Identification of Separate Polysomes Active in the Synthesis of the Light and Heavy Chains of Myosin*

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ABSTRACT: Cytoplasmic extracts of 14-day embryonic chick skeletal muscle were centrifuged through sucrose gradients and polyribosomes were separated into four size classes. Those fractions were then used to catalyze the *in vitro* synthesis of proteins using [^{14}C]amino acids as a source of radioactivity. After incubation, carrier myosin was added to the four fractions and each was subjected to conditions which first favor dissociation and then reassociation of myosin heavy and light chains.

The fractions were subsequently purified for myosin by low ionic strength precipitation and DEAE-cellulose chromatography. Myosin heavy and light chains were then separated using LiCl and citrate, and the radioactivity in each fraction was determined. The results showed that the light chains were synthesized on the size class of polyribosomes containing four to nine ribosomes whereas the heavy chains were synthesized separately on a much larger size class of polyribosomes containing 60–80 ribosomes. These results were confirmed by electrophoresis on polyacrylamide gels of the myosin light chain fractions from the *in vitro* incorporation. Light chains were not synthesized when embryonic liver was used as a source of polyribosomes.

It is generally believed that myosin has associated with it one or more low molecular weight proteins and that this association is necessary for the biological activity of the contractile protein (Dreizen *et al.*, 1967; Dreizen and Gershman, 1970; Gaetjens *et al.*, 1968; Gershman and Dreizen, 1970; Young, 1969). The low molecular weight "light chains" can be dissociated from the main "heavy chains" by a variety of means. These include treatment of myosin with urea (Tsao, 1953), alkaline pH (Gaetjens *et al.*, 1968; Connell and Olcott, 1961; Frederiksen and Holtzer, 1968; Gershman *et al.*, 1966), acetylation (Locker and Hagyard, 1967a,b), succinylation (Oppenheimer *et al.*, 1967), heat treatment (Locker and Hagyard, 1967a; Locker, 1956), 5 M guanidine hydrochloride (Tsao, 1953; Gershman *et al.*, 1966; Dreizen *et al.*, 1966), and more recently with salt treatment at neutral pH (Dreizen and Gershman, 1970; Gershman and Dreizen, 1970; Stracher, 1969). The light-chain fraction contains proteins in the molecular weight range of 17,000–30,000, which comprise from 5 to 17% of the molecular weight of intact myosin (Dreizen *et al.*, 1967; Frederiksen and Holtzer, 1968; Gazith *et al.*, 1970; Gershman *et al.*, 1966; Locker and Hagyard, 1967a; Kominz *et al.*, 1959). They have a well-defined amino acid composition, and although most evidence suggests that the fraction is heterogeneous both with respect to an individual muscle and with respect to different types of muscle (Locker and Hagyard, 1967a,c), the heterogeneity may be more apparent than real (Weeds, 1969).

Until recently it has been possible only to show that removal of the light chains from a preparation of myosin is accompanied by complete and irreversible loss of the biological activity of the myosin. In the past year, however, several reports have appeared describing the reversibility of the above reactions (Dreizen and Gershman, 1970; Gershman and Dreizen, 1970; Frederiksen and Holtzer, 1968; Stracher, 1969). This finding supports the notion that the light chains are an integral part of biologically active myosin and not merely tightly bound contaminants. It is therefore of considerable interest to study the biosynthesis of myosin heavy and light chains. In particular we would like to know whether the myosin light chains are synthesized along with the myosin heavy chains either on a polycistronic mRNA or as a promyosin molecule which is cleaved, or, alternatively, if they are synthesized on different polyribosomes utilizing different mRNAs. This question is fundamental to any inquiry regarding the metabolic activities of these two components of myosin synthesis. Accordingly, we report here studies in the *in vitro* synthesis of myosin light chains utilizing a cell-free protein synthetic system derived from embryonic chick skeletal muscle. We find that the myosin light chains are synthesized on small polysomes (4–9 ribosomes) while the large myosin chain is synthesized on large polysomes containing 60–80 ribosomes (Heywood *et al.*, 1967; Heywood and Rich, 1968).

