

# COMPOSITIONAL STUDIES OF MYOFIBRILS FROM RABBIT STRIATED MUSCLE

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## ABSTRACT

The localization of high-molecular-weight (80,000–200,000-daltons) proteins in the sarcomere of striated muscle has been studied by coordinated electron-microscopic and sodium dodecyl sulfate (SDS) gel electrophoretic analysis of native myofilaments and extracted and digested myofibrils. Methods were developed for the isolation of thick and thin filaments and of uncontracted myofibrils which are devoid of endoproteases and membrane fragments. Treatment of crude myofibrils with 0.5% Triton X-100 results in the release of a 110,000-dalton component without affecting the myofibrillar structure. Extraction of uncontracted myofibrils with a relaxing solution of high ionic strength results in the complete disappearance of the A band and M line. In this extract, five other protein bands in addition to myosin are resolved on SDS gels: bands M 1 (190,000 daltons) and M 2 (170,000 daltons), which are suggested to be components of the M line; M 3 (150,000 daltons), a degradation product; and a doublet M 4, M 5 (140,000 daltons), thick-filament protein having the same mobility as C protein. Extraction of myofibrils with 0.15% deoxycholate, previously shown to remove Z-line density, releases a doublet Z 1, Z 2 (90,000 daltons) with the same mobility as  $\alpha$ -actinin, as well as proteins of 60,000 daltons and less, and small amounts of M 1, M 2, M 4, and M 5; these proteins were not extracted with 0.5% Triton X-100. The C, M-line, and Z-line proteins and/or their binding to myofibrils are very sensitive to tryptic digestion, whereas the M 3 (150,000 daltons) component and an additional band at 110,000 daltons are products of proteolysis. Gentle treatment of myofibrils with an ATP relaxing solution results in the release of thick and thin myofilaments which can be pelleted by 100,000-g centrifugation. These myofilaments lack M- and Z-line structure when examined with the electron microscope, and their electrophoretograms are devoid of the M 1, M 2, Z 1, and Z 2 bands. The M 4, M 5 (C-protein doublet), and M 3 bands, however, remain associated with the filaments.

Striated muscle offers some unique advantages for studies of the synthesis and assembly of organelles, due to its predominant content of a highly ordered arrangement of myofibrillar proteins (12, 13). To

gain a better understanding of the mechanisms underlying myofibrillogenesis and of the assembly and disassembly steps which must occur during protein turnover in the adult, one must establish

the composition and precise localization of all fibrillar proteins, particularly some of the as yet uncharacterized protein bands of high subunit molecular weight (80,000–200,000 daltons) that are displayed on electrophoretograms of whole myofibrils (8, 9, 41, 42, 37, 38, 36). These bands could be cytoplasmic contaminants, degradation products, or structural and regulatory proteins of the myofibril.

Recently, several minor myofibrillar proteins have been isolated and partially characterized. These include  $\alpha$ -actinin (7, 15), M-line proteins (34, 6, 29, 30), and C protein (45, 37, 38). Removal of the Z line by various methods, including brief trypsin treatment (14) or sodium deoxycholate (DOC)<sup>1</sup> extraction (9), resulted in the solubilization of  $\alpha$ -actinin. Further evidence for  $\alpha$ -actinin localization in the Z line comes from antibody studies (28). Examination of glycerinated muscle labeled with antisera to purified C protein reveals antigenic sites in the A band which are distributed as nine transverse strips spaced 420–440 Å apart on each half of the A band (37). Several additional proteins have also been noted on electrophoretograms of myosin preparations (45, 9, 39). The relationship of any of these proteins to muscle contraction or myofibrillogenesis is, at most, poorly understood.

Although these reported proteins could, in fact, be intact structural and regulatory components of the myofibril, the possibility also exists that some of these components might correspond to proteolytic fragments of sarcomere constituents. This idea is suggested by experiments in which the degradation of paramyosin (44) and troponin (18) into smaller molecular fragments was reduced by the addition of protease inhibitors during the isolation of these proteins. On the other hand, some of these gel bands might be cytoplasmic contaminants which exist in the fibrillar fraction.

In the present study, we have emphasized the importance of first defining the myofibrillar components by isolating the intact organelle free from sarcoplasmic contaminants rather than extracting and isolating proteins from crude fibrillar preparations. The analysis of isolated thick and thin filaments prepared from such fibrils should furnish direct information regarding the

localization of the minor protein components in the sarcomere. Although Huxley has demonstrated that myofilaments can be released from fibrils under relaxing conditions and examined by electron microscopy (21), there have been few additional studies directed at a compositional analysis of isolated filaments. Morimoto and Harrington have analyzed a preparation of thick filaments and have concluded that such filaments are composed exclusively of myosin and C protein (35, 36). It has been noted, however, that it is difficult to produce complete dispersal of myofibrils into filaments under conventional relaxing conditions (8, 9, 35). We have analyzed the structure and composition of an easily released class of myofilaments and have used this information to complement results obtained from the selective elution and proteolytic digestion of myofibrils. The results permit an unambiguous identification of all protein bands on SDS gels between 80,000 and 200,000 daltons, and provide an approach to the analysis of myofibrillar assembly, stoichiometry, and turnover in adult muscle.

## MATERIALS AND METHODS

### (a) Solutions

PRB (pyrophosphate relaxing buffer): 0.1 M KCl, 2 mM MgCl<sub>2</sub>, 2 mM EGTA, 1 mM DTT, 2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 0.01 M Tris-maleate buffer, pH 6.8. Low salt buffer: same as PRB, but pyrophosphate omitted. High salt relaxing buffer (RB): 0.5 M KCl, 2 mM MgCl<sub>2</sub>, 2 mM EGTA, 2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 0.1 M Na-phosphate buffer, pH 6.8, and 1 mM DTT. ATP-RB: low salt buffer supplemented with 2 mM ATP.

### (b) Isolation and Purification of Myofibrils

Rabbits were sacrificed, exsanguinated, and the back muscles immediately excised and placed in PRB. Connective tissue was trimmed away, the muscle was cut into 1-cm<sup>3</sup> pieces, and PRB was decanted and replaced. The muscle was then disrupted in approximately 5 vol of PRB by a blade-type homogenizer at a speed of 14,000 rpm for 30 s, and the homogenate was filtered through two layers of cheesecloth. This filtrate was then spun at 800 g for 10 min and the pellet subsequently washed eight times, each wash with 10 vol of low salt buffer. The pellet was rehomogenized if undisturbed bundles of myofibrils were observed by phase contrast microscopy. After eight cycles of resuspension and centrifugation, the 800-g pellet was washed once each in solutions of 0.02% Triton X-100 and 0.02% DOC prepared in low salt buffer. Finally, the pellet was washed four times with low

<sup>1</sup> *Abbreviations used in this paper:* DOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; EGTA, ethyleneglycol-bis-( $\beta$  aminoethyl ether)-*N,N'*-tetraacetic acid; BSA, bovine serum albumin.

salt buffer. Myofibrils were sometimes stored at  $-20^{\circ}\text{C}$  in 50% glycerol in low salt buffer.

### (c) Preparation of Myofilaments

Myofilaments lacking M-line and Z-line components were prepared as follows: Sedimented myofibrils prepared as described in section *b* were resuspended in 10 vol of ATP-RB with repeated passage through a Pasteur pipette. Filaments released at this step were then separated from undispersed fibrils by centrifugation at 800 *g* for 10 min, after which the supernatant was recentrifuged at 800 *g* for 10 min. This second supernatant was then spun at 100,000 *g* for 2–3 h in a 50 Ti Beckman rotor (Beckman Instruments, Inc., Fullerton, Calif.). The pellet contained thick and thin filaments.

### (d) Detergent and High-Ionic-Strength Extraction of Myofibrils

Detergent extracts were obtained by addition of the desired amount of 10% (wt/vol) of either DOC or Triton X-100 to the extracting solutions. The high-ionic-strength extracts were prepared by 10-min extraction with high salt RB. The extracts were then separated from the residue by centrifugation at 800 *g* for 10 min, followed by filtration through 0.45- $\mu\text{m}$  Millipore filters, and spun at 100,000 *g* in a Beckman Ti-50 rotor for 2 h to remove any filaments released during extraction. After dialysis, the detergent extracts were concentrated approximately 10-fold by inclusion of the sample in a dialysis bag immersed in Ficol. Extracts for certain electron-microscopic studies were performed on bundles of fibers teased from oriented strips of muscle which had previously been immersed in 50% glycerol in low salt buffer.

### (e) Tryptic Digestion of Myofibrils

Myofibrils were digested with crystalline trypsin (Armour "Tryptar", Armour Pharmaceutical Co., Chicago, Ill.) in 0.1 M KCl, 2 mM  $\text{MgCl}_2$ , 2 mM EGTA, 0.01 M Tris-maleate, 1 mM  $\text{NaN}_3$ , pH 7.0 (digestion buffer), at room temperature. The ratio of solution volume to 800  $\times$  10 min fibril pellet volume was 10:1, and the trypsin concentration was 0.001 mg/ml, providing a protein ratio of 1:4,000 (wt/wt) for trypsin:fibril. Digestion was terminated after 30 min by the addition of a fourfold weight excess of soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, N. J.) and spinning at  $2^{\circ}\text{C}$  for 10 min at 800 *g*. The digested pellets were washed again with trypsin inhibitor (0.004 mg/ml) in the digestion buffer, followed by four more washes without trypsin inhibitor.

