AN IMMUNOCHEMICAL STUDY OF THE DISTRIBUTION OF MYOSIN IN GLYCEROL EXTRACTED MUSCLE*

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Plates 58 and 59

Recent investigations of the distribution of myosin and actin in the skeletal muscle fiber have relied largely on extraction techniques for the differential removal of specific proteins. This work has been reviewed by Hanson and Huxley (1). Because such extraction techniques may remove much soluble material in addition to myosin or actin, conclusions based on density changes within fibers after extractions have seemed somewhat questionable (2). We have, therefore, attempted a more positive localization of myosin in glycerol fibers by the use of the fluorescent antibody method (3, 4).

Methods

Myosin was prepared from chicken breast muscle by the procedure of Mommaerts and Parrish (5). The first precipitate of myosin was dissolved and reprecipitated 3 times at an ionic strength of 0.05. Traces of material insoluble at ionic strengths of 0.27 to 0.30 were removed in intermediate steps. Antisera were prepared in rabbits by injecting myosin in saline solutions or with alum or aquaphor adjuvants. Sera of good precipitating power were obtained when a total dose of 33 mg. of myosin was given in two brief courses a month apart. The γ-2 globulin fractions of two antisera were labelled by conjugation with fluorescein isocyanate (3). A control solution of normal rabbit γ-2 globulin was similarly labelled. The solutions were purified by dialysis in saline and in 25 per cent glycerol at pH 7.4 to remove free dye.

Chickens were killed by decapitation under pentobarbital anesthesia. Relaxed muscles from the thigh were tied at rest length and extracted in 50 per cent glycerol at 0° to −15°C. In some instances, muscle strips were fixed in 2 per cent formaldehyde briefly before glycerol extraction. After several days of extraction, homogenates or teased fibers were prepared in 25 per cent glycerol and were then treated with the fluorescent globulin solutions for periods ranging from 2 hours to 2 weeks at 5°C. We found to our surprise that antibody staining would occur not only in saline solutions but also in glycerol concentrations of 25 to 50 per cent at low ionic strengths. This made it possible to treat fibers for long periods in labelled globulin solutions without loss of A band density by extraction. After staining, the fibers were washed in saline or 25 per cent glycerol, buffered to pH 7.4, at +5° or −5°C. for a day

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or more. The preparations were first examined in glycerol by phase contrast and fluorescence microscopy. In addition some material was fixed in formaldehyde, embedded in diethylene glycol distearate, and sectioned at 0.1 to 1.0 μ.

OBSERVATIONS

Muscle fibers treated with the control globulin solution showed after washing only an extremely faint and inconstant staining of the Z lines. Repeated dialysis of the labelled globulins revealed that traces of free dye were present, which presumably were responsible for this effect. Fibers treated with labelled antibody solutions were found to be very heavily stained both grossly and microscopically. The A bands, as identified in phase contrast (Figs. 3 and 5), were brilliantly green in the fluorescence microscope (Figs. 1, 2, 4). The I bands were quite unstained, but a very faint Z line fluorescence was detected in some of the fibers, as in the controls. In certain lots of muscle, the fine structure of the A bands showed considerable variation both in phase contrast and in fluorescence. Fig. 1 illustrates the broad, homogeneously stained A bands of what is thought to be relaxed muscle (6). In fibrils which had H bands of varying widths, the H bands seemed weakly stained, but the denser edges of the A bands were very strongly stained (Figs. 2 and 4). The M line, when present, was moderately stained. In areas of marked contraction the closely spaced dense bands seen in phase contrast were strongly fluorescent. In thin sections it was at times possible to resolve such contraction bands into fluorescent doublets.

Other materials which we have briefly examined include glycerol extracted chicken heart, liver, kidney, and the trunks of chick embryos. The fibrils of heart muscle were very clearly stained, but liver and kidney were not stained. In the embryo, as early as the third day, sharply stained striated fibrils were found along the lateral aspect of each somite (Figs. 6, 7, 8). Tissues fixed briefly in formaldehyde before glycerol extraction gave similar results.

