IMMUNOHISTOCHEMICAL LOCALIZATION

OF CONTRACTILE PROTEINS

IN *LIMULUS* STRIATED MUSCLE

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ABSTRACT

Limulus paramyosin and myosin were localized in the A bands of glycerinated *Limulus*
striated muscle by the indirect horseradish peroxidase-labeled antibody and direct and
indirect fluorescent antibody techniques. Localization of each protein in the A band varied
with sarcomere length. Antiparamyosin was bound at the lateral margins of the A bands
in long ($\sim$ 10.0 μ) and intermediate ($\sim$ 7.0 μ) length sarcomeres, and also in a thin line
in the central A bands of sarcomeres, 7.0–$\sim$6.0 μ. Antiparamyosin stained the entire A
bands of short sarcomeres (<6.0). Conversely, antimyosin stained the entire A bands of
long sarcomeres, showed decreased intensity of central A band staining except for a thin
medial line in intermediate length sarcomeres, and was bound only in the lateral A bands
of short sarcomeres. These results are consistent with a model in which paramyosin com-
prises the core of the thick filament and myosin forms a cortex. Differential staining observed
using antiparamyosin and antimyosin at various sarcomere lengths and changes in A band
lengths reflect the extent of thick-thin filament interaction and conformational change in
the thick filament during sarcomeric shortening.

INTRODUCTION

Before the isolation of paramyosin from extracts
of *Limulus polyphemus* (L.) skeletal muscle (7), the
distribution of this protein was thought to be
limited to smooth and obliquely striated muscles
of several invertebrate species. In invertebrate
smooth muscles the presence of paramyosin has
been determined by extraction procedures (24),
X-ray diffraction (1, 14, 41), or the ultrastructural
appearance of thick filaments (12, 13, 27, 41). In
lamellibranch opaque adductors (12, 13, 29) and
Mytilus anterior byssus retractor muscle (ABRM)
(30, 42), paramyosin was found in filaments

ranging from 500 to 1500 A in diameter, oriented
parallel to the length of the cells, but not organized
into ordered repeating patterns. Identification of
these as paramyosin filaments was based on their
periodicity (145 A with a 725 A repeat) which is
identical with that obtained from native paramyo-
sin by X-ray diffraction (1, 11, 41) and from re-
constituted paracrystals by electron microscopy
(11–13, 23, 29, 34, 42).

The unique "catch" properties of these inverte-
brate smooth muscles have been related to the
high paramyosin content of their thick filaments

(22, 40, 43). The role of paramyosin, however, in effecting the catch state has not been determined (2, 40, 42), although several mechanisms have been proposed. An early yet still current theory maintains that paramyosin-paramyosin interactions, brought about by small variations in pH within the muscle fibers, are responsible for the maintenance of the energy-independent state of tonic contracture known as catch (22, 39, 40). Others have preferred to view the catch state as one in which there is slow making and breaking of bonds between the heavy meromyosin cross-bridges of thick filaments and the thin actin-containing filaments (17, 29–31). This mechanism implies the presence of an external myosin coat on the thick filaments which would prevent interaction among the centrally located paramyosin molecules of adjacent thick filaments. Evidence in support of this arrangement of proteins within the thick filaments is the observed alteration in appearance of thick filament surface substructure after preferential extraction of myosin from isolated filament preparations (10, 42). Another explanation of the catch mechanism, based on interpretations of the various banding patterns seen on negatively stained paramyosin paracrystals, suggested that slight configurational changes of medullary paramyosin molecules might effect stabilization of the cortical heavy meromyosin-actin linkages (42). This again requires a paramyosin core with a myosin cortex.

In obliquely striated muscle from which paramyosin has been extracted, such as lamellibranch translucent or "red" adductors (18, 42) and annelid body wall muscles (24), thick filaments may range up to 500 A in diameter. These filaments contain myosin (42) and usually do not display the periodicity characteristic of paramyosin (18). Catch has not been reported in these obliquely striated muscles.

Limulus striated muscle, unlike invertebrate smooth and obliquely striated muscles, is organized into sarcomeres which are delimited by discontinuous Z bands and contain A, I, and H bands. The thick filaments are tapered, and in "rest length" sarcomeres (approximately 7.5 μ) have diameters ranging from 220 A in the center of the A band to 170 A near their ends. As yet, there is no evidence for the existence of catch in this muscle (M. Dewey, unpublished observations).

If paramyosin and myosin coexist in *Limulus* striated muscle as suggested for other paramyosin-

containing invertebrate muscle, immunohistochemical localization of these proteins within sarcomeres at various lengths should provide information regarding the relationship between the two proteins during shortening of a fiber.

This report describes the immunohistochemical localization of the contractile proteins paramyosin, myosin, and actomyosin within the A bands of *Limulus* striated muscle at different sarcomere lengths.

MATERIALS AND METHODS

Preparation of the Antigens

PARAMYOSIN: Paramyosin was prepared from acetone-dried *Limulus* striated muscle in the following manner: KI-extracted proteins were precipitated at pH 6.0, the precipitate was suspended in 0.6 M KCl and Tris buffer, pH 7.3, and subsequently was fractionated by ammonium sulfate precipitation (20–35% saturation), followed by three acid precipitations at pH 6.0 in phosphate buffer. The purified protein (3.04 mg protein/ml by Kjeldahl assay) was stored at 4°C in 0.6 M KCl, 60 mM potassium phosphate buffer, pH 7.35, for a 3-wk period. Assay for antigenicity by double diffusion in agar against rabbit anti-*Limulus* paramyosin serum remained positive during the 3-wk period. The stock was diluted with 0.2 M KCl in buffer, pH 7.35, to obtain 1 mg paramyosin/ml in 0.33 M KCl before injection.

ACTOMYOSIN AND MYOSIN: Ethylenediaminetetraacetate (EDTA)-washed actomyosin was prepared as previously described (5, 6, 9) and myosin was separated from such paramyosin-free actomyosin by a modification (5) of the method of Weber (44). Actomyosin (6.08 mg protein/ml by Kjeldahl assay) in 0.6 M KCl, 5 mM histidine buffer, pH 7.3, was stored for up to 6 months at −10°C after dilution with an equal volume of glycerol. Before use for injection, it was removed from glycerol by several precipitations in ion-free water and resolubilizations in 0.6 M KCl-5 mM histidine buffer, pH 7.35. Dilution for injections was made by addition of 0.3 M KCl to obtain 1 mg actomyosin/ml in 0.35 M KCl. Myosin (1.35 mg protein/ml) was stored for 1–2 wk in 0.6 M KCl-5 mM histidine buffer, pH 7.3, at 4°C, and was diluted to 0.65–1.0 mg myosin/ml by addition of 0.6 M KCl. Two different myosin preparations having essentially identical properties on the ultracentrifuge were used as antigens. No loss of antigenicity was found by double diffusion assay after 2 wk at 4°C.

