SOME ASPECTS OF THE STRUCTURAL ORGANIZATION OF THE MYOFIBRIL AS REVEALED BY ANTIBODY-STAINING METHODS

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ABSTRACT

From observations of fluorescent antibody staining and antibody staining in electron microscopy, evidence is presented for the following: (*a*) Direct contact of the actin and myosin filaments occurs at all stages of contraction. This results in inhibition of antibody staining of the H-meromyosin portion of the myosin molecule in the region of overlap of the thin and thick filaments. (*b*) Small structural changes occur in the thick filaments during contraction. This leads to exposure of antigenic sites of the L-meromyosin portion of the myosin molecule. The accessibility of these antigenic sites is dependent upon the sarcomere length. (*c*) The M line is composed of a protein which is weakly bound to the center of the thick filament and is not actin, myosin, or tropomyosin. (*d*) Tropomyosin as well as actin is present in the I band. (*e*) If actin or tropomyosin is present in the Z line, it is masked and unavailable for staining with antibody.

INTRODUCTION

The sliding filament model for muscle is now generally accepted (15, 16). The essential feature of this model is that the myofibril consists of two types of filaments, thick and thin. The thick filaments are found in the A band and they extend from one end of the A band to the other. The thin filaments extend from the Z line to the H zone. The two types of filaments are arranged in hexagonal array where they overlap in the A band. Cross-bridges are present on the thick filaments and represent the sites of interaction with the thin filaments. There is an increase in thickness of the thick filament in the center of the H zone forming the M line.

The major protein constituents of the myofibril are actin, myosin, and tropomyosin. There is now considerable evidence that the thick filaments contain mysoin and that the thin filaments contain actin (12, 26). The manner in which tropomyosin fits into the structure of the myofibril is not clear. Suggestions have been made that it may be present in association with the I band (11, 18, 28) and that it may be involved in the structure of the Z line (12).

Antibody staining techniques have been used in fluorescent microscopy (18, 35, 36) and electron microscopy (25–27) to determine the distribution of the muscle proteins, actin, and myosin. The localization of antibody to H-meromyosin and L-meromyosin, the tryptic digestion products of myosin (17, 19, 33), has likewise been studied. Preliminary observations have been made which suggest that tropomyosin is present in the I band (12). Using antibody-staining techniques, the findings which are consistent with the requirements of the sliding filament model are: (*a*) anti-

myosin was found to stain the A band in fluorescent microscopy and the thick filaments specifically in electron microscopy; (*b*) antiactin was found to stain the I band in fluorescent microscopy and the thin filaments specifically in electron microscopy. The findings which provide the major inconsistencies with the requirements of the sliding filament model are: (*a*) Although fluorescent antimyosin, in general, localized throughout the A band, anti–H-meromyosin localized strongly in a central line in the A band and less strongly laterally in the A band; whereas anti–L-meromyosin localized only laterally in the A band. (*b*) In contracted fibrils, fluorescent antimyosin staining became discontinuous in the A band. An unstained central region increased in width with increased contraction. (*c*) Fluorescent antiactin stained the I band but not the region in the A band up to the H zone where thin filaments interdigitate with the thick filaments. Fluorescent antiactin also stained very heavily in a fine line in the center of the A band of relaxed sarcomeres where no thin filaments are present.

It is the purpose of this investigation (*a*) to make use of the antibody-staining technique to obtain additional information concerning the structural organization of the myofibril, and (*b*) to arrive at an understanding of the inconsistencies between the sliding filament model and the results of antibody staining.

MATERIALS AND METHODS

Preparation of Antibody

Actin (4, 21), myosin (22), and tropomyosin (1, 2) were prepared from chicken breast muscle. Purified actin and myosin were stored at −24°C in 50% glycerol containing 5×10^{-2} M KCl, 5×10^{-4} M $MgCl_2$, 5×10^{-3} M PO_4 buffer pH 7.0. Tropomyosin was stored as the dried powder. Albino rabbits weighing 8 to 10 lb. were initially given two injections. A solution of 10 mg of protein in saline buffered with M/60 PO_4 pH 7.4 was injected intraperitoneally. This same solution was emulsified with an equal volume of Freund's adjuvant (complete, Difco Laboratories, Inc., Detroit, Michigan) and 5 mg of protein were injected subcutaneously at the back of the neck. Intraperitoneal injections of 10 mg of protein in buffered saline were continued once a week thereafter. Rabbits were bled once or twice a week from the lateral ear vein after a maximal antibody titer was obtained. Final bleeding of the rabbits was by cardiac puncture. The antiserum against each protein antigen was pooled and the γ_2-globulin fraction was obtained by ethanol fractionation (6). Normal γ_2-globulin was similarly obtained. The isolated globulins were stored frozen in saline made up in M/60 PO_4 buffer pH 7.4. The antibody against L-meromyosin (ethanol-resistant fraction) and H-meromyosin was that previously used (18).

Fluorescent Labeling

Both normal and immune globulins were labeled with fluorescein isothiocyanate (23). The conjugates were dialyzed overnight against saline (0.15 M NaCl) made up in M/60 PO_4 buffer pH 7.4. All conjugates were then passed through Sephadex (G-25, medium) (obtained from Pharmacia Fine Chemicals Inc., New Market, New Jersey). They were dialyzed against 25% glycerol containing 7.5×10^{-2} M KCl, 7.5×10^{-4} M $MgCl_2$, 7.5×10^{-3} M PO_4 buffer pH 7.0 (hereafter referred to as "buffered glycerol") and were stored at −24°C.

Antibody Absorption

In order to find the ratio of antigen to antibody necessary for complete absorption of the antibody, a series of mixtures was made with increasing antigen to antibody ratio. These mixtures were used to stain fibrils. The ratio at which no further change in antibody-staining pattern occurred was considered optimal. Mixtures of antigen and antibody in optimal ratio were then treated for removal of excess antigen as described below. No precautions were taken to remove all traces of the antigen, the object being to remove only the greater part of the excess antigen. All final antibody-staining solutions were in buffered glycerol. These were stored at −24°C until needed.

Antimyosin was absorbed with H-meromyosin and L-meromyosin. The meromyosins were prepared according to the directions given by Lowey and Cohen (17) with a 50 min digestion time. The ethanol-resistant fraction of L-meromyosin was used for absorption. The optimal mixtures of antimyosin and L-meromyosin and of antimyosin and H-meromyosin were dialyzed against a solution containing 5×10^{-2} M KCl, 5×10^{-4} M $MgCl_2$, 5×10^{-3} M PO_4 buffer pH 7.0. Abundant precipitate formed in the solution containing L-meromyosin. Both solutions were centrifuged ½ hr at 29,000 RPM in a Beckman Spinco model L preparative ultracentrifuge in a number 30 rotor. The L-meromyosin–absorbed antimyosin was dialyzed against buffered glycerol and stored at −24°C until used. F-actin in a solution containing 5×10^{-2} M KCl, 5×10^{-4} M $MgCl_2$, 5×10^{-3} M PO_4 buffer pH 7.0 was added to the H-meromyosin–absorbed antimyosin. Approximately 30 mg of F-actin were added for 20 mg of absorbed antibody. The mixture was allowed to stand overnight in the refrigerator and was then centrifuged for 1 hr at 29,000 RPM in the model L preparative ultracentri-

fuge. The supernatant was dialyzed against buffered glycerol and stored at −24°C until used for staining.

Likewise, F-actin was mixed with either antiactin or antitropomyosin in various ratios to obtain the optimal ratio for absorption. Mixtures consisting of the optimal ratios were dialyzed against 5×10^{-2} M KCl, 5×10^{-4} M $MgCl_2$, 5×10^{-3} M PO_4 buffer pH 7.0 and were then centrifuged at 29,000 RPM for ½ hr. The supernatants were dialyzed against buffered glycerol and stored at −24°C until used for staining.

Tropomyosin was mixed with either antiactin or antitropomyosin in various ratios. Mixtures of the optimal ratios were allowed to stand in the cold for 1 day and were then dialyzed against 0.01 M acetate buffer pH 5.2 containing 16 g of $(NH_4)_2SO_4$ per liter (1). The precipitate formed was centrifuged for ½ hr at 29,000 RPM and the supernatant was dialyzed against buffered glycerol and stored at −24°C until used for staining.

Preparation and Staining of Fibrils

Chicken breast muscle was excised in long strips parallel to the fiber axis and tied to plastic rods at rest length. These were placed in 50% glycerol previously deionized with Amberlite MB-1 resin. They were stored at 2 to 3°C for 2 days. The glycerol was then changed and the muscle was stored at −24°C for at least 3 wk before use. When needed, portions of these fiber bundles were cut off and placed in buffered glycerol for ½ hr. The muscle was shredded with a sharp needle drawn parallel to the fiber axis and allowed to stand for ½ hr. The shredded muscle was then blended in a Servall omnimixer. The fibril suspension was centrifuged at top speed in an international clinical centrifuge for 10 min. The packed fibrils were washed twice by resuspension in fresh solution. Final suspension was made to 10 times the volume of the packed fibrils. All procedures were carried out at 2 to 3°C.

All fluorescent antibody concentrations were in the range 10 to 15 mg/ml. For antimyosin staining, 3 drops of fluorescent antimyosin were added to 2 drops of the fibril suspension. In general, this ratio was also maintained in staining with the absorbed antibody. With the absorbed antibody, a ratio of 9 drops of antibody to 2 drops of fibrils was occasionally used. For anti-H-meromyosin and anti-L-meromyosin staining, 5 drops of antibody were added to 2 drops of fibril suspension. For antiactin and antitropomyosin staining, 5 drops of antibody were added to 2 drops of fibril suspension. This ratio was maintained for all the absorbed preparations. All antibody staining took place overnight at 2 to 3°C. Material was prepared for electron microscopy as previously described (26).

