THE USE OF FLUORESCEIN-LABELED HEAVY MEROMYOSIN FOR THE CYTOLOGICAL DEMONSTRATION OF ACTIN

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INTRODUCTION

The interaction of actin and myosin is considered to be a necessary condition for the contraction of striated muscle. A cytological demonstration that myosin can bind to a discrete part of the sarcomere, which from other evidence contains actin, was made by Huxley (1, p. 261). He observed an increase in phase contrast density when myosin-extracted fibrils were treated with myosin in solution. Heavy meromyosin (HMM), a protein fragment soluble at low ionic strength which is derived from the tryptic splitting of myosin, was also seen to bind. From electron microscope observations, Huxley (2) noted that this binding appears to be polarized.

The present observations are an effort to increase the cytological sensitivity by which the binding of myosin is visualized and to give additional information on the localization of actin in striated muscle.

MATERIALS AND METHODS

The rabbit HMM used in these experiments was prepared according to Szent-Györgyi (3) and was the non-dialyzable, 0.05 M KCl soluble fraction of the original myosin digest. Fluorescein isothiocyanate (Sylvana Chemical Co., Orange, New Jersey) was plated from acetone onto the wall of a flask, after which HMM in 0.1 M KCl, buffered to pH 8.5 with 0.05 M bicarbonate, was added. The reaction proceeded for 12 to 18 hours at 0°C, after which the mixture was exhaustively dialyzed against 0.05 M KCl at pH 7. A fluorescein isothiocyanate to HMM weight ratio of 1:20 (recommended for antibody conjugation) was used and the level of HMM in the reaction mixture was about 8 mg/cc.

Glycerinated rabbit psoas muscle fibrils in 0.1 M KCl at pH 7 were usually stained with fluorescein-coupled heavy meromyosin (F-HMM) and washed in the test tube. Staining was frequently done at pH 8.5 since it gave the same pattern as at pH 7, but more F-HMM appeared to be bound. *Drosophila* fibrils were stained and washed on the slide.

A solution designated as Mg-pyrophosphate contained 0.1 M KCl, 0.01 M sodium pyrophosphate, 0.002 M $MgCl_2$, and had a final pH of 7. Mersalyl[1] was used at levels of 0.5 mM or less in 0.1 M KCl at pH 7.

Observations were made with an immersion objective using an aperture of about 0.9. Excitation was by darkfield illumination using an HBO200 mercury arc with Zeiss U. G. and B. G. filters. Photomicrographs were taken at initial magnifications of 400 to 650 × on Kodak 35 mm Tri-X film with exposures of 2 to 4 minutes and developed in Microdol-X.

OBSERVATIONS

Fluorescein was coupled to free amino groups of HMM using fluorescein isothiocyanate. With the procedures used, a large part of the ATPase activity of HMM (90 per cent) was lost and the total amount of fluorescein-labeled material which could be absorbed from solution by myofibrils was also small. It has been noted previously (4, 5) that coupling the amino groups of myosin causes a loss of ATPase activity. This large decrease in activity and in binding limits the conclusions which can be made unless other methods can demonstrate that

[1] Salicyl - (γ - hydroxymercuri - β - methoxypropyl)-amide-O-acetate (Winthrop Laboratories, New York City).

the binding is similar to that of myosin and of HMM.

Szentkiralyi (6) has defined some conditions for the binding and release of HMM with rabbit *myofibrils*, and her study provides a basis for staining with F-HMM as well as for determining whether F-HMM binding to fibrils is similar to that of HMM.

Dissociation of F-HMM from the myofibrils when treated with Mg-pyrophosphate is probably the most important experimental criterion for determining that F-HMM binds to actin in the same manner as HMM. Szentkiralyi (6) has observed that about 80 per cent of the unlabeled HMM bound to glycerinated fibrils can be released by pyrophosphate. Good, but never complete, release of F-HMM occurred with some preparations on treatment with Mg-pyrophosphate; other preparations showed no detectable response. The pattern of staining was, however, similar with both types of F-HMM except for one old preparation of lyophilized HMM in which M staining at long sarcomere lengths could be seen.