### (f) SDS-Gel Electrophoresis

Sodium dodecyl sulphate (SDS) gel electrophoresis was performed on 5% acrylamide gels containing a 1:37 weight ratio of bisacrylamide to acrylamide. Acrylamide

(Eastman Kodak Co., Rochester, N. Y.) was recrystallized from chloroform, and methylene bisacrylamide (Eastman) was recrystallized from acetone. A stock gel solution of 22.2 g of acrylamide and 0.6 g of bisacrylamide dissolved in  $\text{H}_2\text{O}$  to 100 ml was stored at  $4^{\circ}\text{C}$  in the dark and filtered (47). The gel buffer was 0.1 M Tris-glycine, pH 8.8, containing 0.4% SDS (Sigma Chemical Co., St. Louis, Mo.). To 10.1 parts of  $\text{H}_2\text{O}$  and 5 parts gel buffer, 4.5 parts of stock gel solution were added and, after deaeration, 0.4 ml of fresh 2% (wt/vol) ammonium persulfate solution and 20  $\mu\text{l}$  TEMED (Eastman) were added per 20 ml of gel solution. Protein samples were prepared by initial dialysis against 0.005 M Tris-HCl buffer, pH 8.0, followed by the addition of 2/3 vol of 0.05 M Tris-HCl (pH 8.0), 0.005 M EDTA, 0.04 M dithiothreitol (DTT), 5% SDS, and 50% sucrose (wt/vol) (11). Samples were placed in a boiling  $\text{H}_2\text{O}$  bath for 60 s, then incubated at  $37^{\circ}\text{C}$  after the addition of a volume of 0.5 M iodoacetamide equal to the volume of the sample plus SDS solution. One part of 0.05% bromphenol blue was added as a tracking dye. The running buffer was 0.025 M Tris-glycine, pH 8.8, 0.1% SDS. Each cylindrical gel was 4 mm in diameter and 11 cm in length. Preliminary runs were made with eight gels for 15 min at 100 V; then the samples were loaded and run at 1/2 mA per gel for about 4 h. Gels were stained and destained according to the method of Fairbanks et al. (11); each gel was stained in 30 ml of 0.025% Coomassie blue, 25% isopropanol, 10% acetic acid for 18 h at room temperature and destained in 10% isopropanol, 10% acetic acid.

### (g) Electron Microscopy

Negative staining was performed with unbuffered 2% uranyl acetate. Some samples, on carbon films spread over copper grids, were washed before staining with 0.1 M KCl, 2 mM  $\text{MgCl}_2$ , 2 mM EGTA, and 0.01 M Tris-maleate (pH 7).

For thin sectioning, samples were washed in 0.1 M KCl, 2 mM ethyleneglycol-bis-( $\beta$  aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), 2 mM  $\text{MgCl}_2$ , 0.01 M  $\text{PO}_4$ , pH 7, fixed with 2.5% glutaraldehyde in this solution for 1 h at  $5^{\circ}\text{C}$ , and then rinsed in the same solution without glutaraldehyde. All samples were postfixed with 1%  $\text{OsO}_4$  in 0.1 M collidine (pH 7.4), stained with 0.5% uranyl acetate en bloc, and dehydrated through graded alcohols and propylene oxide before being embedded in Araldite. Sections were cut with a diamond knife and stained with lead citrate and uranyl acetate. An AEI EM6B (AEI Scientific Apparatus Inc., Elmsford, N.Y.) was used at 60 kV with an objective aperture of 50  $\mu\text{m}$ .

### (h) Protein Samples

The C protein used was the generous gift of Dr. G. Offer (Kings College, London). The following gel calibration standards and markers were used: myo-

globin, chymotrypsinogen-A, cytochrome *c*, lactic dehydrogenase, and bovine serum albumin (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.); urease,  $\beta$ -galactosidase, and papain (Sigma); and muscle phosphorylase (Worthington). Actin was prepared according to the method of Dowben et al. (4) and tropomyosin and troponin according to the procedure of Hartshorne and Mueller (19). M Protein was prepared according to the method of Masaki and Takaiti (30) except that the 10-min high-ionic-strength extract described in Materials and Methods section *d* served as the starting point.  $\alpha$ -actinin was prepared according to Arakawa et al. (1) using myofibrils prepared as described in Materials and Methods section *b* as the starting point.

#### (i) *Protease Assay*

Denatured [<sup>3</sup>H]acetyl hemoglobin was used as a protease substrate. Bovine hemoglobin (Sigma) was acetylated with [<sup>3</sup>H]acetic anhydride as described by Hille et al. (20). The reaction mixture in 0.5 ml contained  $5.4 \times 10^4$  cpm, corresponding to 0.15 mg hemoglobin. Proteolytic activity was assayed in acetate buffer at pH 3.5 and in phosphate buffer at pH 7.0. The final concentration of both buffers was 0.05 M. The reaction was terminated by the addition of trichloroacetic acid (TCA, 5% final concentration). Radioactivity soluble in TCA was assayed in Bray's scintillation solution with a model 3390 Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). A correction for quenching was made by means of an external standard. Tritium was measured with an efficiency of about 35%.

#### (j) *Protein Determination*

Protein was determined by the Lowry method (24) for the protease measurements; a modified biuret method (17) was used for the other measurements. In order to increase the assay sensitivity for these protein determinations, we added 3 ml of sample to 0.3 ml of a 10-fold concentration of the normal biuret reagent. Bovine serum albumin (BSA) was used as a standard in both methods.

### RESULTS

#### (a) *Calibration of the Gel System*

The electrophoresis system used in these experiments and in those described previously (8, 9) was designed to allow entry into the gels of very high-molecular-weight protein bands, i.e., bands with weights greater than 200,000 daltons, and to resolve closely migrating bands between 80,000 and 200,000 daltons. Fig. 1 shows that the log of molecular weight as a function of mobility is linear between 200,000 and 60,000 daltons. Lower

molecular weights can be fitted to a line of lesser slope. Furthermore, a change in the mobilities of proteins with respect to the tracking dye was observed under certain conditions. For example, when a given preparation was loaded onto the gel in a larger volume, the protein bands invariably ran in farther with respect to the dye than if the same sample was loaded on the gel in a smaller volume, even with the same total amount of protein. Wherever possible, identical volumes of sample were loaded on adjacent gels if protein mobilities were to be compared. However, to compare gel bands of similar mobility, it was usually necessary to coelectrophorese different samples on the same gel.

#### (b) *Fibrillar Purification*

In our previous communications (9, 48), methods for preparing relatively pure and uncontracted myofibrils were presented. Even in these preparations, however, occasional membranous material adhering to the fibrils could be noted in thin-sectioned fibrillar pellets, although the low concentrations (0.02%–0.05%) of the detergents Triton X-100 and DOC did promote removal of most of the contaminating membranous material. Increasing the DOC concentration above 0.1% caused all vesicular material to be solubilized, but these higher concentrations caused the extraction of some Z- and M-band components (8, 9). In contrast, the nonionic detergent, Triton X-100, removes almost all of the nonfibrillar, membranous structure within glycerinated myofibers without modification of the fibrillar ultrastructure (Fig. 2). A component of either the basement membrane or sarcolemma is resistant to solubilization with 1% Triton X-100, but most of this material is removed in the course of fibrillar isolation, as judged by electron microscopy of thin-sectioned fibrillar preparations (Fig. 3).

If myofibrils are isolated and washed eight times as described in Materials and Methods section *b* and then extracted with 0.5% Triton X-100, the extract contains a predominant protein of 110,000-dalton subunit molecular weight and lesser amounts of material with a lower molecular weight. Fig. 4 illustrates the SDS gel patterns of skeletal muscle fibrils from rabbit back before and after 0.5% Triton X-100 extraction. The faint band at 110,000 daltons is completely removed even from gels more heavily overloaded than the

example presented in Fig. 4 C. The 110,000-dalton, Triton-extractable protein shows a distinctly lower mobility than the low-ionic-strength, high pH-extractable protein (2, 1), the DOC-extractable Z protein (9), or muscle phosphorylase (not shown). Identical amounts of Z- and A-band proteins which are extractable with DOC (9) can still be released from myofibrils previously incubated in 0.5–1% Triton X-100. Removal of the 110,000-dalton band with Triton X-100 made possible an unambiguous analysis of tryptic digests (see below), since a myosin degradation band was seen to migrate on the SDS gels very close to the 110,000-dalton Triton-extractable protein.

Immersion in Triton X-100 also removes the protease activity which contaminates myofibrillar preparations. Assays of hemoglobin-splitting activities of muscle homogenates and isolated myofibrils are summarized in Table I. As the proteases are solubilized, their activity is enhanced, even after Triton X-100 removal. Although glycerol extraction of myofibrils reduces endopeptidase activity, it does not remove the 110,000-dalton protein or many contaminating membranes.