Microscopy

For fluorescent microscopy, a Reichert research microscope with a Zeiss cardiod condenser and a 100× achromatic oil immersion objective containing an ultraviolet filter was used. An Osram HBO-200 lamp provided illumination. The incident light was filtered through a 1 mm Corning 5840 and a 1 mm Schott UG-1 filter combination. Kodak 103a-G spectroscopic plates and Kodak D-19 developer were used. Exposures varied from 5 to 15 min, depending on fluorescence intensity. An AO objective with a magnification of 97 and a matching AO condenser were used for phase-contrast microscopy.

For electron microscopy, a Siemens Elmiskop I microscope was used. Accelerating voltage was 80 kv with a 400 μ condenser II aperture and a 50 μ objective aperture.

RESULTS

Antimyosin

Fluorescent antimyosin has been shown to stain the A band of relaxed striated myofibrils specifically (18, 35, 36). Occasionally, variations in the distribution of antibody in the A band were observed. In the present investigation it was found that the staining pattern obtained with antimyosin was dependent on the amount of antibody used to stain a given quantity of fibrils. With a large excess of antibody, fluorescent staining was observed throughout the A band. When the amount of antibody used was decreased, four fluorescent bands were consistently obtained, two medial and two lateral in the A band (Fig. 1 *a*). Further decrease of antibody resulted in disappearance of the lateral bands before the medial bands (Fig. 1 *b*).

In electron microscopy the specific staining of only thick filaments with antimyosin has been observed (25, 26). The thick filament stained uniformly along its length except for a small central portion (Fig. 1 *c*). This central portion would correspond to the unstained area between the two medial fluorescent bands observed in Fig. 1 *a* and *b*.

Antimyosin Absorbed with H-Meromyosin or L-Meromyosin

The myosin molecule can be split into two parts by trypsin (17, 19, 33). These are the meromyosins. H-meromyosin is a globular protein and carries the ATPase activity of the original myosin molecule (20, 33) as well as its ability to combine with

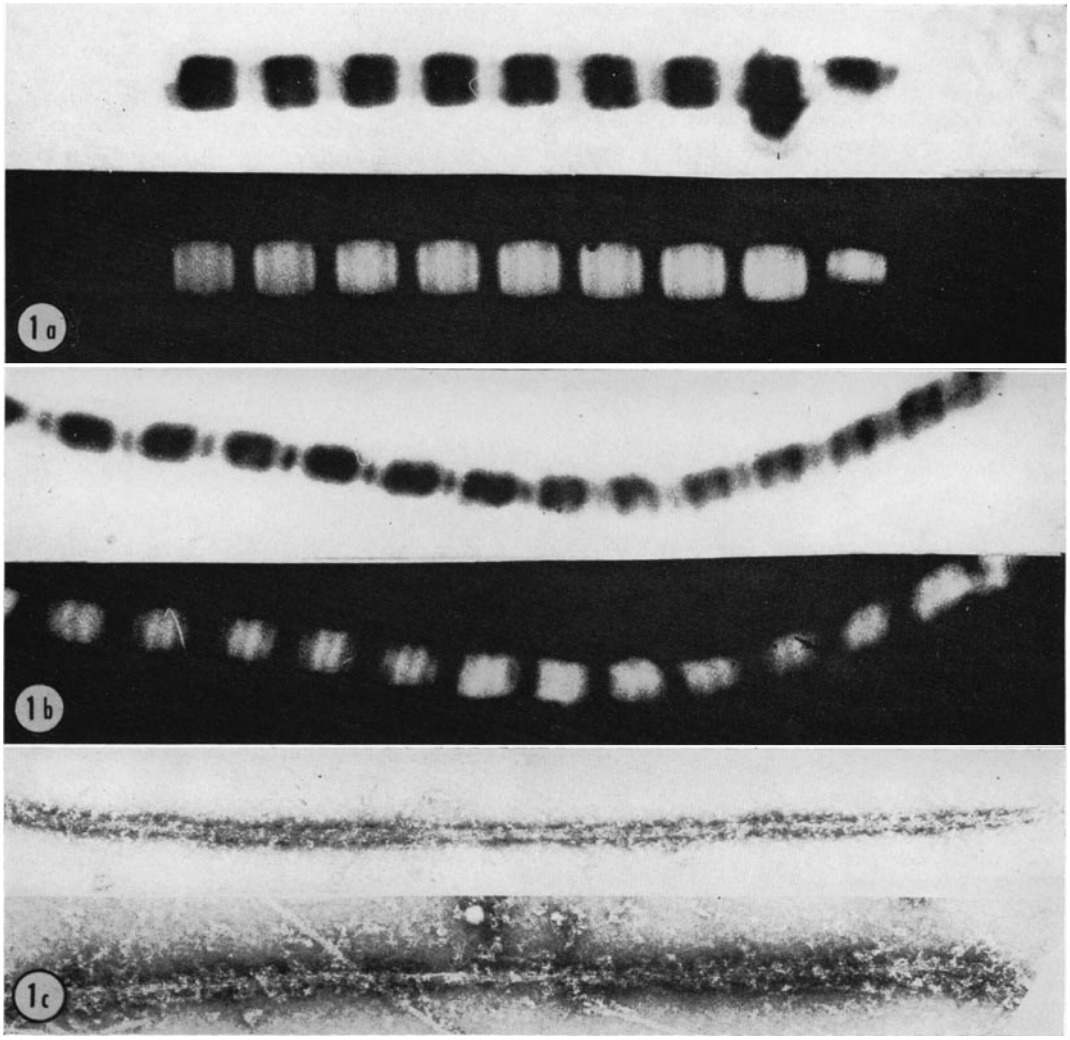


FIGURE 1 Antimyosin staining.

FIGURE 1 *a* The same relaxed fibril is shown in phase-contrast (upper) and fluorescence (lower) for identification of fluorescence-stained areas. Note four fluorescent bands in the A band, two medial and two lateral. Phase-contrast, × 4400; fluorescence, × 4500.

FIGURE 1 *b* Relaxed fibril treated the same as that in Fig. 1 *a* (except that a lower antibody to fibril ratio was used). Note loss of fluorescent lateral doublet. Phase-contrast, × 4400; fluorescence, × 4500.

FIGURE 1 *c* Staining of separated thick filaments in electron microscopy (26). Normal γ-globulin treated (upper) and antimyosin treated (lower) shown by negative staining. Note adherence of antibody to the thick filament everywhere except along smooth central zone. × 130,000.

actin (12, 20, 33). L-meromyosin is a rod carrying most of the helical part of the original myosin molecule (34). As has been previously reported (18, 36), fluorescent antibody prepared against the purified meromyosins localized as shown in Figs. 2 *d* and 3 *d*. Anti–H-meromyosin showed strong central line staining, with light staining throughout the rest of the A band. Anti–L-meromyosin

showed strong lateral staining in the A band (18, 36). These localizations can now be compared with those obtained by absorption of antimyosin with the purified meromyosins. If antimyosin is absorbed with a large excess of purified L-meromyosin, as already described, the antibody against antigenic sites on the L-meromyosin portion of the myosin molecule is removed. The remaining antibody is specific for the antigenic sites on the H-meromyosin part of the myosin molecule. Likewise, by absorbing antimyosin with excess H-meromyosin, antibody to antigenic sites on the H-meromyosin is removed. The remaining antibody is specific for the antigenic sites on the L-meromyosin portion of the myosin molecule. As can be seen in Fig. 2 *b*, the two lateral bands have been removed by absorption of antimyosin with L-meromyosin. In Fig. 3 *b* it is evident that the two medial bands have been removed by absorption of antimyosin with H-meromyosin. Slight staining is still visible in the medial bands, probably owing to incomplete removal of antibody to antigenic sites on the H-meromyosin. In order to be sure that these localizations were not a result of dilution of the antibody, fibrils were stained with a threefold increase in the amount of antibody used. This did not change the staining pattern as is seen in Figs. 2 *c* and 3 *c*. Only the intensity of staining increased. Therefore the medial bands observed in antimyosin staining are due to staining of the available antigenic sites on the H-meromyosin part of the myosin molecule, and the lateral bands are due to staining of available antigenic sites on the L-meromyosin part of the myosin molecule.

The staining pattern obtained for antimyosin absorbed with H-meromyosin is identical to that for antibody prepared against L-meromyosin (compare Fig. 3 *b* or 3 *c* and 3 *d*). The staining pattern observed for antimyosin absorbed with L-meromyosin differs from that for antibody prepared against H-meromyosin (compare Fig. 2 *b* or *c* and *d*). In the fluorescent patterns of Fig. 2 *b* and *c*, a medial doublet is present. In the fluorescent pattern of Fig. 2 *d*, a strong central line staining is present, with decreasing fluorescent staining laterally in the A band.

Antimyosin Staining of Contracted Fibrils

The antimyosin staining pattern was studied as a function of sarcomere length (Fig. 4 *a* through *d*). As the sarcomere length decreases, the two medial bands disappear and the lateral bands become wider and more prominent. On further contraction, the lateral bands become narrower again. An observation which does not show up clearly in the pictures is that the lateral staining in the A band increases in brightness as contraction proceeds. Fig. 4 *a* is a 2.3 μ sarcomere. According to the sliding filament model, the thin filaments in this case are only part way into the A band. There is a central area of noninterdigitation of the thick and thin filaments in the A band. The medial doublet is visible. Fig. 4 *b* is a 1.9 μ sarcomere. The thin filaments from opposite sides meet in the center of the A band. There is no area of the A band in which thick and thin filaments do not interdigitate. The medial doublet disappears and the lateral doublet becomes wider and brighter. Fig. 4 *c* is a 1.5 μ sarcomere. The thin filaments from opposite sides overlap in the center of the A band. The lateral doublet becomes narrower. Fig. 4 *d* is a 1.2 μ sarcomere. The picture is similar to that obtained for the 1.5 μ sarcomere.

