PARAMYOSIN IN INVERTEBRATE MUSCLES

I. Identification and Localization

MYRA ELFVIN, RHEA J. C. LEVINE, and MAYNARD M. DEWEY

From the Department of Anatomy, the Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129, and the Department of Anatomical Sciences, State University of New York at Stony Brook, New York 11794

ABSTRACT

By sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunodiffusion, we identified paramyosin in two smooth invertebrate "catch" muscles (*Mytilus* anterior byssus retractor and *Mercenaria* opaque adductor) and five invertebrate striated muscles (*Limulus* telson levator, *Homarus* claw muscle, *Balanus* scutal depressor, *Lethocerus* air tube retractor, and *Aequipecten* striated adductor). We show that (*a*) the paramyosins in all of these muscles have the same chain weights and (*b*) they are immunologically similar.

We stained all of these muscles with specific antibody to *Limulus* paramyosin using the indirect fluorescent antibody technique. Paramyosin was localized to the A bands of the glycerinated striated muscles, and diffuse fluorescence was seen throughout the glycerinated fibers of the smooth catch muscles.

The presence of paramyosin in *Homarus* claw muscle, *Balanus* scutal depressor, and *Lethocerus* air tube retractor is shown here for the first time. Of the muscles in this study, *Limulus* telson levator is the only one for which the anti-paramyosin staining pattern has been previously reported.

Until 1967, paramyosin was believed to be limited to molluscan smooth "catch" muscles (1, 12), obliquely striated (11) and cross-striated adductors (10, 28), and to annelid obliquely striated body wall muscles (17). Then the presence of paramyosin in the cross-striated muscle of primitive arthropods, the horseshoe crabs, was reported by de Villafranca and Leitner (4) and Ikemoto and Kawaguti (15), and this protein was localized to the thick filaments (21). More recently, paramyosin was isolated from the asynchronous flight muscles of several species of insects (2), and we localized this protein to the A bands of *Lethocerus* flight muscle (22). Thus, the distribution of paramyosin is phylogenetically widespread, and this protein is present in muscles that are distinctly different, both structurally and physiologically.

In order to understand the significance of the presence of paramyosin in such different muscles, it is necessary to determine the following: (*a*) its distribution; (*b*) whether or not it is similar in different muscles of different animals; and (*c*) its quantitative relationship to myosin. Additional data regarding the phylogenetic and anatomical distribution of paramyosin are needed in order to differentiate between possible functional and/or phylogenetic bases for its presence. Different molecular weights have been reported for paramyosins isolated from various muscles (3, 35). The validity of these determinations is questionable, however, since Stafford and Yphantis

(30) demonstrated partial chain loss due to proteolytic degradation of paramyosin during isolation procedures. Determination of molecular weights of different paramyosins, without resorting to extraction procedures, may establish whether or not these differences are real. Immuno-chemical studies can provide further information regarding similarities among paramyosins. If paramyosins are indeed different, then this may be reflected in the differing morphological and physiological properties of muscles containing this protein. On the other hand, if paramyosins are similar, then such differing morphological and physiological properties of these tissues may be, in part, a function of the packing of paramyosin in the thick filaments. Knowledge of the molecular ratios of paramyosin to myosin in these muscles can provide the basis for determination of such packing arrangements.

We have begun a comparative survey of invertebrate muscles by studying (*a*) a series of arthropod muscles which were not previously known to contain paramyosin; and (*b*) a series of molluscan muscles, from which paramyosin had been previously isolated. In this paper, we show for the first time the presence of paramyosin in several arthropod muscles, including: *Homarus* (lobster) slow claw, *Balanus* (barnacle) scutal depressor, and *Lethocerus* (giant water bug) air tube retractor. We also show that the paramyosins of these species, of *Limulus* (horseshoe crab), and of the following molluscan muscles: *Aequipecten* (scallop) striated adductor, *Mytilus* (mussel) anterior byssus retractor (ABRM), and *Mercenaria* (clam) opaque adductor, are all similar in chain weight, and all cross-react with antibody to purified *Limulus* paramyosin. Fluorescent antibody staining confirms this immunologic similarity and localizes paramyosin to the A bands of the striated muscles. The companion paper in this series (23) establishes the molecular ratios of paramyosin to myosin in all of these muscles.

MATERIALS AND METHODS

Preparation of Glycerinated Muscle Bundles

Small bundles of *Limulus* telson muscle were glycerinated at various lengths as described previously (21, 6). *Lethocerus* abdomens, stored in 1:1 glycerol-standard salt solution (18), were donated by Dr. Frank A. Pepe (University of Pennsylvania Medical School, Philadelphia, Pa.) and Dr. Benjamin Walcott (SUNY at Stony Brook, N. Y.). The air tube retractor muscle was dissected free of surrounding tissues just before use. Muscle length was determined by the extent of air tube protrusion before and during abdomen glycerination. *Aequipecten* striated adductor, *Mercenaria* opaque adductor, *Mytilus* ABRM, and *Homarus* claw muscles were glycerinated *in situ* at various lengths. All of the above muscles, except that of *Lethocerus*, were kept moist with *Limulus* wash solution (LWS) (40 mM KCl, 6.7 mM KH_2PO_4, brought to pH 7.3 with KOH; [22]) during preparation for glycerination. *Balanus* specimens, obtained from Pacific Bio-Marine Supply Co. (Venice, Calif.), were dissected as follows. The mantle, onto which the scutal depressor muscle inserts, was carefully separated from both the shell and scutes, and the animal was discarded. The shell was then broken between muscle origins with the aid of an electric bone saw, hammer, and chisel. The mantle was cut between muscle insertions. The resulting preparations consisted of a piece of shell, a scutal depressor muscle, and a piece of mantle. After removal of fat and loose connective tissue, the muscles were divided into small bundles, and the bundles tied at either end, separated from the muscle, and tied at varying lengths to sticks for glycerination. The muscles were washed and kept moist during dissection with artificial seawater obtained from Carolina Biological Supply (Burlington, N. C.). All of the above muscles, except *Lethocerus* air tube retractor, were glycerinated in glycerol-LWS (1:1). Bundles of chicken breast muscle and frog skeletal muscle were tied on sticks and glycerinated in glycerol-standard salt solution and glycerol-LWS (1:1), respectively.

