

THE USE OF SPECIFIC ANTIBODY IN ELECTRON MICROSCOPY

III. Localization of Antigens by the Use of Unmodified Antibody

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ABSTRACT

Antibody staining was observed in the electron microscope by means of untagged antibody and osmium fixation. The antibody was visualized as a change in morphology due to its deposition on the antigenic structures. Glycerinated chicken breast muscle was stained with antimyosin, anti-H-meromyosin, and antiactin. The staining patterns obtained by electron microscopy were consistent with those previously demonstrated by fluorescence microscopy. A second method was used for confirmation of antibody staining. This consisted of extraction of unstained portions of the sarcomere with 0.6 M potassium iodide, 10^{-4} M adenosine triphosphate solution. Stained regions of the sarcomere remained intact because of insolubility of the combined antigen and antibody.

INTRODUCTION

The localization of protein antigens by means of specific antibody has been extended to electron microscopy using antibody doubly labeled with mercury and fluorescein (1, 2). Double labeling permits comparison of serial sections by fluorescence and electron microscopy. The technical difficulties involved do not, at present, permit the attainment of high resolution electron micrographs.

In some problems lack of high resolution may not be of primary importance. The use of doubly labeled antibody may then be advantageous because of the ability to compare directly the results of electron microscopy and fluorescence microscopy. However, in the study of muscle structure the highest resolution possible is desired. High resolution electron micrographs of striated muscle have been obtained using osmium fixation

with heavy metal counterstaining (3, 4). Changes in morphology in different regions of the sarcomere due to adherence of antibody to the antigenic structures will be examined in this report. Striated muscle is especially suitable for this method of antibody localization since the contractile elements consist of highly organized filaments. Antibody adhering to these structures should produce definite morphological changes. For this reason, it is not necessary to have a heavy metal tag to differentiate the antibody from the other tissue proteins.

As the resolution is increased, erroneous localizations due to non-specificity of the antibody become more important. In the work reported here, we are concerned only with the ability to visualize the antibody staining without regard to the meaning of the localizations obtained. A

critical analysis of the meaning of the localizations and of the antibody specificity will be presented in a further publication.

Two methods were used to demonstrate antibody staining. First, changes in morphology due to the adherence of antibody to antigenic structures were observed; and second, the unstained parts of the fiber were extracted with salt solutions in which the combined antigen and antibody was insoluble. Myofibrils treated with fluorescein-labeled antibody against myosin, the meromyosins, and actin show characteristic localizations in the fluorescence microscope (5). These localizations can be correlated with those obtained by electron microscopy.

MATERIALS AND METHODS

Chicken breast muscle was extracted in 50 per cent glycerol at 0°C and stored at -24°C as previously described for chicken neck muscle (2). Muscle fibers were dissected into pieces approximately 0.2 mm in diameter and 4 to 5 mm long. They were washed with 25 per cent glycerol, *m*/60 PO₄ buffer pH 7.4, and stained with normal γ_2 -globulin or antibody γ_2 -globulin in 25 per cent glycerol, *m*/60 PO₄ buffer pH 7.4, overnight at 0-5°C. The fibers were then washed at least 24 hours with 25 per cent glycerol, *m*/60 PO₄ buffer pH 7.4. Aliquots of this material were in addition extracted in 0.6 *M* KI-ATP (6, 7) for 2 hours and washed with the buffered glycerol for 2 to 4 hours. All the muscle was fixed with 1 per cent OsO₄ "Palade fixative" pH 7.4 for ½ hour at room temperature. It was then washed for 10 to 15 minutes with *m*/60 PO₄ buffer pH 7.4 and taken through a graded ethanol series into 100 per cent ethanol. The fibers were then brought into the Araldite mixture using the procedure described by Finck (8). The muscle was cut into small pieces, 1 to 2 mm in length, while still in the Araldite. It was then placed in size 000 capsules with Araldite and polymerized for 2 days at 55°C.

