THE EFFECT OF VARIOUS PROTEIN FRACTIONS ON Z- AND M-LINE RECONSTITUTION

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ABSTRACT

Extraction of thin, glycerinated bundles of rabbit psoas muscle with a low ionic strength solvent results in removal first of M lines and then of Z lines. When these extracted myofibrillar bundles are allowed to interact, at adjusted ionic conditions, with the dilute myofibrillar extract or with the fractions obtained at 40% ammonium sulfate saturation from either the myofibrillar extract or from the Bailey extract of natural actomyosin, reconstitution of Z lines occurs. The ammonium sulfate fraction from the Bailey extract of natural actomyosin restores the tetragonal lattice structure of the Z line. Other structural features such as I-band tufts or cross-bridges, M lines and H-zone binding also occur with some of the proteins used for recombination. Although it has not yet been possible to identify exactly the protein(s) constituting the Z line, it appears unlikely that tropomyosin or troponin alone is the major protein of the Z line. A more likely candidate is α-actinin or a combination of α-actinin with another protein(s). In addition, this study demonstrates that basic morphological differences exist between cross-sections through the Z-line lattice and cross-sections through tropomyosin crystals.

INTRODUCTION

In a recent communication (28), we described the removal of rabbit skeletal muscle Z lines by extraction with a low ionic strength solvent and the subsequent reconstitution of Z lines by adjustment of ionic conditions. Implications of earlier work (Huxley, 17; Corsi and Perry, 6) on normal Z lines and the studies by Fawcett (10) on abnormal Z lines were that tropomyosin was probably located in or very near the Z line.

On the other hand, it has been proposed that α-actinin is a component of the Z line (3). This view has been substantiated by Masaki et al. (20) with the use of fluorescein-labeled anti-α-actinin. In addition, the susceptibility of Z lines to various agents such as trypsin (1, 9, 27) and lipase (12, 13) has been reported. Detailed structural studies of the Z line have also been published (11, 18).

The series of experiments reported here extends the work on Z-line reconstitution to other proteins or protein fractions and also includes effects on M lines and A and I bands. In addition, comparisons are made between the fine structure of cross-sections through the Z line, both before extraction and after recombination, and the fine structure of sections of crystalline tropomyosin.

MATERIALS AND METHODS

Rabbit psoas muscle, glycerinated in the usual manner for at least 30 days, was teased into thin 250–300-μ bundles and extracted at 0°C with a solution containing 2 mM Tris (pH 7.6) and 1 mM dithiothreitol. Long periods of glycerination (24–36 months) apparently cause sufficient damage so that extraction with this solvent results in breakage in the center of the A band. The teased myofibrils were transferred to fresh Tris-dithiothreitol solution and stored under nitrogen. After 10 days, the supernatant was removed and the myofibrils were rinsed in the extracting solution prior to fixation in 2.5% glutaraldehyde or prior to use in recombination experiments.

All recombination experiments were conducted at 0° and normally were of 65–72 hr duration. Each experiment consisted of a control in which the extracted myofibrils were immersed in the protein of choice in a 2 mM Tris (pH 7.6), 1 mM dithiothreitol solution. The remaining samples in a particular experiment consisted of these same components plus various combinations of 0.1 M KCl and 1–5 mM $CaCl_2$ or $MgCl_2$. Other levels of KCl were tested but found to be less satisfactory than the 0.1 M level. Following the period of recombination and before fixation, each sample was washed in a solvent identical with that in which the recombination occurred for that particular sample.

Proteins used for recombination experiments included the following: tropomyosin and troponin, both prepared from natural actomyosin following the Bailey sequence of organic solvents (2) as described by Hartshorne and Mueller (15); the dilute and concentrated unfractionated low ionic strength extracts of the teased myofibrils; the fractions obtained at 40% (P40) or between 40 and 60% (P40–60) ammonium sulfate saturation and 0°, from either the Bailey extract of natural actomyosin (NAM) or from the extract of the teased myofibrils; α-actinin prepared by the method of Seraydarian et al. (26). Where concentration of a particular fraction was desired, a Diaflo (Amicon Corporation, Cambridge, Mass.) membrane, type UM-1, was used. Protein concentrations were determined by micro-Kjeldahl analysis.

The procedure of Bailey (2) was used to crystallize tropomyosin from the P40–60 fraction. The crystals were then sedimented by centrifugation, the supernatant was decanted, and the pellet was stabilized by carefully layering 2.5% glutaraldehyde onto the material. After 2–3 min, the pellet was removed from the centrifuge tube, covered with glutaraldehyde, and subdivided for subsequent processing.

After glutaraldehyde-osmium tetroxide fixation, the tissue was dehydrated in increasing concentrations of acetone and embedded in Epon-Araldite. Sections were stained with 2% uranyl acetate in methanol and poststained with lead citrate. A JEM-7 or a Philips 200 electron microscope was operated at 80 kv with a 30 μ objective aperture. All phase microscopy was done with a Zeiss GFL microscope equipped with a 100 × oil immersion objective. Observations were recorded on 4 × 5 Polaroid Type 55 P/N film.

RESULTS

Appearance of Extracted Myofibrils

The morphology of teased, glycerinated rabbit psoas muscle after having been extracted for 10 days in 2 mM Tris (pH 7.6), 1 mM dithiothreitol is shown in Fig. 1. This treatment results in the early removal of M lines; subsequently Z lines are also removed. These observations on sectioned materials were confirmed in the phase microscope as shown in the insets. Inset *a* shows the prominent Z lines of teased psoas before extraction. Inset *b* shows the appearance of teased psoas after extraction. Although the thickness of these bundles somewhat impairs resolution under phase microscopy, it is quite apparent that Z lines are removed by this treatment. To observe the morphological changes accompanying the extraction, all samples were examined with the electron microscope and frequently were monitored by phase microscopy.