Antiactin

Antiactin was tested for specificity by layering solutions of various concentrations of F-actin, tropomyosin or myosin over it and by observing if a precipitate formed at the interface. F-actin layered over antiactin showed no precipitate. However, observation of a mixture of F-actin and antiactin in electron microscopy revealed aggregation of the F-actin filaments with the antibody. Mixtures of F-actin with normal γ-globulin showed no aggregation. Therefore, specific reaction of antiactin with F-actin did occur though a precipitate was not seen. Tropomyosin layered over antiactin showed a precipitate at the interface. Myosin layered over antiactin did not show a precipitate. F-actin, tropomyosin, and myosin layered over normal γ-globulin were all negative and served as controls.

Antibody prepared against actin has been described as localizing throughout the I band of the striated myofibril and in a fine line in the center of the A band (18, 36).

It has not been clear whether the Z line is stained or not. As can be seen in Fig. 5 *a*, the Z line is not stained with antiactin. Photography of such a fine unstained band is difficult, due to bleeding from the neighboring stained I bands. Antiactin was absorbed with a large excess of tropomyosin as previously described. This re-

moved any antibody to antigenic sites on tropomyosin. The staining pattern observed with this absorbed antibody is shown in Fig. 5 *b*. The I band staining is diminished but the M line staining is still very strong. Likewise, antiactin was absorbed with excess F-actin to remove any antibody to antigenic sites on actin. As can be seen in Fig. 5 *c*, this also resulted in a diminution of staining in the I band and no decrease in the M line staining.

In electron microscopy the specific staining of the thin filaments is always obtained with antiactin (25, 26). I segments consisting of Z lines with attached thin filaments can be seen, in Fig. 5 *d*, to stain along the entire length of the thin filaments with an approximately 400 A period. In general, during separation of the filaments the antigen responsible for the strong M line staining in the myofibril is lost (26). However, occasionally the M line antigen is retained on the thick filament, and staining of the thick filament at its midpoint can be observed (Fig. 5 *e*).

Antitropomyosin

Antitropomyosin was tested for specificity by layering solutions of various concentrations of F-actin, tropomyosin, or myosin over it and by observing whether a precipitate formed at the interface. Tropomyosin layered over antitropomyosin gave a strong precipitate. F-actin layered over antitropomyosin gave a weak precipitate, and myosin gave no precipitate. Layering of these antigens over normal γ-globulin gave no precipitate and served as controls.

Preliminary investigations suggested that antitropomyosin stains the I band (18). Further studies have verified this, as can be seen in Fig. 6 *a*. Staining is exclusively in the I band. In addition, lack of Z line staining can be observed in some cases. This has also been observed by H. Holtzer (private communication). Antitropomyosin was absorbed with tropomyosin, as previously described. This removed any antibody to antigenic sites on the tropomyosin. Absorption resulted in almost complete removal of the I band staining. The micrograph obtained in Fig. 6 *b* was one of a very few obtainable since the fluorescence was barely detectable in the microscope. Likewise, antitropomyosin was absorbed with a large excess of F-actin. This removed any antibody to antigenic sites on F-actin. There was some diminution of the I band staining (Fig. 6 *c*). In addition, suggestions of a nonuniform distribution of tropomyosin in the I band can be seen. This is being investigated further.

Staining of separated filaments in electron microscopy was difficult to see since the antibody did not appear to go on in any periodic fashion.[1] There seemed to be a general increase in amount of material surrounding the thin filaments, but it was difficult to be certain of this. In the sectioned material shown in Fig. 6 *d*, the increase in amount of material in the I band is somewhat more clearly seen.[1]

DISCUSSION

In addition to confirming results previously reported (18, 35, 36), the results given here add

[1] Obtained in collaboration with H. E. Huxley.

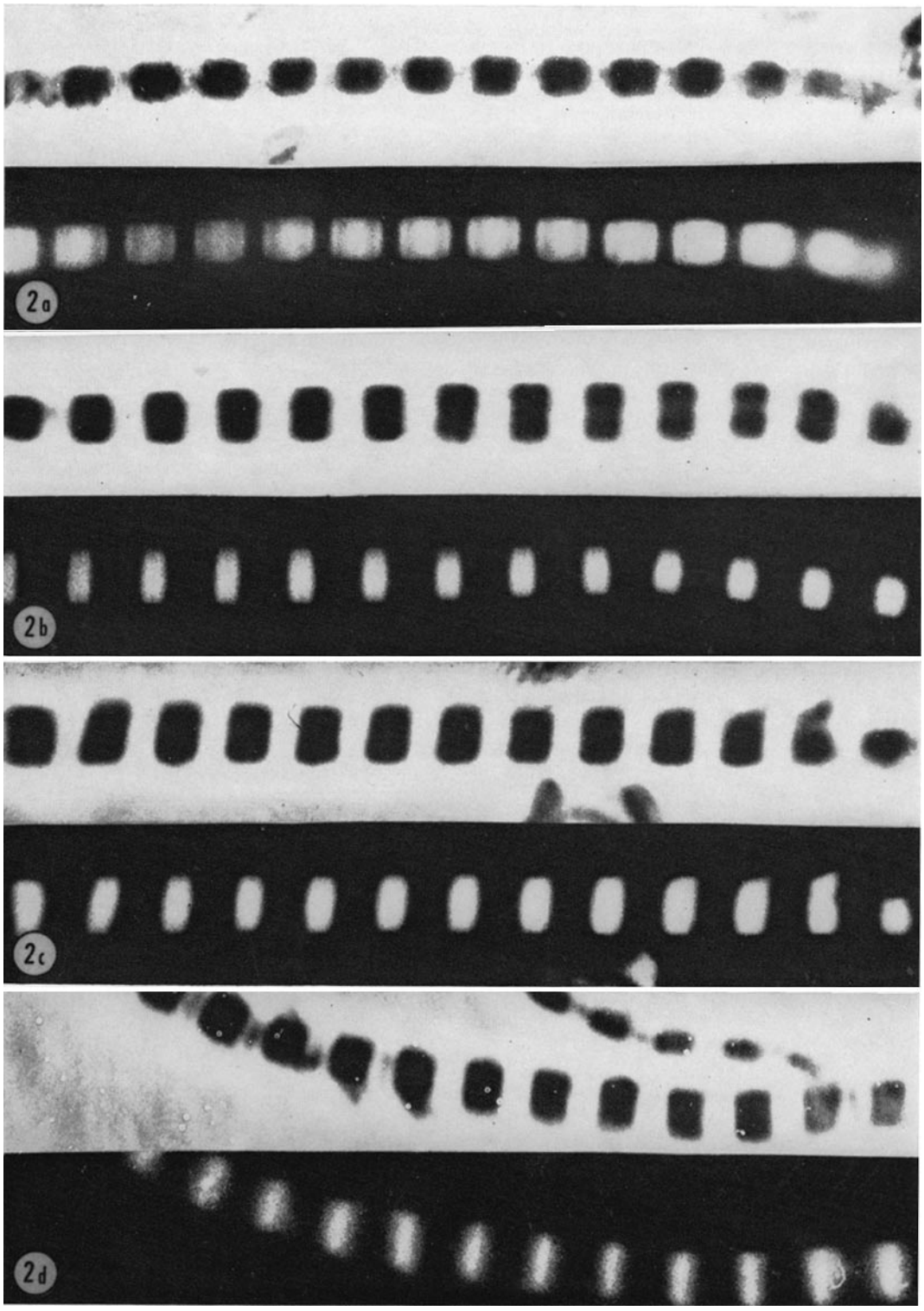
FIGURE 2 Anti–H-meromyosin staining. Phase-contrast, × 4400; fluorescence, × 4500.

FIGURE 2 *a* The same relaxed fibril is shown in phase-contrast (upper) and fluorescence (lower) for identification of fluorescent-stained areas. Antimyosin-stained fibril for comparison with the following micrographs.

FIGURE 2 *b* Antimyosin absorbed with L-meromyosin was used for staining. Antimyosin to fibril ratio is the same as that in Fig. 2 *a*. Note loss of lateral doublet in fluorescence (lower).

FIGURE 2 *c* Fibril treated as in Fig. 2 *b* except that antimyosin to fibril ratio was increased threefold. No change in pattern occurred.

FIGURE 2 *d* Fluorescent anti–H-meromyosin-stained fibril. Note sharp central line staining and decreasing intensity of staining to the lateral edges of the A band (18).


2a
2b
2c
2d

the following observations concerning the localization of specific antibody to the muscle proteins: (*a*) The antimyosin–staining pattern depends on the amount of antibody used for staining. The distribution of antimyosin consists of four bands, two medial and two lateral, in the A band of a relaxed sarcomere. The four bands obtained with antimyosin change with sarcomere length. (*b*) The specificity of localization of the available antigenic sites of H-meromyosin has been improved using the absorption technique. This localization coincides with the medial bands obtained with antimyosin. (*c*) The lateral bands observed with antimyosin represent available antigenic sites of L-meromyosin and correspond to the staining pattern previously observed for anti–L-meromyosin. (*d*) The M line staining with antiactin cannot be removed by absorption with tropomyosin or actin. The M line material is weakly bound to the thick filaments. (*e*) The I band staining with antiactin can be diminished but not entirely removed by absorbing with tropomyosin or actin. The I band staining with antitropomyosin can be almost entirely removed by absorbing with tropomyosin and diminished by absorbing with actin. Antitropomyosin absorbed with actin sometimes gives a nonuniform distribution of stain in the I band. (*f*) The Z line is not stained with antiactin or antitropomyosin. What information concerning the structural organization of the myofibril can be obtained from these additional results?

