THE DISTRIBUTION OF MUSCLE ANTIGENS IN CONTRACTED MYOFIBRILS DETERMINED BY FLUORESCEIN-LABELED ANTIBODIES

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ABSTRACT

Chick myofibrils in different states of contraction were treated with fluorescein-labeled antibodies. The rabbit antibodies were prepared against chick myosin, light and heavy meromyosins, and actin. For any one state of contraction, a single myofibril was photographed through the phase contrast microscope, stained with one of the antisera, and photographed through the fluorescence microscope. The cytological changes in the sarcomeres accompanying contraction as observed under phase were correlated with changes in the distribution of the precipitated antibodies as observed under the fluorescence microscope. The changing patterns observed through the fluorescence microscope were compared with those predicted by the sliding filament model of contraction.

INTRODUCTION

Many properties of cross-striated myofibrils are believed to be a function of the distribution of the contractile proteins myosin, actin, and, to a lesser extent, tropomyosin. For example, the discontinuity of myosin molecules along the length of the relaxed myofibrils is thought to determine the cytology of the A band (1, 4, 5, 12, 15, 19). Huxley and Hanson (4, 14, 15), on the basis of salt extraction experiments and electron microscopy, have proposed that many characteristics of the I band and the presence or absence of the H band correlate, in rabbit muscle, with the distribution of actin along the myofibril. Similarly, the sliding filament theory of contraction (13, 15) proposes that overlap between thick (myosin?) and thin (actin?) filaments observed in electron micrographic sections is prerequisite to the interaction of the proteins responsible for shortening. Clearly a better understanding of where in the sarcomere the contractile proteins are localized in different functional states should further our understanding of how structure and function of the myofibril are interdependent.

Fluorescein-labeled rabbit antibodies against chicken myosin, light meromyosin (L meromyosin), heavy meromyosin (H meromyosin), actin, and tropomyosin are each bound to discrete regions of mature and embryonic sarcomeres (12, 19). While there are unresolved problems concerning the specificity and homogeneity of these antisera—and of the antigens used in their preparation—still their unique localization within a sarcomere allows them to be used to trace these antigens in myofibrils in different states. This report describes the changes in the localization of these antisera and, consequently, the changes in the localization of the reacting muscle antigens in relaxed, mildly contracted, and strongly contracted myofibrils.

MATERIAL AND METHODS

Glycerinated Muscle

Glycerol-extracted chicken breast muscle was prepared as described by Szent-Györgyi and Holtzer (21). To obtain relaxed muscle, bundles of muscle fibers were tied to sticks at body length before excision. To obtain contracted muscle, bundles were allowed to contract freely after excision. Relaxed and contracted material was stored at −15°C for at least 2 weeks before use.

Myofibrils

A fiber bundle was homogenized in 10 ml M/60 PO_4 buffer, pH 7.6, 25 per cent *v/v* glycerol (phosphate-glycerol), for 2 minutes at 0°C in an Omni mixer at 80 volts. The fibrils were then washed three times in 25 per cent phosphate-glycerol by centrifugation and resuspension.

Localization of Antibodies

The fluorescein-labeled antibodies against chicken myosin, L and H meromyosin, and actin were those used in previous studies (3, 10, 12, 19). A drop of phosphate-glycerol suspension of myofibrils was placed on a microscope slide. The fibrils were treated with labeled antibody for 10 minutes by drawing a drop of a solution of antibody in phosphate-glycerol under the coverslip. Unbound antibody was removed by continuously drawing phosphate-glycerol under the coverslip for 10 minutes. Both phase contrast and fluorescence photomicrographs of the same myofibril were taken at a final magnification of 1000 times before and after exposure to the different antibody solutions. Control myofibrils were treated with fluorescein-labeled normal gamma globulin by the same procedures. Fluorescence photomicrographs were taken on spectroscopic plates (Kodak 103a G), using an Osram HBO 200 arc as a light source, 1 mm Corning 5840 plus 1 mm Schott UG 1 as exciting filters, and 1 mm each of Wratten K2 and 2B as barrier filters. An alternative arrangement consisted of 6 mm Schott BG 12 exciting filters and 1 mm each of Zeiss Sp orange 2 and Sp yellow. A Cardioid condenser, N.A. 1.2, was used.

