

THE M BAND

Studies with Fluorescent Antibody Staining

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ABSTRACT

The M band can be extracted from fibrils suspended in 5 mM Tris buffer, pH 8.0, for 15 min. The M band is completely removed only from fibrils of sarcomere lengths greater than 2.1 μ . Extraction does not alter the fluorescent antimyosin staining pattern of the A band, thus providing strong evidence that no alteration of the structural integrity of the thick filament has occurred. Fluorescent antibody staining of the M band of unextracted fibrils can be prevented specifically by absorbing the fluorescent antibody with extracted M band material prior to staining. This verifies the specificity of the extraction procedure.

One of the bands observed in the striated myofibril is the M band. The M band is located in the middle of the A band and is formed by material which adheres to the thick filaments, forming cross bridges between them (1). Antibody prepared against different muscle proteins has been shown to stain the M band in addition to other regions of the sarcomere (2-6). Using absorption and immunochemical techniques, Pepe (5) showed that the M-band staining cannot be attributed to staining of antigenic sites present on actin, myosin, or tropomyosin. Therefore, the M-band material is a different protein. In addition, Pepe and Huxley (7) showed that the M protein was loosely attached to the thick filaments, since, when the thick and thin filaments were separated by homogenization, the M protein did not remain adhering to the thick filaments. With less vigorous homogenization some of the M material remained bound to the separated thick filaments, and specific antibody staining of the bound M material was observed in electron microscopy (8). The presence of antibody to the M protein in antisera prepared against other muscle proteins as antigens must be due to the presence of small amounts of the M pro-

tein as an impurity in these antigen preparations.

Samosudova (9) was able to show removal of the M band from fibrils without effect on the myosin filaments as observed in sections in electron microscopy. The M band was removed with 5 mM Tris (pH 8.0) buffer after the procedure of Perry and Corsi (10). It is possible that alterations in the structural integrity of the myosin filaments not detectable in electron microscopy could occur. Stromer et al. (11) were able to remove both the M-band and Z-band material with the use of 2 mM dithiothreitol without effect on the rest of the sarcomere. Using appropriate conditions, they were also able to replace the extracted material. Recently, the M protein has been isolated (12).

The main purposes of this investigation are (a) to study the differences in extractability of the M band from myofibrils at different sarcomere lengths, (b) to identify the extracted material as M-band material by using it to selectively absorb antibody known to stain the M band, and (c) to use antimyosin staining to check for possible alterations in the structural integrity of the myosin filaments not detectable in electron micros-

copy after removal of the M band material. Antiactin stains both the M band and the I band (5). Therefore, extraction of the M band should result in elimination of only the M-band staining. Also, absorption of antiactin with the extracted M protein should remove only the M-band staining and not the I-band staining of unextracted fibrils. Antimyosin stains the myosin filaments in the A band in a characteristic pattern (5, 13) which would be expected to change if there is any alteration in the structural integrity of the myosin filaments.

MATERIALS AND METHODS

Preparation of Fibrils

Adult chicken breast muscle was used. The muscle was kept cold from the time it was removed from the chicken. It was excised in long strips parallel to the fiber axis, tied at rest length to plastic rods, and placed in a 50% glycerol solution containing 5×10^{-4} M MgCl_2 , 5×10^{-2} M KCl, and 5×10^{-3} M phosphate buffer at pH 7.0 for 2 days at 2°–3°C. The glycerol had previously been passed over Amberlite MB-1 ion exchange resin. The glycerol solution was then changed and the muscle strips were stored at –24°C for at least 3 wk before use.