The embedded tissue was sectioned on a Porter-Blum microtome with a glass knife. The sections were

placed on grids, immersed in 1 per cent phosphotungstic acid in absolute alcohol for 1 minute for staining, and washed for 1 minute in alcohol.

An RCA EMU-3C electron microscope with a 50 μ objective aperture and 50 kv accelerating voltage was used throughout this work.

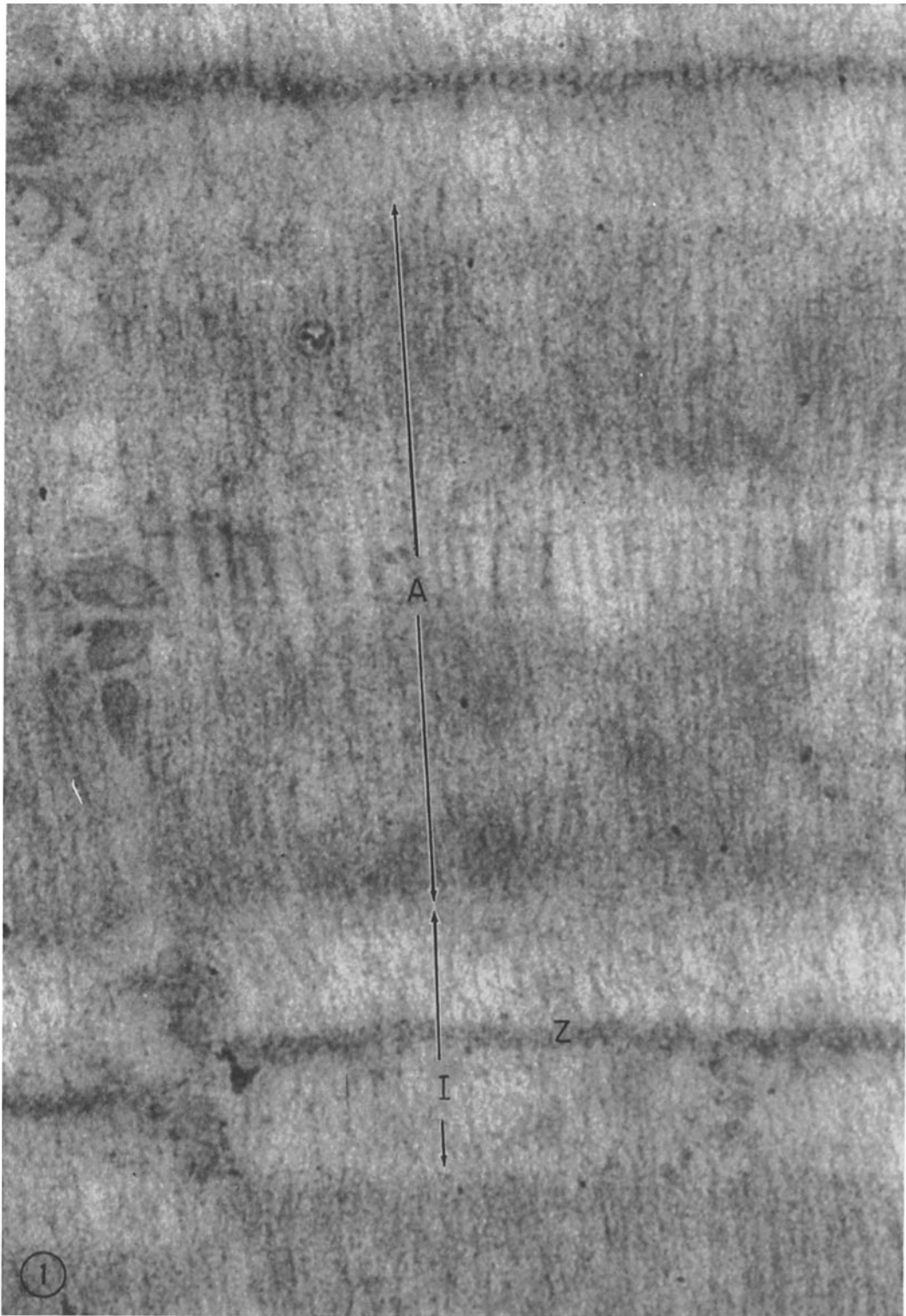
RESULTS AND DISCUSSION

Antibodies prepared against myosin, actin, and the meromyosins (H- and L-meromyosin) are bound to specific regions of the sarcomere. The characteristic localizations are: Antimyosin localizes heavily throughout the A band; antiactin localizes heavily in the middle of the A band and more lightly in the I band; anti-H-meromyosin localizes heavily in the middle of the A band and more lightly throughout the rest of the A band; anti-L-meromyosin localizes laterally in the A band. These same antibody preparations were used for the following study, and the localizations observed in the electron microscope are correlated with those described above.

On staining a strip of muscle cut approximately 0.2 mm in diameter, it was found that the antibody usually did not penetrate the whole thickness. Therefore, the outer fibrils were heavily stained and the centrally located fibrils were less heavily stained, or unstained. This provided direct comparison, on the same grid, of stained and unstained material. The outer stained areas were distinguishable from the inner unstained areas by their differences in morphology. That this difference was not due to artifacts of fixation was established by the fact that it was absent in unstained material. Also different specific antibodies gave different staining patterns for the outer stained fibrils, while the centrally located unstained fibrils showed consistently the normal morphology of unstained material. In addition, use of 0.6 *M* KI-ATP, which extracts unstained portions of the sarcomere while retaining insoluble stained