"TROPOMYOSIN": Tropomyosin was obtained by ammonium sulfate fractionation (35–60% saturation) of the acetone muscle extract after paramyosin had been removed and was subsequently purified by

acid precipitation and/or diethylaminoethyl (DEAE)-Sephadex chromatography. Tropomyosin was stored at 4°C in 1 M KCl, 20 mM Tris-HCl, 1 mM cysteine buffer at pH 7.3, at a concentration of 4.84 mg protein/ml.

Production of Antisera

Antibodies to each of the contractile proteins—paramyosin, myosin, and actomyosin—were made in male white New Zealand rabbits. The immunization schedule was as follows: 1 ml of diluted paramyosin (see above) was administered intravenously (via an ear vein) once weekly, over a 3-wk period, to each of three rabbits. 1 ml of actomyosin (freshly removed from glycerol and diluted before each injection) was likewise administered, also over a 3-wk period, to each of three different rabbits. 1 ml of diluted myosin, per injections was administered subcutaneously to each of two other rabbits according to the same schedule. A mixture of polyadenylic acid and polyuridylic acid (500 μg each, combined in 1 ml of ion-free distilled water) was used as an adjuvant (J. M. Greene, personal communication) and was administered by the same route immediately after injection of the antigen. Bleeding by cardiac puncture was begun 1 week after the last injection of antigen and was continued at weekly intervals. The antisera were separated from whole blood, and antibody titers were determined by the precipitin ring test. All antisera were positive to a dilution of 1:1054.

Antisera Specificity

The specificity of each antiserum was tested by double diffusion in agar and by extinction of staining after absorption of each antiserum with its reciprocal antigen before use in either the direct or indirect immunohistochemical experimental procedures. Before use, all antisera were routinely absorbed with acetone-dried Limulus ova powder. Antiparamyosin also was absorbed with reprecipitated (by acid precipitation) tropomyosin and (by addition of ion-free distilled water) actomyosin. Antimyosin and antiactomyosin were absorbed with both reprecipitated (by dialysis for several hours against 10 volumes of 0.01 M phosphate buffer, pH 6.0) paramyosin and tropomyosin.

Glycerination of Muscle Bundles

Horseshoe crabs, with carapaces measuring between 8 and 16 inches in diameter, were obtained from the Marine Biological Laboratories at Woods Hole, Mass. For dissection, the animals were placed so that the prosomal and opisthosomal carapaces lay flat.

There are six muscles on either side of the opisthosomus which move the telson. With the telson at right angles to the body, the dorsal muscles on either side are maintained at their maximum physiological extension, while the ventral muscles on either side remain at minimum length. The posterior dorsal opisthosomal carapace was removed from the underlying connective tissue and muscles. The connective tissue was gently dissected away and the muscles were exposed. The exposed tissue was kept moist by a constant drip of Limulus wash solution (LWS) (6.7 mM KH_2PO_4, 40 mM KCl, 0.01% streptomyosin, Sigma Chemical Co., St. Louis, Mo., pH 7.35). Small bundles, 1–5 mm in diameter, from both lengthened and shortened muscles were tied at the in situ length onto thin glass rods or wooden applicator sticks, cut free from the animal, and placed in a solution of 50% glycerol (Baker analytic grade [J. T. Baker Chemical Co., Phillipsburg, N. J.] which had been run through a column of Amberlite MB1 ion exchange resin [Mallinckrodt Chemical Works, St. Louis, Mo.]): 50% LWS. Streptomycin (0.01%) was present in all solutions used with glycerinated muscle. After 24 hr at 4°C, the glycerol solution was changed and the tissue in fresh solution was placed in the freezer and was allowed to remain a minimum of 6 wk in glycerol at −10°C before use.

Preparation of Myofibrils from Glycerinated Muscle Bundles

Bundles to be used for antibody staining were homogenized into fibrillar suspensions in the microcup of a VirTis 45 homogenizer (VirTis Co., Inc., Gardiner, N. Y.) at low speed for 1 min, in either LWS containing 2–5 mM EDTA or in 25% glycerol in the above solution depending upon the staining procedure to be used. In the former procedure, cellular debris was removed by centrifugation of the suspension through a sucrose solution of 1.2 density (w/w) in the SW 39L rotor of a Beckman Spinco Model L Ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 40,000 rpm for 1 hr at 0°C. The myofibrils were washed in three changes of LWS lacking EDTA in a Sorvall RC-2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 10,000 rpm for 20 min at 0°C, rewashed twice in ion-free distilled water, and resuspended in ion-free distilled water. This type of preparation was used for both indirect fluorescent antibody and indirect horseradish peroxidase antibody staining. In the latter procedure, the fibrils were washed three times by centrifugation at 10,000 rpm for 20 min in 25% glycerol in LWS without EDTA. This type of preparation was used for either direct or indirect fluorescent antibody staining.

Antibody-Staining Procedures

Both the Coon's direct (3) and indirect fluorescent antibody (45) techniques and the indirect technique of Nakane and Pierce (33) utilizing horseradish peroxidase conjugated to goat anti-rabbit gamma globulin were used for contractile protein localization.

Horseradish peroxidase was conjugated to the 7S fraction of goat anti-rabbit gamma globulin (Pentex Biochemical, Kankakee, Ill.) according to the method of Nakane and Pierce (33), and 4-chloro-1-naphthol was used to detect horseradish peroxidase activity (32). Goat anti-rabbit gamma globulin conjugated to fluorescein isothiocyanate (FITC), purchased from Pentex Biochemical, was purified by fractionation on a DEAE-cellulose column. All goat anti-rabbit antibodies were absorbed with homogenized, glycerinated Limulus striated muscle before use. For direct staining, rabbit gamma globulin was obtained from whole antiserum by ammonium sulfate fractionation (four times, 50% saturation) and was conjugated to FITC by a modification of the technique of Riggs et al. (38) (P. Yau, Honor's Thesis, Smith College).

Fluorescent antibodies were used to stain fibrils, by either the direct or indirect techniques, in suspension in 25% glycerol in LWS under a cover slip, or by the indirect technique in preparations which were allowed to dry on glass slides after several washes in distilled water. This procedure was followed in order to minimize loss of fibrils during subsequent steps. These preparations were mounted in glycerol after antibody staining. All slides were examined and photographed at a magnification of X 1000 in the Zeiss Ultraphot on either Polaroid Type 57 sheet film or Kodak Spectroscopic (103 aG) plates (Eastman Kodak Co., Rochester, N. Y.), using an HBO-200W mercury vapor lamp, a BG-38 exciting filter and Nos. 65, 50, and 44 barrier filters (Carl Zeiss, Inc., New York). Phase-contrast photographs also were made of each fluorescent antibody-labeled fibril photographed.