Antimyosin, Anti–Meromyosin and Anti–H-Meromyosin

Antimyosin has been described as staining the A band specifically. However, the details of the staining pattern observed in the A band have varied (9, 18, 35, 36). As shown above, if the amount of antimyosin used to stain relaxed myofibrils is progressively decreased, four bands consistently become visible, two bands lateral and two medial, in the A band. Further decrease in the amount of antibody used for staining consistently results in diminution and eventual disappearance of the lateral band staining before the medial band staining. The staining pattern is therefore dependent on the amount of antibody used for staining. With large amounts of antibody the intensity of staining obliterates the nonuniformity of the stain in the A band. Disappearance of the lateral bands before the medial bands, on dilution, suggests that the antibody responsible for the lateral band staining is present in a concentration lower than that responsible for the medial band staining.

Antibody prepared against the whole myosin molecule contains antibody to all the antigenic groups on the myosin molecule. These include the antigenic groups of the H-meromyosin part and the L-meromyosin part. Therefore, using antibody specific for each of these parts separately, we can determine the relationship of these four bands to the distribution of the available antigenic sites of each part. Such specific antibody for a portion of the myosin molecule may be prepared in two ways: (*a*) by injecting the purified meromyosin and producing antibody to it; or (*b*) by absorbing antimyosin with one of the meromyosins thus removing all antibody directed against that part of the myosin molecule and leaving antibody specific for the other part. If there are no antigenic sites common to both parts, these two methods should give the same results. If there are antigenic

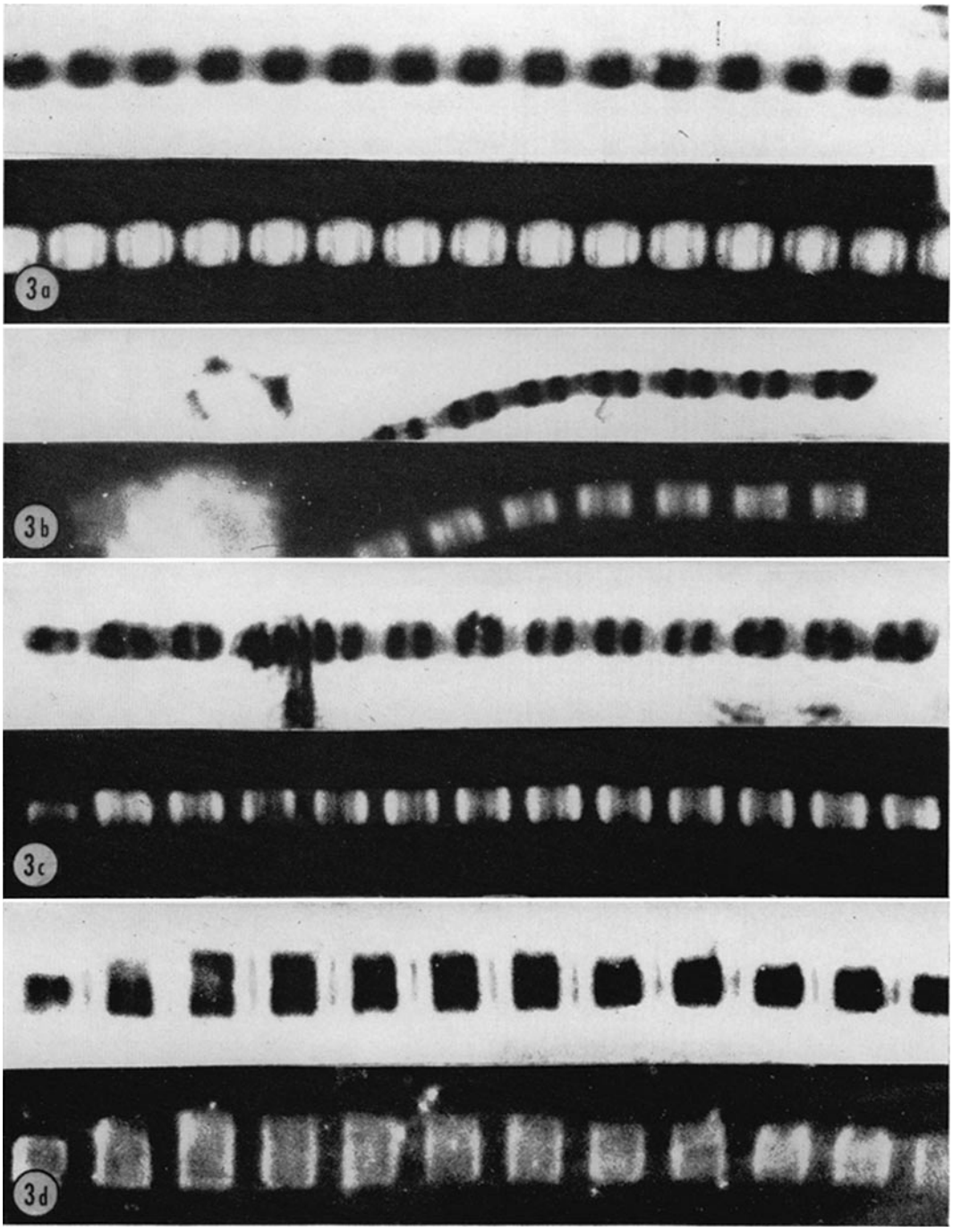
FIGURE 3 Anti–L-meromyosin staining. Phase-contrast, × 4300; fluorescence, × 4400.

FIGURE 3 *a* The same relaxed fibril is shown in phase-contrast (upper) and fluorescence (lower) for identification of fluorescent-stained areas. Antimyosin-stained fibril for comparison with the following micrographs.

FIGURE 3 *b* Antimyosin absorbed with H-meromyosin was used for staining. Antimyosin to fibril ratio is the same as that in Fig. 3 *a*. Note loss of intensity of medial doublet in fluorescence (lower).

FIGURE 3 *c* Fibril treated as in Fig. 3 *b* (except that antimyosin to fibril ratio was increased threefold). No change in pattern occurred.

FIGURE 3 *d* Fluorescent anti–L-meromyosin–stained fibril. Note similarity to staining pattern obtained with the absorbed antibody.


3a
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3c
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sites common to both parts, the results may be different; however, the sum of the patterns obtained for each part cannot include more than the pattern obtained for antimyosin. Using the absorption method, the lateral band staining with antimyosin is identifiable as specific for L-meromyosin, since absorption with purified L-meromyosin removes only the lateral band staining. Likewise the medial band staining is specific for H-meromyosin since absorption with purified H-meromyosin removes only the medial bands. No difference was observed in the localization of the L-meromyosin using the absorbed antimyosin or antibody prepared against purified L-meromyosin. However, the localization obtained for H-meromyosin was different in these two methods. Absorption of antimyosin resulted in localization of H-meromyosin in a medial doublet in the A band. Antibody prepared against purified H-meromyosin gave a strong M line staining with progressively decreasing intensity of staining from the M line to the lateral edges of the A band. If we disregard the strong M line staining, staining of the rest of the A band corresponds to staining of both the H-meromyosin specific medial doublet and the L-meromyosin specific lateral doublet, with the medial doublet staining more intensely. This is consistent with gel diffusion analysis of the antibody components in this antibody preparation (18). Antibodies to both H-meromyosin and L-meromyosin were found. Tunik and Holtzer (36) have reported that the M line staining obtained with anti–H-meromyosin could be removed specifically by absorption with crude $MgCl_2$-precipitated actin. This suggests that the M line staining is due to antibody to an impurity and not to H-meromyosin. The observations reported in this paper, with absorbed antimyosin, confirm this. No M line staining is seen with antimyosin or with antimyosin absorbed with either purified H-meromyosin or L-meromyosin. If there is any antibody to the M line in the antimyosin preparation, it is present in undetectable amounts. In the case of antibody prepared from purified H-meromyosin, it is present in large amounts. This probably represents a concentration of the impurity during preparation of the H-meromyosin from myosin. Injection of this higher concentration of impurity led to a strong antibody response relative to that for the other constituents.