FIGURE 1

Glycerinated chicken breast muscle fixed in 1 per cent OsO₄ "Palade fixative" pH 7.4. Araldite was used as an embedding medium. The sections were stained by immersion in 1 per cent phosphotungstic acid in alcohol. Note the thick filaments in the center of the A band with relatively empty space between them. Also note the closely spaced, thin filamentous structure of the rest of the A band. A, A band; I, I band; Z, Z line. \times 80,000.



portions of the fibril confirmed the antibody localizations.

The characteristic features of normal chicken breast muscle fixed and stained for electron microscopy as described can be seen in the electron micrograph Fig. 1. The center of the A band, or the H zone, is characterized by relatively thick filaments with low density areas between them. The rest of the A band shows fine filamentous structures more closely packed than the thick filaments in the H region. Our concern in this paper will be with the ability to observe changes in morphology due to the adherence of antibody to the antigenic components of the fibril.

The morphological changes due to staining with antimyosin can be seen in Fig. 2, which shows a sarcomere from the outer heavily stained area of the fiber. Notice the thickening of the filamentous structures throughout the A band; also the wider separation of these thick filamentous structures as compared with the separation of the fine filamentous structures occurring in the control. The antimyosin has therefore localized throughout the A band, a finding which is consistent with the results of fluorescence microscopy (5, 9, 10). In Fig. 3 can be seen a more centrally located area which is less heavily stained. This is more like the normal unstained muscle in morphology. The question whether the antibody is on the filaments or between them cannot be answered by these micrographs. Higher resolution electron micrographs are needed.