Methods

Preparation of Myosin. Myosin was prepared from adult and embryonic chick (White Leghorn) leg muscle by either of two methods. The first was according to Finck (1965) with the elimination of steps involving ammonium sulfate precipitation of myosin and the addition of a high-speed centrifugation of the purified myosin in the presence of magnesium and ATP as described by Naus *et al.* (1969). Myosin was also isolated by the pyrophosphate method of Baril *et al.* (1966). Crude extracts of muscle were made in pyrophosphate buffer and then centrifuged at 150,000*g* for 4 hr using a Beckman Ti50 rotor and L2-65 centrifuge. The supernatant was layered onto a precycled DEAE-cellulose column and myosin eluted in stepwise fashion according to the authors. Fractions containing myosin were pooled and dialyzed against myosin buffer (0.5 M KCl–0.001 M EDTA–0.01 M Tris pH 7.4). When

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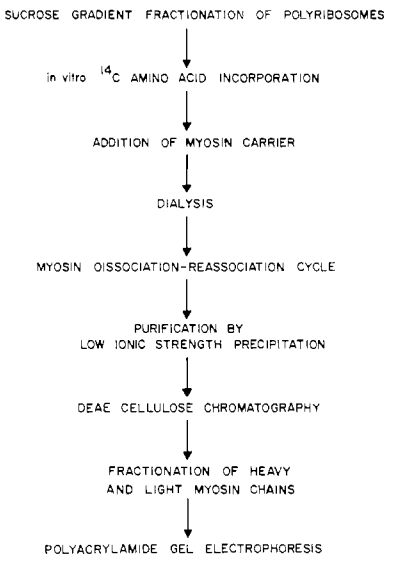

FIGURE 1: Protocol of the experiment to demonstrate the *in vitro* synthesis of myosin light chains. See text for details.

necessary they were concentrated by low ionic strength precipitation and redissolution in the desired volume of the same buffer.

Both methods yielded myosin which sedimented as a single homogeneous peak in the analytical ultracentifuge both in the presence and absence of magnesium and ATP. The myosin underwent no change in viscosity in the presence of magnesium and ATP and possessed the expected ATPase activity (Kielley, 1955; Perry, 1955; Finck, 1965; Baril *et al.*, 1966). Routinely the method of Baril *et al.* (1966) was used because it involved fewer steps and less time, and gave greater yields of myosin.

Dissociation and Reassociation of Myosin Heavy and Light Chains. Light and heavy myosin chains were separated from one another by the K_2CO_3–citrate method of Gaetjens *et al.* (1968) during which 0.001 M DTT[1] was present at all times or by the LiCl–citrate method of Gershman and Dreizen (1970), also in the presence of reducing agent. In experiments where dissociation and reassociation of heavy and light chains were involved, a modification of the method of Gershman and Dreizen (1970) was used. Equivalent volumes of myosin in 0.5 M KCl and 0.002 M DTT–8.0 M LiCl were mixed at 0° and the mixtures were then immediately dialyzed against two quick changes of myosin buffer (30 min each), and then dialyzed for 16 hr against two changes of 1 l. of myosin buffer. This method was chosen because the reassociation of the chains is accompanied by restoration of most of the ATPase activity of the myosin (P. Dreizen and L. C. Gershman, 1969, personal communication; Gershman and Dreizen, 1970). We wished to ensure that the physical separation and reassociation of heavy and light chains occurred; accordingly, control samples of myosin were monitored in the analytical ultracentrifuge. Schlieren patterns obtained during sedimentation velocity runs indicated that first dissociation, then reassociation, of light chains had occurred.

For comparison in one experiment, dissociation was accomplished by the K_2CO_3 method of Gaetjens *et al.* (1968) and reassociation was carried out by back-titrating samples after 20 min at 3° to pH 7.0 with 1.0 N HCl, followed by dialysis against myosin buffer. In this case also control samples of myosin monitored in the analytical ultracentrifuge showed that dissociation and reassociation had taken place.

Preparation and Fractionation of Polyribosomes. Embryonic chick skeletal and liver extracts were prepared and analyzed on linear sucrose gradients according to Heywood *et al.* (1967, 1968) using MSB buffer (0.01 M Tris (pH 7.4)–0.25 M KCl–0.01 M $MgCl_2$). The cytoplasmic extracts from ten 14-day-old embryos were pooled and layered onto six 35-ml gradients: these were centrifuged for 2 hr at 27,000 rpm at 4° using a Beckman SW27 rotor in an L2-65 centrifuge. The gradients were subsequently analyzed and fractionated, and the polyribosomes collected according to Heywood *et al.* (1967).