Three hypotheses were considered by Marshall et al. (18) to account for the wide separation, in the A band, of antibody prepared against H-meromyosin and L-meromyosin. One possibility entertained was that the M line staining may be the result of an antigenic impurity. This possibility was least appealing at the time, but has proved to be the case. Other possibilities suggested were that the myosin molecule is present in the myofibril in an extended form about 6000 A long with the H-meromyosin portion in the center of the A band and the L-meromyosin portion extending to the edge of the A band; or that the myosin actually exists as widely separated subunits in the fibril, which combine on extraction. In view of the fine structure of the thick filaments (12), neither of these possibilities is tenable any longer. Cross-bridges (containing the H-meromyosin) are present all along the filament except for an approximately 0.2 μ portion in the center. Artifi-

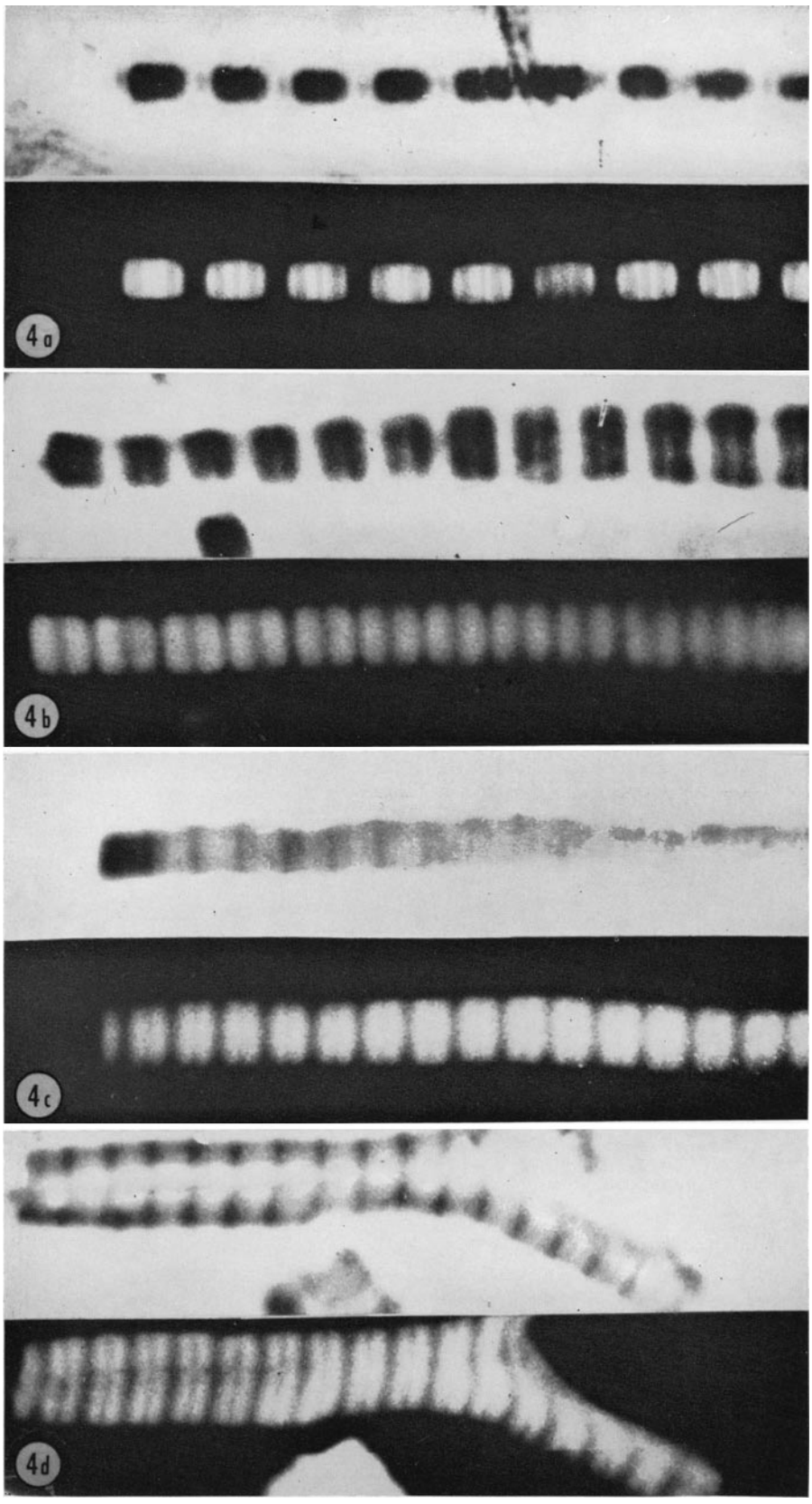
FIGURE 4 Antimyosin staining of contracted fibrils. The same fibril is shown in phase-contrast (upper) and fluorescence (lower) for identification of fluorescent-stained areas. Phase-contrast, × 4400; fluorescence, × 4500.

FIGURE 4 *a* A fibril with 2.3 μ sarcomere length. Note four fluorescent bands, two medial and two lateral, in the A band.

FIGURE 4 *b* A fibril with 1.9 μ sarcomere length. Note disappearance of the two medial fluorescent bands and increase in width of the lateral flourescent bands in the A band.

FIGURE 4 *c* A fibril with 1.5 μ sarcomere length. Note decrease in width of lateral fluorescent bands.

FIGURE 4 *d* A fibril with 1.2 μ sarcomere length. Staining pattern is similar to that in Fig. 4 *c*.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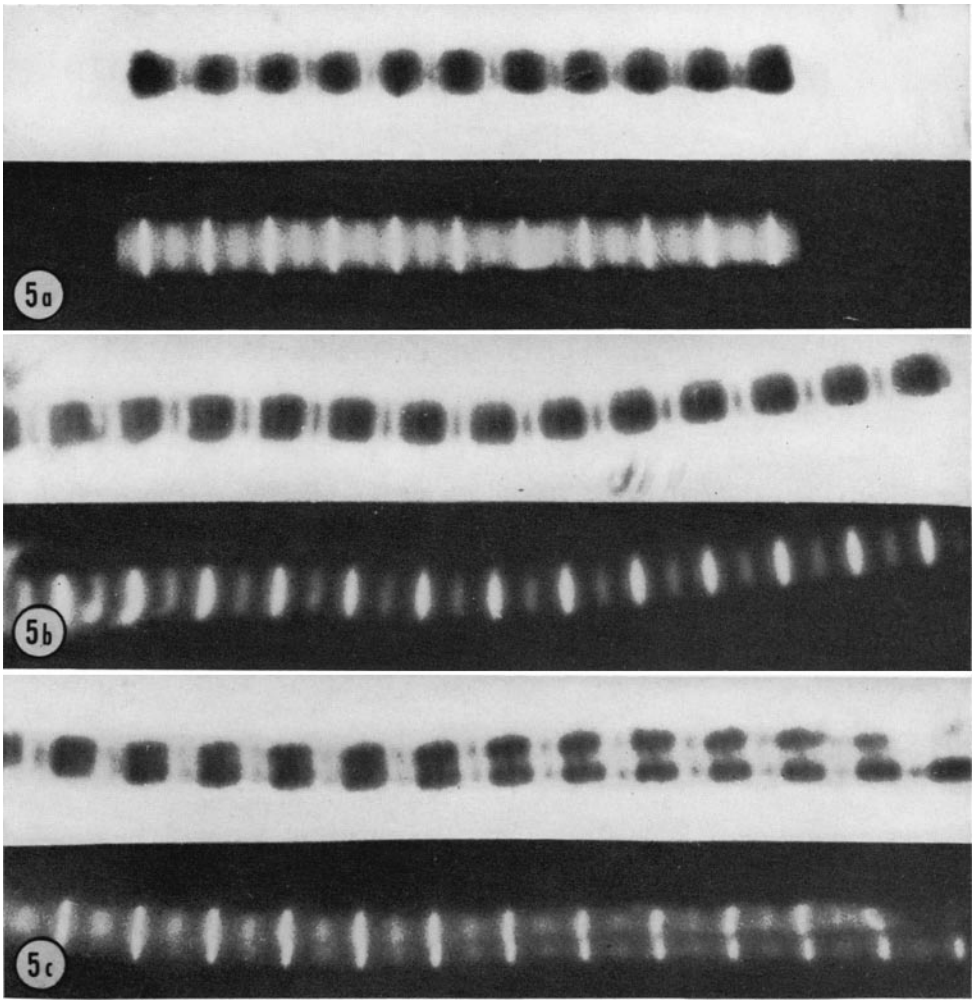


FIGURE 5 Antiactin staining.

FIGURE 5 *a* Fluorescent antiactin-stained fibril shown in phase-contrast (upper part of figure) and fluorescence (lower part of figure) for identification of fluorescent-stained areas. Note strong M line staining and weaker I band staining in fluorescence (18). Also note lack of Z line staining. Phase-contrast, × 3900; fluorescence, × 4000.

FIGURE 5 *b* Fluorescent antiactin absorbed with tropomyosin was used for staining. Note decrease in I band staining with no apparent decrease in the M line staining. Phase-contrast, × 3900; fluorescence, × 4000.

FIGURE 5 *c* Fluorescent antiactin absorbed with F-actin was used for staining. Note decrease in I band staining with no apparent decrease in the M line staining. Phase-contrast, × 3900; fluorescence, × 4000.

cially grown filaments were observed to grow outward from this central smooth portion, and cross-bridges were seen on all sizes, even those consisting of a very small number of myosin molecules. The filaments seemed to grow by aggregation of myosin molecules from solution, the myosin molecules retaining their lengths of approximately 1500 A (12, 17).