Preparation of Myofibrils from Glycerinated Muscle Bundles

Myofibrils (or muscle homogenates in the case of *Mytilus* ABRM and *Mercenaria* opaque adductors) to be used for gel electrophoresis were prepared by homogenization of muscle bundles in 20 ml of LWS in a Sorvall Omnimixer (Dupont Instruments, Sorvall Operations, Newtown, Conn.). The fibrils were washed three times by centrifugation in the same solution, and a suspension of fibrils in LWS of 1:10 (vol/vol) was obtained. A 0.5-ml aliquot of this suspension was used for determination of protein concentration by the biuret technique (9), and the protein concentration of the suspension was adjusted to 2 mg protein/ml by addition of 0.01 M phosphate buffer, pH 7.0. A 2-ml aliquot of the suspension was dialyzed vs. phosphate buffer for 2 h at 4°C. 0.1 ml of 10% dithioerythritol (DTE) and 0.1 ml of 10% sodium dodecyl sulfate (SDS) were added to 1 ml of dialyzed myofibrillar suspension. The mixture was boiled for 3 min to denature the protein. 400 μl of the denatured protein preparation were mixed with 400 μl of tracking dye (50% sucrose plus a few grains of bromphenol blue). Both this mixture and the denatured myofibrillar preparation were stored at −18°C.

Myofibrils or *Mytilus* and *Mercenaria* fibers for antibody staining were prepared immediately before use by homogenization of muscle bundles in 25% glycerol, 75% LWS, containing 0.01% streptomycin (Sigma Chemical Co., St. Louis, Mo.). The fibrils and fibers were washed three times by centrifugation. The pellet was suspended in 10 vol of LWS-glycerol (3:1).

Antibody Specificity

Antibodies against native *Limulus* paramyosin were prepared in rabbits (21). The gamma globulin (IgG) fraction was obtained from the whole serum by ammonium sulfate precipitation. The IgG fraction was absorbed with both native *Limulus* tropomyosin, and native *Limulus* actomyosin. In immunodiffusion, this IgG fraction formed precipitin lines only against *Limulus* paramyosin (both the native protein and paramyosin denatured by boiling for 3 min in 0.01 M sodium phosphate buffer, pH 7.0, with 1% SDS + 1% DTE). Specific antibody to *Limulus* paramyosin was isolated from this IgG fraction by affinity chromatography, according to Pepe (25, 27). Both the specific antibody and the IgG fraction were used for fluorescent antibody staining procedures.

Specific antibody against denatured *Limulus* paramyosin (denatured as described above) was prepared in the same manner. Antidenatured *Limulus* paramyosin formed precipitin lines only against both native and denatured *Limulus* paramyosin. This antibody was used in immunodiffusion experiments to (*a*) identify the protein in the presumed paramyosin band on gels; and (*b*) demonstrate immunologic similarity among the paramyosins from different muscles.

We also prepared antibody to denatured *Limulus* myosin (denatured in the same way as paramyosin). The IgG fraction of the serum was absorbed with *Limulus* paramyosin. This fraction formed precipitin lines only against native and denatured *Limulus* myosin. This antibody was used in immunodiffusion experiments to determine whether myosin was present in the presumed paramyosin bands.

For the immunodiffusion studies, the proteins in the presumed paramyosin bands were eluted (19) from gels of three of the muscles studied (*Limulus, Homarus*, and *Mercenaria*). In addition, protein eluted from the myosin heavy chain bands of *Limulus* gels was used as a control for the specific activity of the antidenatured *Limulus* myosin.

Antibody Staining

Three drops of myofibril suspension were incubated at 4°C overnight with one drop of the isolated specific antinative *Limulus* paramyosin at 0.6 mg/ml. One drop of myofibril-antibody mixture was placed on each cleaned slide and covered with a glass no. 0 cover slip. Excess antibody was washed out by introducing LWS-glycerol at one side of the cover slip and drawing this solution beneath the cover slip by capillary action. Four such washes were made and the fibrils were incubated for 30 min in a 1:10 dilution of the 7S fraction of fluorescent goat anti-rabbit IgG (Pentex Division Miles Research Labs) which was purified by elution from a diethylaminoethyl-cellulose column, and absorbed against glycerinated *Limulus* or *Lethocerus* muscle (21). Fibrils were washed again with four changes of LWS-glycerol and examined and photographed at × 1,000 magnification on a Zeiss Ultraphot, with both phase and fluorescent optics, on Polaroid Type 57 sheet film. Negatives were made on M plates (Eastman Kodak Co., Rochester, N. Y.) with a Polaroid MP-3 camera.

Control experiments included: (*a*) absorption of specific anti-*Limulus* paramyosin with *Limulus* paramyosin before indirect staining; (*b*) staining with normal rabbit IgG, followed by fluorescent goat anti-rabbit IgG; or (*c*) staining only with fluorescent goat anti-rabbit IgG.

SDS Polyacrylamide Gel Electrophoresis

6% polyacrylamide gels containing 0.1% SDS were prepared. Gels of all preparations were loaded with 20 μg of muscle protein. Myosin and paramyosin isolated from *Limulus* and paramyosin isolated from the opaque adductor of *Mercenaria* (5) were donated by G. de Villafranca (Smith College, Northampton, Mass.). The *Mercenaria* paramyosin was prepared according to Johnson et al. (16). All gels containing purified proteins were loaded with 1.25 μg of the protein, which had been boiled for 3 min after addition of SDS and DTE, combined with tracking dye, and stored in the same manner as the myofibrillar preparations.

Gels were loaded with myofibrillar protein prepared from the *Limulus, Homarus, Balanus, Lethocerus, Aequipecten, Mytilus*, or *Mercenaria* muscles. All were also run with added purified *Limulus* paramyosin. In the case of *Mercenaria* muscle, gels were also run with added *Mercenaria* paramyosin. Controls were always run together with experimental gels. Control gels were loaded with: (*a*) vertebrate (chicken or frog) myofibrillar proteins; (*b*) vertebrate myofibrillar protein plus purified *Limulus* paramyosin; (*c*) purified *Limulus* or *Mercenaria* paramyosin; and (*d*) *Limulus* and *Mercenaria* paramyosins, together. Purified *Limulus* myosin and paramyosin, both aged after denaturation, also were run together and separately. This was done to show that none of the breakdown products of myosin heavy chains have the same mobility as the band we identify as paramyosin.

Electrophoresis was done on a Canalco Model 1200 unit, using a Canalco Model 100 power supply (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) at constant current. Approximately 6 mA were delivered to each gel during the duration of the run. The gels were stained

with Coomassie brilliant blue for 1.5 h, destained in 7.5% acetic acid-5% methanol, and stored in 7% acetic acid. The gels were photographed on Kodak M plates with the MP-3 camera, and were scanned on the EC Model 910 densitometer (E-C Apparatus Corp., St. Petersburg, Fla.) at 550 nm. The scans were traced on a Corning Model 840 (Corning Scientific Instruments, Medfield, Mass.) integrating recorder with both scanning and integrating circuits operating simultaneously.