Since nuclei are present in the myofibrillar fraction, we examined the protein components in isolated nuclei. Nuclei were separated from crude myofibrils (before detergent treatment) by sedimentation in 2.2 M sucrose as described previously (48). SDS gel electrophoresis of the pelleted nuclei revealed that nuclear proteins cannot account for any of the high-molecular-weight bands examined in the present studies (Etlinger, unpublished).

### (c) Protein Analysis of Myofibrils Based upon Extraction Experiments

Based on the above studies, myofibrils were first washed with 0.5% Triton X-100 before additional extractions were performed. Proteins extracted by high salt RB are shown in Fig. 5, gel A. For convenience, the protein bands which electrophorese with slightly higher mobility than the myosin heavy chains are numbered M 1 to M 5; the apparent molecular weights are as follows: M 1, 190,000 daltons; M 2, 170,000 daltons; M 3, 150,000 daltons; M 4 and M 5 doublet, 140,000 daltons. All of these proteins are extracted coordinately from isolated uncontracted fibrils upon A-band solubilization; no other proteins migrating between 80,000 and 200,000 daltons

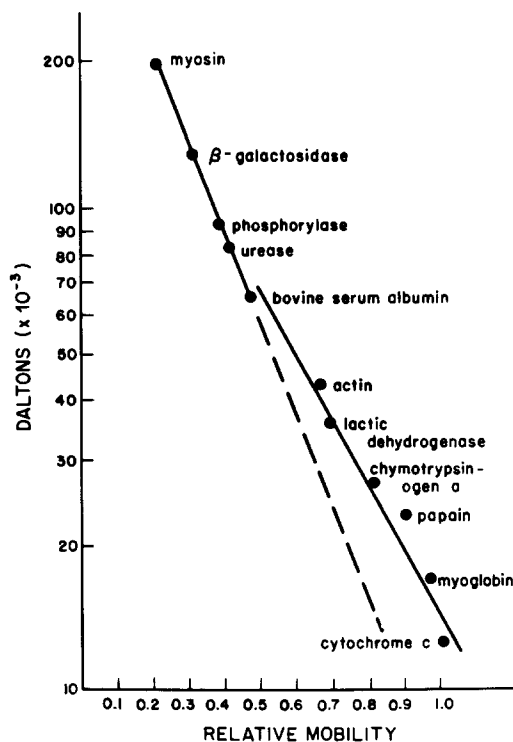
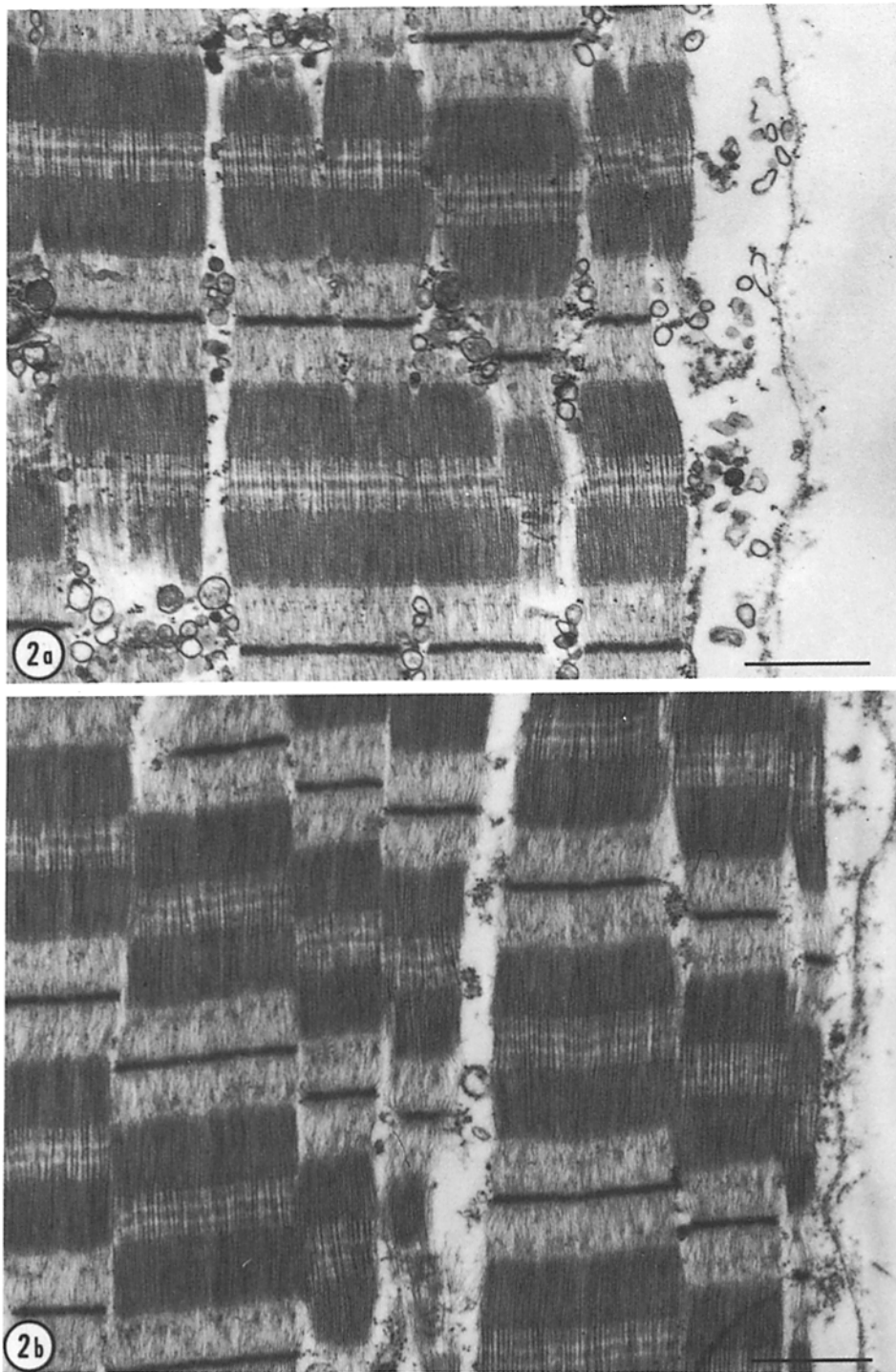


FIGURE 1 Calibration of SDS-gel electrophoresis. Electrophoresis was performed as described in Materials and Methods. To obtain the relative mobility, we measured the migration of a particular protein band relative to the tracking dye position (marked with India ink before gel staining). Molecular weight values were obtained from Weber and Osborn (47).

are solubilized by this procedure. For ultrastructural analysis, glycerinated muscle was used since myofibrillar orientation is preserved in this preparation (Fig. 6). After extraction with high salt RB, no A- or M-line structures are seen on examination by electron microscopy (Fig. 6 c). While extraction of isolated myofibrils results in immediate removal of A-band density as routinely noted by light microscopy, extraction of fiber bundles permitted a dissection of the extraction process since solubilization occurs more slowly, particularly in fibers more deeply located in the bundles. Thus, treatment with high salt RB revealed M-line extraction before solubilization of the bulk of the A band (Fig. 6 b).

DOC extraction of myofibrils solubilizes a different group of proteins (Fig. 5, gel B). In addition to proteins M 1, M 2, M 4, and M 5, bands in the 90,000-dalton region and strong bands



**FIGURE 2** Effect of 1% Triton X-100 on the ultrastructure of glycerinated muscle fibers. (a) Glycerinated myofibers. (b) Glycerinated myofibers (as in a) extracted with 1% Triton X-100. Length of calibration bar equals 1  $\mu\text{m}$ . Strips of rabbit back muscle (0.5 cm thick) were dissected and tied to wooden sticks. The muscle strips were then soaked in PRB for 1 h, followed by 10-min immersions in four changes of 50% glycerol in low salt buffer for 48 h at 4°C with two changes of solution; they were then stored at -20°C. For subsequent Triton extraction, small fiber bundles, approximately 0.1 cm thick, teased from the glycerinated strips, were immersed for 30 min at 4°C in low salt buffer containing 1% Triton X-100, followed by two washes in low salt buffer.