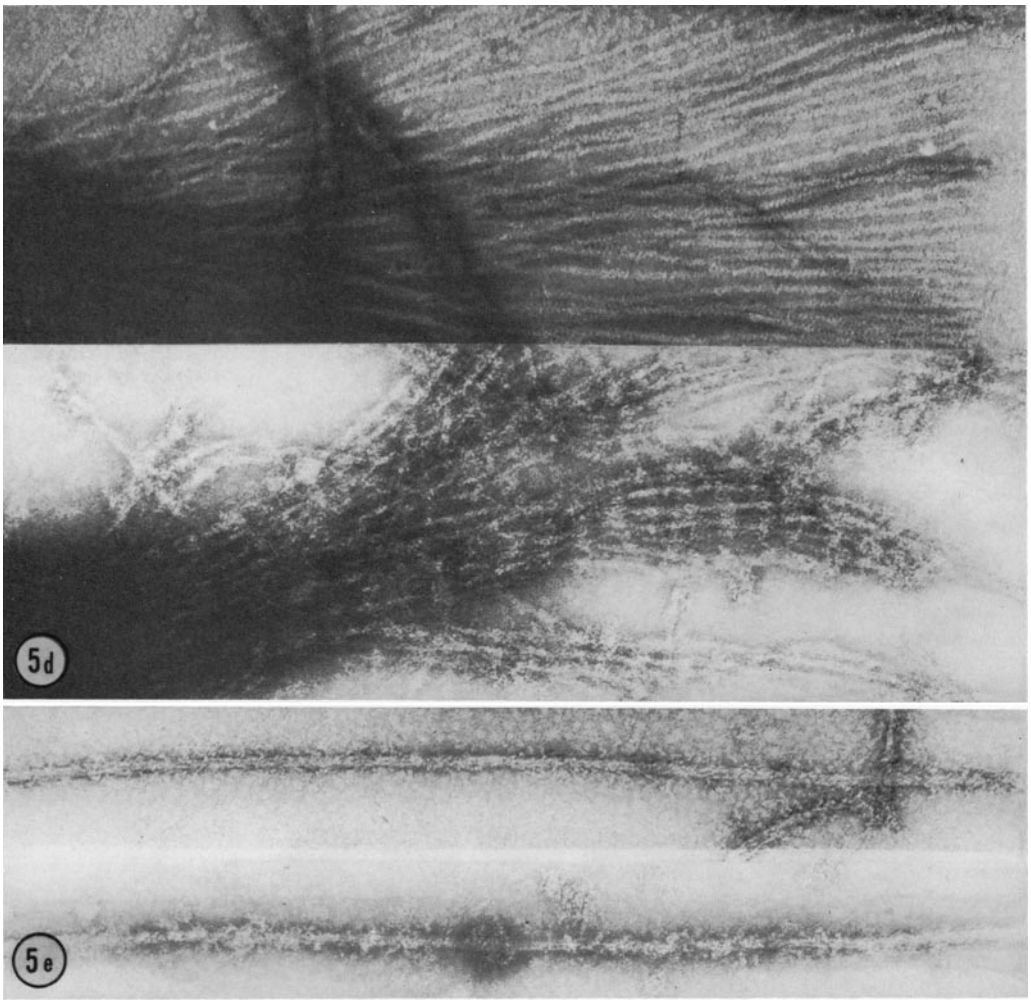


FIGURE 5 *d* Staining of separated I segments in electron microscopy (26). Normal γ-globulin treated (upper) and antiactin treated (lower) shown by negative staining. Note approximately 400 A period of attachment of antibody along the entire length of the filaments. × 130,000.

FIGURE 5 *e* Staining of separated thick filament with antiactin (lower) and with *n*-γ-globulin (upper). Note adherence of antibody at the center of the thick filament corresponding to the M line position in the myofibril. × 100,000.

We now know what the four bands obtained in the A band with antimyosin staining represent in terms of distribution of available antigenic sites. What relationship does this have to the structural organization of the filaments in the myofibril? Since no extension of the myosin molecules or separation of the subunits is likely in the myofibril, the availability of antigenic sites will depend on: (*a*) the way the myosin molecules are packed within the filament; and (*b*) the way they interact with other constituents of the myofibril. The unstained center of the medial doublet seen in fluorescent staining corresponds to the unstained central portion of the thick filament seen in electron microscopy. No cross-bridges are present in this region. The medial doublet in fluorescent staining is seen only in relaxed fibers and corresponds to the area in which there is no overlap and therefore no interaction of thin and thick filaments. The interaction occurs through the H-meromyosin part of the myosin molecule. On contraction of the myofibril to a sarcomere length

of approximately 2 μ, the medial doublet disappears. At this stage of contraction all of the thick filament is overlapping with the thin filaments and presumably all of the available H-meromyosin is interacting with the actin. Wherever actin and myosin interdigitation occurs, inhibition of antibody staining of H-meromyosin antigenic sites occurs. If this inhibition was due to nonspecific steric hindrance to the entrance of antibody between the filaments wherever they interdigitate, the lateral band staining specific for L-meromyosin would not occur. These results suggest that interaction of actin and myosin filaments at all stages of contraction occurs by direct contact of the H-meromyosin portion of the myosin molecule with the actin filament and not by long-range forces between the filaments (7). This direct contact results in specific inhibition of H-meromyosin staining.

Inhibition of fluorescent anti–H-meromyosin staining in the region of overlap of the filaments is essentially complete. This suggests that in this region essentially all the cross-bridges are involved in interaction with the thin filaments. As discussed by Huxley (13), interaction of the filaments during contraction may occur as a result of (*a*) a relatively weak force acting over a long distance (of the order of 100 A); (*b*) a relatively strong force acting over a short distance (of the order of 10 A); or (*c*) some situation intermediate. Action of the force over a short distance would mean that movement of the cross-bridge and/or conformational changes would be small. In this case, not all of the cross-bridges would be able to interact with the surrounding actin filaments, and a vernier effect would be necessary for translation of the filaments to occur (13). Interaction of a fraction of the cross-bridges would obtain both during contraction and in rigor, since interaction is limited by the small movement and/or conformational change in the cross-bridge. If the extent of movement and/or conformational change can occur over a large distance, all the cross-bridges can be involved in interaction with the thin filaments both during contraction and in rigor. Therefore the results of antibody staining are consistent with the action of a relatively weak force over a long distance (of the order of 100 A) during contraction (5).

As seen in fluorescent microscopy, antibody to L-meromyosin stains only laterally in the A band, where no staining for H-meromyosin occurs. In this region, the thin and thick filaments overlap. Anti–L-meromyosin has never been observed to stain separated filaments in electron microscopy (25–27). The smooth central portion of the thick filament which probably represents close packing of the ends of the myosin molecules containing the L-meromyosin portion is not stained in either electron microscopy or fluorescent microscopy. How can these observations be explained? As described by Huxley (12), the organization of the myosin molecules in the thick filaments is most probably such that the L-meromyosin part is

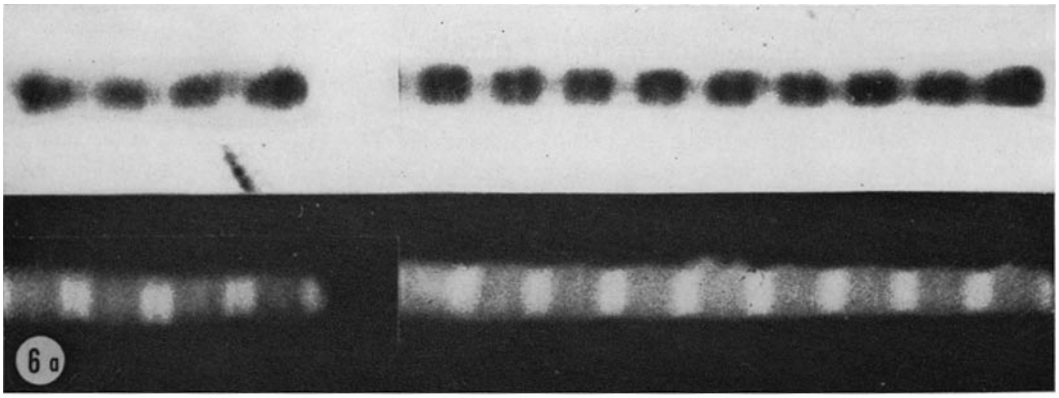
FIGURE 6 Antitropomyosin staining.

FIGURE 6 *a* Fluorescent antitropomyosin-stained fibril shown in phase-contrast (upper) and fluorescence (lower) for identification of fluorescent-stained areas. Note I band staining in fluorescence. Also note lack of Z line staining. Phase-contrast, × 4600; fluorescence, × 4700.

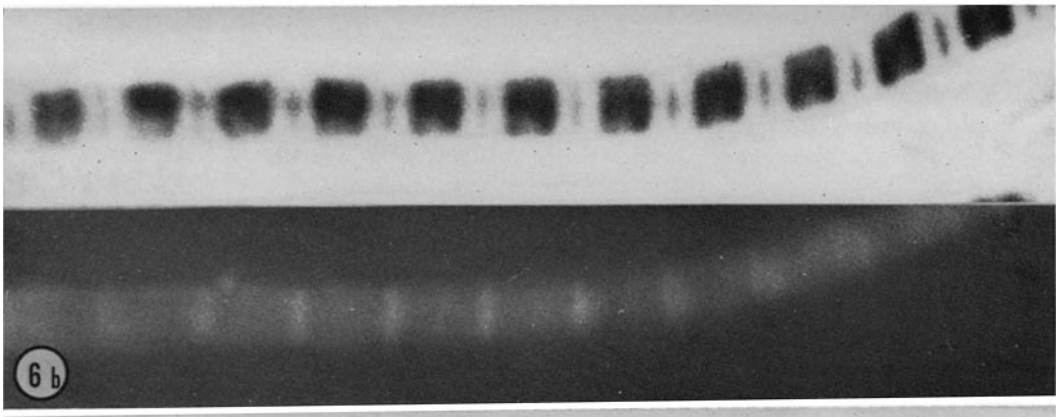
FIGURE 6 *b* Fluorescent antitropomyosin absorbed with tropomyosin was used for staining. Note almost complete removal of I band stain. Phase-contrast, × 4600; fluorescence, × 4700.

FIGURE 6 *c* Fluorescent antitropomyosin absorbed with F-actin was used for staining. Some decrease of I band staining has occurred. Also note indication of nonuniform staining of the I band. Phase-contrast, × 4600; fluorescence, × 4700.