Fibril preparations which were allowed to dry on glass slides (again, in order to minimize tissue loss) after several washes in ion-free distilled water were stained by the indirect horseradish peroxidase-labeled antibody. These preparations were mounted in glycerin jelly after incubation in the substrate medium and washing, and were also examined and photographed at a magnification of X 1000 in the Zeiss Ultraphot using Kodak M plates. A comparison was made of the localization of each antibody by both types of labeling techniques in fibrils at various sarcomere lengths. All magnifications were calibrated with a stage micrometer.

Measurements of sarcomere and A band lengths were made from all photomicrographs and were tabulated. Linear regression curves relating sarco-

mere length to A band lengths were computed for each of the techniques used, and were compared with similar curves computed from measurements of thick filament and sarcomere lengths from electron micrographs of fresh-fixed Limulus striated muscle (Fig. 10).

RESULTS

Properties and Purity of the Antigens

Assays of antigen purity were run to assure lack of contamination of these proteins by other species.

The paramyosin preparation used as an antigen sedimented as a single, symmetrical peak with no shoulders in the Beckman Model E analytic ultracentrifuge (Fig. 1 a). This peak remained discrete even after 144 min at 60,000 rpm. Comparable preparations exhibited single diffuse bands in disc-gel electrophoresis and had no adenosine triphosphatase (ATPase) activity, indicating no contamination with actomyosin.

The EDTA-actomyosin preparation which was used as an antigen showed no contamination with paramyosin in the ultracentrifuge (Fig. 1 b). The ATPase properties and superprecipitation behavior of this protein were as previously reported (6, 9).

Myosin used for antibody production was obtained by dissociation of ultracentrifugally pure, EDTA-washed actomyosin. This preparation showed a slight leading peak in the ultracentrifuge (Fig. 1 c). Other preparations comparable to the one used show a slight leading shoulder which probably represents the dimer. The various characteristics of this protein are currently being investigated.

Specificity of the Antisera

In double diffusion slides, antiparamyosin serum formed precipitin lines only against paramyosin (Fig. 2 a). Likewise, antisera to both actomyosin and myosin formed precipitin lines only against actomyosin (Fig. 2 b, c). Antisera which had been previously absorbed with their respective reciprocal proteins did not form any precipitin lines after 1 wk incubation.

Extinction of specific staining, using either the direct or indirect fluorescent antibody or the indirect horseradish peroxidase antibody techniques, was achieved by incubation of either "wet" or "dry" myofibrillar preparations with antisera which had been previously absorbed with their
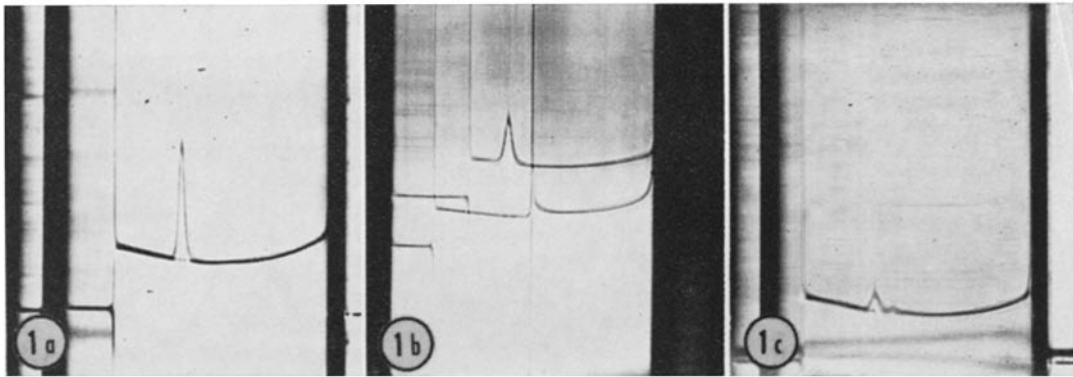
FIGURE 1 Schlieren patterns of *Limulus* contractile proteins used for antibody production. Fig. 1 *a*, schlieren pattern of the *Limulus* paramyosin used for antibody production, preparation No. 44. Picture taken after 144 min at 60,000 rpm; bar angle, 70°; temperature, 2.3°C; solvent, 0.6 M KCl, 60 mM potassium phosphate buffer, pH 7.4; protein concentration, 3.04 mg/ml; and in a double sector cell. $s_{20,w}$ = 3.55 svedbergs. Fig. 1 *b*, schlieren pattern of the *Limulus* actomyosin used for antibody production, preparation No. 201 and a sample of paramyosin (upper trace, wedge window, pooled from preparation Nos. 29 and 30), for comparison to show that the actomyosin has no trace of paramyosin in it. Pictures taken after 64 min at 60,000 rpm; bar angle, 75°; temperature, 6.9°C; solvent, 0.6 M KCl, 5 mM histidine buffer, pH 7.1 (actomyosin), and 0.5 M KCl, 50 mM Tris buffer, pH 8.3 (paramyosin); protein concentration, 6.08 mg/ml (actomyosin) and 2.60 mg/ml (paramyosin). Both preparations are in standard cells (actomyosin) plus wedge window (paramyosin). $s_{20,w}$ = 8.43 svedbergs (actomyosin) and 3.09 svedbergs (paramyosin). Fig. 1 *c*, schlieren pattern of the *Limulus* myosin used for antibody production prepared from actomyosin preparation No. 223. Picture taken after 64 min at 60,000 rpm; bar angle, 65°; temperature, 6.2°C; solvent, 0.6 M KCl, 5 mM histidine buffer, pH 7.3; protein concentration, 1.35 mg/ml; and in a double sector cell. $s_{20,w}$ = 6.91 svedbergs. The slight leading peak probably represents aggregation of myosin monomers.

reciprocal proteins. Likewise, specific staining was absent in preparations exposed to normal rabbit serum, either conjugated to FITC, or alone and followed by staining with goat anti-rabbit gamma globulin conjugated to either FITC or horseradish peroxidase.

Appearance of the Myofibril Preparations

When examined in suspension by phase-contrast optics, all myofibrils appeared to be in good condition in either ion-free distilled water or 25% glycerol in LWS. There was no evidence of swelling. Generally, all of the sarcomeres in any one myofibril appeared to be of similar lengths. Sarcomere lengths did vary, however, from myofibril to myofibril, in all preparations.

Slight disruption of myofibril structure was observed occasionally in preparations which were dried on slides from ion-free distilled water. Indirect fluorescent antibody staining of these fibrils was less intense than when the staining was performed in liquid media under a cover slip. Locali-

zation of the proteins, however, did not differ between the two techniques. Staining with horseradish peroxidase-labeled antibodies was easily achieved only in dried myofibril preparations, as wet preparations were easily dislodged from slides or lost when incubated free in solution.

Localization of Contractile Proteins

All of the contractile proteins thus far studied—paramyosin, myosin, and actomyosin—were localized in the A bands of glycerinated *Limulus* striated muscle. Both myosin and actomyosin showed identical localization within the A bands, presumably due to staining of myosin antigenic sites. Antiactomyosin serum, however, occasionally stained Z bands as well, even after absorption against the nonreciprocal contractile proteins (e.g., paramyosin and tropomyosin). Paramyosin and myosin each had a distinctive pattern of antibody binding at any specific sarcomere length, which was the same with either the fluorescent or

horseradish peroxidase antibody-labeling technique. At different sarcomere lengths the pattern of antibody binding by each of the proteins changed, indicating preferential exposure of specific antibody-binding sites in the A bands at different stages of fiber shortening.