Fibril suspensions were prepared in two different solutions: solution A contains 0.1 M KCl, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0; solution B contains 25% glycerol, 7.5×10^{-4} M MgCl_2 , 7.5×10^{-2} M KCl, and 7.5×10^{-3} M phosphate buffer, pH 7.0. Fibrils prepared in solution A were later used for extraction as described below. Solution B is the same as that generally used in previous fluorescent antibody-staining work (5, 13). To prepare the fibril suspensions, a portion of the glycerinated muscle strip was removed from the deep freeze, placed in either solution A or solution B, and allowed to soak at 0°–2°C for about 10 min. The strip was then placed in fresh solution and shredded with a sharp needle drawn parallel to the fiber axis. The shredded muscle was homogenized on ice in a Sorvall omnimixer (Ivan Sorvall Inc., Norwalk, Conn.) with three bursts of 6 sec each, with a 2–3 min cooling period between the bursts. The suspension was centrifuged in an International clinical centrifuge (International Equipment Co., Needham Heights, Mass.) at approximately 1500 g for 10 min in a cold room kept at 2°–3°C. The packed fibrils were then washed twice by resuspension in fresh solution followed by centrifugation. Fibrils were finally suspended in fresh solution to 10 times the volume of packed fibrils. Fibrils homogenized in solution A were washed and finally suspended in either solution A or solution B. Fibrils homogenized

in solution B were washed and finally suspended in solution B.

The fibrils prepared in the two solutions were observed in phase-contrast microscopy to compare the fibrillar structures. The two fibril preparations were then stained with fluorescent antimyosin and fluorescent antiactin, as described below, and observed in fluorescence microscopy. In this way the staining of fibrils prepared in solution A could be compared to that of fibrils prepared in the more generally used solution B.

Antibody Staining

Preparation and fluorescent labeling of the antibody are the same as those previously used (5, 13). The fluorescent antimyosin and antiactin solutions each contained 10–15 mg of protein/ml. The antibody was dialyzed against the solution in which the fibrils to be stained were suspended. 2 drops of fluorescent antimyosin were used to stain 1 drop of fibril suspension prepared as described above. 1 drop of fluorescent antiactin was used to stain 1 drop of fibril suspension. These same ratios of fluorescent antibody to fibrils were used in all staining experiments. Antibody staining took place overnight at 2°–3°C. Overnight staining was used so that the entire following day could be used for studying the staining patterns and taking pictures. No differences were observed in the staining patterns obtained after either 1 hr staining or overnight staining, except that brighter staining occurred overnight. After staining, the fibrils were placed on slides and washed with fresh solution by drawing it under the cover slip with filter paper.

Microscopy

For fluorescence microscopy a Reichert Zetopan microscope with a cardioid condenser and a 100 X 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. Exposure times varied from 2 to 6 min, depending on fluorescence intensity. For phase-contrast microscopy, an AO objective with a magnification of 97 times and a matching AO condenser were used. Kodak M plates and Kodak D-19 developer were used. Exposure time was $\frac{1}{2}$ sec.

Extraction of M-Band Material

Fibrils to be extracted were prepared by homogenization in solution A as described above. For extraction the fibril suspension was centrifuged and the

pellet was resuspended in 5 mM Tris buffer (tris-[hydroxymethyl]aminomethane) at pH 8.0 to 20 times its volume. Extraction was allowed to proceed for 15 min at 0°C. After extraction the suspension was centrifuged and the supernatant (extracted material) was dialyzed against solution B overnight in preparation for further use. The fibril pellet was washed twice by resuspension in solution B. Final suspension of the extracted fibrils was then made in solution B to 10 times the volume of packed fibrils. These fibrils were observed in phase-contrast microscopy to see if any noticeable alterations in fibril structure had occurred as a result of the 15 min extraction. The extracted fibrils were stained with fluorescent antimyosin to see if there were any differences in the staining pattern of the A band compared to that of unextracted fibrils. Extracted fibrils were also stained with fluorescent antiactin to check for the presence or lack of M-band staining. Both fluorescent antibodies were in solution B. Staining was allowed to proceed overnight before the fibrils were observed in fluorescence microscopy. Fibrils were likewise extracted with the 5 mM Tris buffer, pH 8.0, for longer intervals of time up to 3 hr and stained as above to see what further changes in the staining pattern would occur with prolonged extraction.