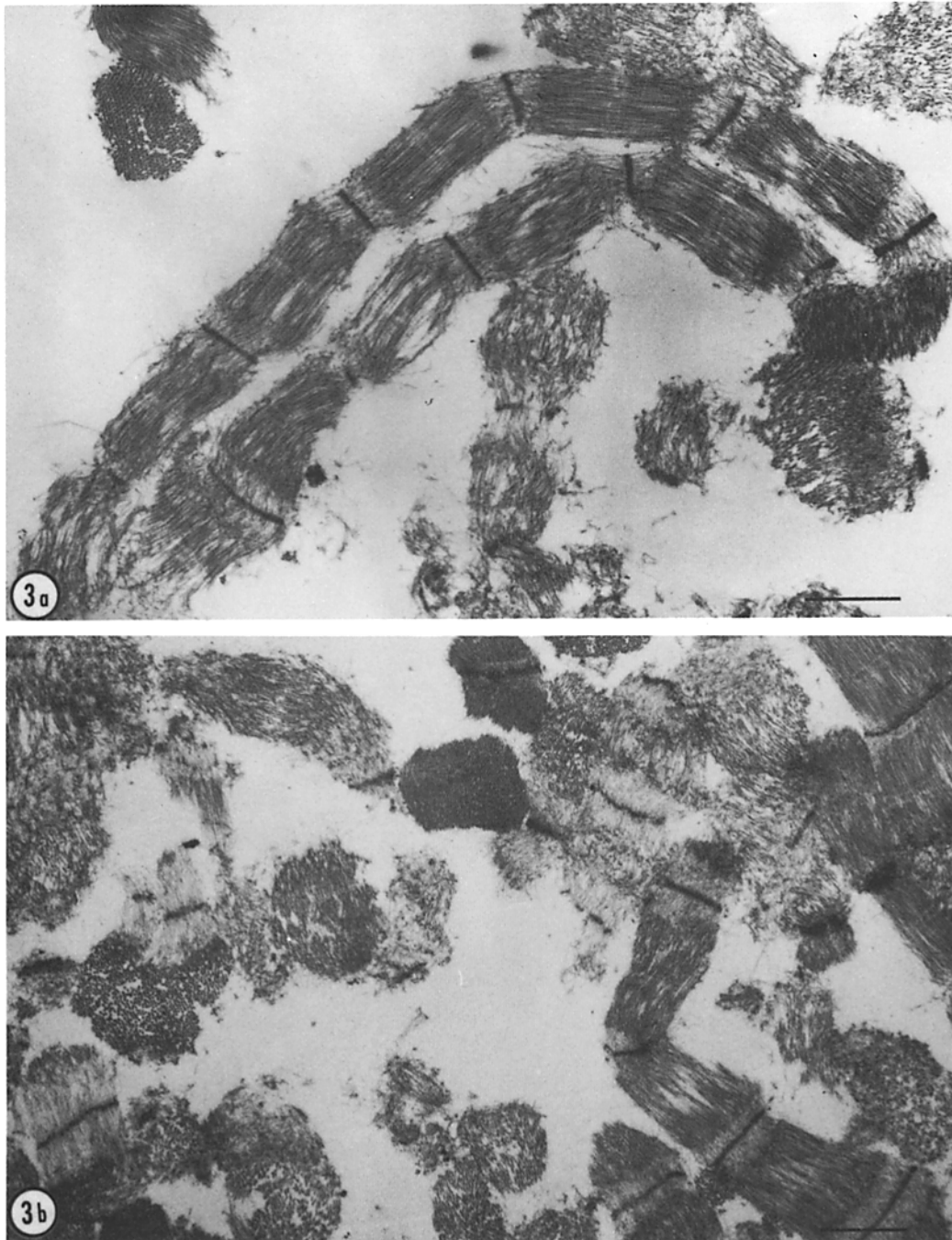


FIGURE 3 Electron microscopy of isolated myofibrils. Myofibrils were prepared from rabbit back muscle as described in Materials and Methods, including extractions with 0.5% Triton X-100. *a* and *b* are micrographs of thin sections from different myofibrillar pellets. The calibration bars equal 1  $\mu\text{m}$ .

with molecular weights of less than 60,000 daltons appear in the extract. Protein in the 90,000-dalton region can be resolved into two components, Z 1 and Z 2 (see Figs. 12, 13), which previously have been shown by sequential extraction and

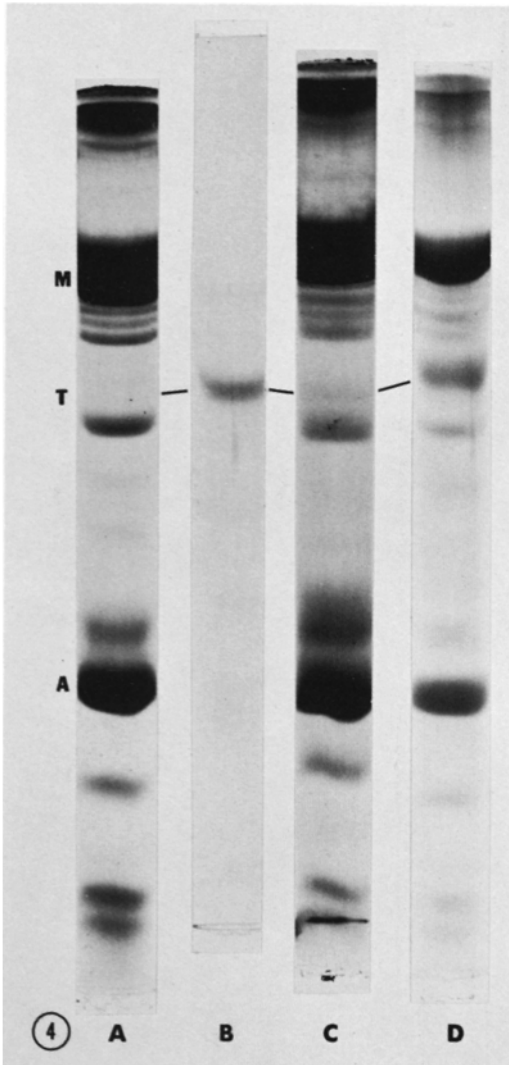


FIGURE 4 SDS-gel electrophoresis of myofibrillar proteins after treatment with 0.5% Triton X-100. (A) Myofibrils after extraction with Triton X-100 (150- $\mu$ g protein). (B) Protein recovered in the Triton X-100 extract (5  $\mu$ g). (C) Myofibrils before Triton X-100 extraction (150  $\mu$ g). (D) Coelectrophoresis of myofibrils (as in A; 50  $\mu$ g) with Triton-extracted protein (as in B, 5  $\mu$ g). The line indicates the position of the major protein band of 110,000 daltons found in the Triton X-100 extract (T). M = myosin heavy subunit. A = actin.

TABLE I  
Protease Removal from Myofibrils\*

pH	Whole muscle homogenate	Glycerinated <sup>†</sup> myofibrils	0.5% Triton X-100 extracted myofibrils	0.5% Triton <sup>§</sup> X-100 extract
3.5	520	75	0	836
7.0	75	7	9	125

Results are expressed as [<sup>3</sup>H]acetyl-hemoglobin splitting activity (cts/mg protein/10 min).

\* Myofibrils were isolated from rabbit back muscle.

<sup>†</sup> One-month extraction at -20°C with 50% glycerol in low salt buffer.

<sup>§</sup> Triton was removed by dialysis and the proteins were concentrated by lyophilization.

coordinate electron microscopy to originate from the Z line (9). The resolution of bands Z 1 and Z 2 is dependent on sufficiently long gels and on the particular conditions of electrophoresis. It is not a result of proteolysis of the sample after the initial isolation. Proteins of lower molecular weight are predominantly solubilized tropomyosin, actin, and the T, I, and C troponin subunits, which will not be discussed in this report. It should be noted that, in this gel system, tropomyosin displays two bands both of which migrate with slightly lower mobility, i.e., higher apparent molecular weight, than actin.<sup>2</sup>

When C protein (45, 37, 38) is coelectrophoresed with proteins released from myofibrils by 0.15% DOC, it is seen (Fig. 5, gel D) to comigrate with bands M 4 and M 5. The estimated molecular weight is about 140,000 daltons. This splitting of the C-protein band is resolved under conditions of low protein loading and sufficiently long gel lengths. Although DOC extracts of whole myofibrils contain protein bands M 1 and M 2, in addition to proteins M 4, M 5, and Z 1 and Z 2, the brief extractions employed in these studies result in only partial removal of each of these bands from the fibrils, i.e. 15-20% decrease in density of each gel band.

<sup>2</sup> This shift in tropomyosin mobility was observed by coelectrophoresing purified tropomyosin with myofibrils. A similar change in tropomyosin mobility was observed when muscle proteins were treated with urea in addition to SDS (42). With the exception of tropomyosin, all of the other bands displayed on gels of myofibrils shown in the present study had similar mobility when electrophoresed in the systems of Weber and Osborn (47) and Laemmli (23).