Z-Line Reconstitution with Various Proteins

The result of the use of unfractionated, nonconcentrated, low ionic strength extract of the myofibrils for Z-line reconstitution is shown in Fig. 2. In this experiment, as in all reconstitution experiments, a solution of 2 mM Tris (pH 7.6), 1 mM dithiothreitol was the basic medium. In this experiment the solution was made 0.1 M with KCl and 1 mM with $CaCl_2$; however, the same results could be achieved without the addition of calcium. Although the myofibrillar extract was quite dilute (0.7 mg/ml), material has been bound in the Z-line region. In addition, small tufts of material are seen in the lateral thirds of the I band. Although the size of the approximately circular tufts is variable, the tufts seem to be associated with one or more thin filaments and are only found in the lateral thirds of the I band. At higher magnification, these tufts appear to be similar to the material bound in the Z-line region. I-band cross-bridges are nearly absent when the dilute extract is used. The term I-band cross-bridges refers to the filament-like structures

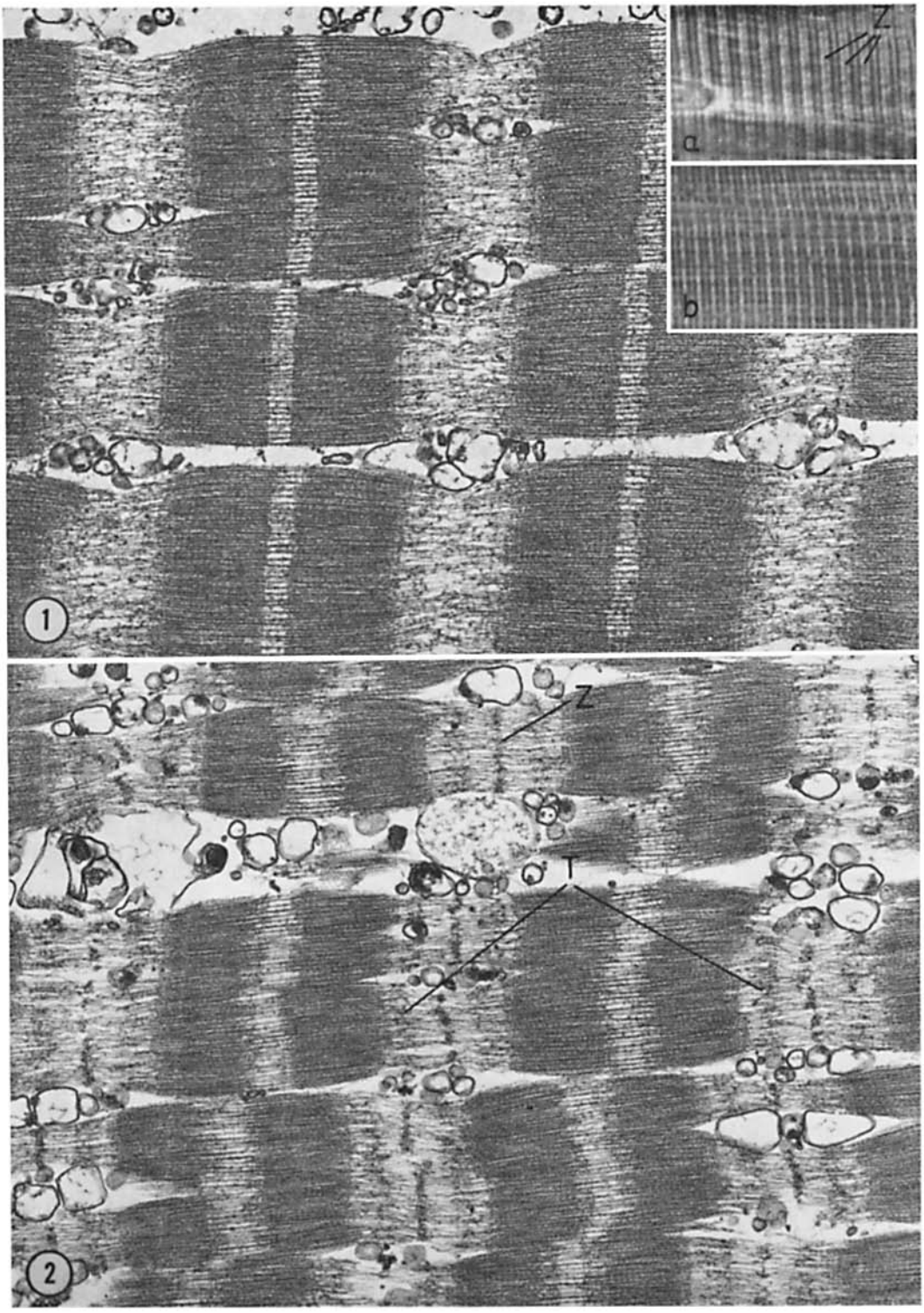


FIGURE 1 Glycerinated rabbit psoas muscle teased into thin bundles and extracted for 10 days with 2 mM Tris (pH 7.6), 1 mM dithiothreitol at 0°C. Note the absence of Z and M lines. This and all other samples for electron microscopy were fixed with glutaraldehyde-OsO_4, embedded in Epon-Araldite, and stained with uranyl acetate-lead citrate. × 27,000. Inset *a* shows a phase contrast micrograph of the teased glycerinated psoas with Z lines (*Z*) prior to extraction. × 1090. Inset *b* is another phase micrograph of extracted psoas and shows the lack of Z lines when compared with *a*. × 1090.