Those regions of the sarcomere to which labeled antibody was bound could be identified by their increased optical density under the phase contrast microscope (7, 21), or by their emission under the fluorescence microscope. The detection of fluorescence was by far the more sensitive method. The phase contrast pattern of an untreated sarcomere was compared with the fluorescent pattern of the same sarcomere after treatment with labeled antibody. Although the centers of fluorescent images coincided with the centers of corresponding phase contrast images, often the widths of the two images did not coincide. Most frequently a given fluorescent band appeared slightly wider in photomicrographs taken through the fluorescence microscope than the same band in photomicrographs taken through the phase microscope. At other times, however, the fluorescent image was narrower. Whether this was due to the distribution of labeled antibody, to the optics of the fluorescence microscope, or to the photographic procedures is uncertain (11).

TABLE I

Morphological Characterization of the Various Stages of Contraction

Stage	Characteristic features
A	I band: present. Z line: dense, faint, or undetectable. A band: wide H band.
B	I band: present. Z line: dense, faint, or undetectable. A band: divided by a dark contraction band, the C_m band.
C	Two dark contraction bands of unequal optical density. The lighter of these may be faint or undetectable. Fibrils break at the lighter band.
D	Closely spaced contraction bands of equal optical density; occasionally lighter bands are also present.

RESULTS

A. *Stages of Contraction*

The different stages of contraction were characterized by the phase contrast appearance of the sarcomeres (Table I; Fig. 1, line 1). A and I bands were readily recognized in stage A sarcomeres; occasionally in relaxed sarcomeres the Z line was difficult to detect. Similarly the A, I, and C_m bands of stage B were readily identified. In stages C and D, however, the A and I bands had disappeared. As a consequence the C_m and C_z bands had to be identified by other means. The following evidence, adopting the terminology of Hodge (6), indicated that in stage C fibrils the light contraction band was the C_z band and the dark contraction band was the C_m band:

1. Stage A and B fibrils fragmented at or near the Z line, never in the A band. Stage C fibrils fragmented at or near the light contraction band.

2. In stages A and B the appearance of the Z line varied from pronounced to undetectable. This also characterized the light contraction band in stage C fibrils.

3. When stage A and B fibrils were transferred

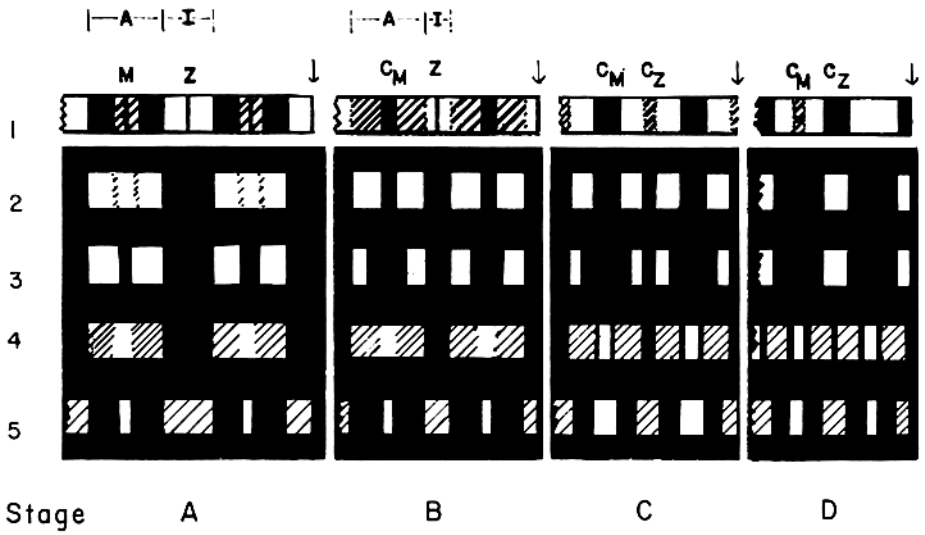


FIGURE 1

Diagrammatic representation of the phase contrast appearance of myofibrils, in various stages of contraction, and of the corresponding fluorescent patterns. No effort has been made to correlate the changes of sarcomere pattern with changes of sarcomere dimensions. Diagonal lines indicate reduced density under the phase contrast microscope and reduced fluorescence under the fluorescence microscope. Arrows indicate the regions of the sarcomere where fibrils fragment. Line 1, phase contrast; line 2, antimyosin; line 3, anti-L meromyosin, line 4, anti-H meromyosin; line 5, actin.

to phosphate-glycerol from low salt solutions, the greatest swelling occurred at the center of the sarcomere. When stage C fibrils underwent the same change in medium, the greatest swelling occurred in the region of the dark contraction band.

