CHANGES IN THICK FILAMENT LENGTH IN LIMULUS STRIATED MUSCLE

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ABSTRACT

Here we describe the change in thick filament length in striated muscle of Limulus, the horseshoe crab. Long thick filaments (4.0 µm) are isolated from living, unstimulated Limulus striated muscle while those isolated from either electrically or K⁺-stimulated fibers are significantly shorter (3.1 µm) (P < 0.001). Filaments isolated from muscle glycerinated at long sarcomere lengths are long (4.4 µm) while those isolated from muscle glycerinated at short sarcomere lengths are short (2.9 µm) and the difference is significant (P < 0.001). Thin filaments are 2.4 µm in length. The shortening of thick filaments is related to the wide range of sarcomere lengths exhibited by Limulus telson striated muscle.

KEY WORDS isolated thick filaments  ·  thick filament length  ·  myosin paramyosin filaments  ·  Limulus striated muscle

Theories of the mechanism of muscle contraction, which postulated conformational changes of either "sarcous elements" (20) or protein polymers (18), were discarded with the acceptance of the "sliding filament" model of Huxley and Hanson (11) and Huxley and Niedergerke (10). This model explains both passive sarcomere shortening and active isotonic contraction of vertebrate striated muscles. The sliding filament model states that the A band and its constituent thick filaments are constant in length. Nevertheless, within less than a decade after the proposal of the sliding filament theory, de Villafranca (1), de Villafranca and Haines (2), and de Villafranca and Marschhaus (3) reported that the A bands of glycerinated Limulus skeletal muscle shortened accompanying ATP-induced contraction. We confirmed the observation of A-band shortening on living, fresh-
could arise either from the cross-bridge period or from the stagger of paramyosin molecules which compose the core of the thick filaments. In either case, this lack of change in the 14.5-nm reflection might be interpreted as an absence of any conformational change in the thick filament since it could be argued that a conformational change involving a decrease in filament length would alter the period at which the cross-bridges come off of the filament backbone or the period of the backbone itself. Three alternative hypotheses which reconcile the observed A-band shortening with the results of X-ray diffraction can be proposed:

(a) Thick filaments are all about 2.5 μm long, instead of our measured 4.9 μm, and continuously realign from the very longest to the very shortest sarcomere lengths causing the A-band length to decrease. This requires end-to-end interaction between thick filaments in sarcomeres of length >7.5 μm.

(b) Thick filament lengths change during sarcomere shortening by dissolution of both cortical myosin and medullary paramyosin (8, 12, 16, 17) from their ends.

(c) Thick filament shortening occurs by a stepwise rearrangement of cortical and core molecules without change in the 14.5-nm reflection.

Here we report measurements of A bands and isolated thick filaments obtained from muscles originally set at long and short sarcomere lengths. We also report the effects of K+ and electrical stimulation of intact fibers on filament length. Our results are consistent with the third hypothesis which involves shortening of thick filaments rather than the model predicting end-to-end interaction between short thick filaments.

MATERIALS AND METHODS
Adult Limulus (horseshoe crabs) were either obtained from the Marine Biological Laboratories (Woods Hole, Mass.) or were caught on the shore of either Belle Terre or Old Field, Long Island, New York. All animals were maintained and fed in chilled (18°C), aerated, artificial seawater (ASW) in the laboratory. Under these conditions they live for several months.

Dissection Procedure
The muscles used in these experiments were the levators of the telson (7) which originate on the dorsal and ventral opisthosomal carapaces. The removal of part of the dorsal carapace and underlying connective tissue exposed the muscles for dissection. Wooden sticks, 20 mm long, were placed alongside the whole muscle, and a bundle of fibers (1-2 mm in diam and 7-10 mm in length) was securely tied to the stick and was cut away from the body of the whole muscle. In this way the in situ lengths were maintained. This procedure induced contractions of the cut ends of the bundle, but these contractions did not spread centrally beyond the ties. The bundles were dissected out with the telson in one of two positions: (a) telson down, at an angle perpendicular to the ventral opisthosomal surface which represents maximum in situ length of the muscle, and (b) telson up, at an angle perpendicular to the dorsal opisthosomal surface which gives the minimum in situ muscle lengths.