In Vitro Incorporation of [^{14}C]Amino Acids into Protein. Four polyribosomal fractions were used to catalyze the *in vitro* incorporation of [^{14}C]amino acids into protein as described by Heywood *et al.* (1967, 1968). A ribosome-free high-speed centrifugal supernatant was used as the source of enzymes and tRNA (Heywood *et al.*, 1968). The incorporation was for 2 hr at 37° and it was stopped by the addition of 50 μg of pancreatic ribonuclease.

Polyacrylamide Gel Electrophoresis. Electrophoresis in sodium dodecyl sulfate gels was carried out according to Weber and Osborn (1969) using 7.5% gels which were calibrated for molecular weight using pyruvate kinase, ovalbumin, pepsin, chymotrypsinogen, trypsin, myoglobin, and lysozyme. Electrophoresis was also carried out in the absence of detergent (Williams and Reisfeld, 1964). In all cases gels and buffer were made up in 10% glycerol to facilitate slicing. Immediately after electrophoresis the gels were either stained or frozen at −20° for slicing. Slices, 0.5 mm in thickness, were made using a Brinkman gel slicer (Brinkman Instruments, Westbury, N. Y.). Three adjacent slices were placed on planchets and dried in preparation for counting of radioactivity in a low-background, gas-flow counter (Nuclear-Chicago).

Preparation of Radioactive Samples for Counting. Samples from the *in vitro* incorporations and subsequent myosin purification steps were precipitated with two volumes of 10% trichloroacetic acid after 0.5 mg of bovine serum albumin was added as carrier. The samples were heated at 95° for 10 min and the precipitates were collected and washed with 5% trichloroacetic acid on Millipore filters (Millipore Corp., 0.45 μ pore size). The filters were placed on planchets, dried, and counted in a Nuclear-Chicago low-background counter, background 1–2 cpm.

Determination of Protein Concentration. The method of Lowry *et al.* (1951) was used. Alternatively, as an approximation the concentration of myosin solutions was estimated using a value of $E_{280}^{1\%}$ of 5.60 for values corrected for Rayleigh light scattering.

Modification of Sulfhydryl Residues. Reduction and carboxymethylation of myosin light chains were carried out according to Crestfield *et al.* (1963).

Materials. Ribonuclease-free sucrose was purchased from Schwartz Bio-Research, Inc.; DEAE-cellulose from Reeve Angel (DE23); ribonuclease from Worthington; potassium pyrophosphate from K & K Laboratories, Inc.; acrylamide and methylenebisacrylamide, electrophoresis grade, from Bio-Rad Laboratories; [^{14}C]amino acids from New England Nuclear Corp. All other chemicals were purchased from Sigma Chemical Co.

Results

Figure 1 shows the basic protocol of the experiments undertaken to demonstrate the *in vitro* synthesis of light and heavy

[1] Abbreviation used is: DTT, dithiothreitol.

TABLE I: Recovery of Radioactivity during the Purification of Myosin.

Purification Step	Polyribosome Fraction: Muscle A	Muscle B	Muscle C	Muscle D	Liver C
I. Incubation mixture and carrier	3200 (280)[a]	6450 (512)	6550 (492)	2380 (172)	5672 (480)
II. Final ionic strength purification	619 (134)	71 (11)	278 (48)	56 (8)	63 (15)
III. Post DEAE-cellulose chromatography	573 (133)	39 (10)	90 (29)	42 (7)	20 (5)
IV. Fractionation					
Heavy chain	499	31	36	21	8
Light chain	15	12	51	12	10

[a] The numbers represent the counts per minute per incubation mixture at various stages of purification. The figures in parentheses are specific activity determinations (counts per minute per milligram of protein).