Molecular weights of the polypeptide chains were determined, with a standard curve constructed according to the method of Weber and Osborn (34). The proteins used as standards were: β galactosidase (130,000 daltons), phosphorylase A (95,000 daltons), catalase (60,000 daltons), and actin (45,000 daltons). These were plotted against mobility normalized to that of actin (Fig. 1).

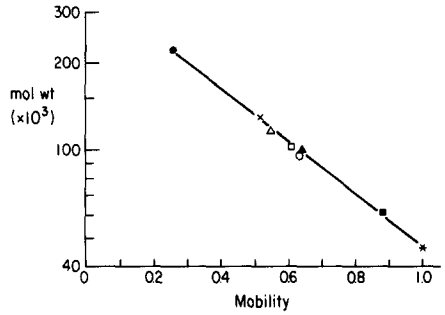


FIGURE 1 Standard curve. The abscissa shows the mobility in 6% SDS gels as related to actin mobility. The ordinate shows polypeptide chain weights (mol wt) in daltons × 10^3. Filled circle = myosin heavy chains; × = β galactosidase; empty triangle = paramyosin in gels of glycerinated muscle preparations and isolated *Limulus* paramyosin; empty square and filled triangle = extracted *Mercenaria* paramyosin; empty circle = phosphorylase A; filled square = catalase; asterisk = actin.

RESULTS

Identification of Paramyosin Bands in SDS-Polyacrylamide Gels

Gels of all of the invertebrate myofibrillar preparations show a band with electrophoretic mobility identical to that of polypeptide chains of purified *Limulus* paramyosin (Fig. 2*a–g*). This band is absent from gels of chicken breast myofibrils (Fig. 2*h*). In the gels of invertebrate myofibrils, added *Limulus* paramyosin increases the staining intensity of the band identified as paramyosin. In *Aequipecten* gels, two closely spaced bands are present near the level of *Limulus* paramyosin chains. Addition of *Limulus* paramyosin enhances the staining intensity of only the lower band, that with greater electrophoretic mobility (Fig. 2*e*). Addition of paramyosin to vertebrate myofibril gels introduces a new band with lower electrophoretic mobility than that which has been identified as phosphorylase (Fig. 2*h*).

Mercenaria paramyosin, when prepared according to the method of Johnson et al. (16), runs as two bands. When this paramyosin is added to the *Mercenaria* muscle homogenate, the two new bands migrate faster than either purified *Limulus* paramyosin or the paramyosin present in the muscle homogenate (Fig. 3*a–d*).

The addition of paramyosin to muscle homogenates increases the staining intensity of the paramyosin band on gels. An increase in the area of only the paramyosin peak was seen in densitometer tracings of gels to which *Limulus* paramyosin had been added (Fig. 3*a*).

In order to determine that the band identified as paramyosin did not result from the breakdown

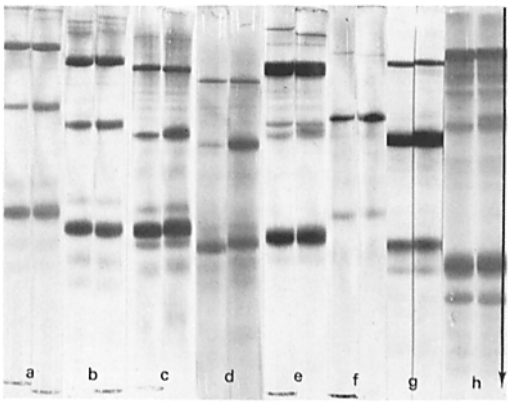


FIGURE 2 Muscle homogenate gel pairs. Right member of each pair has added *Limulus* paramyosin. (*a*) *Limulus* telson; (*b*) *Homarus* claw; (*c*) *Balanus* scutal depressor; (*d*) *Lethocerus* air tube retractor; (*e*) *Aequipecten* striated adductor; (*f*) *Mytilus* ABRM; (*g*) *Mercenaria* opaque adductor; (*h*) chicken breast. Direction of electrophoresis indicated by the arrow.

of myosin heavy chains, we aged denatured *Limulus* myosin and coelectrophoresed it with similarly aged denatured *Limulus* paramyosin. While aged *Limulus* myosin has apparently broken down and migrates as four to five distinct bands of high molecular weight (Fig. 4*c*), all of these show mobilities different from that of the aged *Limulus* paramyosin which migrates as a single band (Fig. 4*a*, *b*) with no change in chain weight.