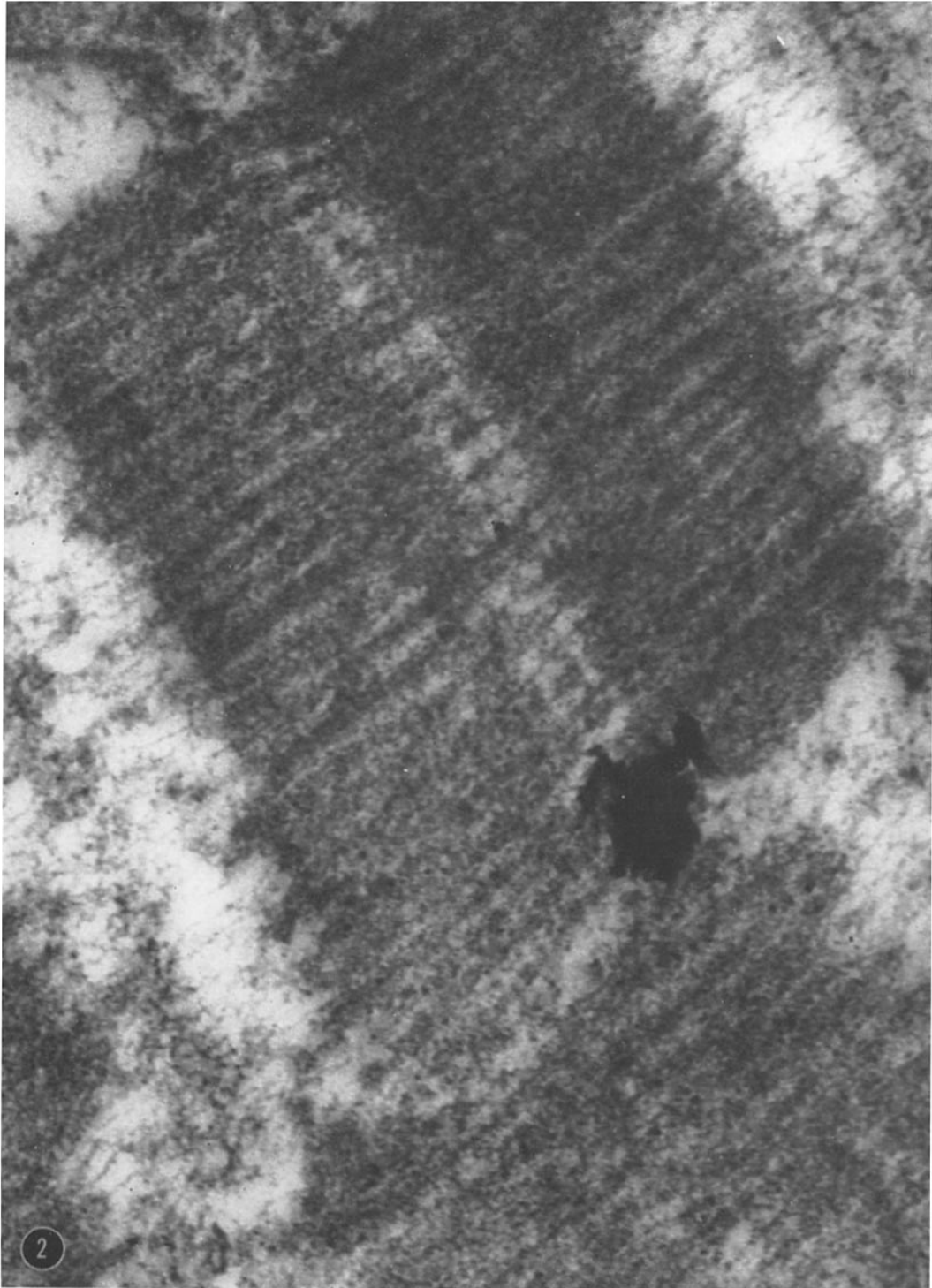
Fig. 4 illustrates the morphological changes produced by staining with antiactin. Both antiactin and anti-H-meromyosin produce the same morphological changes in the sarcomere. Note the dense material present between the thick filaments in the middle of the A band as com-

pared with the lack of material in this area in the control. The regions of the sarcomere which show heavy staining in the fluorescence microscope also show staining in the electron microscope. Those regions corresponding to the more lightly staining regions of the sarcomere as seen in the fluorescence microscope do not show staining in the electron microscope. For example, anti-H-meromyosin, which stains heavily in the center of the A band and more lightly throughout the rest of the A band by fluorescence, shows staining only in the center of the A band in the electron microscope. Antiactin, which stains heavily in the center of the A band and more lightly in the I band by fluorescence, also shows staining only in the center of the A band in the electron microscope.