myosin chains. Polyribosomes collected from sucrose gradients in the four fractions, A–D (Figure 2), were used to direct the *in vitro* synthesis of protein. The products were then analyzed for the presence of myosin. After the incubation solid KCl was added to give a concentration of 0.5 M and 10 mg of unlabeled carrier embryonic chick muscle myosin was then added to each of the four incorporation tubes. The mixtures were subsequently dialyzed overnight against two changes of 0.5 M KCl at 3°. The four fractions containing radioactive proteins and unlabeled carrier myosin were then subjected to conditions which first favor dissociation (LiCl) and then reassociation (dialysis) of myosin heavy and light chains as described in Methods. After dialysis pure myosin was isolated from the four fractions using several steps (Heywood and Rich, 1968). The samples were first diluted with water to an ionic strength of 0.03 M. This precipitated the myosin which was collected by centrifugation at 10,000*g* for 20 min at 3°. The pellets were dissolved in myosin buffer and the ionic strength was then lowered to 0.28 M to precipitate actomyosin which was centrifuged off at 20,000*g* for 30 min. The ionic strength was further lowered to 0.03 M, again precipitating myosin. The pellets resulting from centrifugation at 10,000*g* for 20 min were next dissolved in 0.02 M $K_4P_2O_7$ (pH 9.5) and dialyzed against the same buffer overnight at 3°. The samples were then chromatographed on DEAE-cellulose. Figure 3 shows the elution profile at this step. Fraction A showed considerable radioactivity as expected since the polyribosomes in this fraction are known to be involved exclusively with the synthesis of the heavy chain of myosin (Heywood *et al.*, 1967, 1968; Heywood, 1969). Fraction B contained much less radioactivity and fraction C likewise. It is significant, however, that fraction C contained more radioactivity than fraction B. Fraction D contained the least radioactivity.

After overnight dialysis against myosin buffer, an additional 5 mg of purified embryonic chick skeletal muscle myosin was again added as carrier to each fraction, and they were then separated into heavy and light chains by the LiCl–citrate method (Gershman and Dreizen, 1970) or the K_2CO_3–citrate method (Gaetjens *et al.*, 1968). Equivalent results were obtained with either method. Portions of the heavy- and light-chain fractions were counted for radioactivity with results as shown in Table I. The data suggested that polyribosome fraction A had directed predominantly, if not exclusively, the

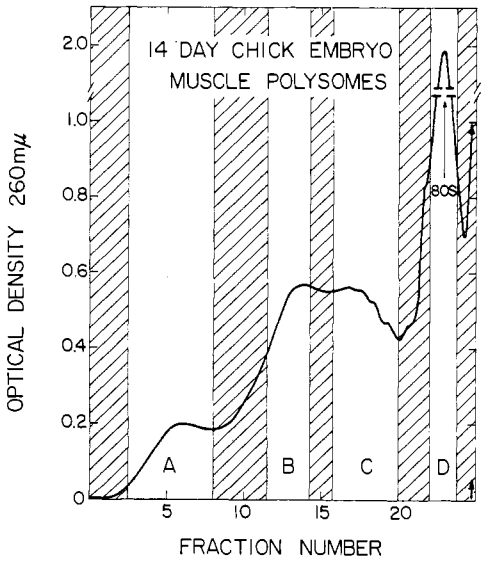

FIGURE 2: Sucrose gradient profile of chick embryonic skeletal muscle polyribosomes. Sedimentation was from right (top of gradient marked by arrow) to left in a 15–40% gradient in MSB buffer. Centrifugation was for 2 hr at 27,000 rpm, 3°. The polysomes were divided into four size classes, A–D, as shown and used in the incubation mixture as described in Methods.

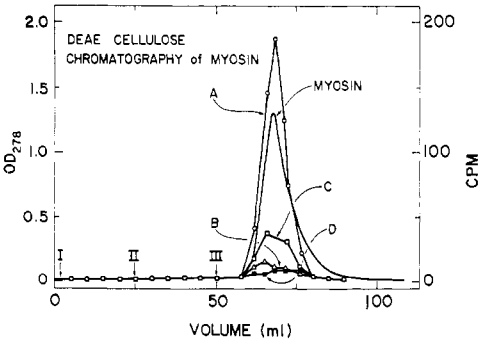

FIGURE 3: DEAE-cellulose chromatography of myosin. The chromatography of the myosin marker is shown by absorbance readings at 278 mμ (solid line) together with radioactive protein from the four incubation mixtures A–D. Elution was in steps using the following buffers: I, 0.02 M $K_4P_2O_7$, pH 8.5; II, 0.005 M $K_4P_2O_7$ (pH 8.5)–0.18 M KCl; III, 0.01 M $K_4P_2O_7$ (pH 8.5)–0.36 M KCl.