Treatment of F-HMM with Mersalyl was effective in reducing the binding of F-HMM to fibrils, but not in completely abolishing it. Pretreatment of the fibrils with Mersalyl also reduced the amount of F-HMM bound. These results are in gross agreement with the *in vitro* study of Perry and Cotterill (7) on the binding of actin to HMM.

Pretreatment of the fibrils with unlabeled HMM abolished all but a very faint trace of F-HMM binding, possibly due to non-protein-coupled fluorescein. Fluorescein isothiocyanate hydrolyzed in the absence of protein stains the A and Z bands to give an image which is the reverse of that seen with F-HMM.

Fig. 1 shows the pattern of staining seen when F-HMM binds to stretched glycerinated rabbit psoas fibrils with a sarcomere length of 2.7 μ. Bands were identified on the assumption that terminal breaks in sarcomeres with detectable I regions occur in the I region close to the Z band. This was verified by polarized light microscopy. The strongly stained doublet is the I region and it is flanked by less strongly stained bands in the A region which are adjacent to an unstained gap at the level of the H band. The less intense line in the I region presumably corresponds to the Z band.

Shorter sarcomeres have a modified pattern as shown in Figs. 2 and 3. Near equilibrium length (2.3 μ) there is some decrease in I length and the H gap disappears. At still shorter sarcomere lengths (2.1 μ), the I region is definitely narrower, the Z line no longer apparent, and a more strongly staining region appears at the level of the M band.

These observations are in good agreement with the model of the sarcomere presented by Hanson and Huxley (1). In this model, actin is localized in filaments which center on the Z line and extend to the edge of the H gap. When the sarcomere shortens below about 2.3 μ, these filaments crumple or overlap at the level of the M line. (Note that the length of the I filaments, determined from Fig. 1, is about 2.3 μ.) Interaction between actin and myosin is presumed to occur in the region where I filaments penetrate the A region.

One can anticipate that in some regions actin

Fig. 1 to 5 are of fibrils from glycerinated rabbit psoas muscle stained with fluorescein-coupled heavy meromyosin (F-HMM). The terminal breaks all occur near the center of the I band. In Figs. 2 and 3, fibrils having different sarcomere lengths were mixed before being stained. The arrow indicates the position of the Z line.

FIGURE 1 The sarcomere length is 2.7 μ and H gaps are clearly visible. × 3,000.