Using the phase microscope it has been shown that the reaction of antibody with parts of the sarcomere protects these parts from extraction with 0.6 M KI-ATP (6, 7). It is therefore possible to use this method to confirm the antibody localizations already described for electron microscopy. When the control, unstained fiber, is treated with 0.6 M KI-ATP, the normal sarcomere structure is lost owing to extraction of the fibrillar protein. This can be seen in the electron micrograph Fig. 5, which bears little resemblance to the unstained, unextracted sarcomere. If the sarcomere is stained with antimyosin before extraction, the structure shown in the electron micrograph Fig. 6 results. The heavily stained A band remains intact, while the I band, including the Z line, is extracted. After extraction, the filamentous structure in the A band is no longer evident as it was in Fig. 2. This may be a result of extraction of material in this region not rendered insoluble by reaction with the antibody. The extraction of a fiber previously treated with anti-H-meromyosin

FIGURE 2

Glycerinated chicken breast muscle stained with antimyosin and then fixed in 1 per cent OsO₄ "Palade fixative" pH 7.4. Araldite was used as embedding medium. The sections were stained by immersion in 1 per cent phosphotungstic acid in alcohol. Note the thick filamentous structures throughout the A band. Also note the wider spacing of these thick filamentous structures in comparison with the spacing of the thin filamentous structures in the unstained control (Fig. 1). $\times 76,000$.



or antiactin results in retention of the heavily stained central line in the A band and extraction of the rest of the sarcomere, parts of which are more lightly stained than this central line. This can be seen in Fig. 7 for the antiactin-stained material.

Antibody staining of striated muscle, using unmodified antibody, can therefore be visualized in the electron microscope, and the visualization

can be confirmed by extraction of unstained portions of the sarcomere.

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FIGURE 3

Same material as shown in Fig. 2. However, this is a sarcomere from a fibril closer to the center of the muscle fiber. Owing to the difficulty of diffusion of the antibody into the fiber, this shows less intense antibody staining than the sarcomere in Fig. 2. $\times 80,000$.