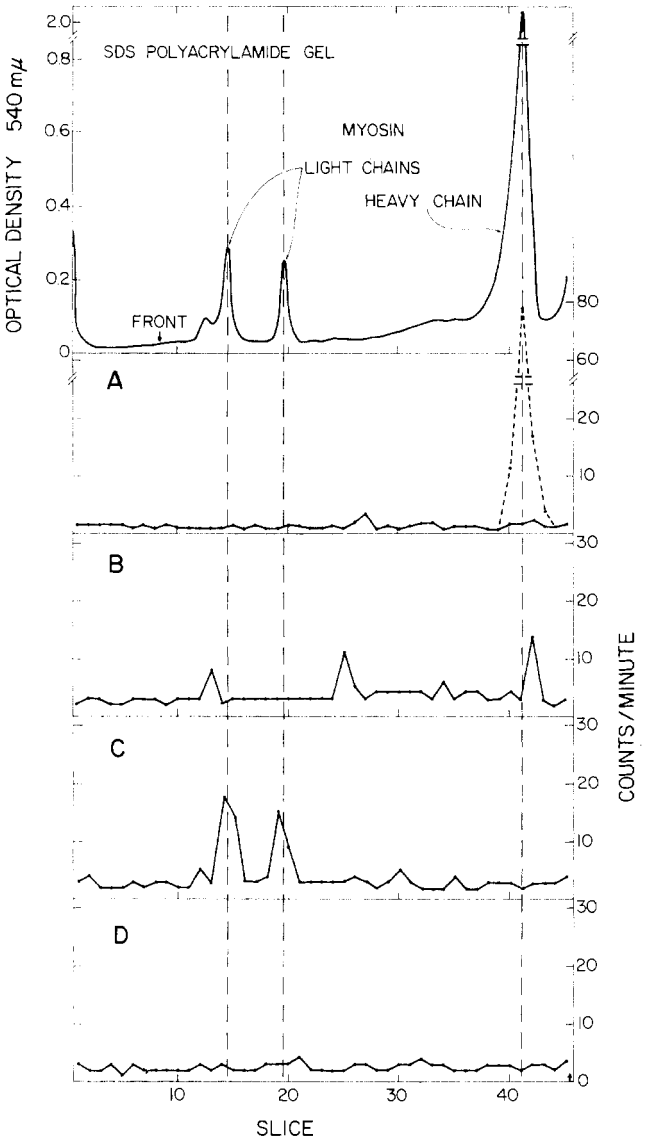

FIGURE 4: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of myosin. Electrophoresis was from right (top of gel marked by arrow) to left, using 7.5% gels, for 5 hr at 8 mA/tube. After electrophoresis the gels were frozen, sliced, and counted as described in Methods. Top panel: electrophoretogram of myosin stained with coomassie brilliant blue. Bottom four panels: electrophoretograms of the myosin light-chain components of incubation fractions A–D (solid lines). Also shown in panel A is the electrophoretic pattern of the heavy-chain component of fraction A (dotted line).

formation of heavy chains and that fraction C made the light chains.

This interpretation needed verification, and this was accomplished by subjecting the light chain fractions from each incubation to polyacrylamide gel electrophoresis. Figure 4 shows the results of electrophoresis in sodium dodecyl sulfate gels. These gels offer the advantage that both heavy and light chains can be electrophoresed and visualized in the same gel. The heavy chains migrate but a short distance into the gel with a mobility corresponding to a molecular weight of about 200,000 (Weber and Osborn, 1969). The light chains migrate as two major and one minor bands with mobilities suggesting molecular weights of 17,000, 23,000, and 14,500, respectively. These figures are in general agreement with values calculated by Dreizen *et al.* (1967) and Locker and Hagyard (1967b).

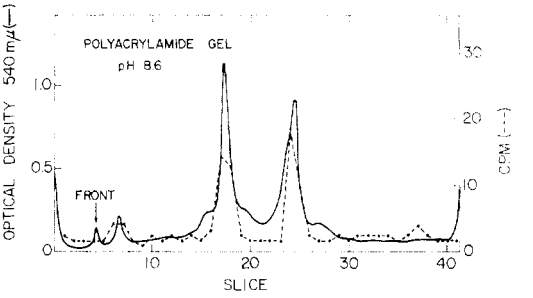

FIGURE 5: Electrophoresis of reduced carboxymethylated myosin light chains at pH 8.6. Electrophoresis was from right to left using 10% gels, for 2 hr at 3.5 mA/tube. Solid line is the electrophoretogram of light-chain marker myosin; after electrophoresis the gel was stained with Amido Black in 7.5% acetic acid. Dashed line is the electrophoretogram of the light chain component of incubation fraction C. After electrophoresis, the gel was frozen, sliced, and counted as described in Methods.