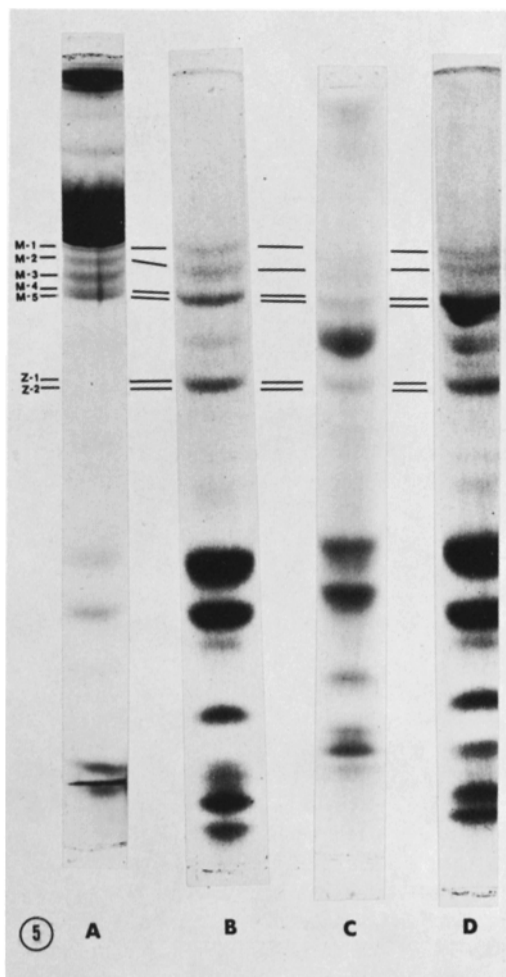


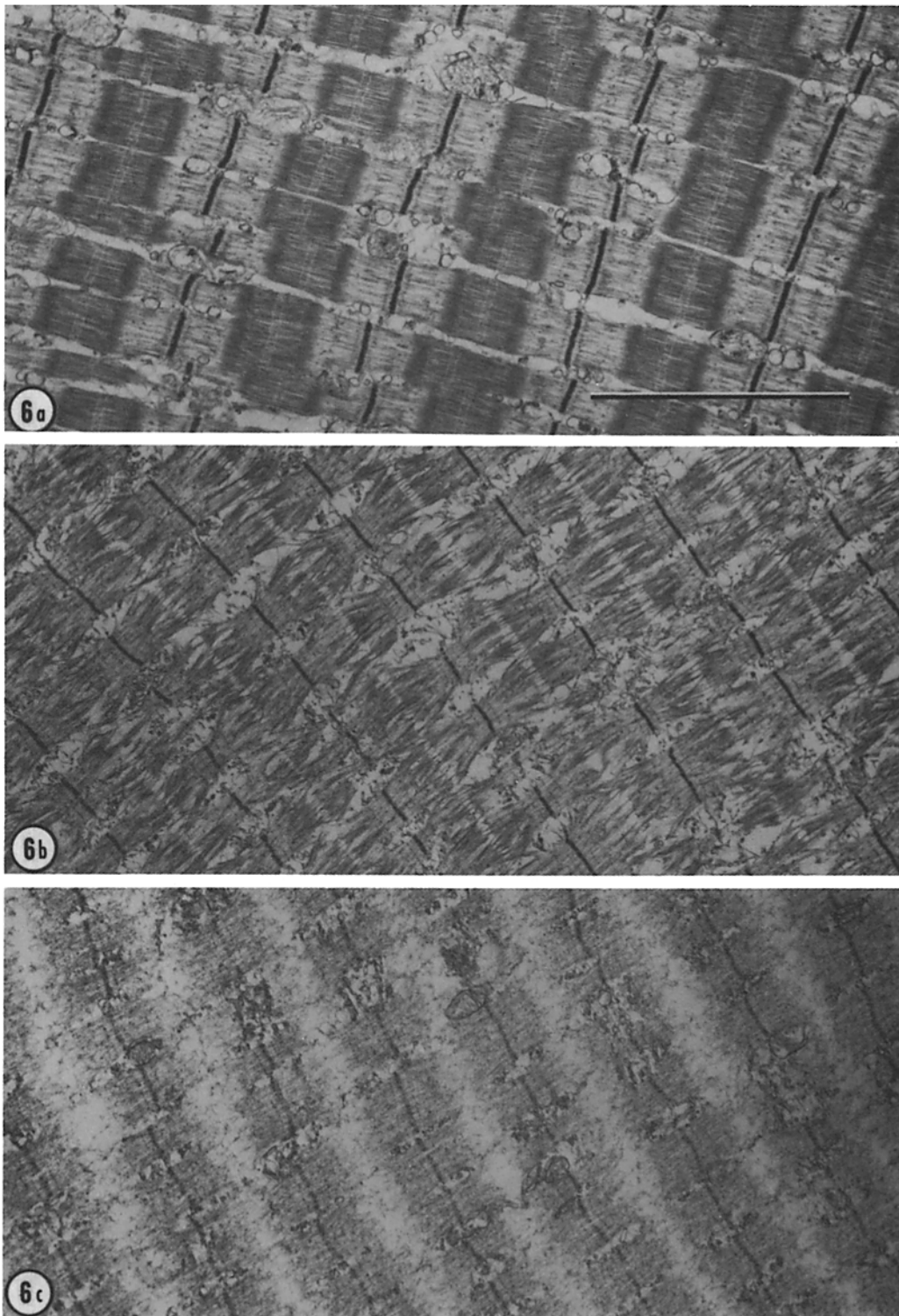
FIGURE 5 Proteins extracted by DOC and by high-ionic-strength buffer. Myofibrils used for extraction were prewashed with 0.5% Triton X-100. (A) Proteins extracted from myofibrils by immersion for 10 min in high salt RB (75- $\mu$ g protein). (B) Proteins extracted from myofibrils by immersion for 5 min in 0.15% DOC dissolved in low salt buffer (30- $\mu$ g protein). (C) Coelectrophoresis of 0.5% Triton-extracted protein (shown in Fig. 4, gel B; 5  $\mu$ g) and DOC extract (this Fig., gel B; 15  $\mu$ g). (D) Coelectrophoresis of the DOC extract (gel B; 30  $\mu$ g) and C protein (10  $\mu$ g). The precise determination of C-protein mobility as bands M 4 and M 5 was made by additional coelectrophoretic experiments performed with small amounts of C protein (1  $\mu$ g and less) which produced small changes in optical density of the M 4 and M 5 extract bands without broadening these bands.

#### (d) Changes in the 80,000–190,000-Dalton Bands after Brief Trypsin Digestion

Since some of bands M 1 through M 5 may be produced by degradation, digestion experiments were performed to ascertain whether or not these bands could be produced by deliberate proteolysis. Mild tryptic digestion carefully terminated with trypsin inhibitor, as described in Materials and Methods section e, revealed the following changes: Band M 3 and an additional band at 110,000 daltons increased in amount, whereas bands Z 1 and Z 2 decreased in intensity and bands M 1, M 2, M 4, and M 5 disappeared (Fig. 7). Coelectrophoresis of digested fibrils with small quantities of added C protein demonstrated that C protein migrates at a gel position from which fibrillar protein is almost completely removed by trypsin (Fig. 8). Coelectrophoresis of digested and undigested myofibrils reveals that M 3 is distinct from M 1, M 2, M 4, and M 5 and that these latter bands do, in fact, disappear upon digestion (Fig. 8). Our experiments reveal that breakdown and/or removal of bands M 1, M 2, M 4, and M 5 from myofibrils is extremely sensitive to trypsin digestion, and that they, unlike M 3, are not tryptic-like proteolytic fragments of myosin.

#### (e) Protein Composition of Myofilaments

The myofilaments prepared by treatment of fibrils with ATP-RB as described in Materials and Methods section c typically constitute approximately 8–10% of the myofibrillar protein. The residual myofibrils were resistant to further disassembly under various conditions (9 and Etlinger, in preparation). Electron-microscopic examination of these myofilaments indicates that most of the thick filaments in these preparations lack M-line structure and that more than 80% of these thick filaments are less than one-half their native length of 1.6  $\mu$ m (Fig. 9). The appearance of these filaments suggests that thick filaments might have, in fact, broken off from the residual fibrils at some point on either side of the center of the A band (Figs. 10, 11). The fragmented thick filaments usually have a tapered appearance at both ends. This is consistent with a mechanism by which the fracture plane occurs at an oblique angle to the filament axis. Fig. 11 shows a broken thick filament adjacent to a rare intact filament found in these preparations. The contour of the broken



**FIGURE 6** Effect of high-ionic-strength buffer on the ultrastructure of glycerinated muscle fibers. (a) Glycerinated myofibers immersed in low salt buffer for 30 min. (b) Glycerinated myofibers extracted for 10 min in high salt RB. Sections were obtained from the middle of the fiber bundle. (c) Same as *b* but 30-min extraction. Glycerinated muscle fibers were prepared as described in Fig. 2. The calibration bar equals 5  $\mu m$ .