We further established that the bands coelec-

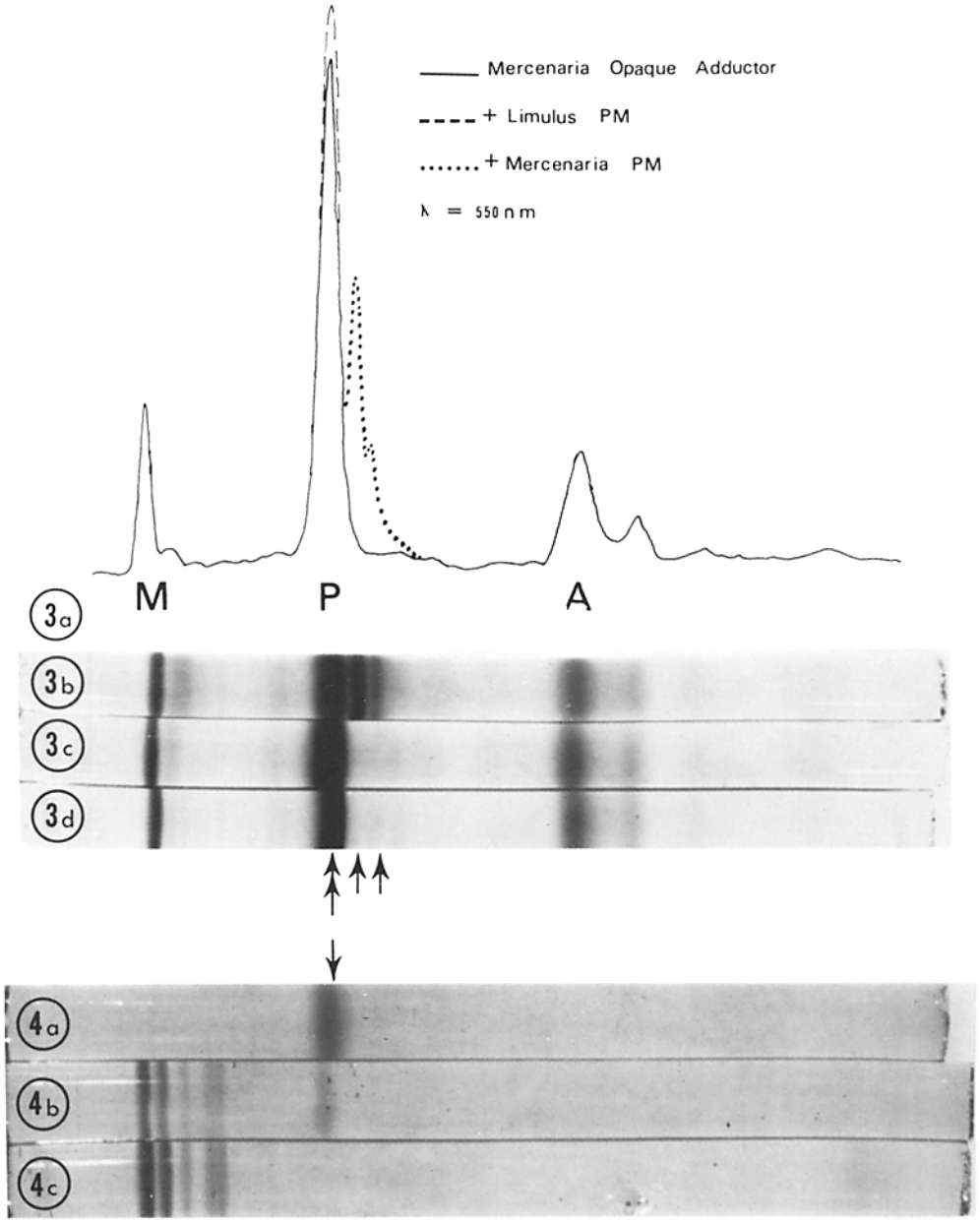


FIGURE 3. *Mercenaria* opaque adductor homogenate. (*a*) Superimposed tracings of scans of the three gels shown below; (*b*) added *Mercenaria* paramyosin (single arrows); (*c*) added *Limulus* paramyosin (double arrow); (*d*) no added paramyosin; *M* = myosin heavy chains; *P* = paramyosin; *A* = actin.

FIGURE 4. Aged myosin and paramyosin isolated from *Limulus* muscle. (*a*) Paramyosin; (*b*) myosin and paramyosin; (*c*) myosin. The myosin appears as four to five high molecular weight bands, all of which migrate much more slowly than the single paramyosin band (arrow).

trophoresing with our *Limulus* paramyosin contain paramyosin chains by the results of double-diffusion in agar. Antibody against denatured *Limulus* paramyosin forms precipitin lines against purified *Limulus* paramyosin and against the proteins eluted from presumed paramyosin

bands cut from gels of other invertebrate muscles. For examples, see Fig. 5. In contrast, antibody against denatured *Limulus* myosin does not react at all with these proteins, but does react with both purified *Limulus* myosin and *Limulus* myosin heavy chains eluted from gels.

Determination of Paramyosin Chain Weights

Estimations of paramyosin polypeptide chain weights were made from our myofibril gels with our standard curve (Fig. 1) according to the method of Weber and Osborn (34). We obtained a value of 115,000 ± 4,000 daltons for both purified *Limulus* paramyosin and the paramyosin present in myofibrils and muscle homogenates of all the invertebrate muscles we studied. *Mercenaria* paramyosin prepared according to the method of Johnson et al. (16) shows a mobility corresponding to polypeptide chain weights of about 102,000 and 97,000 daltons (Fig. 3*b*).

Paramyosin Localization

The IgG fraction of rabbit anti-*Limulus* paramyosin serum binds to all the invertebrate myofibrillar and whole fiber (*Mytilus* ABRM) preparations examined, but not to vertebrate myofibrils. Fluorescent localization of paramyosin is always restricted to the A bands of those myofibrillar preparations with identifiable sarcomeres (Figs. 6–10). All controls were negative.

The isolation of specific antibody using *P*-aminobenzoate (PAB)-cellulose (25, 27) affects the fluorescent staining pattern of *Limulus* sarcomeres only. Paramyosin localization in all of the other invertebrate preparations is the same with

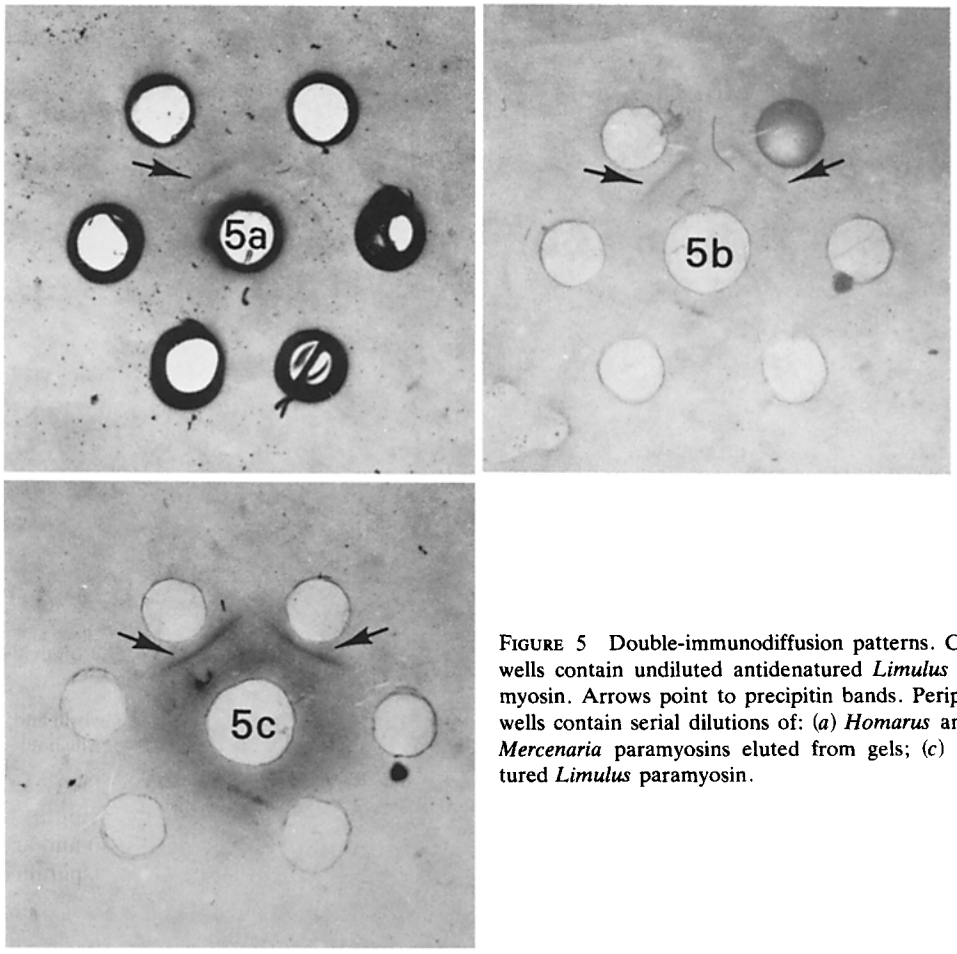


FIGURE 5 Double-immunodiffusion patterns. Center wells contain undiluted antidenatured *Limulus* paramyosin. Arrows point to precipitin bands. Peripheral wells contain serial dilutions of: (*a*) *Homarus* and (*b*) *Mercenaria* paramyosins eluted from gels; (*c*) denatured *Limulus* paramyosin.

either the IgG fraction of anti-*Limulus* paramyosin or PAB-cellulose isolated antibody.

