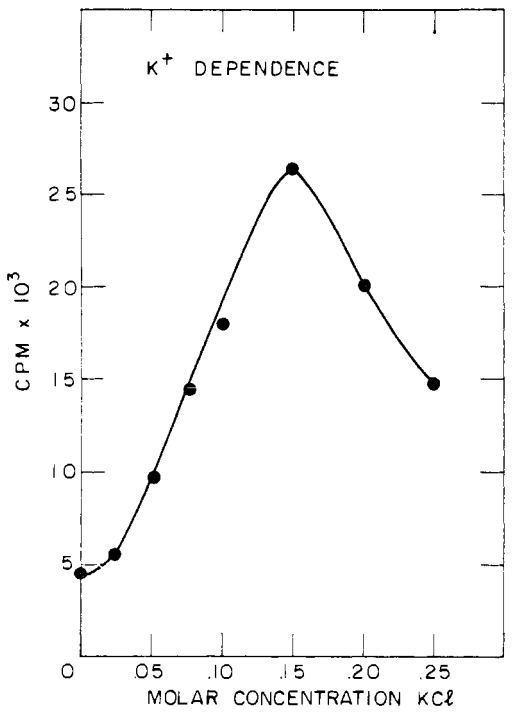

FIGURE 8: The effect of KCl concentration of [^{14}C]amino acid incorporation by chick embryo skeletal muscle polysomes isolated by precipitation at low salt concentration. The precipitate was resuspended in 1-ml incubation buffer containing 0.01 M $MgCl_2$–0.01 M Tris (pH 7.4), 6 mM β-mercaptoethanol, 2 mM ATP, 10 mM phosphoenolpyruvate, 0.5 mM GTP, 0.3 μM of each amino acid containing 0.5 μCi of [^{14}C]amino acids, 50 μg of pyruvate kinase, 0.2 mg of chicken liver tRNA, 0.4 mg of pH 5 enzyme fraction from chick muscle, 0.5 mg of polysomes, and various amounts of KCl. After incubation at 37° for 1 hr, the radioactivity in the hot trichloroacetic acid precipitable material was assayed.

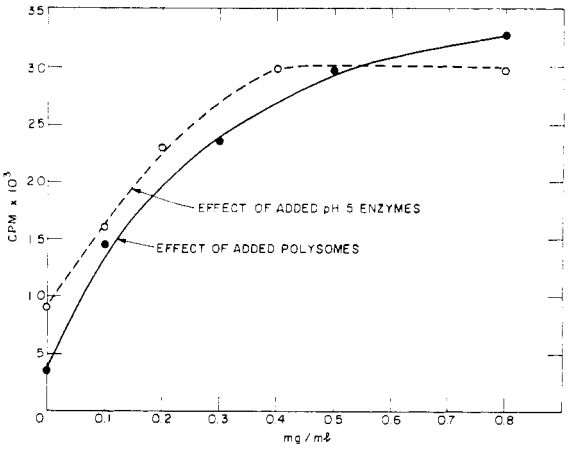

FIGURE 9: The effect of varying additions of pH 5 fraction enzymes (with 0.5 mg of polysomes) or of varying additions of polysomes (with 0.4 mg of pH 5 enzymes) on [^{14}C]amino acid incorporation by chick skeletal muscle polysomes isolated by precipitation at low salt concentration. Incubation conditions were the same as for Figure 8.

arately for radioactivity. In this way, the nascent protein bound to ribosomes and the soluble protein released from polysomes were quantified. The amount of soluble protein released by polysomes, expressed as the percentage of radioactivity found in the supernatant fraction to the total trichloroacetic acid precipitable counts, is shown in Figure 11 as a function of incubation time. The fraction of radioactivity in the supernatant fraction gradually increased reaching 48% of the total trichloroacetic acid precipitable counts at 60 min. It can be concluded that the polysomes steadily release protein into the soluble fraction at a significant rate as *in vitro* amino acid incorporation continues.

The activity of the *in vitro* system can be approximately quantified as follows. Each milliliter of incubation mixture contained about 6 mμmoles of amino acids containing 0.5 μCi of ^{14}C. About 46,000 dpm (30,000 cpm assuming a counting efficiency of 65%) or about 1.4 mμmoles of amino acids/mg of rRNA was incorporated into trichloroacetic acid precipitable material. Such calculations indicate that an average of 20–30 amino acid residues were incorporated per ribosome. By way of comparison, 0.008 mμmole of amino acids/mg of RNA was incorporated into trichloroacetic acid precipitable material using endogenous muscle mRNA in the system described by Breuer *et al.* (1964).

Discussion

Both skeletal muscle and heart polyribosomes have a characteristic population of rapidly sedimenting polysomes which are not found in liver or brain preparations. These polysomes resediment as large polysomes, making it unlikely that they are aggregates of smaller units. These large polysomes appear to be involved in the biosynthesis of myosin (Heywood *et al.*, 1967).