DISCUSSION

The findings described above indicated that our antisera were specific for muscle, but did not prove conclusively that the antigen or antigens detected were myosin. Minor impurities present in the initial myosin preparation might have caused a disproportionately high antibody response in the rabbits. To test this possibility, we have begun an analysis of the antisera by simple diffusion in agar gels (7, 8), using purified myosin and various crude extracts of muscle as challenging antigens. The results to date suggest that the sera contain a major component and one or two minor components. The major component reacts with an antigen which has been provisionally identified as myosin on the basis of its low apparent diffusion coefficient, its partition during salt fractionation of crude muscle extracts, and its anomalous behavior in aged myosin solutions.

We have also analyzed by gel diffusion a fluorescent antibody solution before and after absorption with glycerol extracted muscle fibers. The absorbed solu-

tion lost its major antibody component and the fibers showed strong staining of the A bands. Whether the minor components in the antiserum were also taken up could not be determined.

Another finding which gave some support to the conclusion that the labelled antisera stained myosin came from a trial of the effects of ATP on teased glycerol fibers. It was found that fibers soaked in the labelled control globulin solution contracted consistently to 20 to 30 per cent of rest length, whereas fibers treated with the labelled antibody solution did not contract. This difference persisted after thorough washing.

These studies must be confirmed with antibody solutions from which all other components have been absorbed before we can conclude that an antibody to myosin alone is responsible for all the details of staining within the A band. However, it seems likely from the evidence at hand that myosin is confined to the A band in the relaxed glycerol fiber and that changes in the pattern of distribution of myosin occur in certain states of contraction. In future work, it should be possible to determine the distribution of myosin in the stages of contraction described by Hodge (6) and to localize other muscle proteins by the use of specific antisera. In studies on the chick embryo, the method should yield more precise cytological information on the differentiation of skeletal and cardiac muscle (9, 10).

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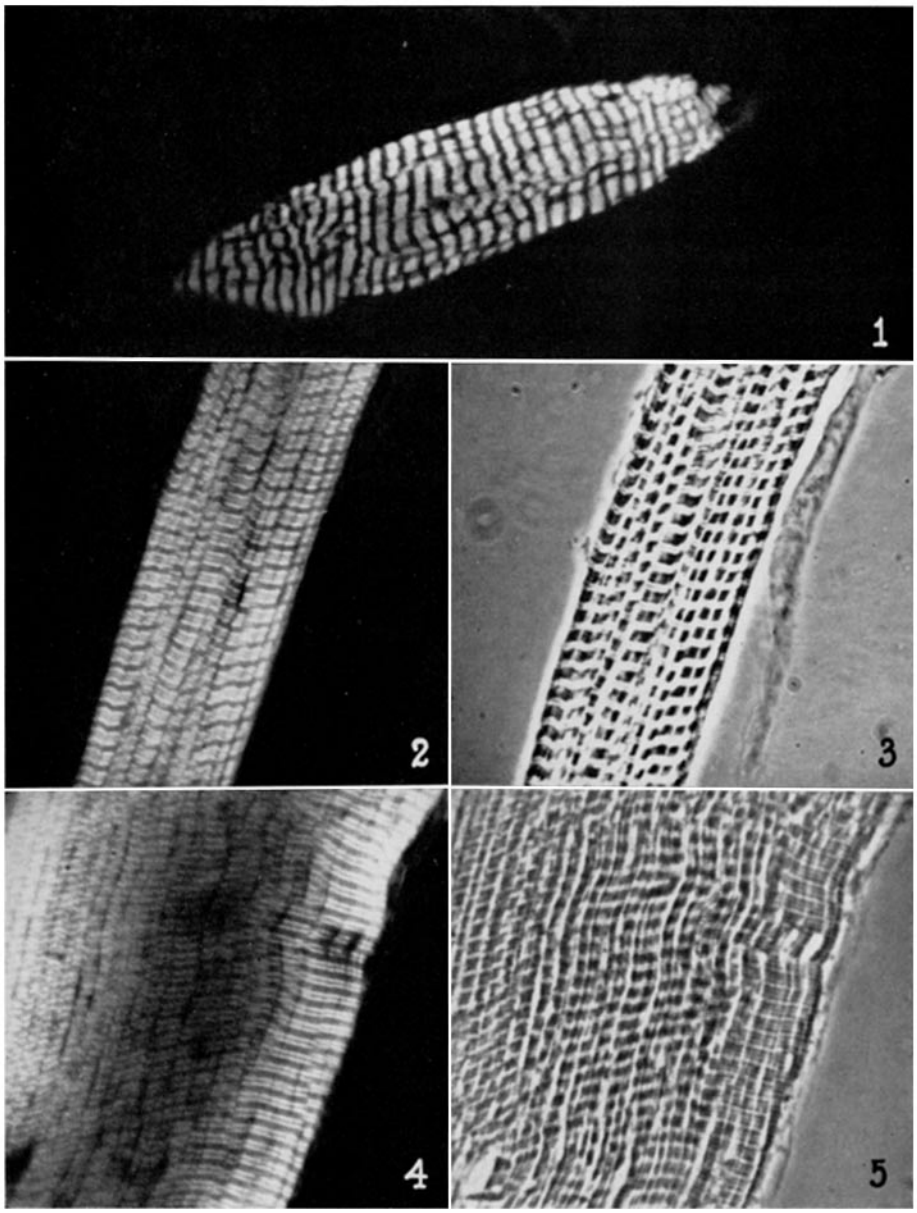
EXPLANATION OF PLATES