Unlike the arthropod muscles described below, the sarcomeres of *Aequipecten* striated adductor, a molluscan muscle, are similar in length to those of vertebrate striated muscle and have short (1.9 μm), regular A bands. These muscles are also similar to insect flight muscle, and both will be referred to as Class I. Specific anti-*Limulus* paramyosin stains long (~3.0 μm) sarcomeres throughout the A bands. Brighter fluorescence occurs at both the lateral edges of the A band and in a single central line (Fig. 6*a*, *b*).

All of the arthropod muscles studied here have long sarcomeres (>5.0 μm) and long A bands (≥ 3.0 μm) as compared to vertebrate striated muscle. We will refer to these muscles as Class II on the basis of their structural similarity.

We have previously described the patterns of A-band staining of *Limulus* sarcomeres at various lengths using absorbed, non-PAB-cellulose isolated specific anti-paramyosin (21). With PAB-cellulose isolated specific anti-*Limulus* paramyosin, the patterns are the same as seen before, in the cases of long (11.0–8.0 μm) and short (<6.5 μm) but not in the case of intermediate-length (8.0–6.5 μm) sarcomeres. In the latter case, the central A-band staining, previously observed with nonpurified antibody (21), is now absent (compare Fig. 7*a–c*).

In *Homarus* myofibrils, the entire A band is stained in short sarcomeres (~5.5 μm). In longer sarcomeres (~7.5 μm), a narrow central region remains nonfluorescent (Fig. 8*a–c*). In phase micrographs paired with the fluorescent ones, this unstained zone is identified as the H band.

In *Balanus* myofibrils, short (~5.0 μm) and long (~9.0 μm) sarcomeres stain only at the lateral edges of the A bands. In intermediate-length (~7.0 μm) sarcomeres, the entire A band is stained except for a narrow central region (Fig. 9*a–f*). The most intense staining borders this unstained central region.

Short sarcomeres (~5.0 μm) of *Lethocerus* air tube retractor myofibrils exhibit diffuse A-band staining. In longer (~11.0 μm) sarcomeres, the central A-band staining is less intense than that seen at the lateral edges of the A band (Fig. 10*a–c*).

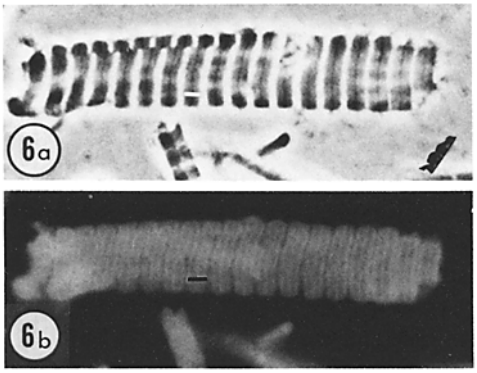
Whole fiber preparations of glycerinated *Mytilus* ABRM, a smooth, molluscan catch muscle (32), contain cells 1.8 mm long and 10 μm in diameter (33). Neither fibrils nor definable sarcomeres are present. These fibers, together with those of *Mercenaria* opaque adductor, constitute a third class of muscle fiber (Class III). Entire fibers stain diffusely with specific anti-*Limulus* paramyosin.

To summarize the results of staining with specific antinative *Limulus* paramyosin: in Class I fibers, at the sarcomere lengths we studied, staining occurs in narrow bands at the lateral edges and in the center of the A band. In Class II fibers, lengthened sarcomeres stain at the lateral edges of the A band. The staining region extends toward the center of the A band as the sarcomeres shorten, until, at short sarcomere lengths, the entire A band is stained. The staining of *Balanus* short sarcomeres differs from this general pattern, being at the lateral edges of the A bands. Diffuse staining occurs throughout Class III fibers.

DISCUSSION

We have established that a protein band seen in gels of homogenates of seven different invertebrate muscles contains paramyosin polypeptide chains. First, this band has a chain weight identical to that of purified *Limulus* paramyosin (115,000 ± 4,000 daltons). Second, this band is absent from gels of vertebrate skeletal muscle homogenates, which do not contain paramyosin. Third, we have shown that this band is not a product of degradation of myosin heavy chains. When myosin breakdown does occur, as illustrated by our gels of aged *Limulus* myosin and paramyosin, the resultant polypeptide fragments never exhibit the same mobility as *Limulus* paramyosin chains. Fourth, and most conclusively, the protein eluted from the band unique to our gels of invertebrate muscle cross-reacts with specific anti-*Limulus* paramyosin. We also show that in the case of *Limulus* muscle this band is not contaminated with myosin fragments, since protein eluted from the paramyosin band of *Limulus* gels does not cross-react with anti-*Limulus* myosin. Similar results were obtained with anti-*Limulus* myosin run against the other paramyosin bands eluted from gels. However, the interpretation of these last results is equivocal, since the immunologic cross-reactivity among the different myosins is not known.

We have established that there are, indeed, basic similarities among the paramyosins in all of the muscles we studied. First, all paramyosins in our gels of muscle homogenates have the same chain weight (115,000 ± 4,000 daltons). This is



FIGURES 6–10 Photomicrographs of myofibrils prepared from glycerinated invertebrate striated muscles and stained with anti-*Limulus* paramyosin by the indirect fluorescent antibody technique. Lines indicate A bands.

FIGURE 6 *Aequipecten* striated adductor myofibrils incubated with specific anti-*Limulus* paramyosin. × 1,000 (*a*) and (*b*) paired phase and fluorescence photomicrographs of myofibrils with long (~3.0 μm) sarcomeres.