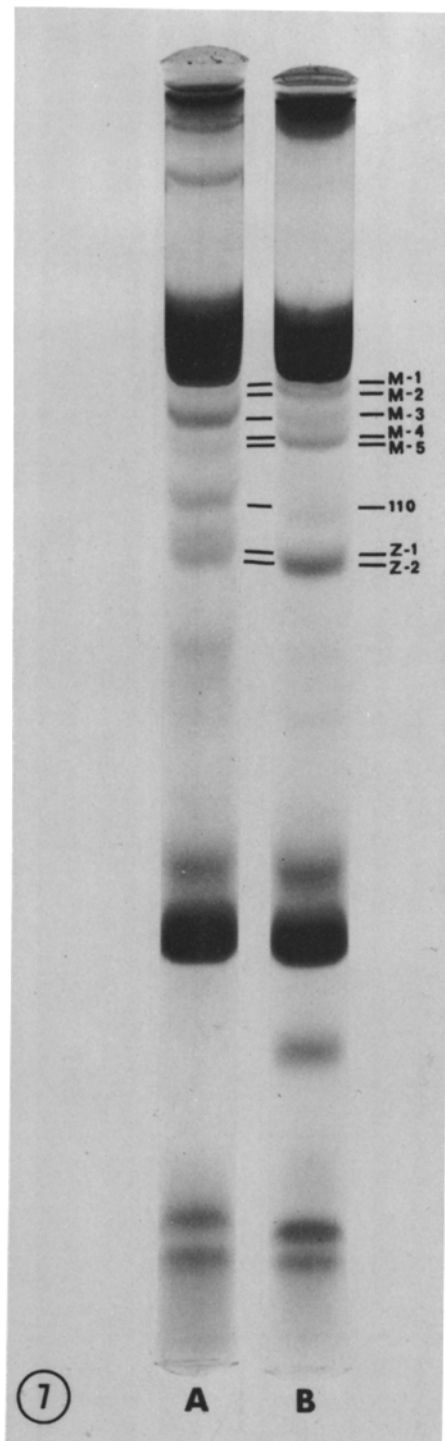


FIGURE 7 Changes in the electrophoretic profiles of myofibrillar proteins produced by mild tryptic digestion of isolated myofibrils. Myofibrils were prewashed with

filament corresponds to the parallel portion of the intact filament.

Likewise, there are no I-Z-I brushes in these preparations and no evidence of Z-line structure at the ends of the thin filaments. Unlike the thick filaments, about 80% of the thin filaments found in these preparations have lengths approximating the native thin-filament length, e.g. 1  $\mu$ m. However, this is also consistent with the absence of Z-line structure on these filaments if the separation occurs immediately before the Z line. Since no remnants of M or Z lines are apparent in these filaments, analysis of the protein composition of released filaments and residual myofibrils should, therefore, help to identify protein components of the M and Z lines.

The electrophoretic profiles of released myofilaments (Figs. 12, 13) lack the M 1, M 2, Z 1, and Z 2 bands that are present in untreated isolated myofibrils or in the myofibrillar residue after filament release in the ATP-RB solution. Since it had been shown previously (9) that the Z 1 and Z 2 bands originate from the Z line (and have electrophoretic mobilities similar to that of  $\alpha$ -actinin), it can be inferred that the remaining unidentified bands M 1 and M 2 correspond to structural components of the M line.

In order to unequivocally establish the identity of the gel bands which are absent from the filament gel profiles, we executed protocols for M-protein (30) and  $\alpha$ -actinin (1) isolation on filaments and intact myofibrils. These proteins have been previously shown by antibody studies to be located in the M line and Z line, respectively (28, 29). Fig. 13 illustrates that no detectable M protein or  $\alpha$ -actinin is obtained when the isolated filaments are used as the starting material for protein isolation. In contrast, the undispersed myofibrils treated in parallel yield  $\alpha$ -actinin and M protein. In order to achieve greater separation of the protein bands, the scans in Fig. 13 were obtained by electrophoresing the samples about 25% farther into the gel in comparison to the other examples illustrated in the present study. Thus, the broad actin peak is found at the bottom of the 11-cm gel. Interestingly, in our studies, the previously published procedures for the purification of M protein (30) and  $\alpha$ -actinin (1) resulted in the

0.5% Triton X-100. (A) Myofibrils digested with trypsin (4,000:1 wt/wt ratio) for 30 min (150- $\mu$ g protein). (B) Same as A, but a fourfold excess of trypsin inhibitor was added before the 30-min incubation.

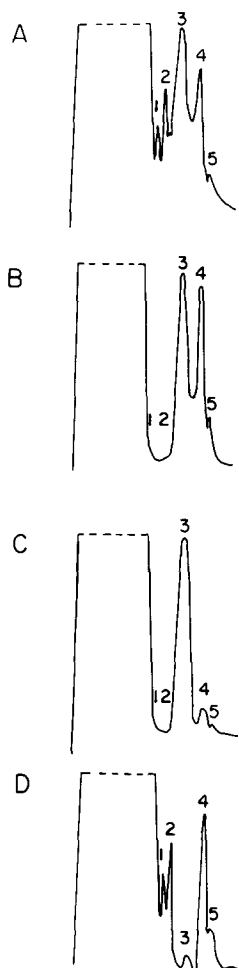


FIGURE 8 Localization of tryptic fragment (M 3) and C protein in myofibrils. (A) Coelectrophoresis of myofibrils digested with trypsin (75- $\mu$ g protein) as described in Fig. 7 A and of control myofibrils (75- $\mu$ g protein) as described in Fig. 7 B. (B) Trypsin-digested myofibrils (150- $\mu$ g protein) as described in Fig. 7 A and C protein (4- $\mu$ g protein). (C) Trypsin-digested myofibrils (150- $\mu$ g protein) as described in Fig. 7. (D) Control myofibrils (150- $\mu$ g protein) as described in Fig. 7 B. Scans were obtained at 540 nM with a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The portion of the scan containing the myosin heavy subunit (off scale) and the region containing bands M 1 through M 5 is shown.

isolation of both components of the doublet gel bands which, we suggest, originate from the M line and Z lines.

In Figs. 12, 13, it can also be seen that bands M 3, M 4, and M 5 remained associated with released filaments. Coelectrophoresis experiments show

that samples of C protein migrate with bands M 4 and M 5.

Finally, Fig. 12 shows that the very high-molecular-weight (VHMW) bands are conspicuously reduced or absent in preparations of released filaments. In contrast, the preparations of myofibrils, both residual and untreated, or of myosin, always show the presence of these low-mobility bands even under electrophoretic conditions which minimize the possibility of protein aggregation. When thick and thin filaments in ATP-RB were centrifuged at 40,000  $g$  for 30 min, the pellet was found to be highly enriched for thick filaments upon examination by electron microscopy. The composition of filaments which pelleted at 40,000  $g$  contained similar proportions of M 3, M 4, and M 5, and very high-molecular-weight protein when compared to either thick and thin filaments before centrifugation or filaments pelleted at 100,000  $g$  for 3 h. This latter protocol resulted in the quantitative sedimentation of both thick and thin filaments. In addition, the 40,000  $g \times 30'$  supernatant contained only thin filaments when examined by electron microscopy. This thin-filament fraction was devoid of the myosin-associated components. Thus, it is concluded that bands M 1 through M 5 and the very high-molecular-weight protein bands are associated with thick filaments in agreement with the high-ionic-strength extraction experiments discussed in section *c*.

## DISCUSSION

We have presented studies on the isolation, purification, and disassembly of uncontracted

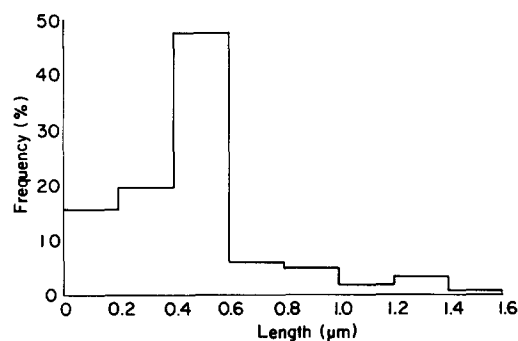


FIGURE 9 Length distribution of thick filaments found in myofilament preparations from fresh muscle. Filaments were prepared as illustrated in Fig. 10. 200 filaments were measured. The mean length was  $5.87 \mu$ m  $\pm$  0.27 (SE). No significant difference in length was seen between different grids or different preparations.