Patterson and Strohman (1970) report two bands of 19,000 and 32,500, while Frederiksen and Holtzer (1968) estimate a molecular weight near 30,000. Figure 4 also shows the only peak found in the electrophoretic pattern of the heavy chains from polyribosome fractions A–D. Only fraction A had radioactive heavy chains, in agreement with the earlier identification of these heavy polysomes as active in heavy-chain myosin synthesis (Heywood *et al.*, 1967; Heywood and Rich, 1968).

Electrophoresis of the light-chain portion of fraction C at pH 8.6 further strengthened the identification. On these gels, myosin light chains migrate predominantly as two peaks of intermediate mobility and one of high mobility (Figure 5). The small fast band varied in its relative proportion to the other bands from one preparation of myosin to the next and decreased in amount when the myosin was washed with 4.7 M ammonium chloride, a treatment which does not alter the enzymatic activity of the myosin (Gershman and Dreizen, 1969, 1970). The radioactive proteins of fraction C coelectrophoresce with the two bands of intermediate mobility and a small peak of radioactivity migrates with the fast band. Thus the radioactive protein products of fraction C migrate with purified light chains in two different types of acrylamide gels, one in the presence of detergent and the other in its absence. In the former gel the mobility is determined by the molecular weight, while in the latter the charge on the protein is also important.

A control experiment was carried out to check on the possibility that nonspecific proteins are synthesized in fraction C which bind tightly to myosin and possess the same electrophoretic properties as the true myosin light chains. In this experiment, 14-day-old embryonic chick liver was used as the source of polyribosomes of size class C (Figure 6). The entire experiment as outlined in Figure 1 was repeated. In this case there were few radioactive counts in the myosin light-chain fraction (Table I, step IV) and the radioactivity was in proteins which did not coelectrophoresce with known myosin light chains. Therefore, the small proteins which are purified with myosin and coelectrophoresce with known light chains seem to be specific to muscle tissue.

Two further experiments were performed to examine more closely the ability of newly synthesized light chains to associate with myosin heavy chains. In the first, the myosin dissociation–reassociation cycle was omitted, and in this case there were significantly fewer counts in fraction C after DEAE-cellulose

chromatography (one-fifth as many as in the original experiment) and correspondingly fewer counts in the light-chain fraction. The other variation involved the use of changes in pH for the dissociation–reassociation cycle (see Methods). Subsequent procedures yielded the same results as when the LiCl method was used for that step, though not as much radioactivity was recovered in the light-chain fraction (50 and 70% of control values in two experiments) as with the latter method.

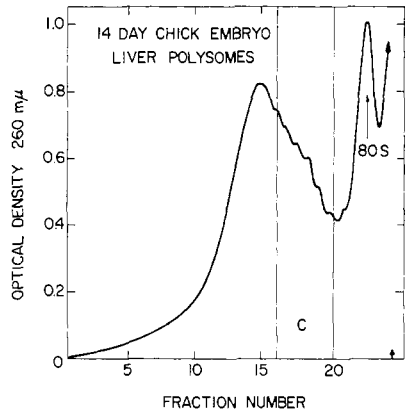

FIGURE 6: Sucrose gradient profile of chick embryonic liver polyribosomes. Sedimentation was from right (top of gradient marked by arrow) to left in a 15–40% gradient in MSB buffer for 2 hr at 95,000*g*, 3°. Polyribosomes in fraction C were collected and used in the incubation mixture as described in Methods.

Discussion

The heavy and light chains of myosin could be synthesized by any of three methods. Their synthesis could be directed by a single, polycistronic mRNA with the two proteins coordinately synthesized. Alternatively, mRNA with a single cistron could be involved with the heavy and light chains covalently linked as a single polypeptide, a "promyosin" which could then be cleaved into the two proteins. This would be analogous to insulin synthesis where the A and B chains are formed from a proinsulin chain (Steiner *et al.*, 1969). Finally, the heavy and light chains could be synthesized separately from different mRNAs on different polyribosomes. It has been demonstrated that the heavy chains are synthesized by the polyribosomes in fraction A (Heywood and Rich, 1968; Heywood *et al.*, 1967, 1968) and that the RNA isolated from those polyribosomes directs the synthesis of heavy chains (Heywood, 1969). However, those experiments do not exclude the possibility that heavy and light chains are synthesized together as a "promyosin." That possibility is excluded by the present experiments. The heavy chains are synthesized in fraction A while the light chains are synthesized in fraction C from polyribosomes containing 4–9 ribosomes. Proteins of the size of the myosin light chains would be expected to be synthesized in that fraction if they are made on monocistronic mRNAs. In eucaryotic cells there appears to be a relationship between the number of ribosomes in the polysomes and the size of the protein. This has been demonstrated for hemoglobin (Warner *et al.*, 1962), the heavy and light chains of antibodies (Becker *et al.*, 1970), as well as for myosin, actin, and tropomyosin (Heywood and Rich, 1968).