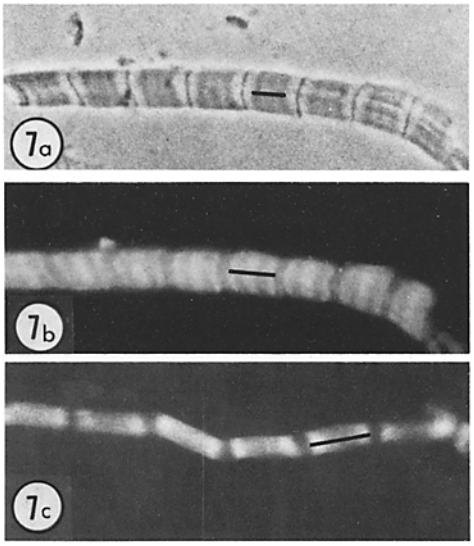


FIGURE 7 *Limulus* telson myofibrils. × 1,000. (*a*) Phase photomicrograph of intermediate-length (7.5 μm) sarcomeres paired with (*b*). (*b*) Fluorescent photomicrograph of same myofibril incubated with anti-*Limulus* paramyosin before PAB-cellulose isolation of the specific antibody. [(*a*) and (*b*) reprinted with permission from *J. Cell Biol.* 1972. **55**:226.]. (*c*) Fluorescence photomicrograph of a myofibril with sarcomeres of lengths similar to those seen above incubated with specific anti-*Limulus* paramyosin that had been isolated by affinity chromatography with PAB-cellulose.

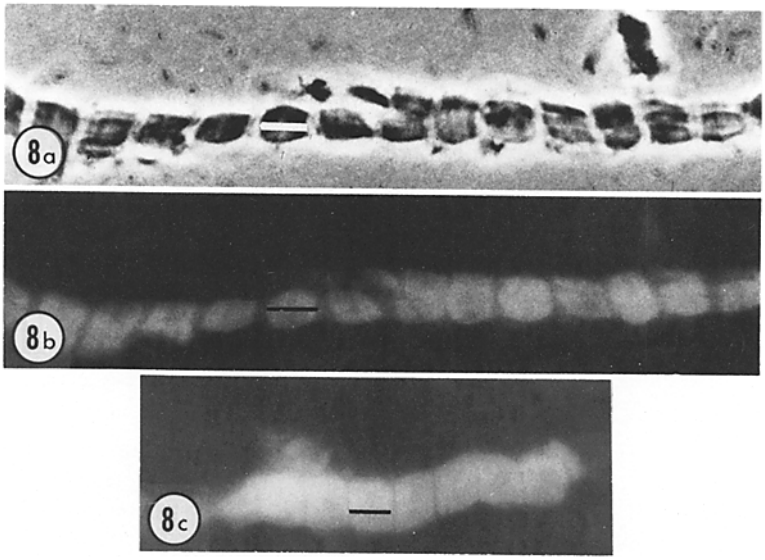
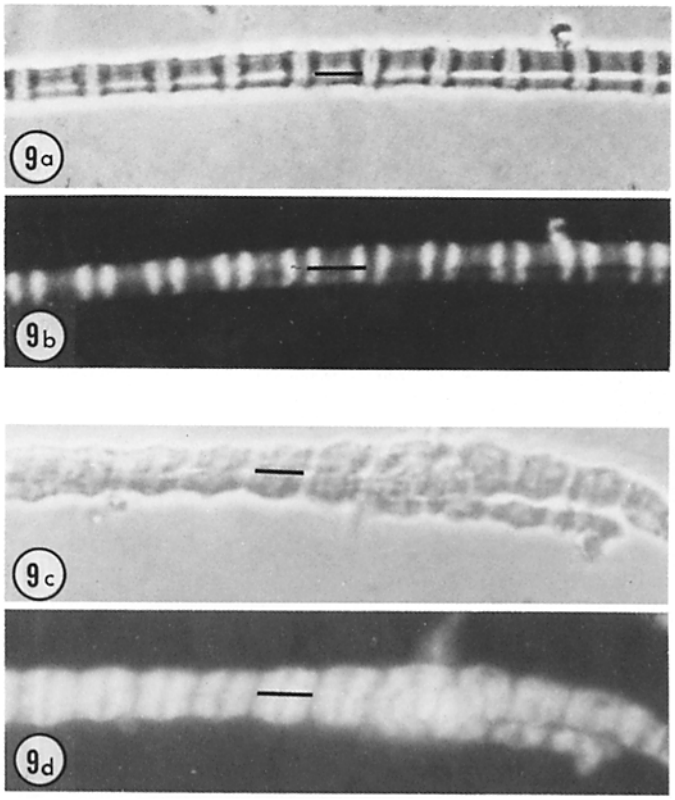


FIGURE 8 *Homarus* claw myofibrils. × 1,000. (*a*) Phase photomicrographs of myofibril with sarcomeres ~7.5 μm long. This picture is paired with (*b*). (*b*) Fluorescence photomicrograph of the same myofibril incubated with PAB-cellulose isolated specific anti-*Limulus* paramyosin. (*c*) Fluorescence photomicrograph of a myofibril with 5.5 μm sarcomeres.



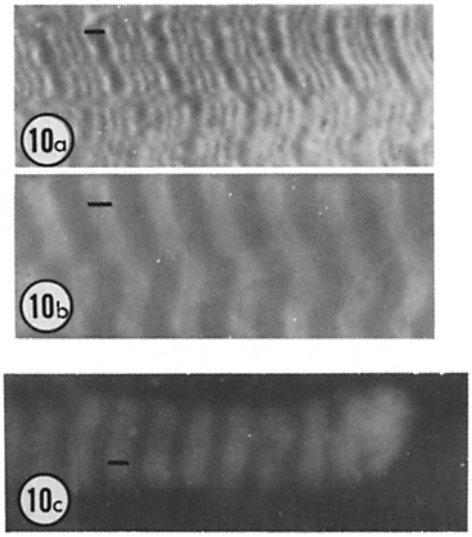


FIGURE 10 *Lethocerus* air tube retractor myofibrils. All myofibrils were incubated with specific anti-*Limulus* paramyosin. × 1,000. (*a*) and (*b*) Paired phase and fluorescence photomicrographs of two myofibrils with long (~11.0 μm) sarcomeres. As evident in the phase picture, this myofibril was pulled out to non-overlap of thick and thin filaments. (*c*) Fluorescence photomicrograph of myofibril with short (~5.0 μm) sarcomeres. Note: This muscle has the shortest A bands of all the Class II muscles studied here.