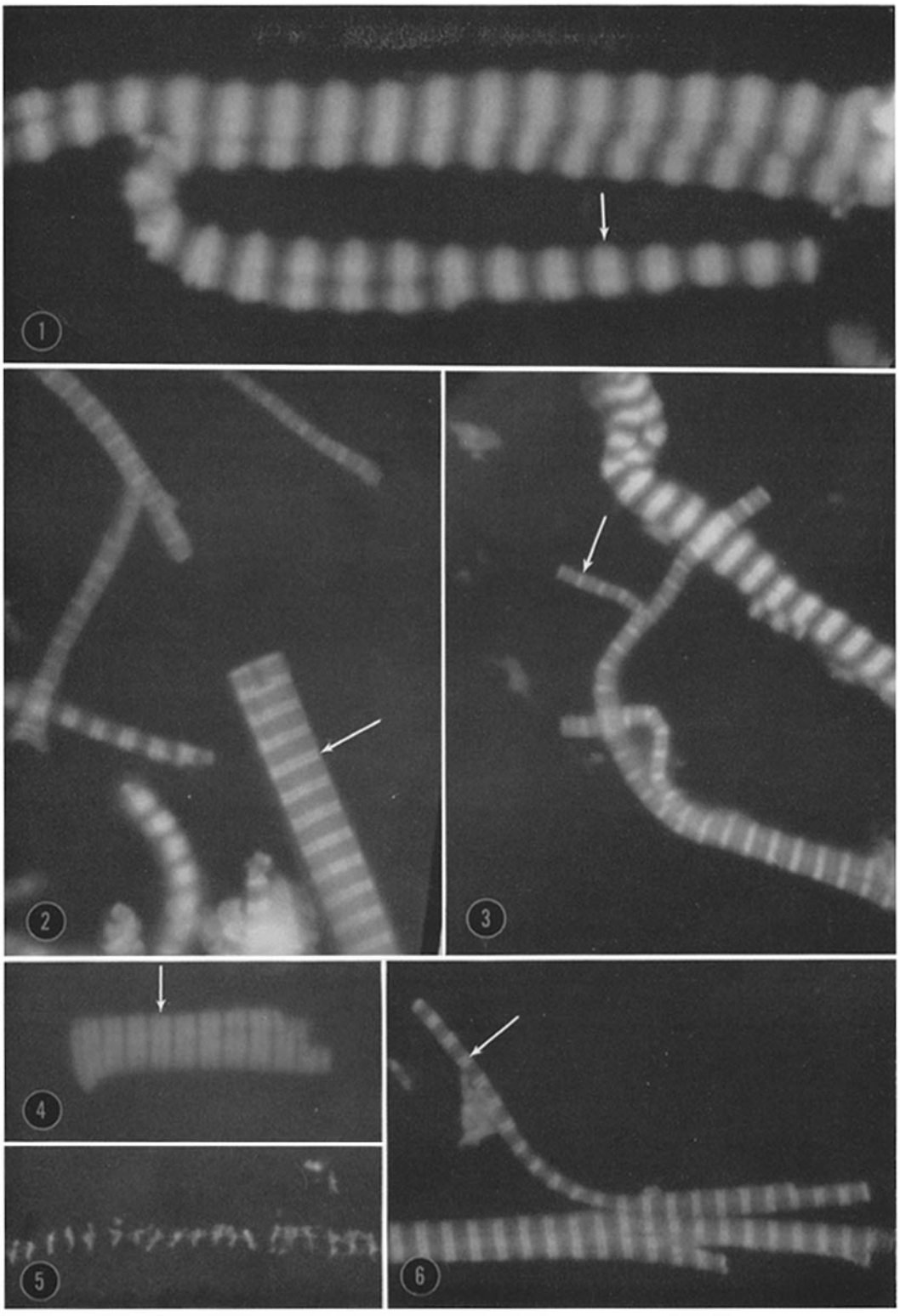
FIGURE 2 The most obvious fibril in this field has a sarcomere length of 2.3 μ and H gaps are not visible. × 1,800.

FIGURE 3 Two fibrils; the one with long sarcomeres shows an unstained H gap, while the other with a sarcomere length of 2.1 μ shows an M line and a narrower I region. × 1,800.

FIGURE 4 Myosin-extracted fibril stained with F-HMM.

FIGURE 5 0.6 M KI extracted fibril stained with F-HMM.

FIGURE 6 Glycerinated chicken breast muscle stained with F-HMM.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will be mechanically blocked from combining with externally applied myosin or HMM. The decrease or lack of staining at the Z line may be of this sort. Brief trypsin digestion which reduces the density of the Z line did not cause a detectable increase in F-HMM stainability. However, the Z region is differentially weakened and separates under the influence of the exciting radiation so that critical observations cannot be made. Additional factors which may affect the number of F-HMM binding sites are (1) the aggregation of actin with other molecules such as tropomyosin or other actin molecules, and (2) the possibility that HMM, which is a large ($\backsim$ 300,000 mol. wt.) molecule with many polar groups, may not be able to approach some binding sites with the proper orientation.

Glycerinated muscle is in rigor and inextensible, indicating that some actin is bound to myosin. In the regions in which this occurs, one would anticipate either complete or partial blocking of F-HMM binding. This is, I think, a reasonable explanation for the decreased I filament staining with F-HMM seen in the region in which A and I filaments are expected to interdigitate. This view is also supported by extraction experiments. Myosin was removed with Hasselbach and Schneider's pyrophosphate solution before the fibrils were stained. The I filaments in these fibrils stain more evenly (Fig. 4), although there was still evidence of reduced staining at the edge of the H region. Considerable variation among fibrils could be seen in such preparations. Extraction with 0.6 M KI prior to staining with F-HMM abolished I filament staining except in the region of the Z band (Fig. 5).