Phase-contrast photomicrographs of the same myofibrils that were treated with fluorescent antibodies showed increased density in the same locations that had appeared fluorescent under UV excitation (Fig. 3 *a*, *b*). This correlation provided identification of the sarcomeric regions stained by fluorescent antibodies.

Localization of Paramyosin

In long sarcomeres (from 10.0 to 7.5 μ), paramyosin was localized to broads band staining only the lateral margins of the A band. The reaction product resulting from the oxidation of 4-chloro-1-naphthol by horseradish peroxidase and hydrogen peroxide was globular in form, and particles of up to 1 μ in diameter were observed. These frequently appeared to overhang the edges of the A bands and extend into the I band (Fig. 4 *a*). The fluorescent antibody also stained the lateral edges of the A bands. The reactive zone appeared somewhat irregular and fairly broad (Fig. 4 *b*).
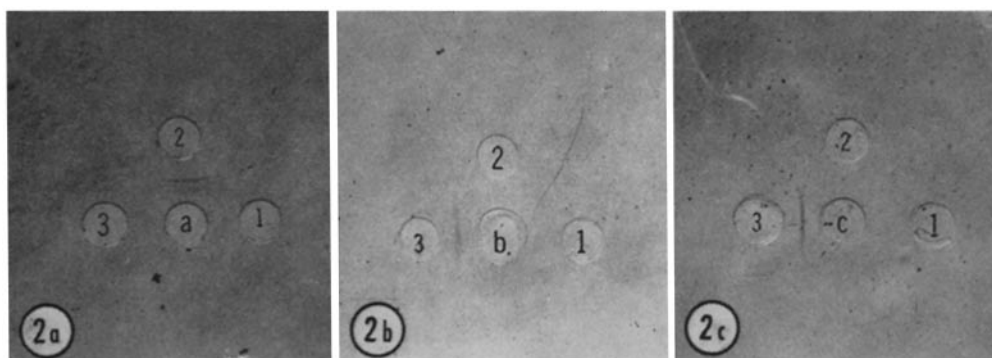

Figure 2   Double diffusion slides showing specificity of antisera to reciprocal contractile proteins. In all slides, the antiserum to be tested was placed in the central well. Well 1 contained *Limulus* tropomyosin, well 2 contained *Limulus* paramyosin, and well 3 contained *Limulus* actomyosin, all at concentrations of 1 mg protein/ml. Slides are unstained. Fig. 2 *a*, a precipitin band is present only between the central well, (*a*), containing antiparamyosin serum, and peripheral well 2, containing *Limulus* paramyosin. No bands are visible between the central well and peripheral wells 1 and 3, which contain, respectively, *Limulus* tropomyosin and *Limulus* actomyosin. Fig. 2 *b*, a precipitin band is present only between the central well (*b*), containing antiactomyosin serum, and peripheral well 3, containing *Limulus* actomyosin. No bands are visible between the central well and peripheral wells 1 and 2, which contain, respectively, *Limulus* tropomyosin and *Limulus* paramyosin. Fig. 2 *c*, a precipitin band is present only between the central well (*c*), containing antimyosin serum, and peripheral well 3, containing *Limulus* actomyosin. No bands are visible between the central well and peripheral wells 1 and 2, which contain, respectively, *Limulus* tropomyosin and *Limulus* paramyosin.
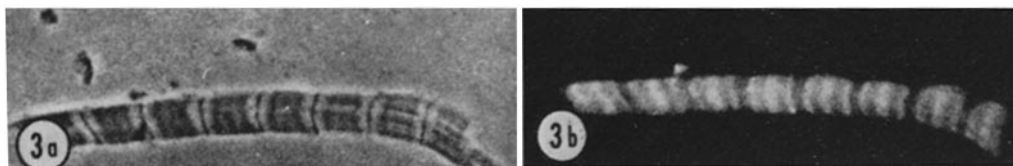
Figure 3   Fibril with 7.5 μ sarcomeres stained with antiparamyosin by the indirect fluorescent antibody technique. × 1000. Fig. 3 *a*, photographed with phase-contrast illumination. The regions of the A band which bind fluorescent antibody show increased density under phase-contrast optical conditions. Fig. 3 *b*, photographed with UV illumination.

Paramyosin localization in sarcomeres of intermediate length (from 7.5 down to 6.0 μ) was again restricted to the A bands. The lateral margins were again highly reactive, but in the horseradish peroxidase antibody-treated preparations, the lateral staining band appeared narrower than it had in the longer sarcomeres. A central staining band was also observed (Fig. 5 a, c). A triplet appearance of stain in the A bands of fluorescent antibody-treated fibrils indicated the continued reactivity at the A band margins, and, as with horseradish peroxidase labeling, the presence of central A band staining (Fig. 5 b). Occasionally, the central staining band was resolved into two
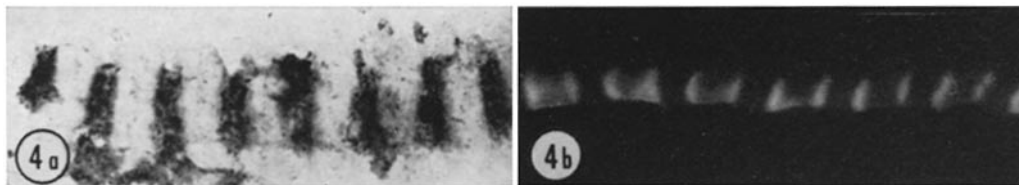