Solutions
ASW was used in these experiments which contained 440 mM NaCl, 10 mM KCl, 10 mM CaSO4, 4 mM MgSO4, 0.4 mM MgCl2 at pH 7.2. An ethylene glycol(bisβ-aminoethyl ether)N,N',N'-tetraacetate (EGTA) solution (0.1 M KCl, 5 mM MgCl2, 1 mM dithiothreitol, 4 mM EGTA, 5 mM ATP in 5 mM Tris buffer, pH 7.0) was used to disrupt the sarcolemma of intact muscle bundles and to dissociate actin from myosin. This solution is identical to the relaxing solution used in glycerinated muscle preparations (1-3). All thick filament isolations were effected in this solution. To examine the effect of activation on thick filament length, intact muscle bundles were briefly treated with high K+ solutions (50, 100, 200, 300, and 450 mM) where KCl was substituted for NaCl in the ASW solution. Limulus wash solution (LWS) (6.7 mM KH2PO4, 40 mM KCl, 0.01% streptomycin, pH 7.4) was used to dilute glycerol to a 50% concentration for glycerination of the muscle. Fixation of the muscle for light and electron microscopy was accomplished with 5% glutaraldehyde (biological grade, Ladd Research Industries, Inc., Burlington, Vt.) in 0.1 M cacodylate buffer with 5% sucrose at pH 7.2. Extraction of the thick filaments which enabled measurements of thin filament lengths was performed with the Hasselbach-Schneider solution (0.5 M KCl, 0.1 M KH2PO4, 0.01 M Na2HPO4, 1 mM MgCl2, 1 mM ATP, pH 7.2) (19).

Isolation of Thick Filaments from Unstimulated Muscle
Muscle bundles were dissected from animals with their telsons in either the up or down position. We have previously shown (7) that fibers fixed in the telson up position have sarcomeres 4.0-6.0 μm long, while those fixed in the telson down position have sarcomere lengths of 9.0-11.0 μm. Tied bundles were placed in several changes of the EGTA solution at 4°C for 18 h, after which time several muscle fibers were stripped off the surface of the bundles and phase micrographs were taken to determine the sarcomere lengths. Subsequently, the bundles were cut into smaller pieces (0.5 mm diam, 3 mm length) in fresh EGTA solution to facilitate penetration of the fresh solution. Examination of these small pieces of muscle with phase optics showed that the sarco-
Isolation of Thick Filaments from K+-Stimulated Muscle

Experimental bundles were prepared in the telson up and down position as previously described. They were then placed in the 450 mM K+ stimulating solution at 4°C for <1 min and then transferred to the EGTA solution. They remained in the EGTA solution for the same amount of time and with the same number of solution changes as the control or unstimulated bundles both before and after being cut into small pieces. Filaments were isolated as previously described.

In another series of experiments, small (0.5 mm diam) muscle bundles were dissected with their carapace origins and tendon insertions intact.

Two types of experiments were performed to show that brief treatment of these intact muscle bundles with high K+ solutions does not dissolve the thick filaments. In the first experiment, the bundles in ASW were adjusted to slack length (5-μm sarcomere length) and the ASW was then replaced with the 450 mM K+ stimulating solution. At peak tension, ~1 min after application, the stimulating solution was replaced with the EGTA solution. Tension declined to zero. The bundles remained in the EGTA solution for the same length of time as in the experiments described previously. Filament isolation was performed as above.

In the second experiment, the same procedure was followed up to the point of peak tension development. At this point, ASW replaced the high K+ solution and the bundles were allowed to equilibrate in flowing ASW for 20 min. Tension declined at the same rate as in the EGTA solution in the previous experiment. After equilibration, the bundles were stretched to a sarcomere length of about 8-9 μm and the ASW was replaced by the EGTA solution. These bundles remained in the EGTA solution for the same length of time as in all previous experiments, and filaments were isolated in the same way.

Isolation of Thick Filaments from Electrically Stimulated Muscle

To determine whether the brief treatment of the intact bundles with the high K+ solution could be altering the intracellular K+ concentration sufficiently to dissolve the thick filaments, intact bundles were stimulated electrically and the thick filaments were isolated.