For stage D sarcomeres the dark contraction bands were the C_z bands, the C_m bands being faint or undetectable under phase. This identification was based on the fact that in stage D sarcomeres the fibrils fragmented at or near the dark contraction band and the greatest swelling occurred in the region between the dark bands.

B. *Distribution of Fluorescent Antibodies in Different Stages of Contraction*

I. *Localization of Normal Globulin:* There was little, if any, binding of fluorescein-labeled normal globulin by the myofibrils. When present, the fluorescence was variable and too weak to be photographed.

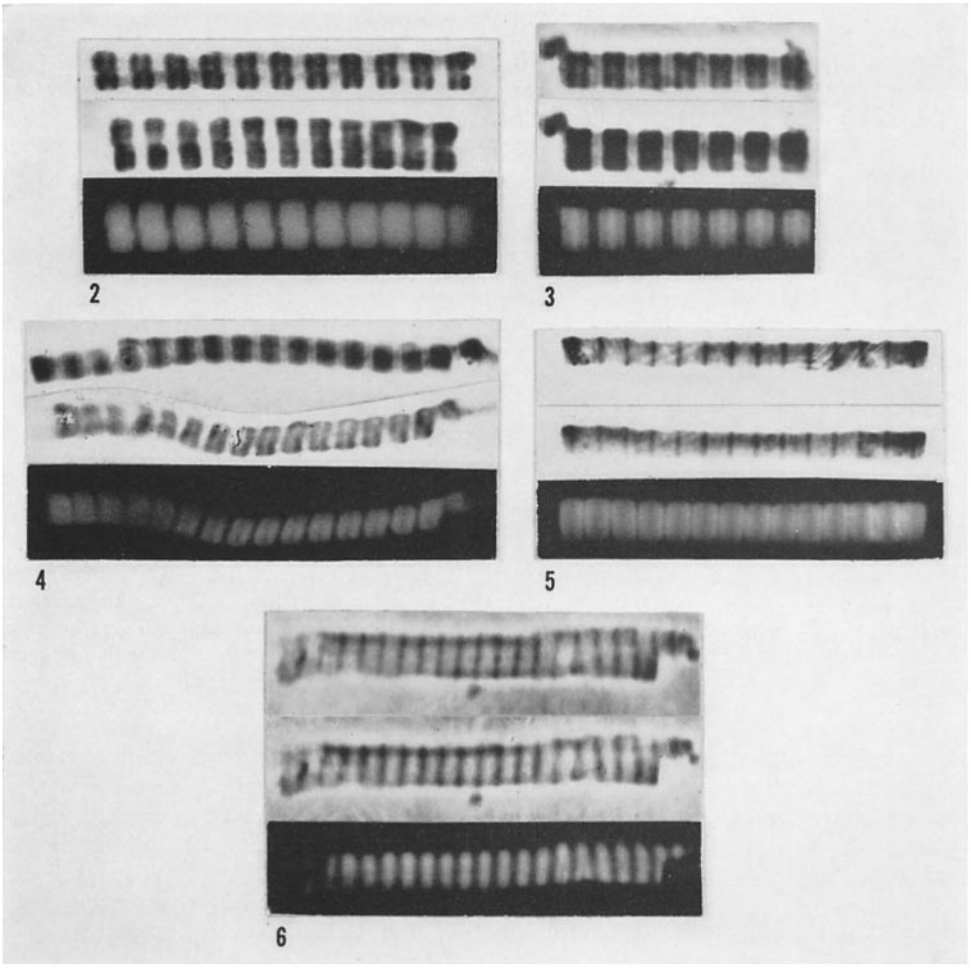
II. *Localization of Antimyosin:* Stage A: As illustrated in Fig. 1, line 2, and Figs. 2 and 3, two fluorescent patterns were encountered: a uniformly fluorescent A band, and a fluorescent A band with an intensely fluorescent central stripe. Note how the bound labeled antibody enhanced the phase density of the H band in Fig. 3.