FIGURE 4   Antiparamyosin staining of long sarcomeres ($\geq$ 7.5 μ). $\times$ 1000. Fig. 4 a, fibrils with long sarcomeres (9.0 μ) stained with antiparamyosin by the indirect horseradish peroxidase technique. The reaction product appears dark. Paramyosin is localized to the very lateral edges of the A band, and the stain appears as a broad band which extends into the I band region. Fig. 4 b, fibril with long sarcomeres (10.6 μ) stained with antiparamyosin by the indirect fluorescent antibody technique. The antibody-binding region appears bright. Again, paramyosin is localized as a broad zone at the lateral edges of the A band.
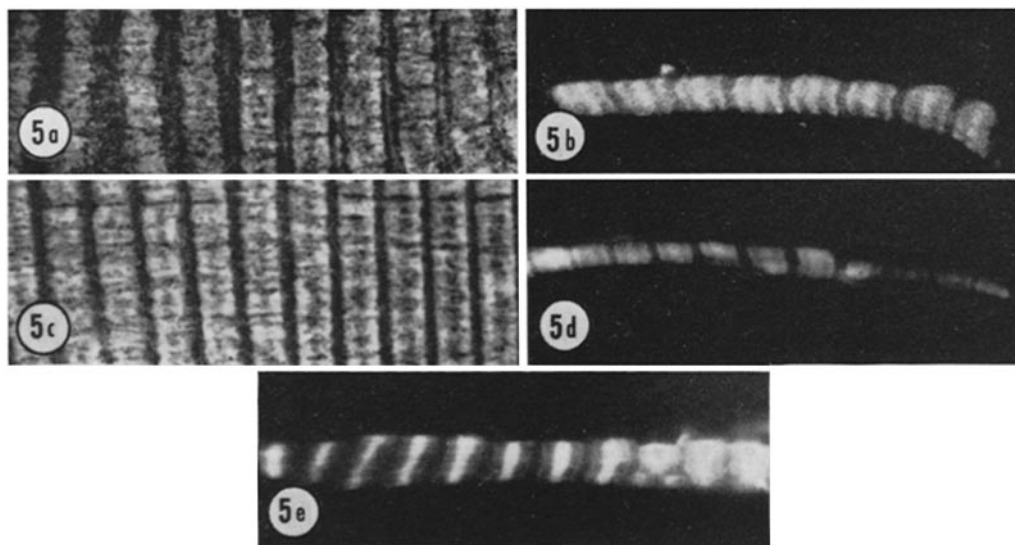

FIGURE 5   Antiparamyosin staining of intermediate length sarcomeres (7.5–6.0 μ). $\times$ 1000. Fig. 5 a, fibril with 7.5 μ sarcomeres stained with antiparamyosin by the indirect horseradish peroxidase technique. Note the narrower staining bands at the lateral edges of the A band as compared with Fig. 4 a, and the appearance of reaction product in the central A band. Fig. 5 b, fibril with 7.5 μ sarcomeres stained with antiparamyosin by the indirect fluorescent antibody technique. Note the fluorescence at the lateral edges of the A band and in a thin line in the central A band. Fig. 5 c, fibril with 6.5 μ sarcomeres stained as in Fig. 5 a. Note the decreased width of the lateral staining bands and the prominent central A band staining. Fig. 5 d, fibril with 6.5 μ sarcomeres stained as in Fig. 5 b. Note the staining present at the lateral margins of the A band and the appearance of a double fluorescent band in the central A band. Fig. 5 e, fibril with < 6.4 μ sarcomeres stained as in Fig. 5 d. Note the decreased staining at the lateral margins of the A band and the apparent broadening of the fluorescent region in the central A band.

narrow staining bands, barely separated by a non-staining region (Fig. 5 d). Often, in intermediate length sarcomeres, the central staining band appeared broader and more prominent than the marginal bands, and the intensity of stain appeared to decrease (Fig. 5 e) gradually, more laterally.

Antiparamyosin stained the entire length of the

A bands in short sarcomeres (those less than 6.0 μ) (Fig. 6). Horseradish peroxidase-labeled antibody preparations showed a denser central line in the A bands of sarcomeres less than 6.0 μ but longer than 5.5 μ, (Fig. 6 a), but this could not always be discerned in the fluorescent antibody-labeled myofibrils (Fig. 6 b). At sarcomere lengths shorter than 5.5 μ the antiparamyosin stained the A bands
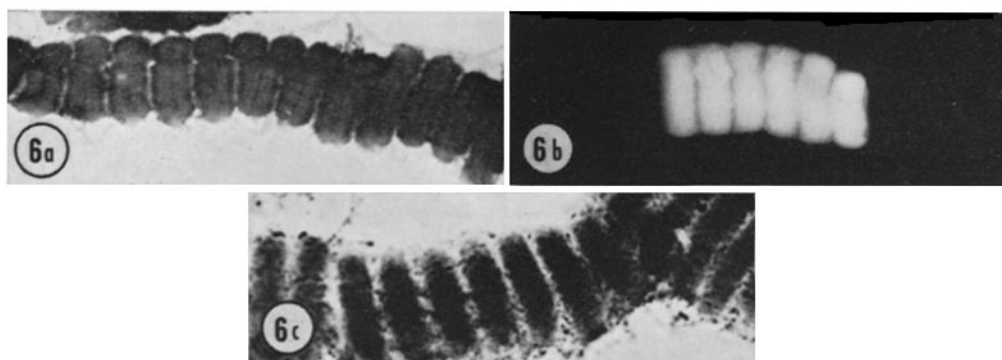

FIGURE 6   Antiparamyosin staining of short sarcomeres (< 6.0 μ). × 1000. Fig. 6 a, fibril with short sarcomeres (5.6 μ) stained with antiparamyosin by the indirect horseradish peroxidase technique. Note the appearance of stain throughout the A band, and the presence of a thin line of reaction product in the center of the A band. Fig. 6 b, fibril with 4.5 μ sarcomeres stained with antiparamyosin by the indirect fluorescent antibody technique. Note fluorescence throughout the A band. Fig. 6 c, fibril with 5.0 μ sarcomeres stained as in Fig. 6 a. Note the appearance of stain throughout the A band, and the absence of the line in the central A band.
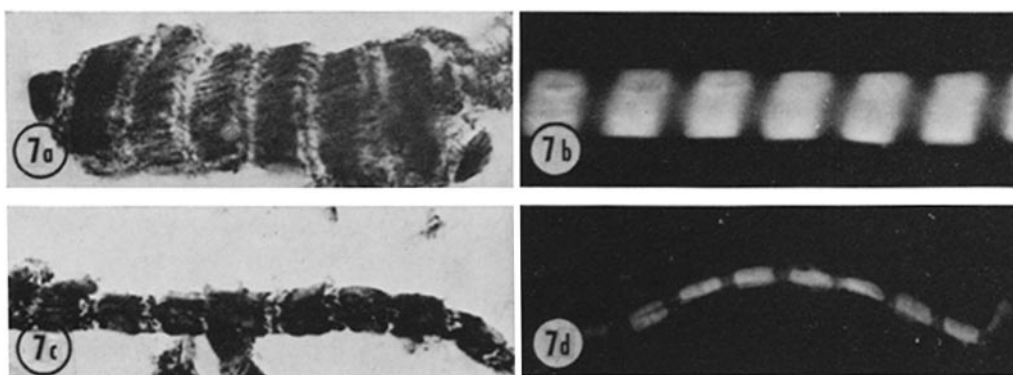

FIGURE 7   Antimyosin and antiactomyosin staining of long sarcomeres (≥ 7.5 μ). × 1000. Fig. 7 a, fibril with 8.6 μ sarcomeres stained with antiactomyosin by the indirect horseradish peroxidase technique. The edges of the A band appear ragged, and the Z line stains. Staining is uniform throughout the A band. Fig. 7 b, fibril with 10.5 μ sarcomeres stained with antimyosin by the direct fluorescent antibody technique. No Z band staining is seen, and stain is found throughout the A band. Fig. 7 c, fibril with 8.5 μ sarcomeres stained with antiactomyosin by the indirect horseradish peroxidase technique. The lateral edges of the A band stain more intensely than the central region. Fig. 7 d, fibril with 7.6 μ sarcomeres stained with antiactomyosin by the indirect fluorescent antibody technique. Staining intensity is decreased in the very central A band (H zone?).

intensely and uniformly, independent of the labeling procedure used (Fig. 6 c).