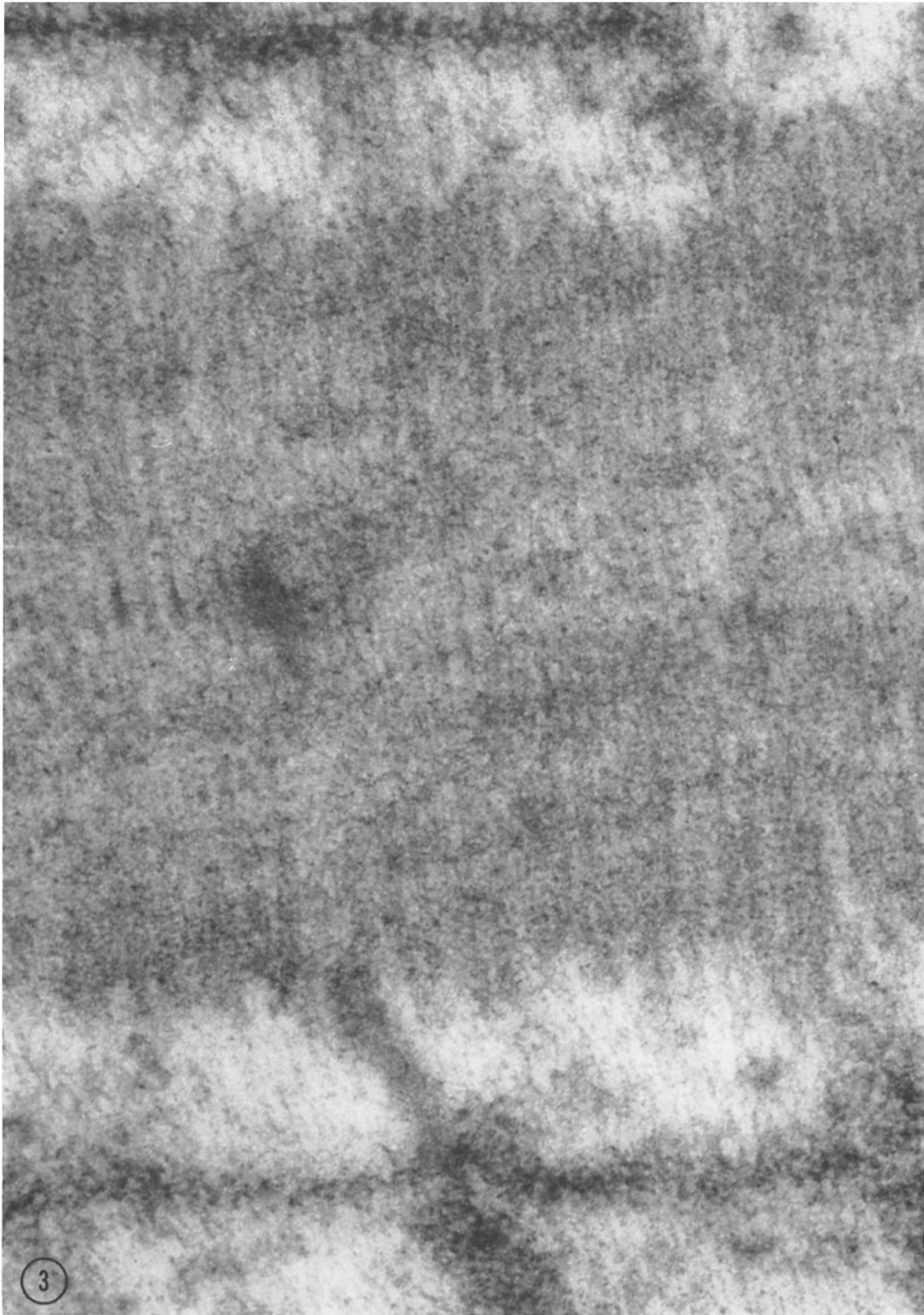


FIGURE 4

Glycerinated chicken breast muscle stained with antiactin and then fixed in 1 per cent OsO_4 "Palade fixative" pH 7.4. Araldite was used as embedding medium. The sections were stained by immersion in 1 per cent phosphotungstic acid in alcohol. Anti-H-meromyosin and antiactin led to the same localization as seen in the electron microscope. This localization corresponded to the heavily stained central region of the A band as observed in the fluorescence microscope (see text). Note the dense material between the thick filaments in the center of the A band. This is to be compared with the same area in the control (Fig. 1), where the thick filaments were separated by relatively empty spaces. Unlike the control (Fig. 1), the center of the A band is of about the same density as the Z line. $\times 80,000$.