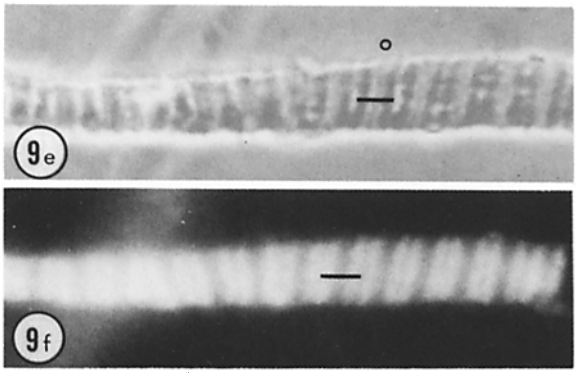


FIGURE 9 *Balanus* scutal depressor myofibrils. All myofibrils were incubated with PAB-cellulose isolated specific anti-*Limulus* paramyosin. × 1,000. (*a*) and (*b*) Paired phase and fluorescence photomicrographs of long (~9.0 μm) sarcomeres. (*c*) and (*d*) Paired phase and fluorescence photomicrographs of intermediate (~7.0 μm)-length sarcomeres. (*e*) and (*f*) Paired phase and fluorescence photomicrographs of short (~5.0 μm) sarcomeres.

identical to the chain weight of *Limulus* paramyosin run on our gels which was isolated by the method of de Villafranca and Haines (5), and is at variance with previous reports of paramyosin molecular weights obtained on extracted proteins from different species (2, 3, 5, 35). We, like Stafford and Yphantis (30), find lower chain weights (102,000; 97,000 daltons) for *Mercenaria* paramyosin isolated according to the method of Johnson et al. (16). Stafford and Yp-

hantis (30) reported that enzymatic degradation of *Mercenaria* paramyosin to β and γ chains (100,000 and 95,000 daltons) occurs during this method of preparation. They modified the isolation procedure to minimize proteolytic degradation and obtained a fraction with 105,000 daltons chain weight, termed α-paramyosin. They believe this to be the intact molecule. Our estimate of *Mercenaria* paramyosin chain weight, from gels of glycerinated muscle homogenates, is still higher than that of α-paramyosin chains, and agrees with the chain weights of paramyosin in all of the invertebrate muscles discussed here. Therefore, proteolysis is an important factor to be considered in the estimation of correct paramyosin chain weight. Our results suggest that the procedure used in preparing *Limulus* paramyosin (5) minimizes alteration of the protein. By dealing exclusively with homogenates of glycerinated muscle, we seem to have avoided proteolysis of paramyosin chains, and our estimates are probably closest to the true chain weight.

Even more stringent evidence for similarity among the various paramyosins is the immunologic cross-reactivity observed with specific anti-*Limulus* paramyosin. This was demonstrated by (*a*) the formation of precipitin lines when the protein eluted from the gel band was diffused against specific antidenatured *Limulus* paramyosin; and (*b*) the fluorescent antibody staining of myofibrils of the striated muscles, with specific antinative *Limulus* paramyosin. These results indicate that there must be considerable homology in the amino acid sequence and/or the tertiary configuration of the different paramyosins.

We have utilized the ability of all of the paramyosins to cross-react with specific anti-*Limulus* paramyosin in order to examine the localization of this protein in sarcomeres of the striated muscles at different lengths. Paramyosin localization to the A bands of all of the striated muscles we studied is strong evidence that this protein is a component of the thick filaments. Specific anti-*Limulus* paramyosin consistently stains the lateral regions of the A bands in all of the muscles we studied, while more central A-band staining varies with muscle type and sarcomere length. In general, extreme lateral A-band staining may be explained as due to exposure of paramyosin resulting from the bending of cortical myosin molecules away from the thick filament surface at the tapered filament ends. This is analogous to the explanation proposed by Pepe (24, 26) regarding lateral A-band staining with anti-chicken myosin in vertebrate striated muscle.

Class I muscles (*Aequipecten* striated adductor and *Lethocerus* flight muscle; reference 22) are similar to vertebrate striated muscle. These stain in narrow bands at the lateral edges and in the central region of the A band. *Aequipecten* long (3.0 μm) sarcomeres also stain diffusely throughout the A band. Exposure of the paramyosin core to antibody binding in the center of the A band of these muscles suggests that there is a region in the middle of the thick filaments in which paramyosin may not be completely covered by myosin.

In the Class II arthropod muscles, more paramyosin becomes available for antibody binding as regions of thick-thin filament overlap increase. This is indicated by broadening of the lateral staining region with decreasing sarcomere length. Exposure of paramyosin along the entire region of overlap between thick and thin filaments indicates that crossbridges are lifted farther away from the filament core, where they interact with actin, than they are in the H zone, which remains unstained. Complete overlap thus results in entire A-band staining of short sarcomeres. *Balanus* short sarcomeres, showing lateral A-band staining, are an exception to this generalization. It is not clear whether this staining pattern is related to the observation that, in such short sarcomeres of *Balanus* muscle, thick filaments penetrate the Z band and terminate in adjacent sarcomeres (14).

The complete misalignment of thick filaments and absence of identifiable sarcomeres in *Mytilus* ABRM (Class III) result in diffuse staining of the entire muscle fiber.

The A-band staining patterns we have observed remain consistent with the hypothesis that paramyosin constitutes the core of thick filaments in paramyosin-containing invertebrate striated muscles (2, 13, 15, 20–22). This agrees with the location proposed for paramyosin in the thick filaments of molluscan smooth catch muscles (3, 7, 29, 31) in which the protein is believed to play a role in the catch mechanism. The nature of that role, however, remains obscure. It has recently been suggested (8), on the basis of in vitro hybrid filament formation, that paramyosin may act to "turn off" catch. The function of paramyosin in noncatch muscles is certainly unknown. As a first step toward understanding the possible role(s) of paramyosin in both types of muscle, we have determined the paramyosin:myosin heavy chain ra-

tios in the thick filaments of the muscles studied here. This work is described in the next paper (23).

The authors are indebted to Dr. Frank A. Pepe and Dr. Benjamin Walcott for *Lethocerus* abdomens and Dr. George de Villafranca for supplying purified *Limulus* and *Mercenaria* paramyosins. We thank Laila Alidina for help in purification of the antibody, and we wish to acknowledge the technical assistance of Harriet Stout and the secretarial help of Alice A. Sydlowski.

This work was supported in part by a grant from the Arcadia Foundation, Norristown, Pa., United States Public Health Service grants GM 20628, GM 21956, and United States Public Health Service grant HL 15835 to the Pennsylvania Muscle Institute. R. Levine is the recipient of a Research Career Development Award from the National Institute of Neurological and Communicable Diseases and Stroke (NS 70476).

Received for publication 10 March 1976, and in revised form 9 June 1976.