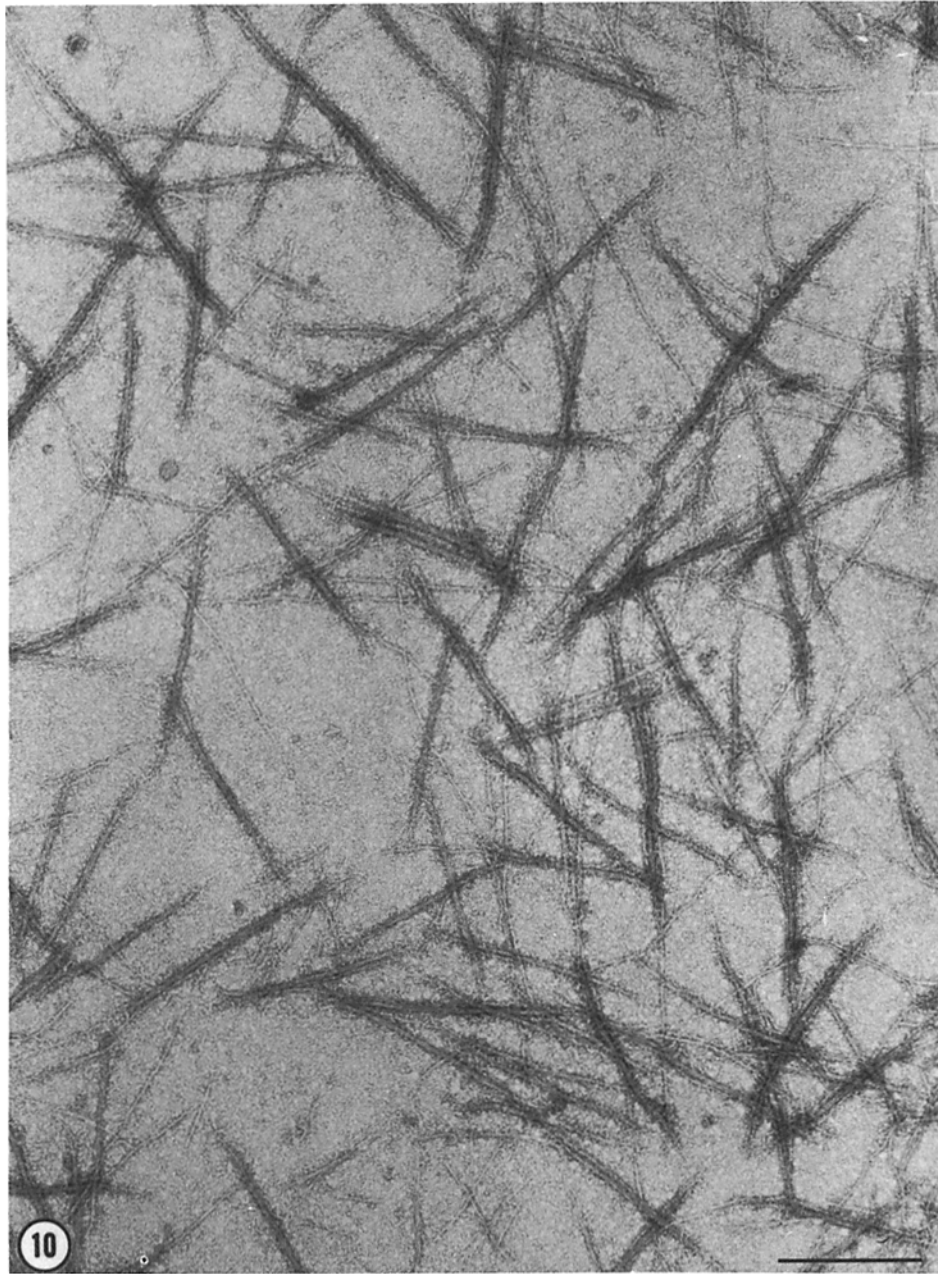


FIGURE 10 Electron microscopy of myofilaments released from myofibrils prepared from fresh muscle. Negative staining with 2% uranyl acetate. Calibration bar equals 0.5  $\mu\text{m}$ .

myofibrils from striated muscle. Contraction of fibrils results in entrapment of subcellular contaminants, which hampers fibrillar purification (48); also, these shortened fibrils are not suitable for subsequent structural analysis. Furthermore, we have observed that not all of the A-band

components are extracted at high ionic strength from supercontracted myofibrils (Etlinger, unpublished). The inclusion of pyrophosphate in addition to EGTA during the initial soaking and homogenization steps of fibrillar isolation facilitates the reproducible preparation of

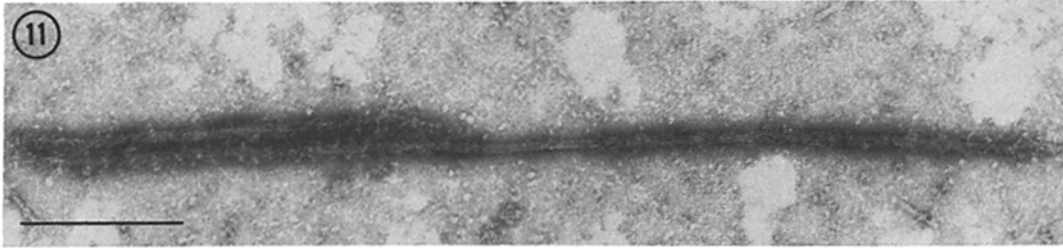


FIGURE 11 High magnification electron micrograph of a rare intact thick filament lying next to a typical thick filament fragment. Negative staining with 2% uranyl acetate. Calibration bar equals 0.2  $\mu\text{m}$ .

uncontracted myofibrils. Presumably, pyrophosphate inhibits contraction by substituting for endogenous ATP as this ATP is diluted during the initial isolation steps. It has, in fact, been shown that isolated myofibrils will contract in the absence of  $\text{Ca}^{++}$  at very low ATP concentrations, e.g., less than 60  $\mu\text{M}$  (27). However, other actions of this polyphosphate cannot be excluded at this time. The methods employed here for both relaxation and subsequent purification of myofibrils result in fibrils which are structurally intact and which retain enzymatic and regulatory activity (48). In addition, when these purified fibrils are electrophoresed in a system containing 10–15% acrylamide (23), both the alkali and 5,5'-dithiobis(2-nitrobenzoic acid) myosin light chains and the T, C, and I troponin subunits can be resolved (Fischman, unpublished observation). It has been shown previously that the use of 1% Triton X-100 does not affect the EGTA sensitivity of the  $\text{Mg}^{++}$  myofibrillar ATPase (43). Our gel studies, at least at the sensitivity used here, indicate no loss of constituent myofibrillar proteins in Triton X-100 solutions, at concentrations which solubilize membrane components and protease(s) in the fibrillar fraction. A membrane-bound,  $\text{Ca}^{++}$ -activated ATPase extracted with Triton X-100 and DOC, and having a mobility on SDS gels similar to that of the 110,000-dalton component found in our Triton extracts, has been reported by other laboratories (25, 26, 32, 33). It is probably the same component, since its solubilization parallels that of the sarcoplasmic reticulum from our preparations.

Protease contamination of myofibrillar preparations has been observed in several recent studies, and digestion products have been shown to appear during the isolation of troponin. This breakdown of troponin is activated strongly at

acidic pH during isoelectric point fractionation (18, 5). In addition, other neutral and acid protease activities have been shown to contaminate actin (3) and myosin preparations (16).

One approach to prevent degradation has been the use of protease inhibitors during isolation, but this usually results in only partial inactivation of total protease activity (18). We have shown that all detectable contaminating protease activity can be removed from the myofibrillar fraction of rabbit skeletal muscle by the use of 0.5% Triton X-100. Residual neutral protease activity not removed by Triton X-100 has, however, been found in myofibrils prepared from heart muscle (40) and in certain rat skeletal muscle preparations (31; Etlinger, unpublished).

Compared to the extensive information now available on the arrangement of tropomyosin, troponin, and actin in the thin filaments, relatively little is known about thick-filament composition and organization. Various studies have appeared in which the authors claim to have extracted or isolated proteins that associate with myosin in the thick filament. In these studies, M-line proteins having subunit weights of 155,000 (29, 30), 140,000–190,000 (9), 100,000 (6, 29, 30), and approximately 40,000 daltons (6, 34) have been reported. Presumably, these proteins associate with or constitute the transverse extensions which cross-link thick filaments in the middle of the A band (22). If one takes into account differences in estimates of molecular weight, our results, which suggest that M-line protein corresponds to components M 1 and M 2, are consistent with the findings of Masaki and Takaiti (30) although those authors resolved only one component at approximately this molecular weight. Bands M 1 and M 2 were not seen in the fragmented thick and thin filaments, and since these single thick filaments show no evidence of M-line bridge structure

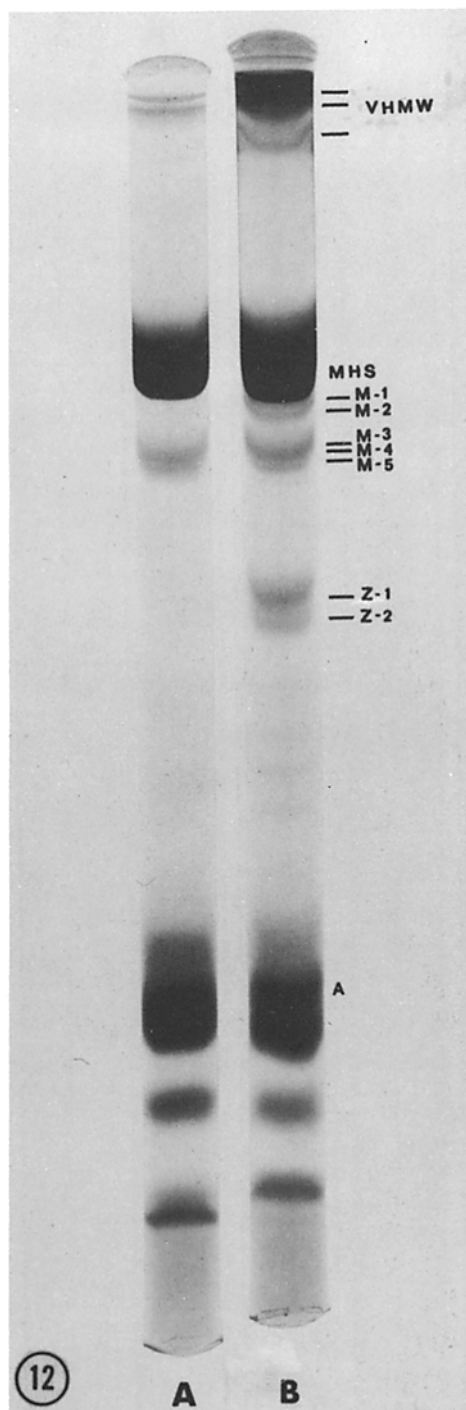


FIGURE 12 Protein composition of easily released myofilaments compared with residual myofibrils. (A) Myofilaments released from isolated myofibrils (130- $\mu$ g protein). (B) Residual myofibrils after a total of five extrac-