Small (0.5 mM diam) bundles were dissected with their carapace origins and tendon insertions intact. These bundles were mounted in a glass chamber with the origin clamped to one chamber wall and the tendon attached to a tension transducer (Grass FT .03, Grass Instrument Co., Quincy, Mass.). ASW was gravity fed into the bath at one end and was removed by a suction overflow at the other end. In addition there was a drain at the bottom of the chamber to rapidly remove the solutions. Silver electrode plates (5 cm long, 1 cm high) were placed on either side of the intact bundles and were attached to a Grass stimulator. The muscle was set to slack length. Stimulation at 65 V, 5-ms duration at 80 Hz produced a tetanic contraction equivalent in magnitude to that produced by the high K+ solution. A laser beam was adjusted to pass through the bundle so that the sarcomere length at the peak of tension could be determined (7).

At the peak of the electrically induced contraction, the ASW was rapidly replaced with the EGTA solution. The stimulus current was maintained for 3-4 min after the introduction of the new solution. At that time, the sarcomere length was about 6 μm. The whole apparatus was then kept at 4°C with several changes of the EGTA solution. As before, the total time the muscle was kept in the EGTA solution was the same as in the previous experiments. In some experiments, during the last hour of the EGTA treatment, the bundle was stretched to a longer sarcomere length of about 9 μm. The thick filaments were then isolated as previously described.

Isolation of Thick Filaments from Glycerinated Muscle

Muscle bundles were dissected in the telson up or down position. Tied bundles were glycerinated in 50% glycerol in LWS at 4°C. After glycerination for 18 h, several fibers were stripped off each bundle for phase microscopy. The sarcomere and A-band lengths were measured. The tied bundles were placed in several changes of EGTA solution to replace the glycerol. After 18 h in the EGTA solution, the muscle was cut into small pieces in fresh solution. Several changes of solution were made during a 3-h period. Sarcomere and A-band lengths of the small pieces were the same as in the stripped fibers. The pieces were homogenized in the EGTA solution. The homogenate was diluted with an equal volume of 0.75% O-(diethylaminoethyl)cellulose (DEAE cellulose) (14) in EGTA solution and centrifuged at 5,000 rpm for 10 min at 0°C. This procedure was repeated three times, and aliquots of the final supernate were placed on grids and negatively stained.

Measurements of Filament Lengths

Phase micrographs were taken of fresh or glycerinated fibers. Sarcomere and A-band measurements were made on microdensitometer tracings (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England, model MKIIIC) of negatives at 10:1 ratio. Electron micro-
graphs of isolated filaments were enlarged to \( \times 30,000 \). Individual filaments were measured directly from prints, and only those filaments whose tapered ends and central bare zones were clearly discernible were measured.

For each experiment, sarcomere and A-band lengths were correlated with the lengths of thick filaments isolated from the same preparation. The Students' \( t \) distribution was used to determine whether the difference found between populations of filaments was significant.

**Tension Measurements**

In experiments described both here and published (7), muscle bundles were treated with a variety of solutions (high K\(^+\) solution, EGTA solution, 50\% glycerol solution, and 5\% glutaraldehyde fixative), and thick filament lengths were measured. It was important, therefore, to examine the effects of these solutions on tension development of the muscle bundles. Small, intact muscle bundles (0.5 mm diam) were mounted in the glass chamber as previously described in the section on electrical stimulation. Test solutions were added after cessation of ASW flow by pouring the solution into the chamber as the ASW was rapidly removed by the bottom drain. At least 75 ml of a test solution was poured into the chamber (three times its volume). ASW could then be allowed to flow into the chamber, again replacing the experimental solution at a rate in excess of 100 ml/min.

**Determination of Thin Filament Length**

To accurately measure the length of the I band, the thick filaments were extracted from muscle fibers and the fibers were examined by light and electron microscopy. Muscle bundles with sarcomeres >7.5 \( \mu \text{m} \) were tied to sticks and soaked in a hypotonic solution (20 mM EGTA in 10 mM phosphate buffer at pH 7.2) for 10 min to disrupt the membranes. The bundles were then soaked in an excess of the Hasselbach-Schneider myosin extraction solution at 4\(^\circ\)C overnight (19). In other experiments, muscle bundles were stretched beyond the point of overlap between the thick and thin filaments. All bundles were fixed and embedded for light and electron microscopy. I bands were measured from microdensitometric tracings of light (\( \times 265 \)) and electron micrographs (\( \times 5,000 \)).