PLATE 58

FIG. 1. Fluorescence photomicrograph of chicken thigh muscle stained for myosin with fluorescein-labeled antibody in 25 per cent glycerol, 0.03 M phosphate buffer, pH 7.4, for 2 days. The A bands are stained rather uniformly. In phase contrast photomicrographs (not reproduced here) the same fiber shows A bands of uniform internal density. Thin section, about 0.2 μ. × 1000, numerical aperture 1.4.

FIGS. 2 and 3. Fluorescence and phase contrast photomicrographs of a 0.5 μ section of a muscle fiber, stained for myosin in buffered 25 per cent glycerol for 4 days. In fluorescence (Fig. 2), A, M, and H bands are seen. Faint staining of Z lines occurs also but is found in control sections as well, and appears to be an artifact caused by traces of free dye. × 1000, numerical aperture 1.4. In dark medium phase contrast (Fig. 3), the A bands show much less internal structure than in fluorescence. × 1000, numerical aperture 1.25.

FIGS. 4 and 5. Fluorescence and phase contrast photomicrographs of the same 1 μ section of a muscle fiber stained for myosin in 0.15 M NaCl solution, pH 7.4, for 12 hours. In fluorescence (Fig. 4), the H bands are wider and the I bands narrower than in the specimen illustrated by Fig. 2. The peripheral fibrils are more strongly stained than those in the core of the fiber, because of the short staining time. Fig. 4, × 1000, numerical aperture 1.2. In dark medium phase contrast (Fig. 5) the peripheral fibrils show internal details of M, H, and A band differentiation not seen in the core fibrils. It is suggested that, in saline solutions without glycerol, extraction of substances other than myosin from the A bands occurs concomitantly with the staining reaction. × 1000, numerical aperture 1.25.