Absorption of Fluorescent Antiactin with M-Band Material

The material removed from fibrils by extraction with 5 mM Tris buffer, pH 8.0, for 15 min (see above) was used to absorb fluorescent antiactin. The ratio of extracted material to antiactin necessary for absorption was determined by preparing a series of mixtures with increasing antigen (extracted material) to antibody ratio. These mixtures were

allowed to stand at least 1 hr at 2°–3°C, and were then used to stain unextracted fibrils. The ratio at which no M-band staining was seen was the proportion finally used. This was 15 drops of extracted material to 1 drop of fluorescent antiactin. The unextracted fibrils used were initially homogenized in solution B. Both the antigen (extracted material) and the antiactin were in solution B. The antibody to fibril ratio was kept the same as used throughout this work (see section on antibody staining), taking into account the dilution due to the presence of the absorbing material. The fibrils were allowed to stain overnight before being observed in fluorescent microscopy. Antiactin diluted with 15 drops of solution B alone was used as a control for the absorption.

RESULTS

Unextracted Fibrils

Good fibrillar structure was observed in fibrils prepared in either solution A (Fig. 1 *a*) or solution B (Fig. 1 *b*). The A bands were dense and showed no evidence of swelling or other structural damage. After staining with fluorescent antimyosin in the same solution in which they were prepared, observation in fluorescence microscopy showed four fluorescent bands in the A band for fibrils at the longer sarcomere lengths (2.4 μ in Fig. 2 *a* and 2.6 μ in Fig. 2 *b*). There was one band near each A–I junction, and the other two bands were in the middle of the A band. At shorter sarcomere lengths (2.0 μ in Fig. 2 *c*) the bands near the A–I junctions were wider and brighter, and the other two bands in the middle of the A band were not seen. These observations are the

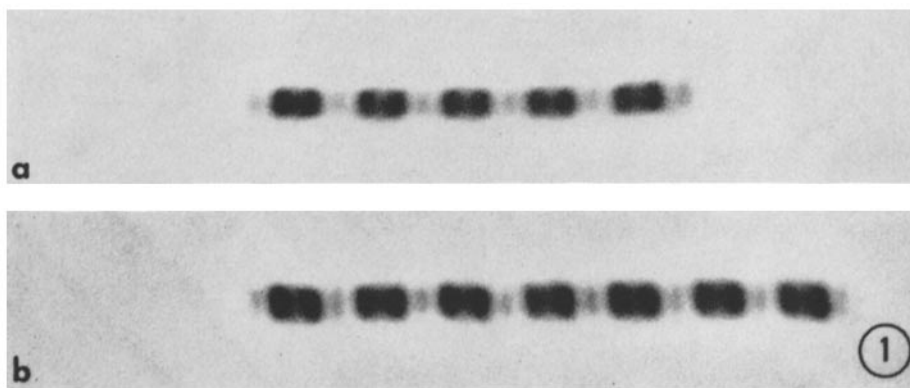


FIGURE 1. Fibrils in phase-contrast microscopy. (*a*) Fibril at 2.7 μ sarcomere length homogenized in solution A. (*b*) Fibril at 2.7 μ sarcomere length homogenized in solution B. \times 4050.