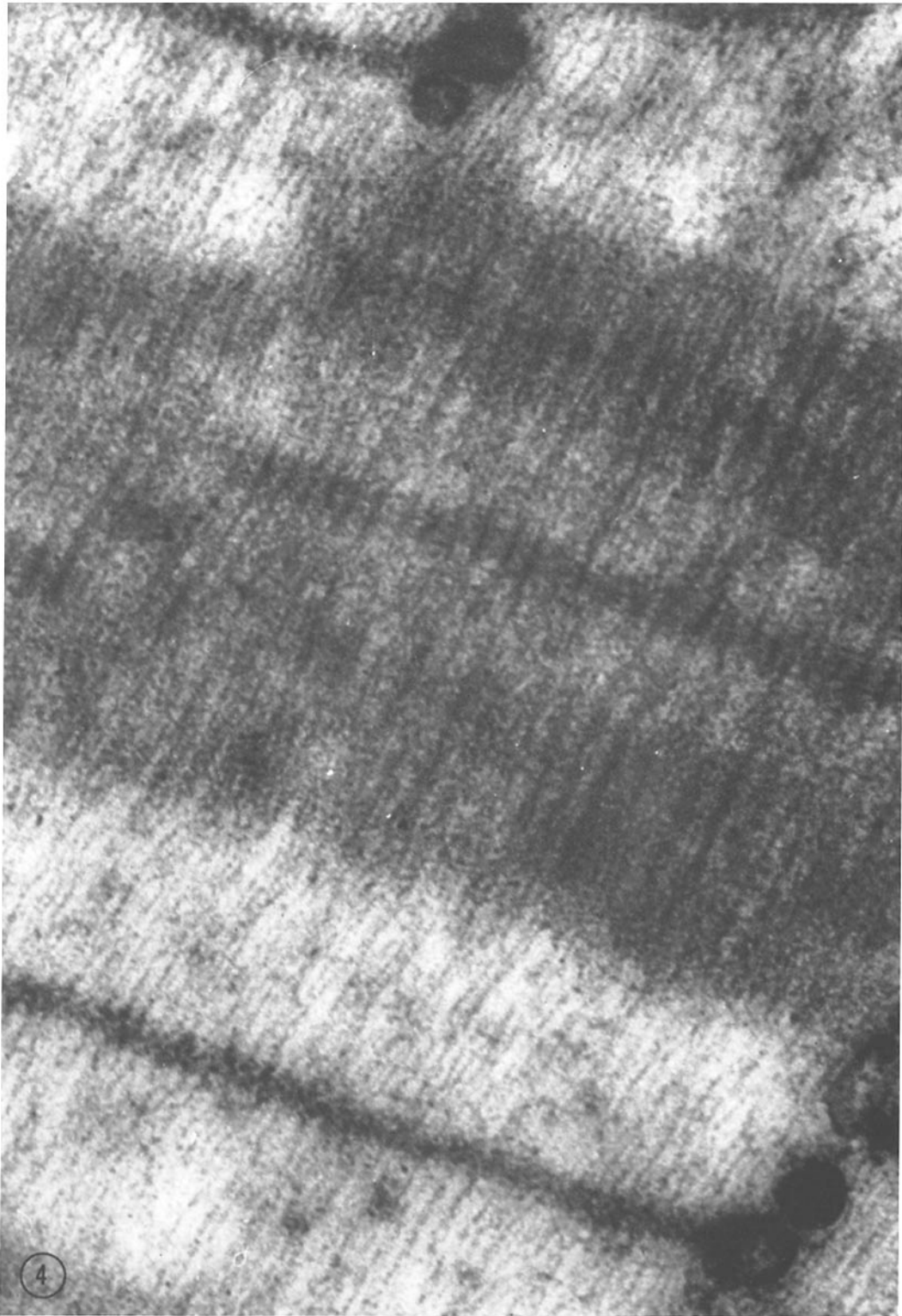


FIGURE 5

Glycerinated chicken breast muscle extracted with 0.6 M KI-ATP and then fixed in 1 per cent OsO_4 "Palade fixative" pH 7.4. Araldite was used as embedding medium. The sections were stained by immersion in 1 per cent phosphotungstic acid in alcohol. 0.6 M KI-ATP extracts most of the contractile protein. This causes disruption of the normal structure of muscle. The unextracted counterpart of this figure is Fig. 1. $\times 80,000$.