**RESULTS**

**Appearance of Isolated Filament Preparations**

Thick filaments isolated from fresh muscle bundles by the procedure described here were relatively free of contaminating actin filaments, and only rarely was Z-band material observed on the negatively stained grids. Clearly, many thick filaments were broken as seen by incomplete fractures and square, untapered ends. The proportion of broken filaments to intact filaments was similar in all preparations. Only those with tapered ends and single, central bare zones were considered intact and were measured.

**Measurements of Thick Filaments Isolated from Unstimulated Muscle**

Both after 1 h in EGTA solution and at the time of homogenization, muscle fragments from unstimulated bundles had similar sarcomere lengths (8.3 \( \mu \text{m} \) [SD 0.8] vs. 7.7 \( \mu \text{m} \) [SD 1.1]) and A-band lengths (4.1 \( \mu \text{m} \) [SD 0.5] vs. 4.0 \( \mu \text{m} \) [SD 0.6]) whether they were taken from bundles tied in telson up or telson down positions, respectively. Thus, during treatment with the EGTA solution, both muscle preparations achieved an "equilibrium" sarcomere length. Likewise, the thick filaments isolated from these fragments did not differ significantly in length: 4.1 \( \mu \text{m} \) (SD 0.5) vs. 4.0 \( \mu \text{m} \) (SD 0.7) (Fig. 1a and b). These filament lengths are, however, significantly shorter than those previously measured in fresh fixed material (4.9 \( \mu \text{m} \) [SD 0.5]) (7) at sarcomere lengths equal to the equilibrium lengths.

**Measurements of Thick Filaments Isolated from K\(^+\)-Stimulated Muscle**

Since unstimulated muscle when placed in the EGTA solution goes to an equilibrium sarcomere length irrespective of its initial sarcomere length, it was not surprising to find that the thick filaments isolated from these muscles were not different (Fig. 1a and b). Therefore, filaments were isolated from muscle bundles that were induced to contract with a high K\(^+\) solution. High K\(^+\) was used since it induces a reproducibly high tension development in these bundles of muscle fibers which do not conduct action potentials.

Thick filaments isolated from muscle bundles which had contracted in response to high K\(^+\) solution were significantly shorter (\( P < 0.001 \)) than those isolated from unstimulated muscle (Fig. 2). These thick filaments measured 3.1 \( \mu \text{m} \) (SD 0.8) if the muscle originally had been tied in the telson up and 2.9 \( \mu \text{m} \) (SD 0.1) if in the telson down position (Table I). Again, as in the unstimulated muscle, the sarcomeres in either case returned to an equilibrium length (~7.9 \( \mu \text{m} \), Table I) in the EGTA solution (Fig. 3a and b). However, the A bands were short, ~3.5 \( \mu \text{m} \) in both cases. To determine the reversibility of filament
Phase-contrast micrographs and densitometer tracings of living, nonstimulated sarcomeres together with electron micrographs (x 30,000) of filaments isolated from the same preparation. (a) Fiber taken from telson levator with telson down (x 1,100). (b) Fiber taken from telson levator with telson up (x 1,300). Note the similarity of measured parameters from these living fibers in EGTA solution.

**Figure 1**
Figure 2. The distribution of isolated intact thick filament lengths from unstimulated muscle in EGTA solution (top) compared to the distribution obtained from muscle first K⁺-contracted and then placed in EGTA solution (bottom). Note that shorter filaments are obtained from the K⁺-contracted muscle.
shortening, and thereby to control for possible irreversible dissolution of the filaments during isolation after high K⁺ stimulation, two experiments were performed on small intact muscle bundles. In the first experiment, the bundles were set at slack length, then induced to contract with high K⁺ solution. At the end of the tension plateau, 60 s after stimulation, the high K⁺ solution was rapidly replaced with EGTA solution. Tension returned to zero, 1–2 min after application of the EGTA solution. The bundle was treated as previously described for the filament isolation. As was found in the previous experiment with large bundles, the thick filaments that were isolated were short and measured 2.8 μm (SD 0.7) in length.

In the second experiment, the bundles were placed in ASW after K⁺ stimulation instead of the EGTA solution. After a 20-min equilibration period, the bundle was stretched and then the filaments were isolated. In this case, the isolated filaments were long, 3.9 μm (SD 0.5), similar to those isolated from unstimulated muscle.

Therefore, K⁺ stimulation of muscle bundles induces a shortening of the thick filaments which we were able to reverse by placing the bundles in ASW and, after equilibration, stretching the bundle.