FIGURE 2 Reconstitution of Z lines with the unfractionated, dilute myofibrillar extract at 0.7 mg/ml in the basic medium adjusted to 0.1 M KCl and 1 mM $CaCl_2$. Z lines (*Z*) are easily seen as are I-band tufts (*T*). × 25,300.

seen only in the region of the I band between the tufting zone and the Z line and extending from one actin filament to an adjacent actin filament.

Very different results were achieved by using the concentrated (4 mg/ml) unfractionated extract (Fig. 3) in the basic medium plus 0.1 M KCl. The most striking feature is the return of M lines which appear to be very similar to those seen in unextracted myofibrils. When viewed obliquely, these reconstituted M lines frequently show the tripartite structure described by Knappeis and Carlsen (19). Since M lines are always removed before Z lines, the presence of M lines in the absence of Z lines serves as an internal control of M-line reconstitution. The H zone also shows a conspicuous increase in density due to binding in the region of heavy meromyosin (HMM) projections where the projections are not in contact with the interdigitated thin filaments.

I bands do not show the expected prominent Z lines but instead exhibit binding mainly in the middle two-thirds, with a marked tendency for the most dense accumulation of material to occur in the center of the I band. The arrangement of this material can be seen in the higher magnification inset taken from the I band of a very thin section of identically treated material. The increased density is mainly due to the presence of cross-bridges between adjacent thin filaments, while little if any evidence of tufts, as seen in the dilute extract, could be found.

The results of reconstitution with a P40 fraction (3 mg/ml) of the myofibrillar extract in basic medium plus 0.1 M KCl and 1 mM $CaCl_2$ are seen in Fig. 4. Z lines and tufts in the lateral thirds of the I band are apparent and very similar to those observed in the sample treated with the dilute extract (Fig. 2). However, the area between the tufts and the Z line has greatly increased in density and is populated by cross-bridges. Also evident, especially when viewed obliquely, is material bound to the M-line region. Both this P40 fraction of the myofibrillar extract and the P40 fraction of the Bailey extract (2) of NAM were shown to be deficient in tropomyosin. This deficiency was evidenced by their inability to inhibit the Ca^{++}-activated ATPase activity of reconstituted actomyosin (25); and these fractions exhibited no EGTA (ethylene glycol bis [β-aminoethyl ether]-N,N'-tetraacetic acid)-sensitizing factor activity (7, 8, 14).

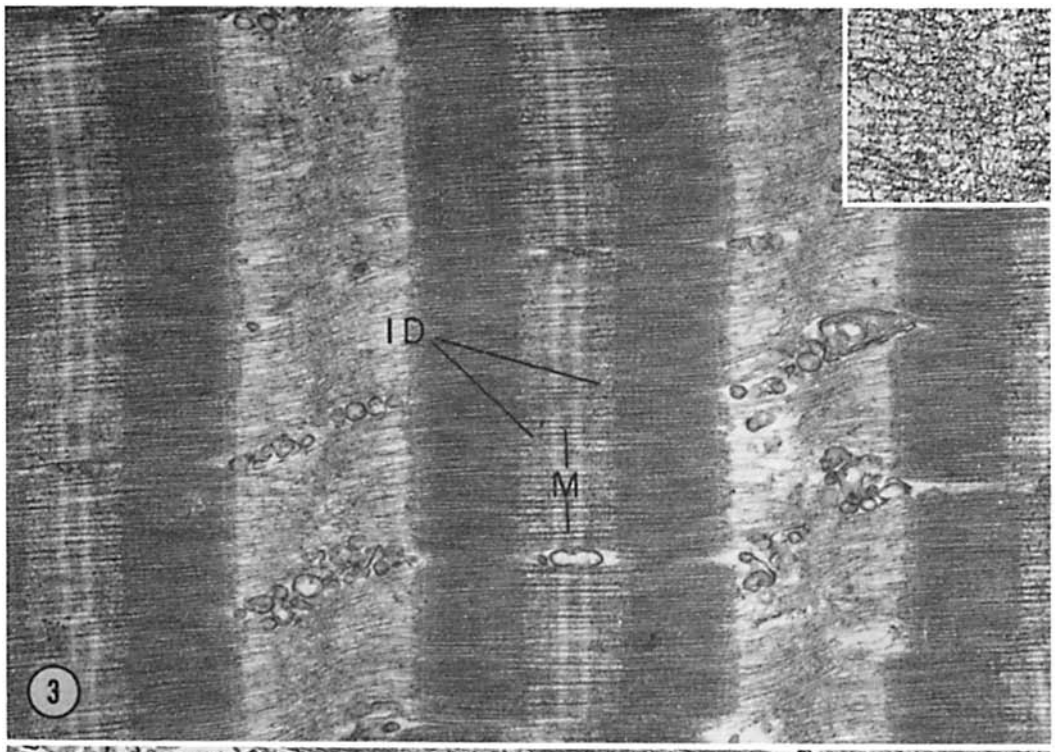
The result of treatment of previously extracted myofibrils with the P40 fraction obtained from a Bailey extract of NAM (15) is shown in Fig. 5. The basic medium was adjusted to 0.1 M KCl. This somewhat thicker section shows the usual heavy binding seen in the Z-line region with this treatment. Tufts along the lateral thirds of the I bands are also consistently observed. A very small amount of material has been attached to the M-line region, but no cross-bridges could be found in the I bands of thin sections of this material.

The use of α-actinin at 8.5 mg/ml in the basic medium plus 0.1 M KCl and 1 mM $MgCl_2$ results in a large amount of binding in the I band and an increased density in the H zone except for the central portion (Fig. 6). The concentration dependence of this reaction is more apparent when Ca^{++} or Mg^{++} are added to the basic medium containing 0.1 M KCl, as shown by the observation that the addition of these cations results in an additive binding in the I band at high levels of α-actinin; but little or no effect occurs at low levels. The inset (Fig. 6) shows a portion of an I band from a sample in the basic medium adjusted to 5 mM $MgCl_2$, 0.1 M KCl. The cross-bridges between thin filaments are clearly present and are responsible for the heavy deposits seen in the I bands in Fig. 6.