REFERENCES

1. BAILEY, K. 1957. Invertebrate tropomyosin. *Biochim. Biophys. Acta.* **24:**612-619.
2. BULLARD, B., B. LUKE and L. WINKELMAN. 1973. The paramyosin of insect flight muscle. *J. Mol. Biol.* **75:**359-367.
3. COHEN, C., A. G. SZENT-GYÖRGYI, and J. KENDRICK-JONES. 1971. Paramyosin and the filaments of molluscan "catch" muscles. I. Paramyosin: structure and assembly. *J. Mol. Biol.* **56:**223-237.
4. DE VILLAFRANCA, G. W. and V. E. LEITNER. 1967. Contractile proteins from horseshoe crab muscle. *J. Gen. Physiol.* **50:**2495-2496.
5. DE VILLAFRANCA, G. W. and V. E. HAINES. 1974. Paramyosin from arthropod cross-striated muscle. *Comp. Biochem. Physiol.* **47B:**9-26.
6. DEWEY, M. M., R. J. C. LEVINE, and D. E. COLFLESH. 1973. Structure of *Limulus* striated muscle: the contractile apparatus at various sarcomere lengths. *J. Cell Biol.* **58:**574-593.
7. ELLIOTT, A. 1974. The arrangement of myosin on the surface of paramyosin filaments in the white adductor of *Crassostrea angulata*. *Proc. R. Soc. Lond. B. Biol. Sci.* **186:**53-66.
8. EPSTEIN, H. F., B. J. ARONOW, and H. E. HARRIS. 1975. Interaction of myosin and paramyosin. *J. Supramol. Struct.* **3:**354-360.
9. GORNALL, A. G., C. J. BARDAWELL, and M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:**751-766.
10. HANSON, J. and J. LOWY. 1960. Structure and function of the contractile apparatus in the muscles of invertebrate animals. *In* Structure and Function of Muscle. 1st edition. G. H. Bourne editor, Academic Press, Inc., New York. Chap. IX. **1:**265-336.
11. HANSON, J. and J. LOWY. 1961. The structure of the muscle fibers in the translucent part of the adductor of the oyster *Crassostrea angulata*. *Proc. R. Soc. Lond. B. Biol. Sci.* **154:**173-196.
12. HANSON, J., J. LOWY, H. E. HUXLEY, K. BAILEY, C. M. KAY, and J. RUEGG. 1957. Structure of molluscan tropomyosin. *Nature (Lond.).* **180:** 1134-1135.
13. HARDWICKE, P. M. D. and J. HANSON. 1971. Separation of thick and thin myofilaments. *J. Mol. Biol.* **59:**509-516.
14. HOYLE, G., J. H. MCALEAR and A. I. SELVERSTON. 1965. Mechanism of supercontraction of a striated muscle. *J. Cell Biol.* **26:**621-640.
15. IKEMOTO, N. and S. KAWAGUTI. 1967. Elongating effect of tropomyosin A on thick filaments in the long sarcomere muscle of the horseshoe crab. *Proc. Jpn. Acad.* **43:**974-979.
16. JOHNSON, W. H., J. S. KAHN, and A. G. SZENT-GYÖRGYI. 1959. Paramyosin and contraction of "catch muscles". *Science* (Wash. D. C.) **130:**160-161.
17. KOMINZ, D. R., F. SAAD, and K. LAKI. 1957. Chemical characteristics of annelid, mollusc and arthropod tropomyosins. *In:* Conference on the Chemistry of Muscular Contraction. Igako Shoin, Ltd., Tokyo. 66-76.
18. KUNDRAT, E. and F. A. PEPE. 1971. The M band: studies with fluorescent antibody staining. *J. Cell Biol.* **48:**340-347.
19. LAZERIDES, E. 1975. Tropomyosin antibody: the specific localization of tropomyosin in nonmuscle cells. *J. Cell Biol.* **65:**549-561.
20. LEVINE, R. J. C., M. M. DEWEY, and D. E. COLFLESH. 1973. Unusual striated thick filaments in *Limulus* skeletal muscle. *J. Cell Biol.* **57:**591-593.
21. LEVINE, R. J. C., M. M. DEWEY, and G. W. DE VILLAFRANCA. 1972. Immunohistochemical localization of contractile proteins in *Limulus* striated muscle. *J. Cell Biol.* **55:**221-235.
22. LEVINE, R. J. C., M. M. DEWEY, M. ELFVIN, and B. WALCOTT. 1974. *Lethocerus* flight muscle paramyosin: antibody localization and electrophoretic studies. *Am. J. Anat.* **141:**453-458.
23. LEVINE, R. J. C., M. ELFVIN, M. DEWEY, and B. WALCOTT. 1976. Paramyosin in invertebrate muscle. II. Content in relation to structure and function. *J. Cell Biol.* **71:**273-279.
24. PEPE, F. A. 1971. Structure of the myosin filament in striated muscle. *Prog. Biophys. Mol. Biol.* **22:**77-96.
25. PEPE, F. A. 1973. The myosin filament: immunochemical and ultrastructural approaches to molecular organization. *Cold Spring Harbor Symp. Quant. Biol.* **37:**97-108.

26. PEPE, F. A. 1975. Structure of muscle filaments from immunohistochemical and ultrastructural studies. *J. Histochem. Cytochem.* **23:**543–562.
27. PEPE, F. A. and B. DRUCKER. 1975. The myosin filament. III. C-protein. *J. Mol. Biol.* **99:**609–617.
28. PHILPOTT, D. E., M. KAULBROCK, and A. G. SZENT-GYÖRGYI. 1960. Filamentous organization of molluscan muscles. *J. Ultrastruct. Res.* **3:**254–269.
29. SOBIESZEK, A. 1973. The fine structure of the contractile apparatus of the anterior byssus retractor muscle of *Mytilus edulis*. *J. Ultrastruct. Res.* **43:**313–343.
30. STAFFORD, W. F. III, and D. A. YPHANTIS. 1972. Existence and inhibition of hydrolytic enzymes attacking paramyosin in myofibrillar extracts of *Mercenaria mercenaria*. *Biochem. Biophys. Res. Comm.* **49:**848–854.
31. SZENT-GYÖRGYI, A. G., C. COHEN, and J. KENDRICK-JONES. 1971. Paramyosin and the filaments of molluscan "catch" muscles. II. Native Filaments: isolation and characterization. *J. Mol. Biol.* **56:**239–258.
32. TWAROG, B. M. 1967. The regulation of catch in molluscan muscle. *J. Gen. Physiol.* **50:**157–169.
33. TWAROG, B. M., M. M. DEWEY, and T. HIDAKA. 1973. The structure of *Mytilus* smooth muscle and the electrical constants of the resting muscle. *J. Gen. Physiol.* **61:**207–221.
34. WEBER, K. and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl-sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:**4406–4412.
35. WOODS, E. F. 1969. Subunit structure of oyster paramyosin. *Biochem. J.* **113:**39–47.