A corrollary of this explanation of the pattern of F-HMM binding is that the amount of F-HMM bound should be a function of the sarcomere length. This may be in disagreement with other work in which the amount of unlabeled HMM bound to fibrils was shown not to depend on sarcomere lengths between 2.26 and 2.75 μ (6).

The localization of actin by F-HMM seems reasonable in terms of the interdigitating filament model. There are, however, differences between the localization of actin as determined with fluorescein-labeled antibody and with F-HMM. In the published work using anti-actin, the I region seems to be evenly stained (8) or to have a more heavily stained Z line (9), and the only definite staining apparent in the A region is a strongly stained "M" line. This M line is visible at sarcomere lengths where one would anticipate an H gap, and the authors point out that there is some question as to the homogeneity of "actin" antigens.

These observations demonstrate that the mechanism of binding of F-HMM to glycerinated myofibrils is similar to that of unlabeled HMM and from the pattern of binding it is likely that F-HMM combines with actin. The specificity of binding is essentially undefined even for unlabeled HMM. For myosin, the formation of a contractile system is a significant property which can be considered.

Experiments with myosin and actin (10-12) or actin-like proteins (13) suggest that their interaction to produce a contractile system is not species specific. Similarly, Huxley (1) noted that myosin from rabbit was bound to fibrils from the blowfly *Calliphora*. Other authors (12) have considered that the requirement for actin in contractile systems is subserved by a variety of compounds

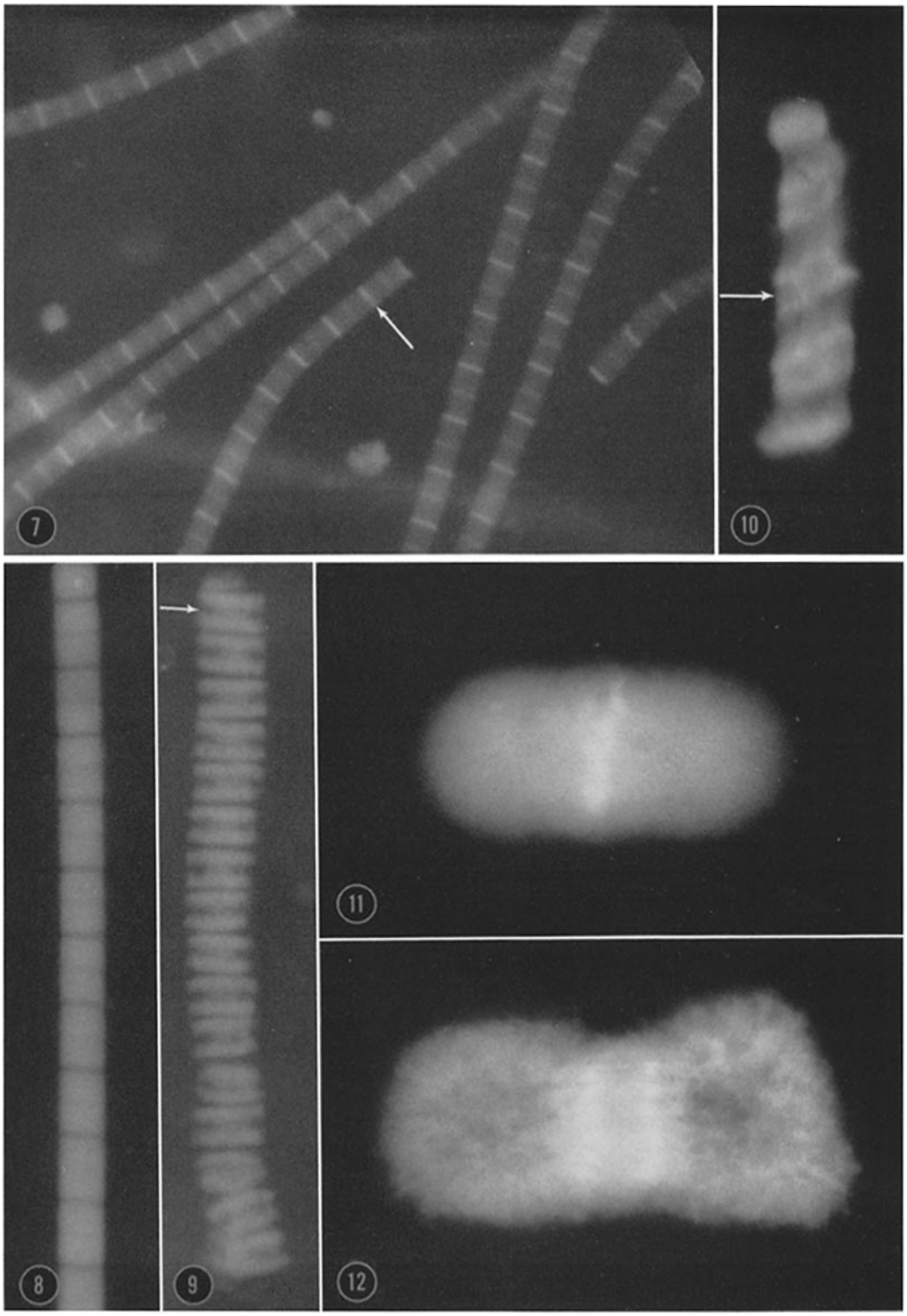
FIGURE 7 Fibrils teased from the indirect flight muscle of *Drosophila* and stained with F-HMM. The M staining was characteristic of the preparation of F-HMM used and not of *Drosophila* fibrils. × 2,300.