A comparison of the results obtained with the various protein fractions is shown in Table I. The columns headed Z lines and tufts show marked similarities in all instances except the Z-line reaction with α-actinin. This category was rated as having a slight reaction because of the occasional observation that short segments of Z lines were beginning to reorganize with this fraction. The results are also quite similar in the two columns headed cross-bridges and HMM binding. The last column indicates that the effect of Ca^{++} or Mg^{++} on the amount of material bound in the sarcomere is nonadditive in all instances except with α-actinin.

Comparisons Between Z-Line Cross-Sections and Tropomyosin Crystals

Because tropomyosin-deficient fractions appeared to possess the ability to reconstitute Z lines under appropriate conditions, it seemed relevant to examine cross-sections of Z lines before removal and after reconstitution and to compare them with cross-sections of similarly fixed, embedded, and



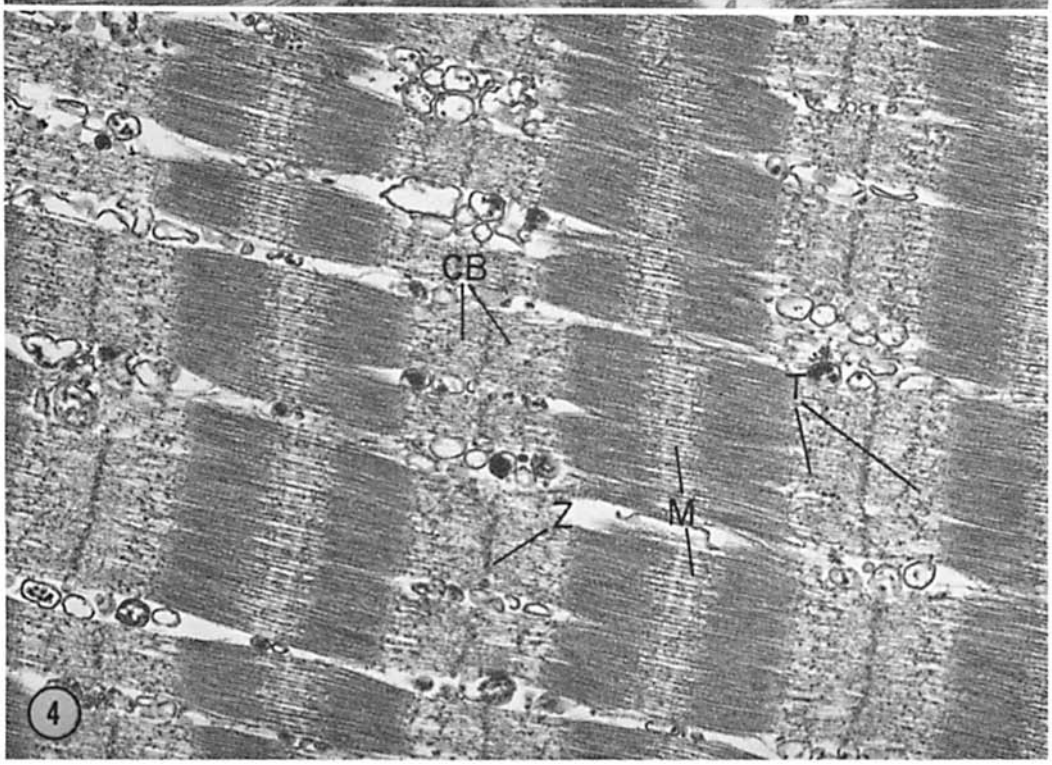
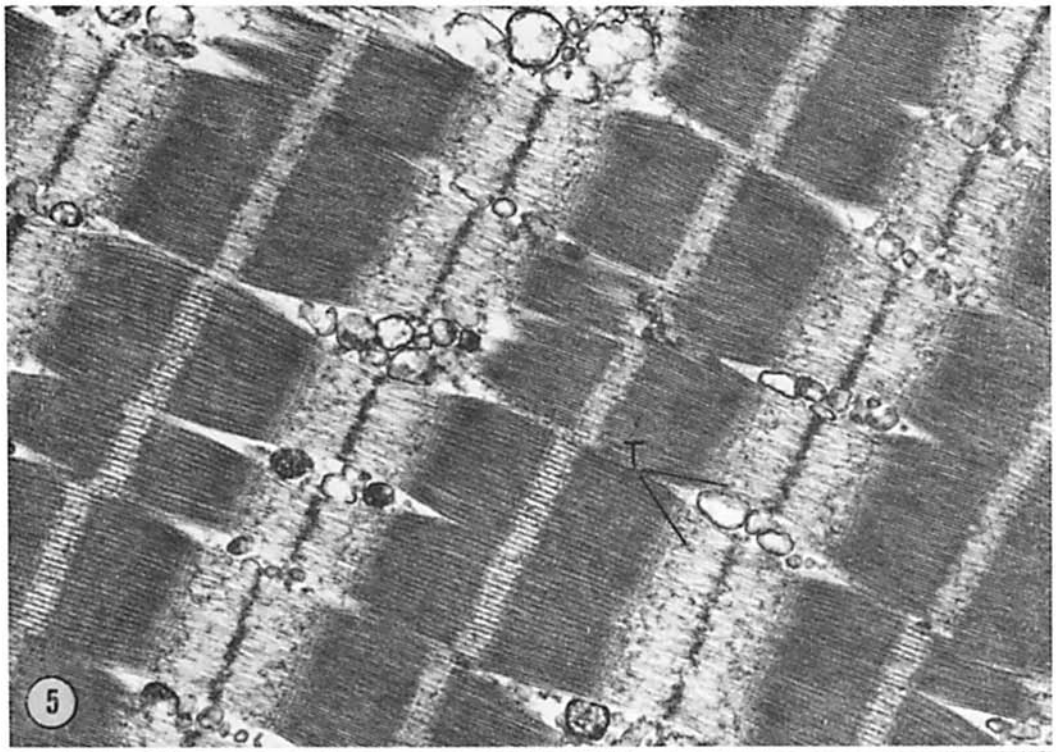