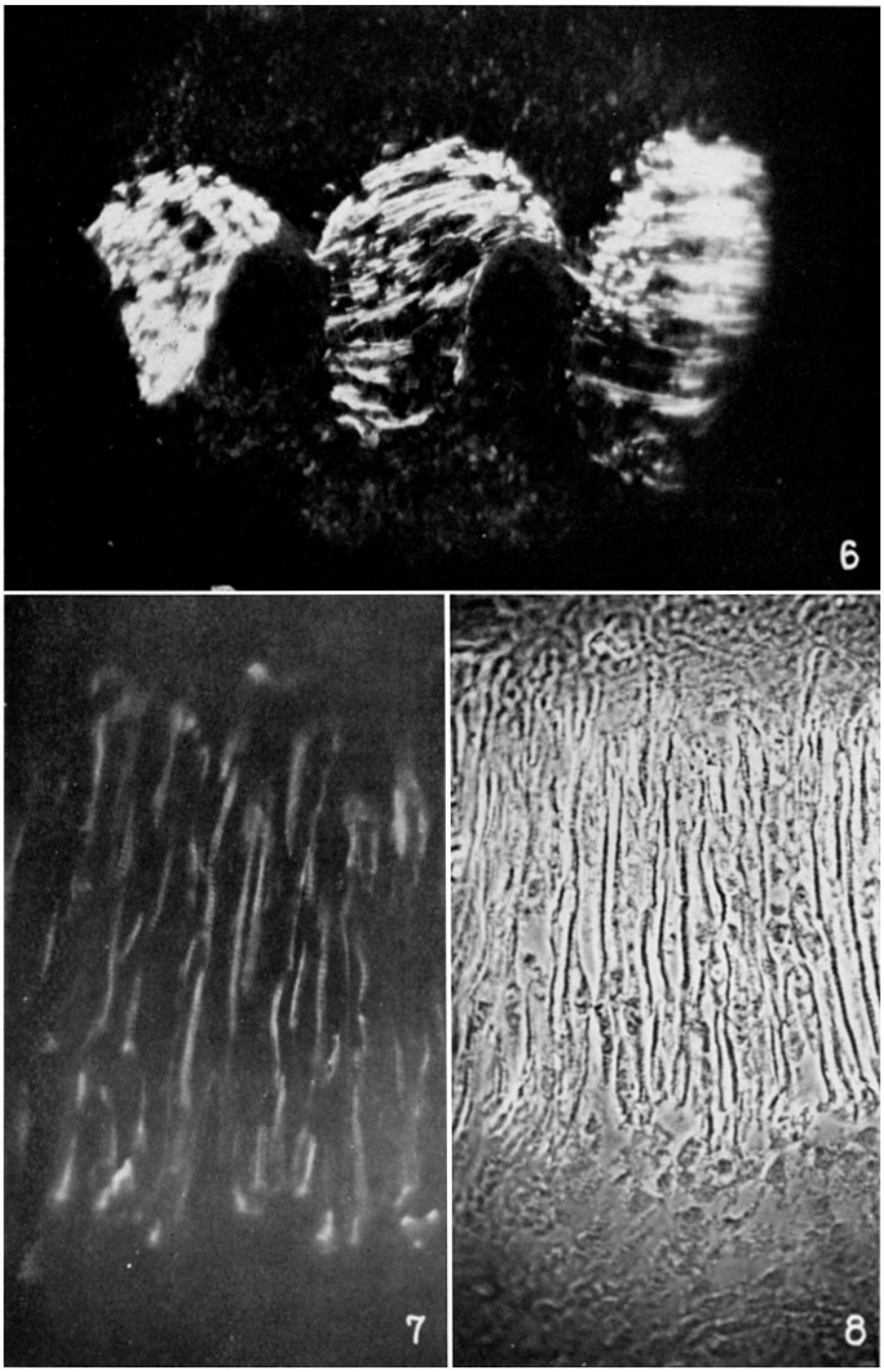


(Finck *et al.*: Myosin distribution in glycerol extracted muscle)

PLATE 59

FIG. 6. Fluorescence photomicrograph of a parasagittal section of a 4-day chick embryo trunk, which has been stained for myosin in buffered 25 per cent glycerol for 4 days. The brilliantly fluorescent fibrils of each myotome are seen coursing over the corresponding dorsal root ganglion, which is quite unstained. (It should be noted also that spinal cord, notochord, cartilage, and mesenchyme were unstained.) × 225, numerical aperture 0.65. Thickness, 1 μ.

FIGS. 7 and 8. Fluorescence and phase contrast photomicrographs of the same section of chick embryo as is illustrated in Fig. 6. (In some instances the individual fibrils had striations with internal details remarkably like those of adult muscle, although, in these examples, such details are not shown clearly.) Fig. 7, × 670, numerical aperture 1.0. Fig. 8, × 660, numerical aperture 1.25.



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