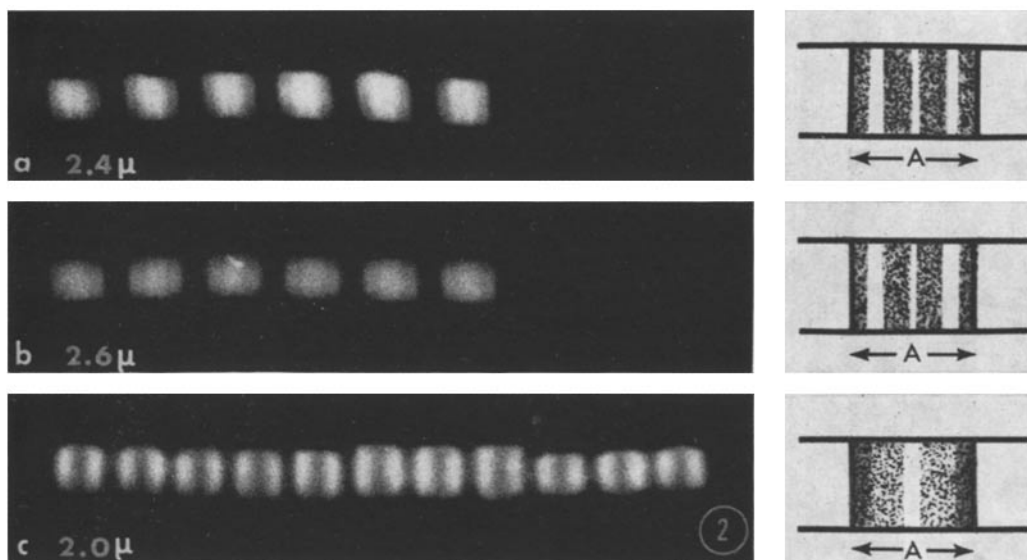


FIGURE 2. Fluorescent antimyosin-stained fibrils with accompanying diagrammatic representation of the A-band staining. (a) Fibril at 2.4μ sarcomere length homogenized and stained in solution A. (b) Fibril at 2.6μ sarcomere length homogenized and stained in solution B. (c) Fibril at 2.0μ sarcomere length homogenized and stained in solution A. This same staining pattern was seen in fibrils of short sarcomere lengths (2.0μ and shorter) homogenized and stained in solution B. $\times 4500$.

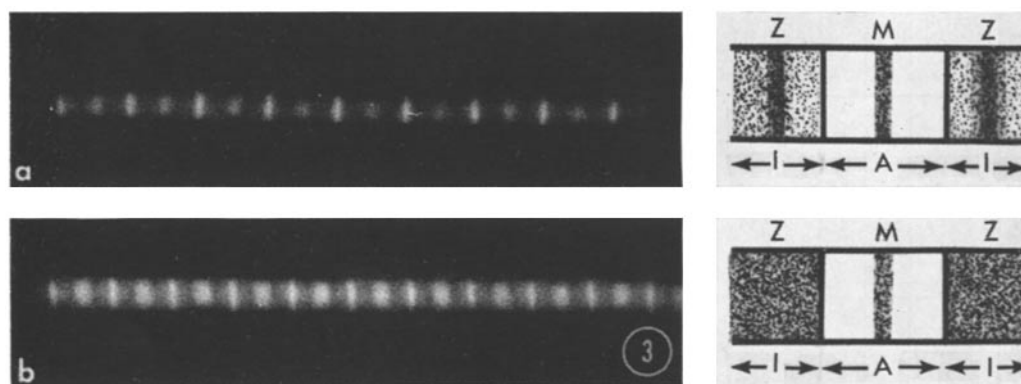


FIGURE 3. Fluorescent antiactin-stained fibrils with accompanying diagrammatic representation of the staining pattern. (a) Fibril homogenized and stained in solution A. Note nonuniformity and low intensity of the I-band staining. Brightest staining in the I band is in the vicinity of the Z band. Also note bright staining in the M band. (b) Fibril homogenized and stained in solution B. Note bright I-band staining and bright M-band staining. $\times 4500$.

same as those previously reported for antimyosin staining of the A band (5, 13). No differences were observed in the A-band staining patterns for fibrils prepared and stained in the two different solutions A and B.

Fibrils prepared in solution A and solution B both showed bright M-band staining when

stained with fluorescent antiactin (Figs. 3 a and 3 b). However, in the case of fibrils prepared and stained in solution A (Fig. 3 a) the I-band staining was less uniform and less intense than that of fibrils prepared and stained in solution B (Fig. 3 b). The brightest staining in the I band occurred in the vicinity of the Z band (Fig. 3 a).

Extracted Fibrils

Extraction was always carried out on fibrils prepared in solution A. Fibrils extracted for 15 min with 5 mM Tris buffer, pH 8.0, maintained good structural integrity as observed in phase-contrast microscopy. Occasionally, there was a slight degree of A-band swelling in the extracted fibrils (14). Fluorescent antiactin staining of extracted fibrils showed that the M band was removed completely from fibrils with sarcomere lengths longer than 2.1μ (Fig. 4 *a* shows a fibril at 2.5μ sarcomere length with no M-band staining). The M band was not removed and was brightly stained in fibrils shorter than 1.9μ (Fig. 4 *b* shows a fibril at 1.7μ sarcomere length with bright M-band staining). The extent of removal of the M band varied within the range of 1.9μ – 2.1μ sarcomere lengths. The I-band staining was not noticeably affected by extraction of the fibrils with 5 mM Tris pH 8.0 buffer for 15 min.