FIGURE 3 Typical appearance of previously extracted fibers reacted with unfractionated, concentrated myofibrillar extract at 4 mg/ml with 0.1 M KCl in the basic medium. M lines (*M*) are very prominent. Lateral thirds of the H zone have increased density (*ID*) probably due to attachment of material in the region of the projections from the thick filaments. × 29,000. I-band cross-bridges are shown at higher magnification (inset) from another, thinner section. × 72,300.

FIGURE 4 P40 (3 mg/ml) of the myofibrillar extract in the basic medium adjusted to 0.1 M KCl and 1 mM $CaCl_2$. Z lines (*Z*), tufts (*T*), I-band cross-bridges (*CB*), and M lines (*M*) are all present after this treatment. × 22,700.



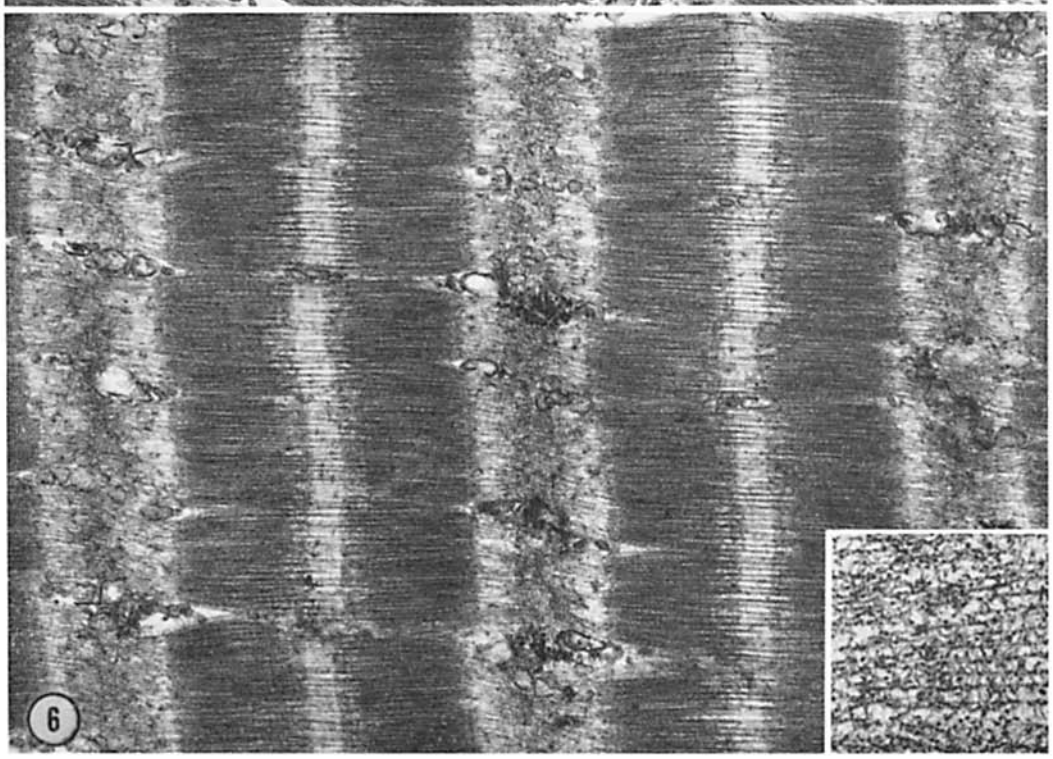


FIGURE 5 Somewhat thicker section showing the prominent Z lines and numerous tufts (*T*) seen when the P40 from the Bailey extract of natural actomyosin is applied at 5.69 mg/ml, with the basic medium adjusted to 0.1 M KCl. × 23,750.

FIGURE 6 Results with α-actinin at 8.5 mg/ml, 0.1 M KCl and 1 mM $MgCl_2$. An increased density is observed in the I band and in the lateral thirds of the H zone. × 28,300. Inset is a portion of the I band from a thinner section and shows cross-bridges between thin filaments. × 68,100.

TABLE I

Effects of Various Proteins on Recombination.

This table summarizes the results obtained with the various proteins when used for recombination experiments under the conditions described in the text. Note the similiarities between results in the columns headed formation of Z lines and formation of tufts and between the columns headed formation of cross-bridges and binding to HMM.

Protein used	Formation of Z lines	Formation of tufts	Formation of cross-bridges	Formation of M lines	Binding to HMM*	Effect of Ca^{++} or Mg^{++}
Dilute myofibrillar extract	++	++	0	0	0	N
Concentrated myofibrillar extract	0	0	++++	++++	++++	N
P40 of myofibrillar extract	+++	++	+++	++	++	N
P40 of Bailey extract of NAM	++++	+++	0	0	0	N
α-actinin	+	0	++++	0	+++	A
Tropomyosin	0	0	0	0	0	0

* H zone binding other than pseudo-H zone, i.e. the HMM bridge-free central portion of the thick filament.