by electron microscopy, it suggested that bands M 1 and/or M 2 correspond to M-line protein. Furthermore, the procedure of Masaki and Takaiti (30) resulted in the isolation of M protein only from myofibrils and not the single filaments. This component was previously shown to react immunologically with the M line (30). Bands of molecular weight similar to that of all of these components have also been noted on SDS gels of partially purified myosin preparations (45, 39). Our results suggest that, when A- and M-band components are extracted from Triton-extracted, protease-free fibrils, no proteins which migrate between C protein (approximately 140,000 daltons) and 55,000 daltons are seen in this A-band extract. Studies (6) on chicken breast muscle drew attention to a band of 100,000 daltons claimed to be extracted from the M line. Compositional differences between chicken breast and rabbit back muscle might account for this difference. Alternatively, the 100,000-dalton component might correspond to a contaminant removed during fibrillar isolation or to the tryptic degradation component with this approximate mobility noted in the present study. It has been suggested that a second protein of 42,000 daltons is an M-line component (6, 34) and that this protein is identical to creatine kinase (46). We have also observed a protein of 42,000 daltons in A-band extracts, and it may be that such a protein, perhaps creatine kinase, has a structural role in the myofibril; however, it is also possible that certain soluble proteins become less soluble when muscle is chilled near 0°C during myofibrillar isolation, and that these proteins preferentially adhere to structures in the sarcomere. Phosphorylase contamination of  $\alpha$ -actinin preparations (1) is likely to be another example of this phenomenon.

The bands purifying with thick filaments are M 3, M 4, and M 5. If M 3 is a degradation product, as suggested above, then only M 4 and M 5 (C protein) are likely to be components of thick filaments. Band M 5 could be a slightly degraded form of band M 4, since small amounts of C

tions with ATP-RB (140- $\mu$ g protein). VHMW = proteins with mol wt greater than 200,000 daltons. MHS = myosin heavy chains (200,000 daltons). M 1 to M 5 = proteins extracted with myosin. A = actin (43,000 daltons). These samples were electrophoresed approximately 15% longer than the other gels included in this study in order to illustrate the separation of the Z 1 and Z 2 components.

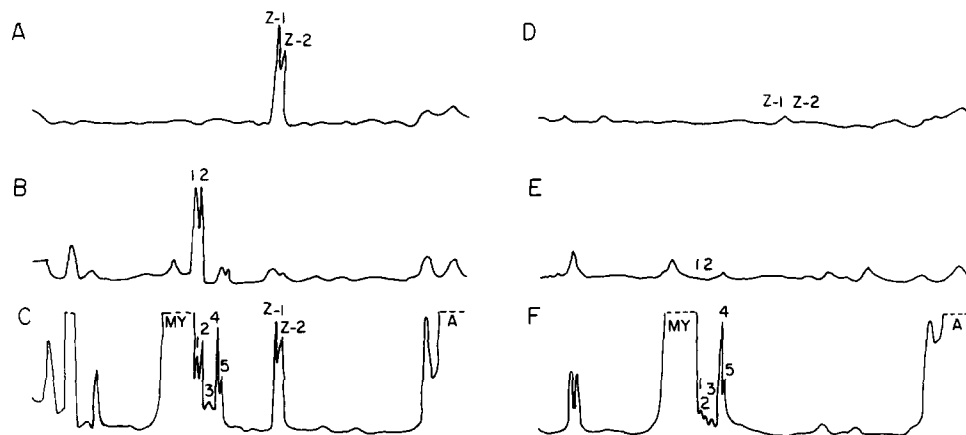


FIGURE 13 Isolation of M protein and  $\alpha$ -actinin from filament and myofibril preparations. A, B, and C were obtained from myofibrils preextracted with 0.5% Triton X-100. D, E, and F were obtained from filaments prepared from 0.5% Triton X-100 extracted myofibrils. A and D were obtained after using a protocol for  $\alpha$ -actinin isolation (1). B and E were obtained after using a protocol for M-protein isolation (30). MY = myosin heavy chain. A = actin. Gel scans were obtained at 540 nM with a Gilford spectrophotometer. Samples were electrophoresed until actin (43,000 daltons) migrated 11 cm to the bottom of the gel, i.e. 1 h after tracking dye migrated out of the gel.

protein were also seen to split and run with M 4 and M 5. Independently of our work, Morimoto and Harrington (35) have reported the separation of thick filaments from thin filaments by glycerol gradient centrifugation. They also claimed that such thick filaments contain only C-protein and myosin subunits. In these studies, however, a 7-day extraction of a crude fibrillar fraction was used which, we found, contained protease activity. We believe that caution should be exercised in the analysis of SDS gels of myofibrils after prolonged extractions, since tryptic digestion of myofibrils produces a protein band, M 3, which is very close in mobility to C protein (M 4); in some gel systems, these two protein components might not be resolved as separate bands.

The very high-molecular-weight material (greater than 200,000 daltons) which is consistently observed on SDS gels of myofibrillar preparations is of interest since this undefined protein can constitute approximately 10% of the total myofibrillar protein (Etlinger, unpublished). The amount of protein in these bands is considerably reduced in easily released thick-filament preparations, yet they are found in myosin extracts of whole myofibrils. The origin and composition of these bands are unknown. Whether these proteins arise from the myofibrils and are related to myosin, e.g., whether they are cross-linked myosin or additional thick-filament components, will require further analysis.

The fact that C protein will coelectrophorese with two closely migrating bands (M 4 and M 5) and that the Z- and M-line protein can be resolved into two bands suggest that the faster-migrating protein might possibly be derived from the more slowly migrating component by proteolysis which occurs before the removal of contaminating protease. Also, we have shown that the M-, Z-, and C-protein bands are extremely susceptible to tryptic digestion. Therefore, we conclude that interpretations of stoichiometric relationships between the protease-sensitive proteins of these molecular weights and other fibrillar components must be viewed with caution. However, the doublet nature of the M- and Z-line protein bands may reflect a secondary separation based on charge which may occur in the present SDS electrophoretic system (Etlinger, in preparation) and which, therefore, might indicate the presence of isoproteins in these mixed fiber type preparations.

The compositional analysis of released myofilaments provides additional evidence that all of the approximately 90,000-dalton protein originates from the Z line, and that single thin filaments released during this procedure break off without attached Z material. This conclusion is based on the fact that there are no I-Z-I brushes in our preparations, and there is no evidence from negative staining that these thin filaments have any attached Z-line structure. In addition, no



detectable  $\alpha$ -actinin could be isolated from the single filaments under conditions which readily yielded this protein from myofibrils. We conclude that the protein subunits of approximately 90,000 daltons are located in the Z-line region, in agreement with the localization of  $\alpha$ -actinin in that region (28, 14). We have previously shown that DOC extraction removes the dense material of the Z line while not extracting its entire filamentous structure. Although the 90,000-dalton proteins are released during Z-line extraction, Z-line removal by DOC was shown to release the dense material immediately while only releasing a small fraction of the Z 1 and Z 2 proteins, thus suggesting the rapid release of another, as yet, unidentified component of the Z line (9). It should be noted that proteins migrating with tropomyosin, troponin, and actin are also extracted with DOC; but these extracted proteins only account for a small proportion of the tropomyosin, troponin, and actin present in the intact myofibril. The possible location of some of these proteins in the Z line cannot yet be eliminated.

In conclusion, the present work establishes a basis for future studies of the changing composition of myofibrils in response to varying physiological conditions, during muscle development and in disease, with respect to both native protein components and degradation products. Our compositional analysis has utilized a preparation of easily released myofilaments comprising approximately 10% of the fibrillar protein. Related studies on myofilament release suggest the possible existence in vivo of two myofilament populations which differ in relative strength of attachment in or on the myofibrils (10). These studies indicate that the loosely held population of myofilaments contain a higher fraction of newly synthesized myosin than the nondissociable fibrillar residue (10). This approach should permit a more detailed kinetic analysis of myofibrillar synthesis, assembly, and turnover than has heretofore been possible.

The authors would like to express their gratitude to Dr. M. Rabinowitz for his encouragement and helpful criticism and to Mrs. R. Gramas and Mrs. G. Wu for their excellent technical assistance.

This work was supported in part by U.S. Public Health Service grants PH 4368133, HL 16637, HL 13505-04, University of Chicago Cancer Research Center grant IPOICA 14599-01, and a grant from The Muscular Dystrophy Association of America, and the Chicago and Illinois Heart Associations.

J. E. acknowledges support from the U.S. Public Health Service Biophysics training grant no. GM 780 and a postdoctoral fellowship from The Muscular Dystrophy Association of America.

Received for publication 12 December 1974, and in revised form 21 August 1975.

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