Stage B: The A band fluoresced throughout its length, with the exception of a central region coincident with the C_m band. Note how the local precipitation of antibody has increased the phase density of the A band near the A-I junction (Fig. 4).

Stage C: The regions between the C_m and C_z bands were fluorescent. The non-fluorescent band bisecting the sarcomere was broader, and the non-fluorescent C_z region narrower, than in stage B. Consecutive A bands appeared to be converging on the non-fluorescent C_z band between them to form the fluorescent C_z band described in the next stage (Fig. 5).

Stage D: Only the regions coincident with the dark contraction band, the C_z band, fluoresced. Frequently the doublet nature of the fluorescent C_z band could be observed along the edges of the myofibril. The doublet nature of the fluorescent C_z band was often revealed by the terminal bands where the fibril broke, which were one-half the width of other C_z bands (Fig. 6).

III. *Localization of Anti-L Meromyosin:* Stage A: The A band fluoresced throughout its length, with the exception of a non-fluorescent band in the center (Fig. 7).



FIGURES 2 TO 6

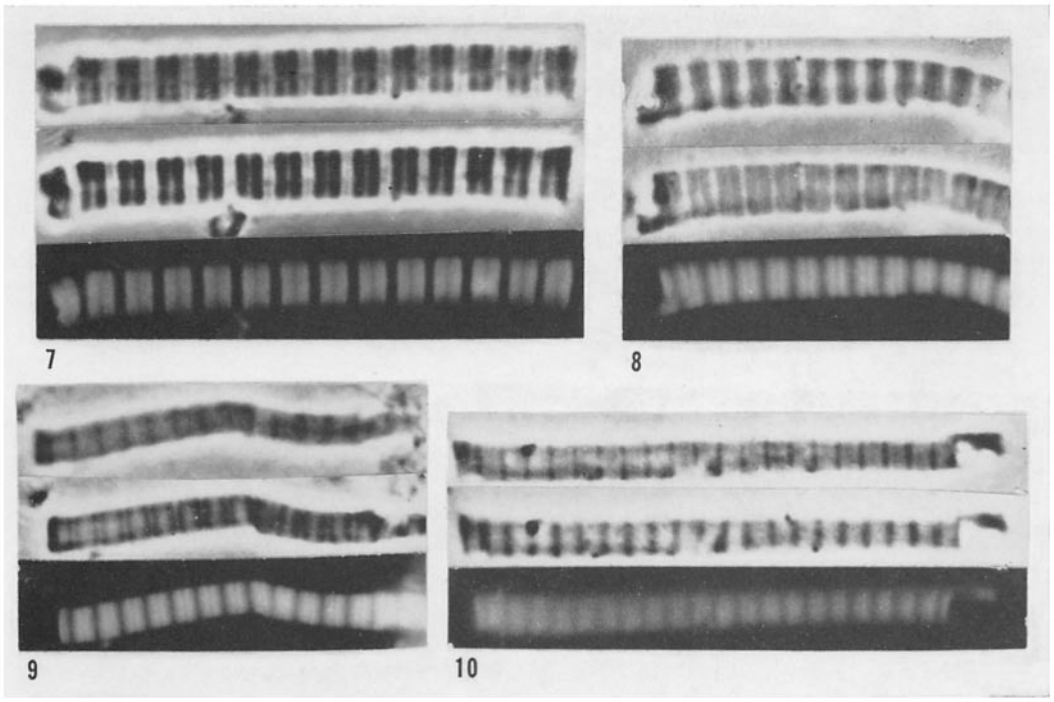
Fibrils before and after treatment with antimyosin. In each figure there is a phase contrast photomicrograph of the untreated fibril (upper), a phase contrast photomicrograph of the same fibril after treatment with labeled antibody (middle), and a fluorescence photomicrograph of the treated fibril (bottom). Figs. 2 and 3, stage A. Fig. 4, stage B. Fig. 5, stage C. Fig. 6, stage D. See text for details. × 2000.

Stage B: The non-fluorescent C_m band was broader than the corresponding C_m band of stage B sarcomeres treated with antimyosin. As a result, a fluorescent doublet centered on the Z region was already indicated in stage B sarcomeres treated with anti-L meromyosin (Fig. 8).