Key to Symbols:

0 = no reaction
\+ = slight reaction
++ = weak reaction
+++ = moderate reaction
++++ = strong reaction
N = nonadditive effect
A = additive effect

stained crystalline tropomyosin. Fig. 7 *a* shows an oblique cross-section through the Z line of glycerinated rabbit psoas muscle before extraction. The "over-under" woven pattern formed by fairly broad strands, and described by Reedy (23), is easily seen in the right-center of the field. Another feature seen at the left-center is that filaments approaching the Z line are arranged in a nearly square pattern and that, as the center of the Z line is approached, a central structural entity is added to the square (arrow). As described by Huxley (17), this appearance may be due to the displacement of two lattices by 45° from each other. The axes of the crossing fibers form very nearly a right angle.

A similar cross-section of material from a sample containing reconstituted Z lines as shown in Fig. 5, viz. with P40 of NAM, reveals the pattern as seen in Fig. 7 *b*. The woven pattern is again seen, and the relatively smooth strands are similar in size and appearance to the ~60–65 A strands seen in Fig. 7 *a*. However, the cross-over points in the reconstituted lattice are 170–180 A apart compared with a distance of ~125–135 A in the native Z-line lattice. The square pattern formed by the incoming filaments in the lower portion of the field in Fig. 7 *b* shows that some filaments are missing, probably indicating that reconstitution is not entirely complete. A very thin section through an embedded tropomyosin crystal reveals the lattice seen in Fig. 8. These strands appear not to be smooth but could be interpreted to be in the form of a coiled strand (arrows) having a diameter within the coil of ~10 A. Such a speculation was originally proposed by Huxley (17). Also, the axes of these strands do not appear to cross at right angles, as is the case in the Z-line lattice, but instead they cross at an angle differing by 5°–15° from 90°. The result is an appearance resembling the stacking of trapezoids base-to-base and top-to-top. This appearance causes one to see alternating distances of ~100–110 A and 155–165 A between cross-over points as one moves from left to right or from top to bottom along a particular strand.

DISCUSSION

Methods of Extraction

From the results presented here and in our previous communication (28), it seems clear that this method of extraction with low ionic strength solvents results in the removal of material from the Z line which, under the appropriate conditions, retains its ability to rebind to this region. Although the pioneering work of Corsi and Perry (6) on myofibrillar extraction at low ionic strength demonstrated that Z lines disappeared during

such treatment, their observations were all made with the phase microscope and did not permit the resolution attainable with the electron microscope. Samosudova (24) did use electron microscopy to monitor changes in glycerol-treated chicken muscle while extraction with 5 mM Tris pH 7.6, was carried out. Although Samosudova (24) did not specifically state her starting material, there is reason to believe that she worked with homogenized myofibrils as did Corsi and Perry (6). At the low ionic strength of the solvents used in both of these studies, we found that an objectionable amount of swelling occurred with isolated myofibrils. Consequently, this starting material was used only for limited investigations.

In these preliminary studies, the myofibrils were obtained after homogenization of the muscle. The morphological extraction sequence was monitored with the phase-contrast microscope and also electron microscopically after staining with dilute (0.5%) aqueous solutions of uranyl acetate. The latter method offered a rapid means of assessing a particular treatment. Although the specimen thickness limited resolution at the ultrastructural level, electron microscopy was considerably more informative than phase microscopy. As anticipated, the extraction process was found to be similar to, but more rapid than, that with the fiber bundles.

Following an unpublished observation by one of the authors,[1] an attempt was made to extract the Z line more selectively from isolated myofibrils with the use of formamide. At formamide concentrations of 10–20% (v/v), Z lines were removed but concomitantly other areas of the myofibril, particularly the A band, were extracted.

[1] Hartshorne, D. J. Unpublished data.

Removal of Z lines by tryptic digestion has been reported in several papers but was not used in these experiments, primarily for two reasons. First, one of our goals was reconstitution of the Z-line structure; since it was known that trypsin would attack thin filaments in the I band (27), the use of this method appeared to be not advisable. Secondly, in addition to removing Z lines, trypsin would probably also hydrolyze to some extent the extracted proteins and possibly interfere with the subsequent recombination and characterization of the Z line.

The use of pancreatic lipase for Z-line removal, as reported by Garamvolgyi and Guba (13), also seemed open to question. Proteolytic enzymes are frequent contaminants of commercial lipase preparations. The precaution of using pH 6 was taken by Garamvolgyi and Guba to minimize the effect of tryptic hydrolysis. Although the rate of tryptic hydrolysis is considerably reduced at pH 6 compared to its optimal pH range, it has been shown, with the use of various substrates, that some hydrolysis will usually occur (21). Therefore, in spite of this precaution, only a small amount of trypsin contamination could produce a marked effect over an extended hydrolysis period as used by Garamvolgyi and Guba. In addition, Goll and Reedy[2] have shown that lipase treated with soybean trypsin inhibitor would not remove Z lines but that untreated lipase would remove them.