FIGURE 6 *d* Staining of fibrils in electron microscopy. A normal γ-globulin–treated fibril is seen in the upper figure and an antitropomyosin stained fibril in the lower figure. Note small general increase of material visible in the I band as a result of antibody staining (obtained in collaboration with H. E. Huxley.). × 140,000.



6a



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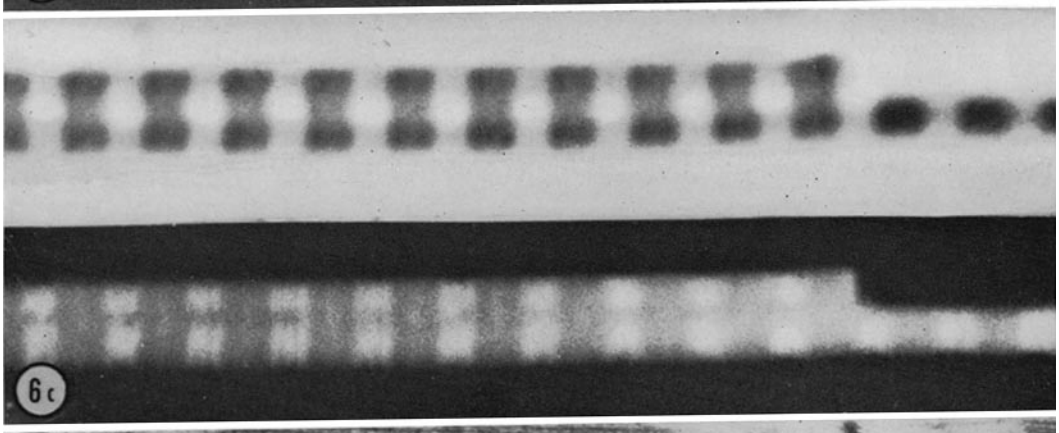

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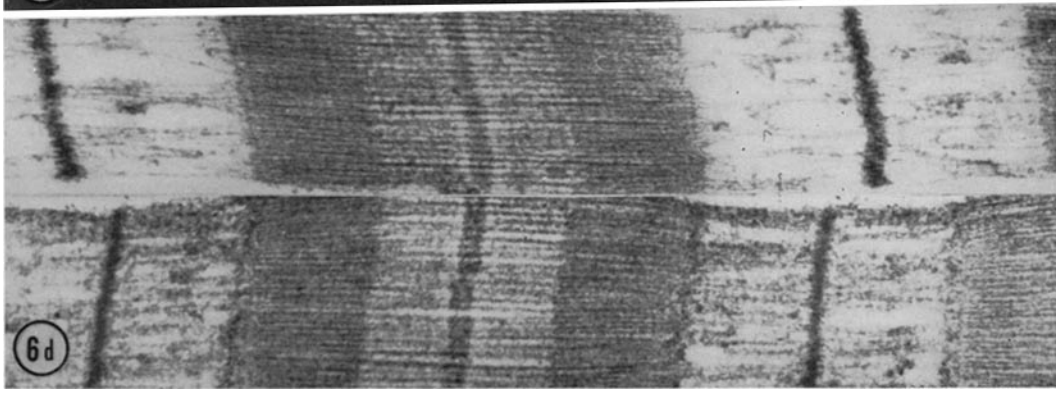

6d

buried inside the filament and forms the core, whereas the globular H-meromyosin part projects out to form the cross-bridges. This would mean that sterically the L-meromyosin part would probably be unavailable for staining throughout the length of the thick filament. Indeed this may be so in the intact, separated, thick filament. The tightly packed central smooth segment consisting of tightly packed ends of the myosin molecule containing the L-meromyosin portions is certainly not available for staining either in the separated filaments or in the intact fibril. Lack of staining in this region may be due to steric hindrance to the antibody as a result of the tight packing, or due to involvement of the antigenic sites in the aggregation of the L-meromyosin portions of the myosin molecules. Only the adherent M line material can be stained in this area when antibody to it is present. An additional feature which reflects the structural organization of the thick filament is the tapered ends. These result from the staggered arrangement of the myosin molecules in the filament. The H-meromyosln cross-bridges on these tapered ends are farther from the surrounding thin filaments than the H-meromyosin cross-bridges on the rest of the thick filament. In order for interaction to take place with the thin filaments at the tapered ends, the packing of the myosin molecules in the filament at the ends must become looser. This would mean that the H-meromyosin sites would be tied up with the actin and that the L-meromyosin sites would become exposed and available for staining at the two ends of the thick filament. This would explain the lateral staining in the A band specific for L-meromyosin only. The availability of the L-meromyosin antigenic sites would depend on the extent to which the compact organization of the filament was loosened. In addition, the observations on contracted fibrils with respect to the lateral staining were that the lateral bands increased in width and became brighter, and, on further contraction, decreased in width again. These observations suggest that, as contraction proceeds, there is a progressive loosening of the structure of the filaments, exposing an increasing number of the antigenic sites on the L-meromyosin part of the myosin molecules. This may result from an increase in distance between the filaments as contraction proceeds (3, 8, 37). These changes are presented diagrammatically in Fig. 7. As further contraction to sarcomere lengths smaller than 2 μ occurs and the thin filaments from opposite sides of the sarcomere overlap (13), the filaments may provide hindrance to the entrance of antibody between the filaments, resulting in a narrowing of the broadened lateral bands. The results of Tunik and Holtzer (36) for fluorescent anti-H-meromyosin and anti-L-meromyosin staining of contracted fibrils are also consistent with this interpretation. Inhibition of staining in the region of overlap of the thin filaments may not be entirely nonspecific. Tunik and Holtzer (36) showed that staining of the M line in this region was not inhibited in highly contracted fibrils. No evidence is available, at present, for specific interaction between the thin filaments from one side of a sarcomere and the opposite half of the thick filament. It may be that inhibition of anti-L-meromyosin staining in this region is due to the position of the thin filaments from one side relative to the antigenic sites on the opposite half of the thick filament.

The changes in antimyosin staining with contraction observed by Szent-Györgyi and coworkers (35) were interpreted as representing migration of myosin in the A band. An important piece of evidence leading to this interpretation was that quantitative estimates of antibody uptake showed no difference between contracted and relaxed fibrils. Decrease of anti-H-meromyosin uptake with concomitant increase of anti-L-meromyosin uptake in contracted fibrils would account for this.

In general, the results presented here are consistent with the sliding filament model. The changes occuring in the thick filaments, which are reflected by changes in the availability of antigenic sites on the L-meromyosin, are an added feature to this model. The loosening of the structural organization of the thick filaments is considered to be a result of both the necessity for actin-myosin attachment to occur throughout contraction and the changes in the center-to-center distance between actin and myosin during contraction. There is considerable evidence for the increase in actin to myosin center-to-center distance during contraction. Carlsen et al. (3), from electron microscopy, reported a 20% increase in this distance with 40% shortening. X-ray diffraction studies of living frog muscles demonstrated a change of 70 A in this distance during contraction of the sarcomere from 3.4 to 1.9 μ, and a 60 A change in mammalian muscle during contraction of the sarcomere from 3.7 to 2.2 μ (8). Increases

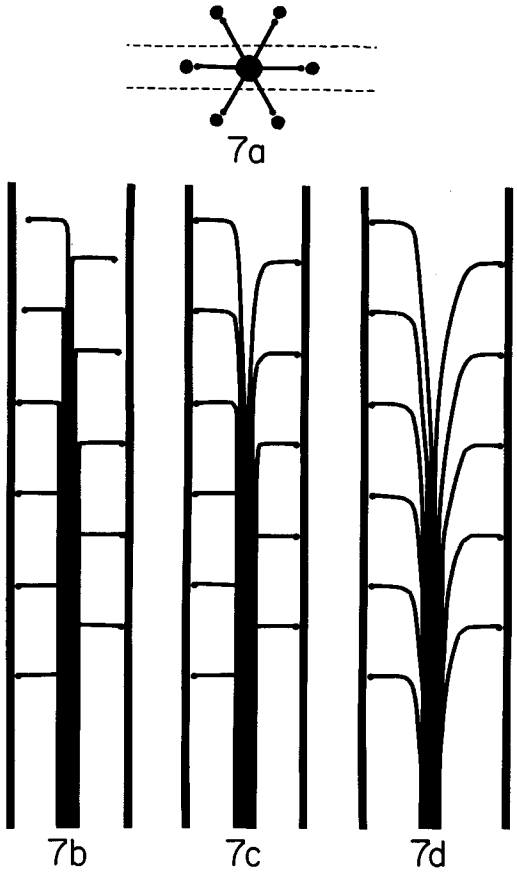

FIGURE 7 Diagrammatic representation of the interaction of thin and thick filaments at the lateral edges of the A band at different sarcomere lengths.

FIGURE 7 *a* Representation of the interaction of a thick filament with the six surrounding thin filaments as seen in cross-section. The cross-bridges extend from the thick filament to the surrounding thin filaments.

FIGURE 7 *b* Representation of a longitudinal section through the thick filament taken as indicated by the dashed lines in Fig. 7 *a*. The tapered end of the thick filament is depicted. This diagram represents the case where no interaction of the cross-bridges with the thin filaments occurs. The cross-bridges protrude from the filament, and the rest of the myosin molecules are closely packed in the thick filament.

FIGURE 7 *c* Representation of the effect that attachment of the cross-bridges to the surrounding thin filaments has on the packing of the myosin molecules in the thick filament at the tapered ends. The packing of the myosin molecules in the filament is loosened, making some antigenic sites of the L-meromyosin part available for staining.