Stage C: Only the regions between the contraction bands fluoresced. The non-fluorescent C_z band was quite narrow, the non-fluorescent C_m band quite broad. The doublet nature of the fluorescent bands converging on the Z line was most clearly seen in this stage (Fig. 9). Note the deposition of antibody observable under the phase microscope around the C_z region. Though considerable changes between stages B and C were observed under phase, the fluorescent patterns were quite similar.

Stage D: The regions coincident with the C_z bands fluoresced. These fluorescent C_z bands exhibited the doublet nature described for antimyosin-treated fibrils (Fig. 10).

IV. *Localization of Anti-H Meromyosin:* Stage A: Only the A band fluoresced. The fluorescence was most intense in the central portion of the A



FIGURES 7 TO 10

Fibrils before and after treatment with anti-L meromyosin. Arrangement of phase and fluorescent photomicrographs as before. Fig. 7, stage A. Fig. 8, stage B. Fig. 9, stage C. Fig. 10, stage D. See text for details. × 2000.

band, in the region corresponding to the H band. A broad, less intensely fluorescent area corresponded to the region of the A band near the A-I junction. This latter region of reduced fluorescence appeared to be the region stained by L meromyosin antisera (Fig. 11).

Stage B: The major difference between stage A and stage B was a narrower non-fluorescent I band. It is worth stressing that with the anti-H meromyosin sera it was the C_m band which fluoresced most strongly, whereas with antibody to myosin or L meromyosin the C_m band was non-fluorescent (Fig. 12).

Stage C: Again the C_m band fluoresced prominently and the C_z band was non-fluorescent. The less fluorescent broad zones were often separated from the fluorescent C_m band by narrow non-fluorescent regions. The broad zones of reduced fluorescence corresponded to the regions rendered fluorescent by the L meromyosin antisera (Fig. 13).

Stage D: The most fluorescent band lay midway between the C_z bands. Adjacent portions of the broad, less fluorescent bands formed a doublet around the C_z area (Fig. 14).

V. *Localization of Antiactin:* Stage A: The I band and particularly the H band fluoresced. The fluorescence of the former was diffuse, that of the latter sharp (Fig. 15).

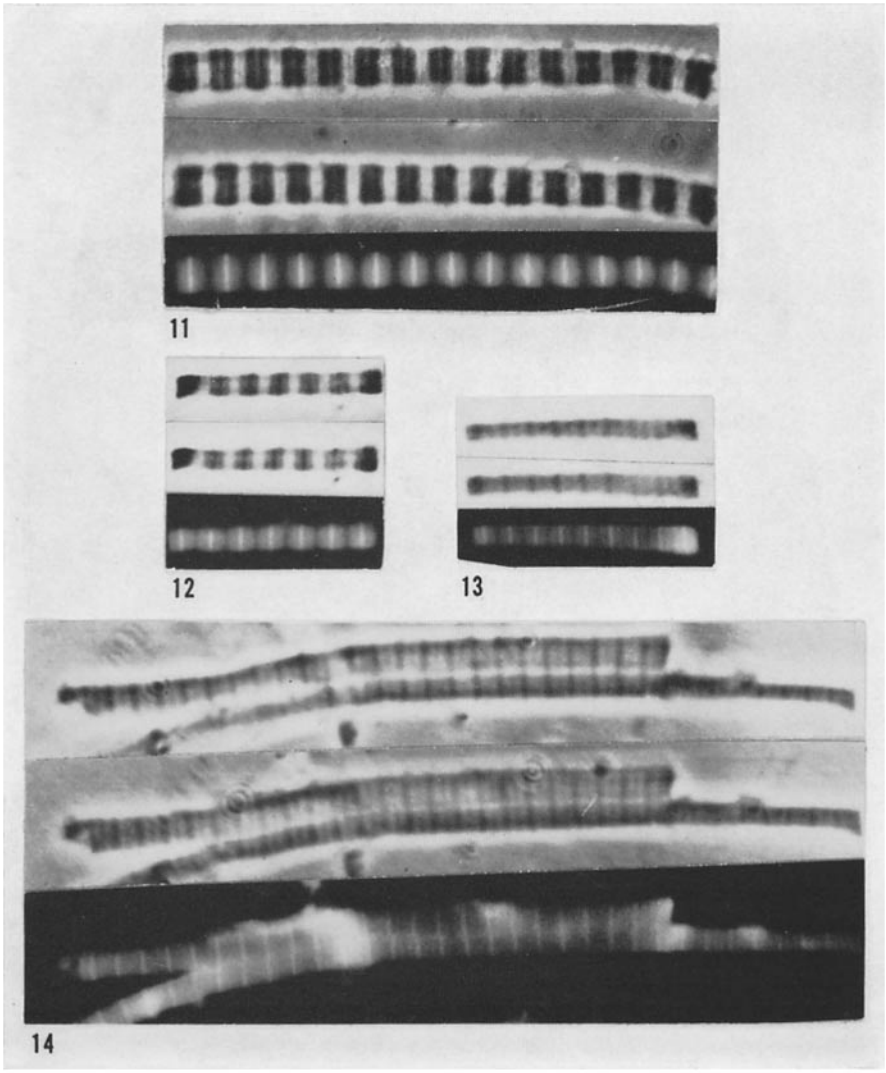
Stage B: Similar to stage A, but the bands were closer together. The bright, sharply defined fluorescent bands corresponded to the C_m bands observed under the phase microscope (Fig. 16).