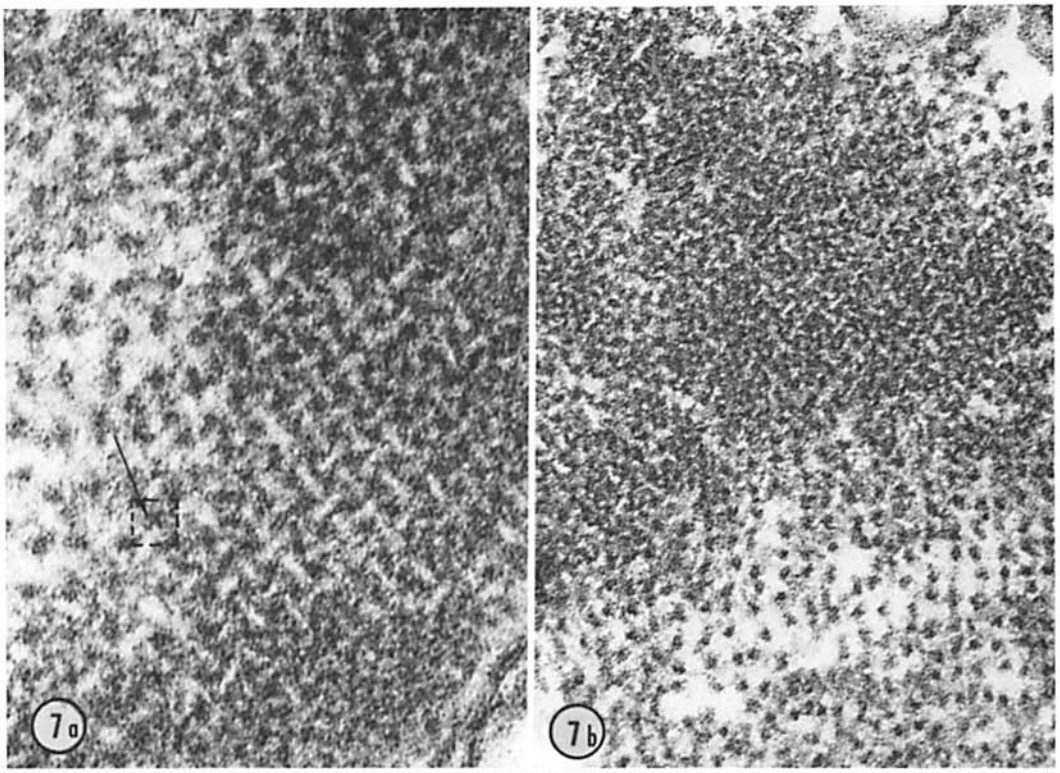
The extraction method with 2 mM Tris-HCl (pH 7.6) 1 mM dithiothreitol as described here was found to be most suitable when applied to fiber bundles. However, this extraction was not

[2] Goll, D. E., and M. K. Reedy. Personal communication.

FIGURE 7 *a* Cross-section of Z line as seen in glycerinated psoas prior to extraction. The "weave" pattern is very evident. Arrow points to the central structural element added to the center of the square formed by filaments entering the Z line. × 302,000.

FIGURE 7 *b* Cross-section of reconstituted Z line from same sample as shown in Fig. 5. The arrangement of the lattice is nearly identical with that of the untreated lattice shown in Fig. 7 *a*. × 133,200.

FIGURE 8 Cross-section of tropomyosin crystal. The base-to-base and top-to-top stacking of trapezoid-like openings in the net is easily seen as one moves from top to bottom in this micrograph. This phenomenon is responsible for the altering wide-narrow-wide spacing as one moves from left to right across this field. Some of the strands forming the lattice give some indication of being coiled or helical (arrows). × 192,800.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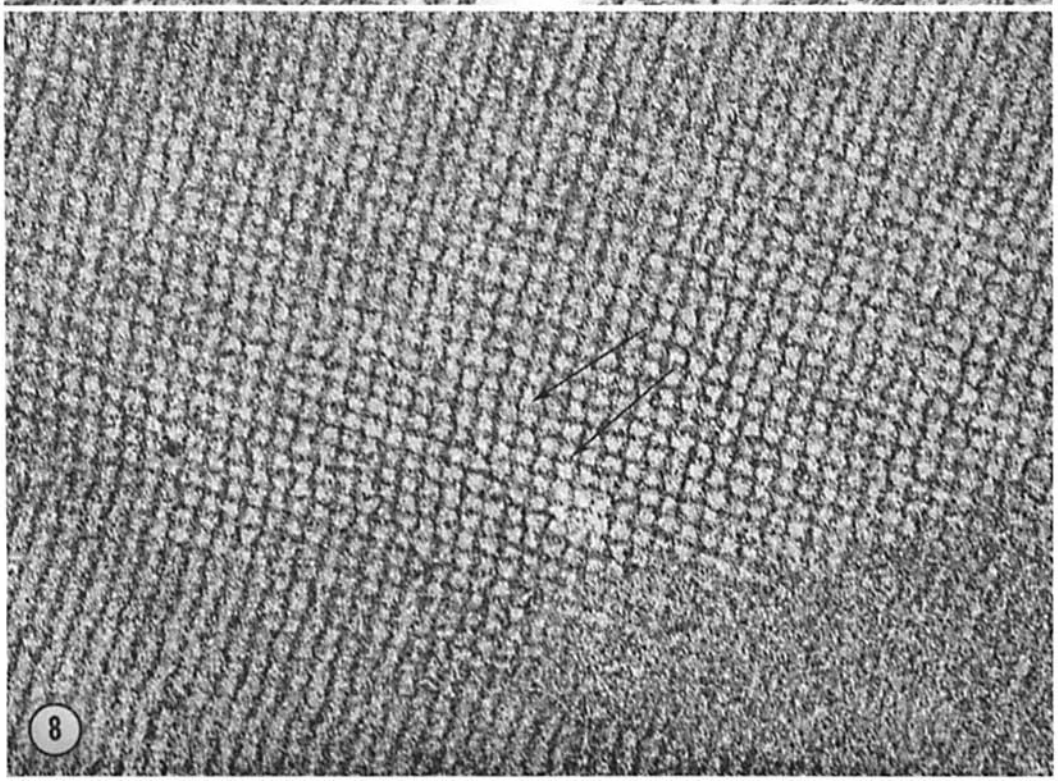
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selective for one particular protein. Extracts always contained tropomyosin, troponin, α-actinin, and possibly other unidentified components. The amount of protein precipitated at 40% ammonium sulfate saturations (P40) from the 10 day extract was 70–80%. This fraction contained little or no tropomyosin. As the extent of extraction was increased, the amount of tropomyosin and troponin extracted also increased, until after exhaustive treatment the P40 fraction accounted for approximately only 60% of the total extract. In this case, the P40 probably also contained some denatured actin. This finding indicated that the extraction of most of the tropomyosin from the myofibril occurred subsequent to Z-line removal.