**Measurements of Thick Filaments Isolated from Electrically Stimulated Muscle**

In spite of the fact that the previous experiments demonstrate that the shortening of thick filaments which occurs during K⁺-induced contracture of the muscle bundles is reversible, some still might argue that treatment with the high K⁺ solution could raise the intracellular K⁺ concentration to a level sufficient to solubilize the thick filament proteins. To control further for this possibility, filaments were isolated from muscle bundles which had been contracted by electrical stimulation.

In one series of experiments, bundles set at slack length where the sarcomere length was 6 μm as measured by laser diffraction, were stimulated electrically and shortened to a sarcomere length of about 5 μm. The bundles were then treated with EGTA solution at peak tension and the filaments were isolated as before. The thick filaments that were isolated were short, 2.9 μm (SD 0.3), and were not significantly different from those isolated from K⁺-stimulated muscle. Therefore, the shortening of the thick filaments is dependent on muscle activation but independent of the method used to achieve the activation.

In another experiment the same procedure was followed except that just before filament isolation after the normal 16 h in the EGTA solution, the bundles were stretched and the filaments were then isolated. In this case, the filaments were short, 3.1 μm (SD 0.5). Thick filament length is restored after induced shortening when the muscle is first allowed to recover in ASW and is then stretched to longer sarcomere lengths. These experiments suggest that filament lengthening may
Fiber taken from telson levator with the telson down (× 1,200). (b) Fiber taken from telson levator with the telson up (× 1,200). Note the similarity of measured parameters of both fibers when in EGTA solution. Compare isolated filament lengths with those in non-K⁺-stimulated fibers (Fig. 1a and b).
require actin-myosin interaction which is abolished by the EGTA solution.

Measurements of Thick Filaments Isolated from Briefly Glycerinated Muscle

Fresh muscle, irrespective of its initial sarcomere length, when soaked in the EGTA solution, goes to an equilibrium sarcomere and A-band length. This does not occur on glycerination as the sarcomere and A-band lengths are maintained at their initial set lengths (7). Therefore, filaments were isolated from briefly glycerinated muscle at long and short sarcomere lengths. Thick filaments isolated from muscle glycerinated at sarcomere lengths of 8.0 μm (SD 1.2) with A bands of 4.5 μm (SD 0.5) were 4.4 μm (SD 0.5) long. These were, on one hand, significantly (P < 0.001) longer than those isolated from unstimulated fresh fibers, and on the other hand, significantly (P < 0.001) shorter than those previously measured in situ in fresh fixed long sarcomeres (7). Filaments isolated from muscle glycerinated at sarcomere lengths of 6.4 μm (SD 1.0) with A bands of 3.4 μm (SD 0.6) were 2.9 μm (SD 0.5). There was a highly significant (P < 0.001) difference between filaments isolated from glycerinated long and glycerinated short sarcomeres (Table I, Figs. 4a and b, and 5).

Following is a summary of results on isolated thick filaments: (a) Long thick filaments (equal to or greater than 4.0 μm) were obtained from Limulus telson muscle which was either fresh and EGTA-treated or EGTA-treated after glycerination at long sarcomere lengths. (b) Both A-band and thick filament shortening occurred after K⁺ and electrical stimulation of fresh muscle at any sarcomere length, or when fibers were set at a short sarcomere length and then glycerinated. (c) Thick filament length is restored after induced shortening, however, when the muscle is allowed to recover in ASW and is then stretched to longer sarcomere lengths. (d) Exposure to the EGTA solution seems to be ineffective in restoring filaments to their longer lengths after K⁺ or electrical stimulation. (e) Incubation in the EGTA solution, on the other hand, seems to reverse the effect of K⁺ stimulation on sarcomere length in the case of fresh muscle. Glycerinated muscle, however, retains the set sarcomere length.