Localization of Myosin and Actomyosin

Protein localization in the A band was identical, using antisera to either myosin or actomyosin.

The A bands of long sarcomeres reacted throughout their length with antimyosin (with the occasional exception of the H zone) (Fig. 7 d). With the horseradish peroxidase technique, the staining in the central A band sometimes appeared less intense than that at the lateral margins of the A band (Fig. 7 c). In sarcomeres where the entire A band stained intensely, the lateral staining regions were slightly irregular (Fig. 7 a). Variations in staining intensity were seen when the direct fluorescent antibody technique was used to localize myosin in long sarcomeres (Fig. 7 b, d). There was a striking decrease in antimyosin stain-

ing of the central A band region in intermediate-length sarcomeres, except for the occasional persistence of the medial staining line. This was more prominent in the fluorescent antibody-labeled myofibrils (Fig. 8 b) than in those incubated in antibody labeled with horseradish peroxidase (Fig. 8 a). The lateral margins of the A bands still stained intensely with either technique. The lateral staining regions as seen in Fig. 8 c and d, were much broader (but still confined within the limits of the A bands) than those seen in myofibrils of comparable sarcomere length incubated with antiparamyosin. Where present, the medial staining line did not show a doublet appearance.

Antibody to myosin stained only the lateral edges of the A bands in short sarcomeres (Fig. 9 a, b). No medial staining was observed with either antibody-labeling method.
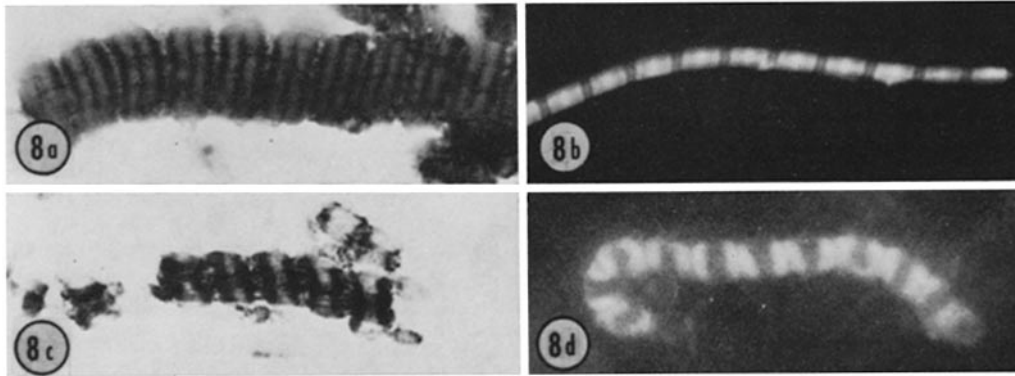

FIGURE 8 Antimyosin and antiactomyosin staining of intermediate-length sarcomeres (7.5 μ-6.0 μ). X 1000. Fig. 8 a, fibril with 6.6 μ sarcomeres stained with antiactomyosin by the indirect horseradish peroxidase technique. Lateral and medial A band staining is prominent. Fig. 8 b, fibril with 6.5 μ sarcomeres stained with antiactomyosin by the indirect fluorescent antibody technique. Compare with Fig. 8 a. Fig. 8 c, fibril with 6.0 μ sarcomeres stained as in Fig. 8 a. The medial staining line is absent and the lateral staining bands are fairly broad. Fig. 8 d, fibril with 6.0 μ sarcomeres stained with antimyosin by the direct fluorescent antibody technique. Compare with Fig. 8 c.
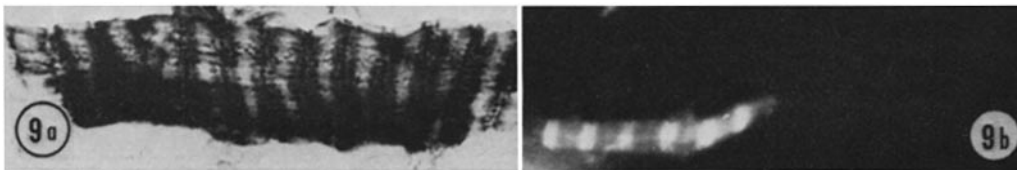

FIGURE 9 Antimyosin and antiactomyosin staining of short sarcomeres (< 6.0 μ). X 1000. Fig. 9 a, fibril with 5.0 μ sarcomeres stained with antiactomyosin by the indirect horseradish peroxidase technique. Reaction product is found only at the lateral edges of the A band. Fig. 9 b, fibril with 5.0 μ sarcomeres stained with antimyosin by the direct fluorescent antibody technique. Compare with Fig. 9 a.

Measurements

In these studies, sarcomere lengths ranged between 10 and 3.6 μ.

Over the range of sarcomere lengths measured, no "contraction bands" were observed, and a discrete A band could always be identified.

A linear decrease in A band length (Fig. 10) was observed in all preparations as the sarcomere lengths decreased from 10 to approximately 7.5 μ. As was mentioned above, in the horseradish peroxidase-labeled myofibrils this was accompanied by a decrease in the width of the bands of reaction product at either edge of the A band.

A further linear decrease in A band length also was observed as the sarcomere lengths continued to decrease from <7.5 to 3.6 μ (Fig. 10).
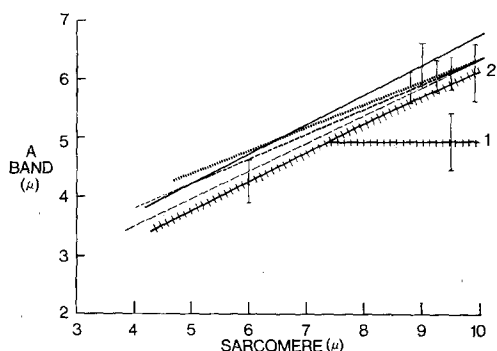

FIGURE 10 Graph showing the relationship of A band length to sarcomere length. Slopes were computed from measurements made on photomicrographs of glycerinated myofibrils and electron micrographs of longitudinally sectioned (sections cut normal to fiber axis) fresh-fixed muscle fibers. The thick filament lengths and over-all A band lengths (including misalignment) were measured independently on medium power (approximately X 10,000) electron micrographs (EM) of individual sarcomeres. Thick-filament lengths were recorded from filaments which passed entirely through the A band, and when at least 10 filaments of the same length were measured per sarcomere. N in this case refers to sarcomeres having been analyzed for thick filament length, not individual filaments measured. Bars indicate standard errors of slopes. N for the other slopes also equals number of sarcomeres. Confidence interval for all slopes: $P < 0.01$. ╫╫╫ 1, length of thick filaments (EM), N = 232; ╫╫╫ 2, length of over-all A band (EM), N = 190; ———, length of over-all A band, fluorescent antiparamyosin, N = 785; ......, length of over-all A band, fluorescent antimyosin, N = 653; ————, length of over-all A band, horseradish peroxidase antiparamyosin, N = 967; — — —, length of over-all A band, horseradish peroxidase antimyosin, N = 1190.