Reconstitution of Z Lines

With regard to the recombination studies, it should be pointed out that, in the approximately 40 experiments conducted so far in this series, the P40 fraction of the Bailey extract of natural actomyosin has shown the most consistent ability to restore prominent Z lines. Why the P40 of the Bailey extract of natural actomyosin gives better results than the P40 of the low ionic strength extract of natural actomyosin is not known. It seems most likely, however, that the distinction is due to different compositions of the two fractions. To pursue this speculative line of thought, it is interesting that, if α-actinin is the major component of the Z line (3), an additional factor must be required to limit its binding only to the ends of the I filaments, and thus forming the Z line and not forming the I-band cross-bridges. (It should be remembered that isolated α-actinin gave relatively poor Z-line reconstitution but extensive cross-bridging in the I band.) It is possible, if an additional factor is involved, that the two constituents (factor and α-actinin) are in different proportions in the two P40 fractions. The use of organic solvents and a different extraction medium for the preparation of the Bailey extract of actomyosin could be responsible for such a difference. Attempts to simulate the effect of the Bailey P40 by using low ionic strength P40 plus varying constituents, including tropomyosin, have failed so far. Because of similarities between the magnitude of cross-bridge production and H-zone binding, it is tempting to speculate that a similar or associated substance might be responsible for both.

The apparent restriction of I-band cross-bridges to the area of the I band on either side of the Z line and the location of tufts only in the lateral thirds of the I band might indicate that the I band is not homogeneous across its width. This observation was made by Pepe (22) in some of his antibody experiments. On the other hand, another possibility is that extraction with low ionic strength solvents does not remove material uniformly from the I band, the result being the exposure of different sites along the thin filaments.

The tufting reaction in the I band appears to be less susceptible to I-band damage than does the restorability of the Z line. When myofibrils are extracted for a prolonged period of time, under our conditions, a gap develops between the ends of the thin filaments in the region formerly occupied by the Z line.[3] Use of these myofibrils for reconstitution experiments with fractions of known behavior results in a seemingly unaffected tufting reaction but no Z lines. Therefore, it seems reasonable to hypothesize the necessity for proximity of thin filaments from adjacent sarcomeres in the Z-line region to serve as centers on which the reconstitution can proceed. The Z line does not appear to be required for the sarcomeres of a myofibril to retain their end-to-end orientation. We have observed no other structural entity which could account for this orientation, but the mechanical packing due to the thickness of the fiber bundle may be responsible for maintaining it. Bundles of myofibrils that are 100 μ thick contain about 6700 layers of myosin filaments. This thickness is probably sufficient to maintain register during the mild stress of teasing, but homogenization usually disrupts such register.

Although several structural and dimensional differences between the Z-line lattice and tropomyosin crystals have previously been pointed out, still another difference was noted in the staining properties. Both native and reconstituted Z lines appear to have material between the lattice strands. When tropomyosin is fixed, sectioned, and stained in the same way, the strands of the lattice stain positively, and the space between the strands appears to be open, a fact previously mentioned by Hodge (16). It should be added that an aliqout of this tropomyosin solution was subjected to the conditions necessary for tactoid formation as reported by Cohen and Longley (4). The resulting tactoids

[3] Stromer, M. H Unpublished data.

had a gross appearance and banding pattern identical with those described by those authors.

Very recently, Caspar, Cohen, and Longley[4] have obtained further results from X-ray diffraction and electron microscope studies of tropomyosin crystals and various polymorphic aggregates. They have concluded that "the tropomyosin crystal net has a different symmetry from that of the Z line. There is no way in which a distortion of the tropomyosin crystal could produce a net with the Z-line symmetry without altering the basic pattern of connections." This independent study, therefore, agrees with our chemical and electron microscope evidence that tropomyosin is not the major component of the Z line.

Reconstitution of M Line

The restoration of prominent M lines by using the concentrated unfractionated myofibrillar extract is plausible if one considers the small amount of material initially present in the in vivo M line and the likely requirement for a certain minimal concentration before reorganization can occur. The explanation for the presence of I-band cross-bridges rather than tufts and Z lines is less straightforward. As indicated previously, α-actinin exhibits a marked concentration-dependent tendency to form cross-bridges in the I band. Therefore, the possibility exists that these cross-bridges are composed of α-actinin. Another less likely possibility is that the material responsible for the tufts and Z lines was inactivated by the handling involved in concentrating the extract.

[4] Caspar, D. L. D., C. Cohen, and W. Longley. Personal communication, submitted to *J. Mol. Biol.*

Because of the experimental design which involved monitoring of removal and reconstitution of Z lines by both phase and electron microscopy, it seems reasonable to assume that the possibility of confusing unremoved Z lines with restored Z lines is quite remote. As pointed out previously, the sequence of extraction in which M lines were removed before Z lines eliminated the possibility that M lines could be found without Z lines unless reconstitution had taken place. Although these experiments do not completely rule out the possibility that another morphological form of tropomyosin, or a complex of tropomyosin with some other substance, may be located in or near the Z line, it seems less likely that tropomyosin alone is the principal Z-line material. Further evidence to support this view has been published by Corsi et al. (5) who found that Z lines resisted removal under conditions which extracted actin and tropomyosin.

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