Measurements of Thin Filament Lengths

Bundles of muscle fibers treated with the myosin extracting solution remained intact and clearly exhibited absence of thick filaments in light and electron micrographs (Fig. 6a). I-band lengths averaged 4.8 μm (SD 1.2). Examination of superstretched muscle by both light and electron
FIGURE 4 Phase-contrast micrographs and densitometer scans of glycerinated muscle together with electron micrographs (× 30,000) of filaments isolated from the same preparations. (a) Fiber glycerinated at the telson down length (× 1,100). (b) Fiber glycerinated at the telson up length (× 1,500). Note differences in sarcomere, A-band, and thick filament lengths between the two preparations.
Figure 5  The distribution of isolated intact thick filament lengths of muscle glycerinated at long (top) and short (bottom) sarcomere lengths. Note that shorter filaments are obtained from muscle glycerinated at short sarcomere lengths.
microscopy shows a gap of lesser density at the margins of the A band (Fig. 6b). This is interpreted as being due to nonoverlap of the thick and thin filaments. Measurements of these "isolated" I bands agree with those obtained from the extracted fibers. Additional confirmation of these measurements has been made from Fourier reconstructions of the refractive index along sarcomeres of glycerinated fibers (6). It is of interest that de Villafranca et al. (4) have reported that this extraction solution removes actomyosin. Our results would suggest that the length of the I band is unaffected by the extraction procedure.

Estimating the Z band to be 0.2 μm long, thin filaments are 2.4 μm long. Thus, with thin filament lengths of 2.4 μm and maximum thick filament lengths of 4.9 μm, we calculate that the maximum excursion of the sarcomere, still permitting overlap, in *Limulus* muscle is 9.8 μm.

**DISCUSSION**

The sliding filament model (10, 11) of muscle contraction as applied to vertebrate striated muscle holds that the thick and thin filaments are of constant length, regardless of contractile state or sarcomere length of the muscle. Corollary requirements include constancy of both A-band and cross-bridge repeat period.

The striated telson levator muscles of *Limulus*, however, seem to deviate from the vertebrate model. As shown here in the case of glycerinated muscle and previously (1, 3, 7), the A-band length varies linearly with the sarcomere length. The longer A bands seen in longer sarcomeres (>7.5 μm) are due to a relative misalignment of the thick filaments which are not held in an ordered array within the A band by centrally located M bridges (7, 12). The decrease in the A band below 7.0 μm, however, seems to be due to a shortening of the now aligned thick filaments. This conclusion is based on measurements of thick filament lengths *in situ* in sectioned muscle (7) and on our results presented here. In accordance with the sliding filament model, however, the thin filaments do move toward the center of the A bands as the sarcomeres shorten. This is indicated by the appearance of double overlap of the 2.4-μm long thin filaments at a sarcomere length of 4.8 μm (7). Therefore, although the basic principle of the vertebrate model that predicts that muscle shortening is due to a relative sliding of thick and thin filaments is applicable to *Limulus* muscle, the model must be modified to take into account the A-band length change and the shortening of the thick filaments observed as the sarcomeres shorten below 7.0 μm.

Thick filaments isolated from unstimulated muscle treated with EGTA solution are of the same length whether the initial sarcomere length was short or long. Equilibrium sarcomere lengths and long A bands were seen in these fibers (Table I) after exposure to EGTA solution and before homogenization. Therefore, we conclude that treatment of whole muscle bundles with the EGTA solution disassociated the thick from the thin filaments and that the sarcomere structure then goes to an equilibrium length. One explanation for this effect of the EGTA solution may be that it first penetrates the fibers due to partial disruption of the sarcolemma by the EGTA. Then the solution exerts its "relaxing" effect on the contractile system.

Short thick filaments were obtained when we stimulated muscle maximally before filament isolation. We first used high external K⁺ to produce contracture since this is the most reliable method of achieving uniform membrane depolarization. It could, however, be argued that exposure of even intact fibers to 450 mM K⁺ could raise the intracellular K⁺ concentration to a level that would solubilize the myosin. In frog muscle, for example, it has been shown that very high intracellular K⁺ concentrations can be achieved with exposure to high external K⁺ (15). In the frog experiments, however, the muscle was exposed to a series of hypotonic high K⁺ solutions for a long time (12-18 h). In contrast, we used brief (<1 min) exposures to isotonic high K⁺. Further, since a second contracture can be produced on the second exposure after a recovery period, it is unlikely that any significant solubilization of myosin occurred during the first contracture. We have also used electrical stimulation to contract small muscle bundles and have obtained the same results as with K⁺-induced contracture.