FIGURE 7 *d* Representation of the result of increasing distance between thick and thin filaments as contraction proceeds. The necessity for maintaining attachment of the H-meromyosin to the surrounding thin filaments in contracted fibrils results in further loosening of the close packed arrangement of the myosin molecules in the thick filament. This leads to further exposure of antigenic sites of L-meromyosin. The length over which the thick filaments tapers at its end increases and a slight shortening occurs.

of up to 100 A between thin and thick filaments have been reported in insect muscle without breakdown of the regular lattice (37). One might expect that loosening of the structure of the thick filaments necessary to preserve contact of actin and myosin over such distances would result in an apparent decrease in length of the thick filaments and consequently in the length of the A band on contraction. The change in length would depend on how much of the part of the myosin molecule making up the basic structure of the filament is bent out of the filament during contraction. The myosin molecule is 1500 A long (17) with a globular region 150 to 250 A long associated with the H-meromyosin part and probably representing the cross-bridge (12, 31, 32, 38). If we take 200 A as the length of the cross-bridge, we are left with 1300 A as the length of the myosin molecule contributing to the basic filament dimensions exclusive of the projecting cross-bridges. The length of the L-meromyosin part of the myosin molecule is approximately 700 A (12, 17); therefore approximately 600 A of the H-meromyosin is included inside the filament. Crystals of L-meromyosin show an axial periodicity of about 430 A with a 100 A wide light band in negatively stained preparations (12, 29). The appearance of this light band may result from overlap of the L-meromyosin molecules arranged in register (12). If this is so, we might expect that at least 100 A and probably more of the L-meromyosin part of the myosin molecule must remain in its original position in the filament to maintain the structural integrity of the filament during contraction. This leaves a maximum of 1200 A of the rod part of the molecule as movable, and 600 A of this movable part is L-meromyosin. Therefore, if we assume that a maximum of 1200 A of the rod part of the molecule can project out of the filament during contraction, the maximum change in length of the thick filament associated with this would be 0.24 μ. This would also result in an increase in the length over which the ends taper. Since approximately 600 A of the rod part of the myosin molecule is part of the H-meromyosin part of the molecule, more than 600 A must project out of the filament in order for antigenic sites of the L-meromyosin part to be exposed for staining. If we assume that a minimum of 700 A projects out of the filament (this would include 100 A of the L-meromyosin part), a shortening of 0.14 μ in the length of the thick filament would result. These changes are represented diagrammatically in Fig. 7. Small changes in length of the A and I filaments as a function of sarcomere length have been reported (3). Variations in length of thick filaments by about 8% were found. These changes have been attributed to the experimental conditions by Page and Huxley (24) who concluded that the filament lengths remain constant in the untreated specimens. In any case, changes in length of this order of magnitude are difficult to demonstrate unequivocally with present methods for preparation of materials for electron microscopy.

X-ray diffraction studies have so far been unable to detect any major changes in the molecular arrangement within the actin or myosin filaments during contraction (8, 37). If we assume that a 1200 A portion of the rod part of the myosin molecule can be bent out so that the loose end moves laterally 60 A, this will represent an axial movement of the loose end by less than 10 A. Lateral movement of the loose end of a 700 A portion of the rod part of the myosin molecule by 60 A would result in axial movement of the loose end by less than 5 A. Such changes would be difficult to detect by X-ray scattering. These changes represent projection of the myosin molecules out of the thick filaments in order to maintain contact with the thin filaments at all stages of contraction. Superimposed on these may be cyclic changes in conformation of the H-meromyosin part of the myosin molecule as proposed by Davies (5). These cyclic changes would represent the force producing interactions resulting in translation of the filaments. Recent refinements in X-ray–scattering instrumentation have led to results which indicate movement of the cross-bridges during contraction (14). Also, differences have been observed in the orientation of the cross-bridges in insect flight muscle by both electron microscopy and X-ray diffraction (30). These differences occurred between muscle relaxed with ATP and muscle in rigor produced by glycerination. The observed effects are probably a result of both the cyclic, force-producing changes and the projection of part of the myosin molecule from the thick filament (resulting in exposure of L-meromyosin).

Antiactin and Antitropomyosin

Antibody prepared against "purified" actin or "purified" tropomyosin was found to be a mixture of antibodies to both action and tropomyosin. No

antibodies reacting with myosin were demonstrable. Both actin and tropomyosin showed localization in the I band. Such localization has been observed for actin in previous work (18, 36). Previous investigations of tropomyosin were limited by the weakness of staining (18). An additional observation concerning the I band staining is that the Z line remains unstained with both antiactin and antitropomyosin. This, however, does not exclude the possibility that one or both of these proteins may be present in the Z line. However, if present, they are unavailable for staining. This is consistent with observations reported by Frank et al. (9) with formalin-fixed fibrils.

Absorption of antiactin with purified actin and purified tropomyosin resulted in partial removal of the I band stain, consistent with the antibody being a mixture of antibodies to actin and tropomyosin; and consistent with the presence of both actin and tropomyosin in the I band. Likewise, absorption of antitropomyosin with purified tropomyosin and purified actin resulted in partial removal of the I band stain. In the case of absorption of antitropomyosin with purified tropomyosin, the I band stain was almost completely removed, a fact which indicates that there is a larger amount of antibody to tropomyosin than antibody to actin present in the preparation. The staining patterns obtained with antitropomyosin absorbed with purified actin in fluorescent microscopy indicate a nonuniformity of distribution of the tropomyosin in the I band. This is being investigated further.

Considerable evidence has now been accumulated indicating the close relationship of actin and tropomyosin in the myofibril (10, 28). The nature of this interaction and its significance with respect to the contractile apparatus are unclear. The difference between the antiactin- and antitropomyosin staining patterns seen in electron microscopy may provide a clue. Antiactin contains a mixture of antibody to actin and antibody to tropomyosin. In electron microscopy it consistently gives a strong 400 A period of staining in the I band (26). The I band contains both actin and tropomyosin. Antitropomyosin likewise contains antibody to both proteins but with a considerably larger amount of antibody to tropomyosin. Antitropomyosin in electron microscopy does not give a strong 400 A period in the I band. Therefore, the 400 A period is associated with antiactin and is probably not due to staining of the tropomyosin. It is difficult to understand how a 400 A period can be obtained by staining of the double actin helix, which has a cross-over point approximately every 350 A and an actin monomer every 55 A along one chain (10). It may be that the interaction of actin and tropomyosin is such that the antigenic sites of actin are only available every 400 A along the filament. If this is the case, then the antibody to tropomyosin which is present in the antiactin must be present in concentration too low to be observable in electron microscopy. The possibility also exists that the 400 A period may be due to other antigenic impurities in the actin and not to actin itself (26).

The extent of antibody staining seen in fluorescent microscopy is sometimes difficult to ascertain, especially where staining intensity is high. The stained areas tend to bleed into surrounding areas during photography. However, in the case of the I band staining observed with antiactin antibody, the staining seems to be limited to the I band, in spite of the fact that the actin filaments do extend into the A band region. This may represent blocking of antigenic sites of actin by its interaction with the myosin in the A band. Staining of separated I segments in electron microscopy (Fig. 5 *d*) (26) showed a 400 A period of adherence of antibody along the thin filaments, all the way to the ends of the filaments. In the relaxed intact fibrils these ends would be in the A band. As yet, it is difficult to conclude unequivocally from electron microscopy whether or not the thin filaments are being stained in the A band. However, fluorescent antibody staining suggests that at least the antiactin staining of the thin filaments in the A band is considerably inhibited.

In addition to I band staining, antiactin antibody showed intense staining of the M line (18, 36). The M line staining was not removed by absorption with either purified actin or tropomyosin. This fact, in addition to lack of reaction of the antibody with purified myosin, leads to the conclusion that the M line staining is due to some antigenic material other than actin, myosin, or tropomyosin. This antigen is weakly bound to the thick filament at the center of the smooth central portion. During separation of the filaments by homogenization in excess ATP, it is generally lost (26). In the cases where it is retained, it can be stained by antibody present in the antiactin antibody preparation. Lack of change in the staining of the M line during contraction of the myofibril (36) suggests that, although weakly bound, the M

line material remains adhering to the center of the thick filament throughout contraction and that its antigenic sites remain available for staining.

SUMMARY

Interpretation of the results of antibody staining must take several points into consideration. (*a*) The antibody response to different antigens varies widely. A large amount of antibody may be produced to a small amount of impurity. Use of the absorption technique is valuable in determining whether this is the case. (*b*) In addition to antibody to impurity, it is necessary to consider the availability of antigenic sites to the antibody stain. The antigenic sites may be present but blocked by interaction with other consituents of the system being studied. (*c*) It is also possible that an apparently unstained area may represent an area of diminished antibody staining relative to surrounding brightly stained areas. (*d*) In electron microscopy, visibility of the antibody depends both upon the amount of antibody adhering to the structure and upon its distribution with respect to the structure. The experiments described in this work were based on these considerations.

The conclusions arrived at can be summarized as follows: (*a*) Direct contact of the actin and myosin filaments occurs at all stages of contraction, resulting in inhibition of antibody staining of H-meromyosin. Essentially complete inhibition in the region of overlap indicates involvement of almost all of the H-meromyosin sites. This suggests that the force producing contraction may act over distances of the order of 100 A. (*b*) Small structural changes occur in the thick filaments during contraction leading to exposure of antigenic sites of L-meromyosin. (*c*) Close association of actin and tropomyosin occurs in the I band. A 400 A periodicity associated with the availability of antigenic sites of actin is present. (*d*) The M line is formed by a protein, other than myosin, actin, or tropomyosin, which is weakly bound to the center of the thick filament. (*e*) There are no available antigenic sites of either actin or tropomyosin in the Z line.

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