Many large polysomes have been observed in thin-section electron micrographs of embryonic muscle (Allen and Pepe, 1965; Fischman, 1967). Although Clavert *et al.* (1949) observed basophilic material in the A bands of stained sections of muscle which was removed by pretreatment with ribonuclease, electron micrographs have failed to reveal a structural association between polysomes and the thick filaments in either embryonic muscle or in mature myofibrils. However, fragments of sarcoplasmic reticulum which is rich in RNA have been found in preparations of isolated myofibrils (Muscatello *et al.*, 1962).

It has long been appreciated that RNA is associated with myosin (Perry, 1952, 1960; Mihalyi *et al.*, 1957; Perry and Zydowo, 1959; Baril *et al.*, 1966), both in myofibrils and in isolated myosin. The RNA associated with myosin has a base composition similar to that of rRNA (Mihalyi *et al.*, 1957). Recently, Zak *et al.* (1967) showed that about 85% of myofibrillar RNA sediments at 28 and 18 S, species characteristic of rRNA in roughly the proportions found in ribosomes, while the remaining 15% is largely rapidly sedimenting RNA. At the present time, it is not known whether this RNA has a specific role in the organization of the myofibrillar proteins or in the function of the contractile mechanisms. It seems likely that some, if not all, of the RNA in preparations of myosin is an impurity resulting from its tendency to coprecipitate.

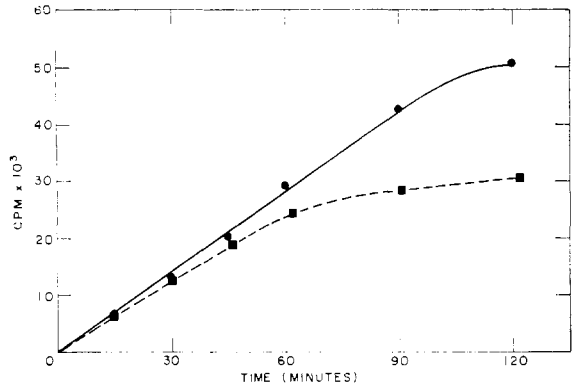

FIGURE 10: The time course of [^{14}C]amino acid incorporation by skeletal muscle polysomes using the incubation conditions of Figure 8 with 0.4 mg of pH 5 fraction enzymes or with 0.6 mg of ribosome-free high-speed supernatant fraction.

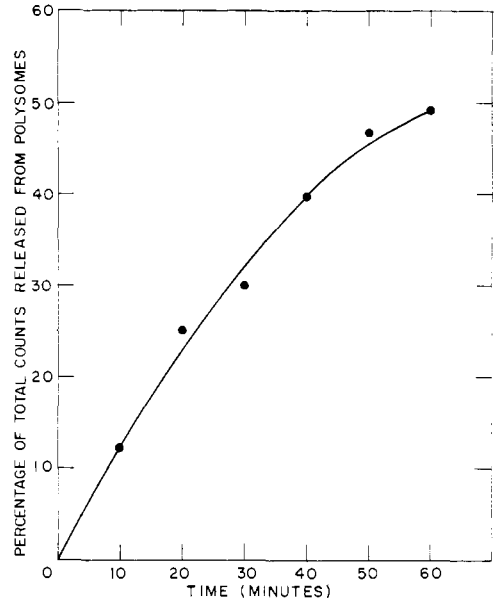

FIGURE 11: After *in vitro* incorporation of [^{14}C]amino acids by chick embryo skeletal muscle polysomes, the radioactivity in total hot trichloroacetic acid precipitable fraction and in the hot trichloroacetic acid precipitable material attached to polysomes was determined. The incubation conditions were the same as in Figure 8; the reaction was terminated by addition of 20 μg of ribonuclease. The graph shows the percentage of precipitable counts released from the polysomes with increasing incubation time.

The proclivity of polysomes to coprecipitate with myosin has a direct bearing upon studies of muscle protein biosynthesis. In tissues containing myosin, the use of low salt concentration buffers such as are frequently employed in the isolation of polysomes, leads to large losses, probably because of the presence of naturally occurring myosin. When a high salt concentration buffer is used, polysomes can be obtained from muscle in high yield. The RNA associated with the polysome–myosin precipitates contained largely 28S and 18S RNA and accounts for almost all of the cytoplasmic RNA of these classes. On the other hand, very little 4S RNA was precipitated. These results correspond well with those of Zak *et al.* (1967).

It is not clear what kinds of interactions are responsible for the coprecipitation phenomena. Myosin forms insoluble complexes with synthetic polynucleotides (which possess little secondary structure) as well as with polyribosomes. In this connection, it is worth noting that a 1:1 mixture of poly A and poly U (which can form double strands owing to hydrogen bonds) was less completely precipitated than either poly A or poly U alone. Although myosin does not coprecipitate with either rRNA or tRNA (small molecules with a great deal of secondary structure), it may form soluble complexes with them. The fact that the yield of polysomes in the precipitate is substantially greater than that of single ribosomes may be due to the presence of mRNA (a large molecule not covered with protein) which may bind more myosin than rRNA. It is possible that the interactions are largely electrostatic between the polynucleotides and clusters of cationic sites on the myosin molecule. The dissolution of the precipitates upon increasing the salt concentration may represent solvation, or may represent weakening of such electrostatic interactions.