In their A-band staining, the extracted fibrils stained with fluorescent antimyosin were not different from unextracted fibrils (compare Fig. 2 *a* and Fig. 5). In some cases there was an impression that the intensity of the staining of the bands near the A–I junctions at the longer sarcomere lengths was less for the extracted fibrils than for the unextracted fibrils.

Fibrils extracted for longer than 15 min and for as long as 3 hr showed some alteration of the structural integrity of the fibril as observed in phase-contrast microscopy. It was noted that as the extraction time increased, the number of fibrils with swelled A bands increased. When the fibrils were stained with fluorescent antiactin, it was observed that in fibrils of sarcomere lengths shorter than 1.9μ the M band was not removed even after extraction for as long as 3 hr. The I-band staining remained essentially unchanged with increased extraction.

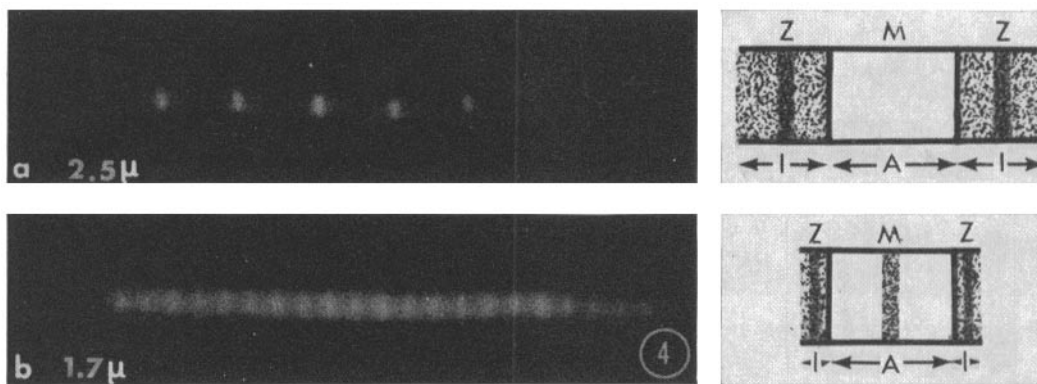


FIGURE 4 Fluorescent antiactin-stained fibril with accompanying diagrammatic representation of the staining pattern. Fibrils were homogenized in solution A, extracted with 5 mM Tris buffer pH 8.0 for 15 min, and stained in solution B. (a) Fibril at 2.5μ sarcomere length. Note complete absence of the M-band staining. Also note similarity of the I-band staining to that seen in Fig. 3 *a*. (b) Fibril at 1.7μ sarcomere length. Note staining of the M band. $\times 4500$.

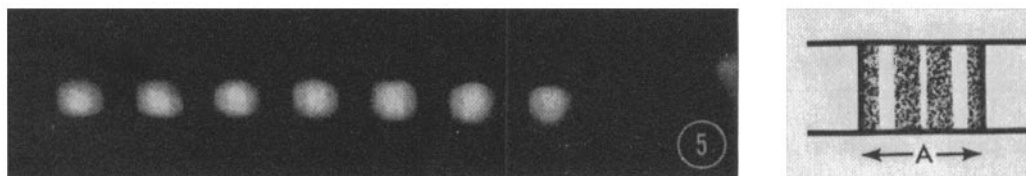


FIGURE 5 Fluorescent antimyosin-stained fibril which, prior to staining, was treated in the same way as the fibrils in Fig. 4. This fibril has a sarcomere length of 2.5μ . Compare the staining pattern to that obtained with the unextracted fibril in Fig. 2 *a* (see text for details). $\times 4500$.