Our results clearly show that the A bands of stimulated muscle and the thick filaments isolated from them are significantly shorter than those from unstimulated muscle regardless of the initial sarcomere length. This is true even though the sarcomeres attained an equilibrium length in the EGTA solutions (Table I). Thus, whatever forces drive the sarcomere length to equilibrium in the EGTA solution do not seem to affect the thick filament length. Further, since short thick filaments have bare zones of the same length (0.2
μm) as long thick filaments, all the observed length changes must have occurred in the region of head-to-tail interaction (16) in each half filament.

Since recovery of long thick filaments after contraction was only possible when muscle was stretched in ASW and not when muscle was similarly stretched during relaxation in the EGTA solution, we think it possible that lengthening of short thick filaments may require some form of actin-myosin interaction. This could mean that the intracellular conditions are different in the two solutions; the EGTA containing solution causing more complete thick-thin filament dissociation than that occurring during relaxation in vivo or in ASW. Lengthening would, therefore, be produced by transmission of the imposed stretch to the thick filaments via their interaction with the thin ones.

To show that thick filament length can be related to sarcomere length, we isolated filaments from briefly glycerinated muscle set at short or long sarcomere lengths. Unlike the situation in fresh muscle, after glycerination and throughout the subsequent treatment with the EGTA solution, sarcomeres and A bands remained at their original set lengths. Therefore, the elastic components of the fiber and fibrils which are at their lowest energy level (or most salubrious configuration) in sarcomeres at equilibrium length are affected by the glycerol treatment. Interestingly enough, our preliminary experiments measuring active tension generated by electrically stimulated Limulus telson muscle indicate that $P_0$ is obtained at the equilibrium sarcomere length. It appears that either interfilament forces dictate the establishment of this equilibrium length or some other components such as the sarcolemma or sarcoplasmic reticulum may be involved in this phenomenon. Since glycerol prevents the return to the equilibrium length, it is likely that the membrane systems are responsible.

Long filaments were obtained from glycerinated bundles with long sarcomeres, and short filaments from those with short sarcomeres. It is interesting to note that the short filaments isolated from glycerinated muscle are the same length as the filaments isolated from stimulated muscle. The filaments isolated from stretched, glycerinated mus-
Fibers, however, are longer than those isolated from unstimulated muscle, but shorter than filaments measured in situ in muscle fixed at long sarcomere lengths. These differences can be explained by the effects of the different solutions on tension development by the muscle. We find that EGTA solution causes significant tension development in a resting muscle bundle. Glycerination, however, induces a much smaller amount of tension, while glutaraldehyde produces still less. Therefore, thick filaments isolated from fresh muscle treated with EGTA solution could be expected to be shorter than those seen after glycerination or fixation at a given muscle length, since the muscle bundles are partially activated by the EGTA solution before the solution can dissociate the contractile system. The difference between the tension development due to glycerination and that due to fixation is small, with glycerination producing greater tension. Therefore, one could expect that the filaments isolated from long sarcomeres after glycerination would be shorter than those measured after fixation.

At this time, due to the ability of the filaments to return rapidly to long lengths, we feel that shortening of the thick filament occurs as a result of...
of a stepwise rearrangement of paramyosin and myosin molecules. That this hypothesis may indeed be correct is suggested by the recent observations by Epstein et al. (9). These investigators produced two types of hybrid (clam paramyosin, rabbit myosin) paramyosin filaments by different procedures. These differed with respect to their actomyosin ATPase activity and appearance of cross bridges on negatively stained specimens. The suggestion was made that since enzymatic activity was inhibited by the aperiodic type of filament, the difference between the two types was due to alterations in the interaction between paramyosin and myosin molecules. Such changes in molecular interaction leading to a rearrangement of paramyosin and myosin, while maintaining 14.5-nm periodicity, may be involved in the filament shortening that we have observed. We are currently attempting to establish the ionic and molecular requirements for in vitro filament shortening.

The authors wish to thank the following people for their technical and secretarial assistance: Joseph Cavallo, David Fuchs, Mrs. Vivian Schotter, and Ms. Alice A. Sydlowski. We thank Dr. Myra Elfvin for helpful discussion.

This investigation was supported by grants from the National Institutes of Health, GM 20628 and HL 15835 to the Pennsylvania Muscle Institute. J. C. Levine is a recipient of a Research Career Development Award from the National Institutes of Neurological Diseases and Stroke (70476).

Received for publication 5 April 1976, and in revised form 30 June 1977.

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