Stage C: The regions corresponding to the contraction bands as observed under the phase microscope fluoresced. The sharp fluorescent band corresponded to the C_m band, the more diffuse zone to the C_z area (Fig. 17).

Stage D: The prominent fluorescent band lay midway between the C_z bands. The dimmer fluorescent band was coincident with the C_z bands.

DISCUSSION

Before interpreting the localization of the antisera in contracted myofibrils it is worth while to review

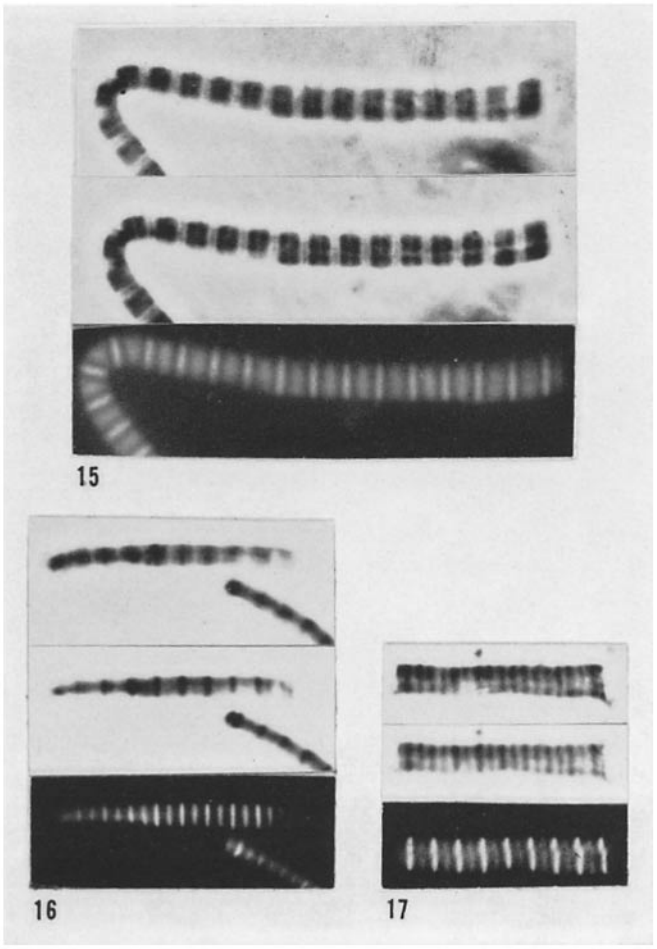


FIGURES 11 TO 14

Fibrils before and after treatment with anti-H meromyosin. Arrangement of phase and fluorescent photomicrographs as before. Fig. 11, stage A. Fig. 12, stage B. Fig. 13, stage C. Fig. 14, stage D. See text for details. × 2000.

their status as specific staining reagents. That the antimyosin molecules do combine selectively with myosin as defined by other approaches is demonstrated by the following. Myosin antibodies (*a*) complex exclusively with antigens in the A band; (*b*) do not react with antigens in sperm tails, cilia, mitotic figures, fibroblasts, or other types of cells (3, 8, 9, 10, 23); (*c*) protect the A band from salt solutions known to extract myosin (7, 21), and *quantitatively* prevent the extraction of myosin from suspensions of mature and embryonic fibrils pretreated with the antibody (8). What is still unknown, however, is whether the myosin antisera contain antibodies to A band antigens other than myosin.