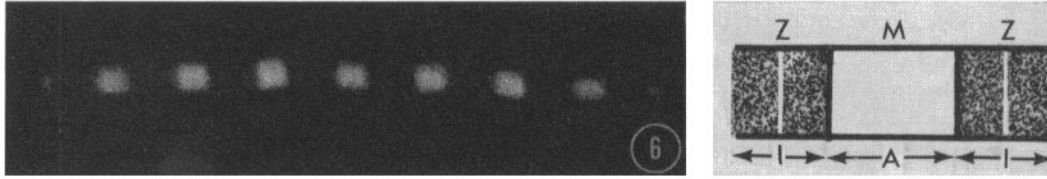


FIGURE 6 Fibril homogenized in solution B and stained with fluorescent antiactin previously absorbed with M material. Note absence of M-band staining and no change in the I-band staining compared to that seen in Fig. 3 b. $\times 4500$.

Absorption of Antiactin

Fluorescent antiactin which, prior to staining, was absorbed with 15 drops of M-material previously extracted from fibrils, did not stain the M band of unextracted fibrils. The I-band staining was not affected by absorption of the fluorescent antiactin (Fig. 6). Dilution of 1 drop of fluorescent antiactin with 15 drops of solution B, the control for the absorption, produced the same staining pattern with unextracted fibrils as was obtained with 1 drop of fluorescent antiactin alone.

DISCUSSION

One of the main purposes of this work was to study the extractability of the M band from myofibrils of different sarcomere lengths. It has been found that the extractability of the M band varies with sarcomere length. In fibrils with sarcomere lengths less than 1.9μ (Fig. 4 b), the M band cannot be removed with 5 mM Tris buffer pH 8 even after 3 hr' extraction time. The M band is completely removed from fibrils with sarcomere lengths longer than 2.1μ in 15 min (Fig. 4 a). This difference in extractability as a function of sarcomere length may be related to some earlier findings reviewed by Pepe (15). He pointed out that although M-band staining has been observed at all sarcomere lengths in glycerinated fibrils, formaldehyde fixation prior to staining limited the M-band staining to only fibrils of short sarcomere length (less than 2.1μ). At sarcomere lengths greater than 2.1μ , where there was no overlap of thin filaments in the M-band region, formaldehyde fixation presumably destroyed the antigenicity of the M band.

Very little is known about the effects of change in sarcomere length on the M-band structure other than that the M-band patterns observed in longitudinal and cross-sections in electron microscopy are the same at all sarcomere lengths

(Pepe, unpublished results). It is known, for instance, that the distance between filaments increases with decrease in sarcomere length (16). What effect does this have on the M band? Do the M bridges increase in length as the distance between the filaments increases, or does the interfilament distance remain constant only in the M-band region? If the M bridges increase in length as the distance between filaments increases, do they do so by stretching of the protein molecules making up the M bridges, or by a slippage of one molecule with respect to another? The relationship between the antibody-staining characteristics of the M band at different sarcomere lengths and the extractability of the M-band protein at different sarcomere lengths to the molecular morphology of the M band is unclear.

An important consideration in the antibody staining of striated fibrils is the effect on the structural integrity of the fibrils caused by isolation of the fibrils from the tissue and by any manipulations such as the extraction procedure used in this work. Alteration of the structural integrity of the fibril may alter the antibody-staining properties of the fibrils. In this work two different solutions were used to prepare the suspension of fibrils. Solution A (0.1 M KCl, 5 mM EDTA, pH 7.0) was used to prepare fibrils to be used for extraction after the procedure originally described by Perry and Corsi (10). In general, antibody staining of both extracted and unextracted fibrils was done in solution B (25% glycerol, 7.5×10^{-4} M $MgCl_2$, 7.5×10^{-2} M KCl, 7.5×10^{-3} M PO_4 buffer pH 7.0) which has previously been used in antibody-staining work (5, 13). Fibrils prepared in these two different solutions were stained with antimyosin and antiactin to check for any possible differences in the staining patterns occurring as a result of the different solutions. No differences were observed in the antimyosin-staining patterns (Fig. 2), but sig-