DISCUSSION

The proteins used as antigens—*Limulus* paramyosin, myosin, and actomyosin—were all free from contamination by other muscle proteins as seen in the analytic ultracentrifuge (Fig. 1 a, b, c). The purity of paramyosin was also seen on disc-gel electrophoresis. Furthermore, dispersion of *Limulus* paramyosin in 20 mM KSCN and reprecipitation with barium yields paracrystals which, when negatively stained with 2% aqueous uranyl acetate, exhibit the D II and D III (2, 23) patterns of predominantly dark bands bisected by less intensely staining ones, with a periodicity very similar to that observed in *Mercenaria* paracrystals (7; G. W. de Villafranca, unpublished results). The absence of ATPase activity in this fraction further confirmed the lack of contamination by actomyosin.

The $s_{20,w}$ of *Limulus* actomyosin, its appearance in sedimentation in the ultracentrifuge (Fig. 1 b compared with wedge window cell sample of paramyosin), and analyses of its ATPase properties and behavior on superprecipitation gave results which were the same as those previously described for purified *Limulus* actomyosin (6, 9), and indicated absence of contamination by paramyosin.

Myosin, prepared from such paramyosin-free actomyosin, also appeared to be free of contamination either by actin or paramyosin. The smaller leading peak seen in the schlieren pattern from the ultracentrifuge (Fig. 1 c) probably represents aggregation of myosin molecules rather than presence of a contaminating protein. *Limulus* myosin, similar to myosin of other poikilotherms, is particularly susceptible to aggregation (19). Tropomyosin, prepared from acetone-dried *Limulus* muscle powder after extraction of paramyosin, showed a single peak with a leading shoulder in the ultracentrifuge. This soluble protein was rich in nucleic acids, but is not completely characterized as yet, and was used in this study only for absorption of antisera to the other muscle proteins and in double diffusion slides to test the specificity of antibodies prepared against the other muscle proteins.

As determined by both double diffusion in agar and absorption with the reciprocal antigens, the antisera employed here exhibited a high degree of specificity toward the proteins to which they were made (Fig. 2 a, b, c).

Both the fluorescent antibody- and horseradish peroxidase-labeled antibody techniques gave similar experimental results. Localization of muscle

proteins was more precise with the fluorescent antibody technique, as this method did not result in the formation of large globules of reaction product. Comparison of fluorescent antibody-treated preparations of dried fibrils with those of fibrils suspended in liquid media showed that while the specific staining, and thus protein localization, was identical in both cases, the intensity of the fluorescent label was greater with the latter technique. The horseradish peroxidase-labeled antibody technique, however, had the advantage of providing more cytologic detail, as the myofibrils were viewed and photographed with bright-field illumination. Differences in widths of staining regions within the A bands were more easily discerned by this technique. Also, storage of mounted preparations for later examination was not a problem.

Our immunohistochemical studies show localization of both paramyosin and myosin in the A bands of *Limulus* striated muscle. These results lead us to agree with other investigators that both proteins are present in the thick filaments (11, 19, 42). We further observe differential staining of myosin and paramyosin in distinct regions of the A bands in sarcomeres of the same length, as well as alterations in the localization of each protein in sarcomeres of different lengths (Figs. 4–9). Our results indicate that both paramyosin and myosin antibody-binding sites are exposed at all sarcomere lengths, but that the locations and extents of these regions in the A band change as the sarcomeres shorten.

After treatment of *Limulus* myofibrils with antimyosin, the staining patterns observed closely resembled those reported by Pepe (35, 37) who demonstrated variations in antimyosin staining of glycerinated chicken breast muscle myofibrils at different sarcomere lengths. Staining occurred over the length of the A band in long sarcomeres, but was restricted to the lateral regions of the A bands in short sarcomeres. Although a "quadruplet" appearance of stain was not evident in antimyosin staining of *Limulus* long sarcomeres, as it was in the chicken muscle (35, 37), we did observe a "triplet" staining pattern in sarcomeres of intermediate length, and a restriction of staining to the lateral A band regions in short sarcomeres. These staining patterns may be explained in accordance with Pepe's analysis of chicken muscle (37), if we assume the presence of myosin at the surface of the thick filaments of *Limulus* muscle. In long sarcomeres, the region of no overlap be-

tween thick and thin filaments (H zone) is wide and heavy meromyosin antigenic sites are available for staining in this region, thus giving the central A band staining. Light meromyosin antigenic sites are available laterally in the A band due to the bending of the myosin molecules off of the paramyosin core. Whole A band staining would then be due to fusion of the heavy meromyosin and light meromyosin specific staining sites.

The triplet appearance of antimyosin stain in sarcomeres of intermediate length may be accounted for by the continued exposure of antibody-binding sites of light meromyosin in the lateral regions of the A bands, but decreased availability of heavy meromyosin sites more medially, except in the most central regions of the A band, as the no-overlap region of the central A band decreases in width. It has been shown that in other striated muscles, antibody binding by heavy meromyosin becomes confined to a progressively narrower region in the central A band as the H zone decreases in width.

In short sarcomeres, where overlap is presumably complete, cross-bridge attachment to thin filaments most likely involves all of the heavy meromyosin portions of the myosin molecules along the length of the A bands. Thus, heavy meromyosin antigenic sites are no longer exposed for staining. Only light meromyosin antigenic sites remain exposed laterally due to increased bending of the myosin molecules away from the body of the filament at its tapered ends, during cross-bridge attachment. This results in the restriction of antimyosin staining to the lateral A band in such sarcomeres.

It would be of great interest to compare the availability of reactive sites of both heavy and light meromyosins in sarcomeres of different lengths. As yet, however, neither subunit has been obtained from *Limulus* myosin.

Antiparamyosin was bound only at the very lateral margins of the A bands in long sarcomeres, but also stained the central A bands in those of intermediate length and was bound along the entire A bands of short sarcomeres. These results are best interpreted if we assume, in agreement with others, that paramyosin comprises the core of the thick filaments. Ikemoto and Kawaguti (21) reported that in striated muscle of *Tachypleus tridentatus*, another xiphosuran, the thick filaments had a core of low electron opacity and suggested that this represented the location of paramyosin

within these filaments, which were then covered by a myosin cortex. In an earlier paper (20), they showed that the in vitro association of myosin molecules to produce the long thick filaments (approximately 4.0 μ) of *Tachypleus* striated muscle depended upon the presence of paramyosin. They indicated, however, that the paramyosin core did not extend into the tapered ends of the thick filaments. Such a molecular organization cannot be true of *Limulus* striated muscles, as we have demonstrated antiparamyosin binding at the lateral margins of the A bands in long sarcomeres. We interpret the exposure of antibody-binding sites of the paramyosin core at the tapered ends of the thick filaments in the following manner.