The localization of anti-L meromyosin on the myofibril and its cross-reaction with myosin in solution or in gel diffusion tests indicate that it could be directed to a region of the myosin molecule (8, 19).



FIGURES 15 TO 17

Fibrils before and after treatment with antiactin. Arrangement of phase and fluorescent photomicrographs as before. Fig. 15, stage A. Fig. 16, stage B. Fig. 17, stage C. See text for details. × 2000.

The nature of the antigens binding anti–H meromyosin is less clear. From its localization in relaxed and contracted myofibrils it is probable that anti–H meromyosin solution contains at least two components: one against antigens in the regions of the A band near the A-I junction (possibly the L meromyosin antigens), and one against antigens concentrated in the H band. The separation of the diffuse fluorescent band from the fluorescent C_m band in contracted fibers and the similar staining of this region with anti–L meromyosin suggests that one component is shared by the two meromyosin antisera. It is of interest that analysis by means of Oudin tubes independently suggests that one component is shared by the two meromyosin antisera (19). Whether the H band antigens correspond to a discrete fragment of the myosin molecule exposed by trypsin digestion (20), or whether it is against a still undefined antigen, such as cholinesterase (2, 18, 22), is not known at present.

Likewise there is evidence that the antiactin sera contain at least two species of antibody molecules: antibody precipitated by antigens in the I band, and the component directed against the H band antigens. The common behavior of the H band and the C_m band when fibrils in different stages of contraction are treated with antiactin and anti–H meromyosin suggests that the two solutions share a common population of

antibody molecules. This finding is in keeping with the observation that antibody reacting with H band material may be selectively removed from anti-H meromyosin sera by absorbing against a crude fraction of $MgCl_2$-precipitated actin (Holtzer, Szent-Gyorgyi, and Abbott, unpublished observations). The remaining component of the antiactin solutions—the component reacting with the I band—is unique to the antiactin. Whether this component is against the whole actin molecule or a fraction or bound impurity of the actin molecule, or whether it is against still another unknown antigen in the I band is being investigated.

The preparation of glycerinated bundles of muscle fibers and their subsequent homogenization may cause contraction and stretching of the fibrils. As a result, a given sarcomere length could be arrived at by any combination of contraction (isometric or isotonic) and stretching (9). The possibility of these changes renders uncertain any exact relationship between stages of contraction and relative or absolute sarcomere length. However, sarcomere length does seem to decrease regularly from stage A to stage D. It is likely that these stages of contraction occur in their lettered sequence. Assuming that these stages are stop-motion samples from a continuous morphological change, then the redistribution or changing state of the muscle antigens responsible for shortening may be deduced from the fluorescence patterns characteristic of each stage.

Two aspects of the fluorescence patterns obtained with myosin antisera are of particular interest in view of the sliding filament model of contraction. These are: (*a*) the absence of fluorescence in the center of the sarcomeres in all contracted fibrils, and (*b*) the formation of doublet C_z bands as the myosin from adjoining sarcomeres encroaches on the common Z line (see 16, 17). Presumably the changes responsible for contraction result in the observed discontinuous distribution of myosin. This could be due either to a "movement" of myosin away from the nonfluorescent regions or to an alteration in the availability of antigenic sites on the myosin molecule. In either case these findings suggest a type of heterogeneity in the A band that as yet cannot be equated with the thick filaments observed under the electron microscope.

The fluorescence patterns obtained with actin and H meromyosin antisera also merit attention. They suggest that (*a*) the I band antigens (revealed by antiactin sera) and the H band antigens (revealed by antiactin and anti-H meromyosin sera) maintain their relative positions throughout all stages of contraction, and (*b*) a discontinuous, non-overlapping distribution of these antigens, or at least of their available antigenic sites, persists throughout all stages of contraction. These findings would not have been anticipated by the sliding filament model of contraction. Further characterization of muscle antigens and their antibodies should lead to a better correlation between the structure of myofibrils revealed by electron microscopy and that revealed by the fluorescence antibody technique.

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