nificant differences were observed in the antiactin-staining patterns (Fig. 3). Although the M-band staining with antiactin was the same in the two solutions, the I-band staining of fibrils prepared in solution A (Fig. 3 *a*) was considerably diminished with respect to that observed for fibrils prepared in solution B (Fig. 3 *b*). This decrease in staining was seen regardless of whether the staining was done in solution A or solution B, the difference being solely a result of the solution in which the fibrils were originally homogenized. The I-band staining that was present in fibrils homogenized in solution A was most intense in the vicinity of the Z band. This would indicate that the I-band material is more completely retained by fibrils prepared in solution B than in solution A. In both solutions, the structural organization of the fibrils as observed with phase-contrast microscopy was the same (Fig. 1). These results emphasize the possible discrepancies that can occur as a result of preparative techniques and which must be considered in the interpretation of antibody-staining results. The nonuniformity of the I-band staining in solution A may reflect a redistribution of I-band material during preparation of the fibrils, or it may represent removal of some nonuniformly distributed antigenic I-band constituent. Previous absorption studies of I-band staining observed with antiactin indicated that there was a nonuniform distribution of antigenic material in the I band (5).

The effect of extraction of fibrils with 5 mM Tris (pH 8) buffer on the structural integrity of the fibrils has been studied by electron microscopy (9, 14). It was found that short extraction removed the M band without effect on the rest of the sarcomere (9) and that longer extraction caused the thick filaments to split especially in the middle of the A band (14). Perry and Corsi (10) showed biochemically that no myosin was removed on prolonged extraction. In all these studies extraction times were 3 hr or longer. In the short extraction times (15 min) used in the major part of this study, complete removal of the M band was indicated by lack of M-band staining in extracted fibrils (Fig. 4 *a*). This was so only for fibrils with sarcomere lengths greater than 2.1 μ as previously discussed. Under these conditions of extraction, the I-band staining was unchanged (compare Fig. 3 *a* and Fig. 4 *a*), indicating that the antigenic I-band material present in fibrils prepared in solution A is not removed during the 15 min extraction period.

Absorption studies were used to identify the extracted material as immunochemically identical to the M material. It was found that absorbing the antiactin with extracted material prior to staining unextracted fibrils eliminated the M-band staining completely while the I-band staining was unaffected (Fig. 6). Any appreciable amount of I-band antigens if present in the extracted material would be expected to remove at least a part of the I-band staining. The results of these absorption studies with the extracted material are consistent with the observations of the staining patterns of the fibrils from which the material was extracted. That is, extraction removed the M band without effect on the I band (see above). These absorption and extraction experiments directly identify the extracted material as being antigenically identical to the M-band material and not to the I-band material.

Even with a 15-min extraction time, slight swelling in some of the A bands of the extracted fibrils was observed. Prolonged extraction increased this effect. The fact that the M-band material could be completely removed in 15 min from fibrils with sarcomere lengths longer than 2.1 μ without appreciable swelling of the A band is significant. This suggests that removal of the M band is not the factor responsible for swelling. The swelling observed after prolonged extraction may result from the additional removal of material between the filaments during the longer extraction times. It is interesting to speculate that this additional material may in some way be involved in the organization of the filaments into a precise lattice (17).

Fibrils extracted for 15 min and stained with fluorescent antimyosin showed essentially the same staining pattern as unextracted fibrils (Fig. 2) except for the impression that the bands along the A-I junction were diminished in intensity (Fig. 5). This was most apparent when comparing extracted and unextracted fibrils at the longer sarcomere lengths around 2.5 μ . This staining represents the staining of antigenic sites specific for the LMM (L-meromyosin) portion of the myosin molecule (5, 13). In this region of the filaments a part of the LMM portion of the myosin molecule is presumably bending out of the core of the myosin filament, making the antigenic sites on this part of the molecule available for antibody staining (5, 13, 17). It is conceivable that extraction partially removed something which aided in the bend-

ing of the myosin molecule out of the filament. Recently a basic scheme for the action of the myosin cross bridge as part of an organized micell which includes the cross bridge and the composite actin filament (actin + proteins associated with the actin filament) has been presented (17). The decrease in LMM staining as a result of the extraction might then be explained in terms of either the disturbance or the partial extraction of the organized micellar aggregate. Depending on how the bridge is involved in the micell, this could result in more of the LMM portion of the myosin molecule being included in the core of the filament.

This work was supported by United States Public Health Service Grant RO1 AM 04806.

Represents work by E. Kundrat in partial fulfillment of requirements for the degree of Ph.D. in Anatomy.

Received for publication 4 June 1970, and in revised form 24 August 1970.

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