In long sarcomeres, there is little overlap; interdigitation and cross-bridge attachment between thick and thin filaments probably occurs only in the lateral regions of the A bands (our preparations, being glycerinated, are in rigor). Myosin molecules not involved in cross-bridge attachment occupy most of the surface of the filaments, and may prevent access of antibody to paramyosin antigenic sites. Only the very laterally located myosin molecules are involved in interaction with actin and thus become lifted away from the body of the thick filament. As the thick filaments are tapered at their ends, we have invoked Pepe's rationale that the myosin molecules interacting with the thin actin-containing filaments are lifted farther away from the rest of the thick filament in this region than are the more medially located myosin molecules when they become involved in the formation of cross-bridge attachments (36, 37). In this way, the inner core (paramyosin) of the thick filaments may be exposed to bind antibody at the lateral margins of the A bands.

The broad bands of stain seen at the lateral margins of the A bands and the intrusion of this stain into the I bands in long sarcomeres treated with horseradish peroxidase-labeled antibody may be due to the piling up of reaction product at these sites, resulting in the overlap of stain into the I bands. Another explanation is based on the observation that the thick filaments in the A bands of long sarcomeres of *Limulus* striated muscle are not in register. Misalignment of thick filaments is most likely related to the absence of M bands in this muscle. The M bands in striated muscles having shorter sarcomeres than *Limulus* muscle probably function to maintain thick filament alignment (36) and may be also functionally

related to the duration of the contractile event. M band protein is immunologically distinct from other muscle proteins (25, 34), and was successfully localized in the central A bands of chicken skeletal muscle by the fluorescent antibody technique (25). Long sarcomeres lacking M bands are found in several different invertebrate muscles, including cockroach femoral muscle (16), crab leg muscle (15), *Tachypleus* retractor muscle (21), and *Limulus* cardiac (28) and skeletal (26) muscle. All of these show some degree of thick filament misalignment in lengthened myofibrils, and thus an apparent increase in A band length is seen in such sarcomeres (26, 27). The addition of staining globules at the tips of out-of-register thick filaments, produced by binding of horseradish peroxidase-labeled antibody in this region, would therefore produce a broad reactive zone (Fig. 11).

Realignment of out-of-register thick filaments occurs as *Limulus* sarcomeres shorten from approximately 10 μ down to approximately 7.0 μ. This is indicated by the apparent decrease in A band lengths seen in intermediate-length sarcomeres, as compared with those of long sarcomeres (Fig. 10), and by the narrowing of the antibody-binding zones at the lateral edges of the A bands (compare Figs. 4, 5, and Fig. 11). Our independent electron microscopic studies of *Limulus* striated muscle at
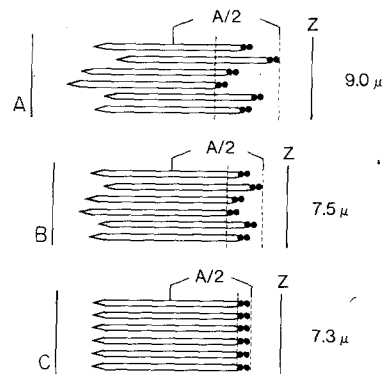

FIGURE 11 Schematic representation of arrangement of thick filaments and staining by the indirect horseradish peroxidase antibody technique which may be responsible for the apparent increase in A band length in sarcomeres over 7.3 μ in length. Black circles represent reaction product of hydrogen peroxide and 4-chloro-1-naphthol after incubation with the horseradish peroxidase-labeled antibody. For clarity, thin filaments have been excluded from this schematic representation. Fig. 11 a, sarcomere length = 9.0 μ. Fig. 11 b, sarcomere length = 7.5 μ. Fig. 11 c, sarcomere length = 7.3 μ.

various sarcomere lengths confirm the relationship between sarcomere length and degree of thick filament alignment. Furthermore, the thick filament length in sarcomeres, ranging from approximately 10.0 to 7.0 μ in length does not vary significantly from 4.9 μ (M. Dewey and R. J. C. Levine, unpublished results). Fourier syntheses, computed from microdensitometric tracings of layer lines obtained from light diffraction experiments on glycerinated *Limulus* single fibers, show the decrease and eventual elimination of broad shoulders at the lateral edges of the A bands as sarcomeres shorten from 9.6 to $\sim$ 7.5 μ (10).

The appearance of antiparamyosin stain in the central A band in intermediate-length sarcomeres is less easily explained. Exposure of antigenic sites of the paramyosin core of the filaments in specifically this region requires rearrangement of the cortical myosin molecules. At intermediate sarcomere lengths, interaction of the more medially located myosin molecules with the thin filaments, thus exposing the paramyosin core, is unexpected. As antimyosin staining, probably due to antibody binding by heavy meromyosin, is also seen in the central A band in intermediate-length sarcomeres, such interaction probably does not occur. In progress and planned experiments, including localization of paramyosin and myosin at the electron microscope level in isolated thick filament preparations of *Limulus* striated muscle, and localization of *Limulus* light and heavy meromyosins in sarcomeres of various lengths, should help to clarify these results.

The antigenic sites of the paramyosin core of the thick filaments are completely exposed to react with antibody throughout the A bands in short sarcomeres, while only the lateral A band regions stain with either antiactomyosin or antimyosin. Such exposure of paramyosin antibody-binding sites may be explained by assuming that, except at the tapered ends of the thick filaments, all of the antigenic sites of the cortical myosin molecules are obscured by their interaction with actin in completely shortened sarcomeres. If the cortical layer is only one myosin molecule in thickness (10, 16, 38), heavy meromyosin-actin interaction and associated increase in lattice spacing between thick and thin filaments would reduce steric hindrance to antiparamyosin binding and allow staining of the thick filament core.

Other conformational changes of thick filament proteins and of the thick filaments themselves,

which also might be partially responsible for the observed antiparamyosin staining of the entire length of the A bands in short sarcomeres, however, cannot yet be dismissed. Both electron microscopic (M. Dewey and R. J. C. Levine, unpublished results) and light diffraction studies (10) indicate that in sarcomeres below 7.0 μ the thick filaments are in good register, yet continued decrease in the length of the A band occurs in the shortening of sarcomeres from approximately 7.0 to <5.0 μ, in agreement with our measurements of A band lengths obtained from phase-contrast and antibody-treated preparations (Fig. 10) and earlier studies (4, 8).

Detailed ultrastructural and optical diffraction studies of the thick filament array and possible changes in thick filament volume at various sarcomere lengths, which may be related to both the observed changes in A band length and the exposure of paramyosin antigenic sites throughout the A bands of short sarcomeres, are in progress.

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