The resuspended polysomes can be utilized in a very active cell-free system which incorporates labeled amino acids into hot trichloroacetic acid precipitable material, which first is attached to polysomes and then is released into the high-speed supernatant fraction. The prolonged period of virtually linear amino acid incorporation may indicate that significant polypeptide chain initiation takes place in addition to chain extension. Using the very large polysomes from chick skeletal muscle, the protein product of the *in vitro* system has been identified as myosin (Heywood *et al.*, 1967).

In media of low salt concentration, it appears that myosin has the ability to form mixed precipitates with polysomes of all types, with polysomes from tissues other than muscle and from bacteria, as well as from mammalian cells. Polysomes can be isolated from the postmitochondrial supernatant fraction of tissues other than muscle by the addition of highly purified myosin and lowering the salt concentration. The polysomes may then be redissolved in high salt concentration medium, and used for *in vitro* [^{14}C]amino acid incorporation studies. Large quantities of active undegraded polysomes can be prepared rapidly using this procedure, which in some cases may permit the synthesis of sufficient protein for isolation and identification.

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The Biosynthesis of Pyridomycin. I*

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ABSTRACT: Biosynthesis of pyridomycin by growing cells of *Streptomyces pyridomyceticus* in a complex medium was studied by observing incorporation of various labeled compounds into this antibiotic. L-Aspartic acid, glycerol, 3-hydroxypicolinic acid, propionic acid, pyruvic acid, L-threonine, and L-isoleucine were incorporated into pyridomycin. L-Lysine, DL-2-aminoadipic acid, L-proline, L-phenylalanine, L-methionine, formic acid, L-serine, and L-alanine were not incorporated into pyridomycin. Degradation studies of pyridomycin according to Figure 1 showed that the radioactivity of L-[^{14}C]aspartic acid and [^{14}C]glycerol was found in both pyridine nuclei of pyridomycin. Sodium pyruvate was also incorporated into 3-hydroxypicolinic acid. These facts indicate that 3-hydroxypicolinic acid is biosynthesized from L-aspartic acid and glycerol or pyruvic acid and the pyridine ring of the C_{11} moiety of pyridomycin is derived from L-aspartic acid and glycerol. This is the first paper reporting the biosynthesis of pyridine nuclei by *Streptomyces*. [^{3}H]3-Hydroxypicolinic acid was introduced into the 3-hydroxypicolinic acid part of pyridomycin.

However, DL-[^{14}C]tryptophan and [^{3}H]3-hydroxyanthranilic acid were not incorporated into pyridomycin. Experiments testing the incorporation of propionic acid and pyruvic acid showed that the C_6 side-chain part of the C_{11} moiety ($C_{11}H_{16}N_2O_3$) containing pyridine was derived from these two precursors. [^{14}C]-Propionic acid was incorporated at high rate and selectively into the terminal C_3 part of this moiety. To the best of our knowledge, this is the first study to observe the participation of propionic acid in the biosynthesis of an amino acid and a peptide. Label from L-threonine was found in the L-threonine moiety of pyridomycin, and that of L-isoleucine was detected almost exclusively in the α-keto-β-methylvaleric acid portion of pyridomycin. From these results, the biosynthetic pathway of pyridomycin was illustrated in Figure 2.

Pyridomycin is an antimycobacterial antibiotic (Maeda *et al.*, 1953) produced by *Streptomyces pyridomyceticus* (Okami *et al.*, 1954). The structure has been recently determined by X-ray analysis (Koyama *et al.*, 1967) and chemical degradation (Ogawara *et al.*, 1968). Pyridomycin, a 12-membered ring compound, is hydrolyzed to α-keto-β-methylvaleric acid, 3-hydroxypicolinic acid, L-threonine, and 4-amino-3-hydroxy-2-methyl-5-(3-pyridyl)pentanoic acid which is further degraded to α-formylpropionic acid and 3-pyridylacetaldehyde as shown in Figure 1. These hydrolysis and degradation products are easily obtained and it is possible to study the biosynthetic route of this antibiotic.

Concerning the biosynthesis of the pyridine ring by higher plants and microorganisms, tryptophan-3-hydroxyanthranilic acid pathway, aspartate–glycerol pathway, aspartate–pyruvate pathway, and mevalonate pathway have been reported (Leete, 1965). Although *Neurospora* and mammals utilize tryptophan as a source of niacin, *Escherichia coli* and *Bacillus subtilis* synthesize the pyridine ring from aspartic acid (Mattoon, 1963; Ogasawara *et al.*, 1967). In higher plants, for example, *Nicotiana rustica*, glyceraldehyde is incorporated into the pyridine ring of nicotine (Fleeker and Byerrum, 1967).

With these considerations in mind, experiments were performed to elucidate the biosynthetic route of the py-

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