The validity of the experiments reported here rests upon the elimination of contaminating proteins by the myosin purification steps and the reliability of the coelectrophoresis of radioactive proteins with known myosin light chains as a criterion for their identification. In these experiments, myosin was purified from the *in vitro* incorporation mixture to a condition of constant specific activity (Heywood *et al.*, 1968). The use of two different methods of electrophoresis, one where separation is only according to size, the other where separation is according to size and charge, markedly reduces the possibility of fortuitous coelectrophoresis of radioactive protein with light chains. It thus supports the conclusion that light chains have in fact been synthesized. The experiment using liver rather than muscle polyribosomes further suggests that whatever is being synthesized is peculiar to muscle tissue.

These experiments do not help to decide if the light chains associated with myosin are true components of the enzymatically active protein or merely tightly bound contaminants. Nonetheless the fact that the heavy and light chains are synthesized separately raises many interesting questions related to possible control mechanisms involved in myosin biosynthesis as well as to myosin physiology. The evidence available at present suggests an important role for the light chains in the biological activity of myosin. Several studies directly suggest that the light chains are intimately involved in the binding of ATP to the myosin ATPase site (Dreizen and Gershman, 1969; Dreizen *et al.*, 1967; Gershman and Dreizen, 1970; Murphy and Morales, 1970). In addition there is information which suggests a correlation between differences in myosin ATPase activity and differences in myosin light-chain composition (Locker and Hagyard, 1967c; Samaha *et al.*, 1970; Seidel *et al.*, 1964; Streter *et al.*, 1966). It is possible that the ATPase activity of myosin is the rate limiting step in myosin activity and that the myosin molecule is the primary determinant of the nature and speed of the contractile process (Mommaerts, 1969). An understanding of the relationship between the synthesis and metabolism of the heavy and light chains is therefore of considerable importance in understanding the assembly of myosin in developing and adult tissues and in understanding of the maintenance of normal muscle contractility. It will clearly be of interest to learn if changes in the light chain fraction of myosin are involved in abnormal processes such as muscular hypertrophy, myopathy, or muscular dystrophy. A basic question concerns the relative turnover rates of myosin light and heavy chains. Since these molecules are synthesized separately, they may be controlled individually. In particular, the light chains are found at the "working" end of the myosin molecule where it attaches to the actin filaments during contraction. Is it possible that the separate synthesis of the light chains allows them to be replaced in normal tissue at a more rapid rate than the myosin heavy chains which interact together to form myosin rods? The answers to some of the above questions will be presented in future publications.

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Threonine Deaminases from *Saccharomyces cerevisiae* Mutationally Altered in Regulatory Properties*

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ABSTRACT: Two mutants of *Saccharomyces cerevisiae* resistant to 5×10^{-3} M thiaisoleucine were found to have threonine deaminases 10- and 100-fold less sensitive than the wild type to L-isoleucine as inhibitor. The apparent affinity for L-threonine as substrate, L-valine as activator, and L-isoleucine as activator was unchanged. Reversal of the isoleucine inhibition required higher concentrations of valine in the mutants as compared to the wild type. The enzyme was shown to exhibit cooperativity with respect to substrate in the absence of effectors, and with respect to activators as well. The kinetics of activation by valine or by isoleucine may be described by a modification of the allosteric equation of Monod, Wyman, and Changeux (1965, *J. Mol. Biol. 12*, 88). A tentative model for the interactions of the enzyme with its substrate and effectors, with two sorts of sites able to bind isoleucine or valine, is proposed.

This model accounts for the activation as well as the inhibition by isoleucine.

Threonine deaminase (L-threonine hydro-lyase ((deaminating)), EC 4.2 2.16) from a variety of sources has been studied for its allosteric properties (Changeux, 1963; Maeba and Sanwal, 1966; Zarlengo *et al.*, 1968; de Robichon-Szulmajster and Magee, 1968; Brunner *et al.*, 1969; Hatfield and Umbarger, 1970a,b; Sharma and Mazumder, 1970). All

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