FIGURE 8 *Drosophila* fibrils with the A substance removed by 0.1 per cent desoxycholate in 0.1 M KCl at pH 7 before staining. × 3,000.

FIGURE 9 *Drosophila* fibril with the A substance removed by treatment with Mersalyl followed by 0.3 M KCl before staining. × 3,000.

FIGURE 10 Glycerinated fibril from *Limulus* muscle with a sarcomere length of 7.4 μ. An average A length for this muscle was 4.5 μ. × 1,800.

FIGURES 11 AND 12 Mitotic apparatuses isolated from the sea urchin, *Strongylocentrotus purpuratus*, after treatment with F-HMM followed by a brief wash. × 1,250.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including deoxyribonucleic acid (DNA), although there is some doubt (14) that DNA-myosin fibers undergo ATP-dependent shortening.

Fig. 6 shows the pattern of staining with rabbit F-HMM and fibrils from glycerinated chicken breast muscle. Fig. 7 shows the pattern of rabbit F-HMM staining with unextracted fibrils teased from the indirect flight muscle of *Drosophila*, while Figs. 8 and 9 show the pattern seen when most of the A substance is removed before staining. F-HMM staining of *Drosophila* fibrils is similar to that of rabbit psoas fibrils in its sensitivity to Mg-pyrophosphate and to Mersalyl. Fig. 10 shows the pattern of staining seen when glycerinated fibrils from *Limulus* muscle were treated with rabbit F-HMM.

It is interesting that isolated sea urchin spindles as well as their chromosomes bind F-HMM (Figs. 11 and 12) although in this instance no attempt was made to establish the specificity of binding.

In conclusion, it seems likely that F-HMM binding has a limited usefulness in well defined situations. The large decrease in enzymatic activity and binding capacity on coupling, the size of the HMM molecule, and particularly the undefined and apparently low specificity of binding are restrictions on its general use. The good differential staining which does not follow the protein distribution of the sarcomere indicates that the binding has some specificity.

A general characteristic of contractile elements within cells may require that myosin interact with an "actin" which is characterized by myosin binding. F-HMM may be a useful indicator for the cytological detection of such "actins."

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