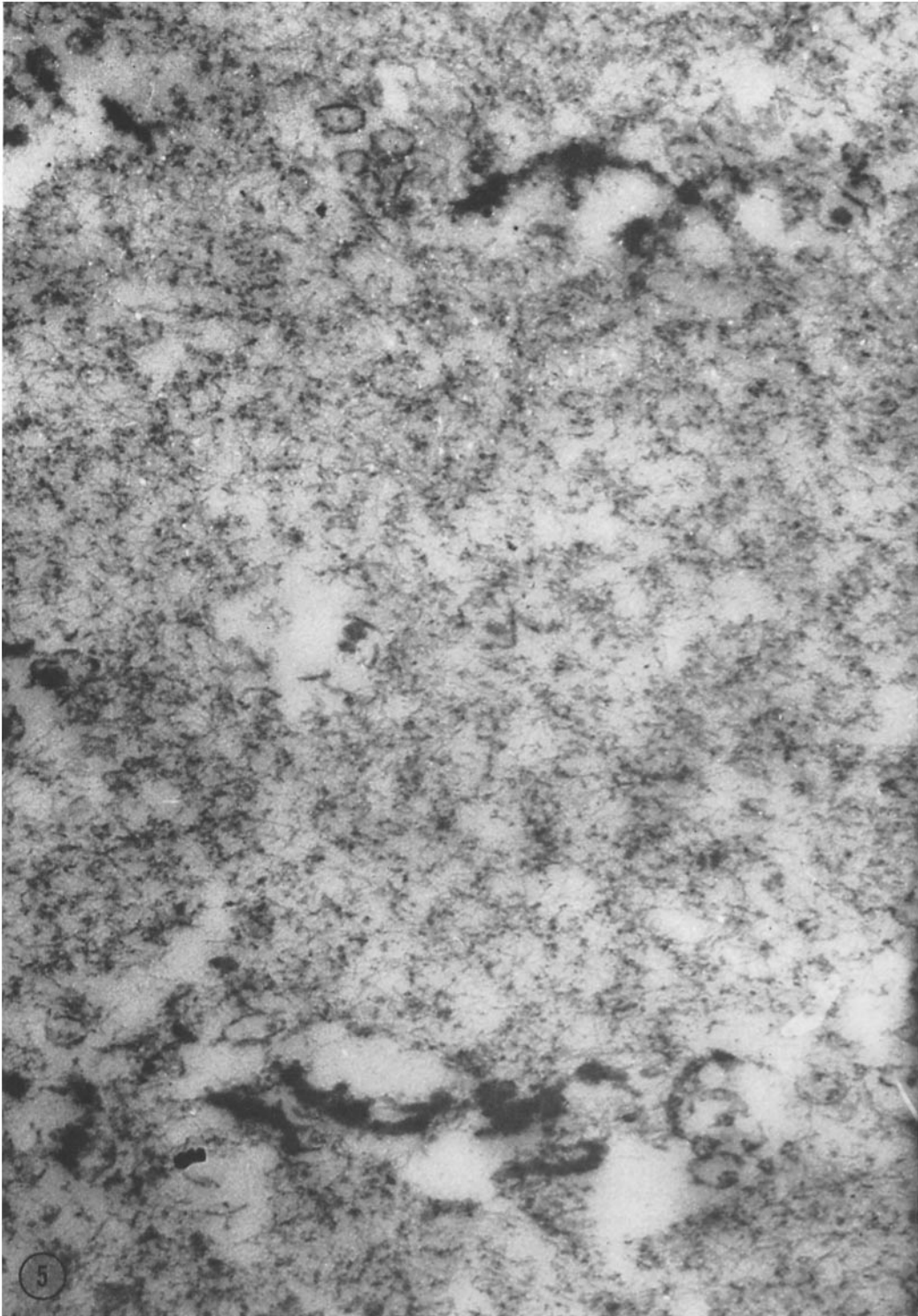


FIGURE 6

Glycerinated chicken breast muscle stained with antimyosin and then extracted with 0.6 M KI-ATP. It was then fixed in 1 per cent OsO₄ "Palade fixative" pH 7.4. Araldite was used as embedding medium. The sections were stained with 1 per cent phosphotungstic acid in alcohol. Staining of the A band with antimyosin prevents extraction of the A band with 0.6 M KI-ATP owing to the insolubility of the antigen-antibody complex in the extracting solution. This figure is to be compared with Fig. 5 and Fig. 2. Also note that in the stained, unextracted material of Fig. 2 the filamentous structure is clearly evident, whereas in the stained, extracted material the filamentous structure has been destroyed. × 80,000.

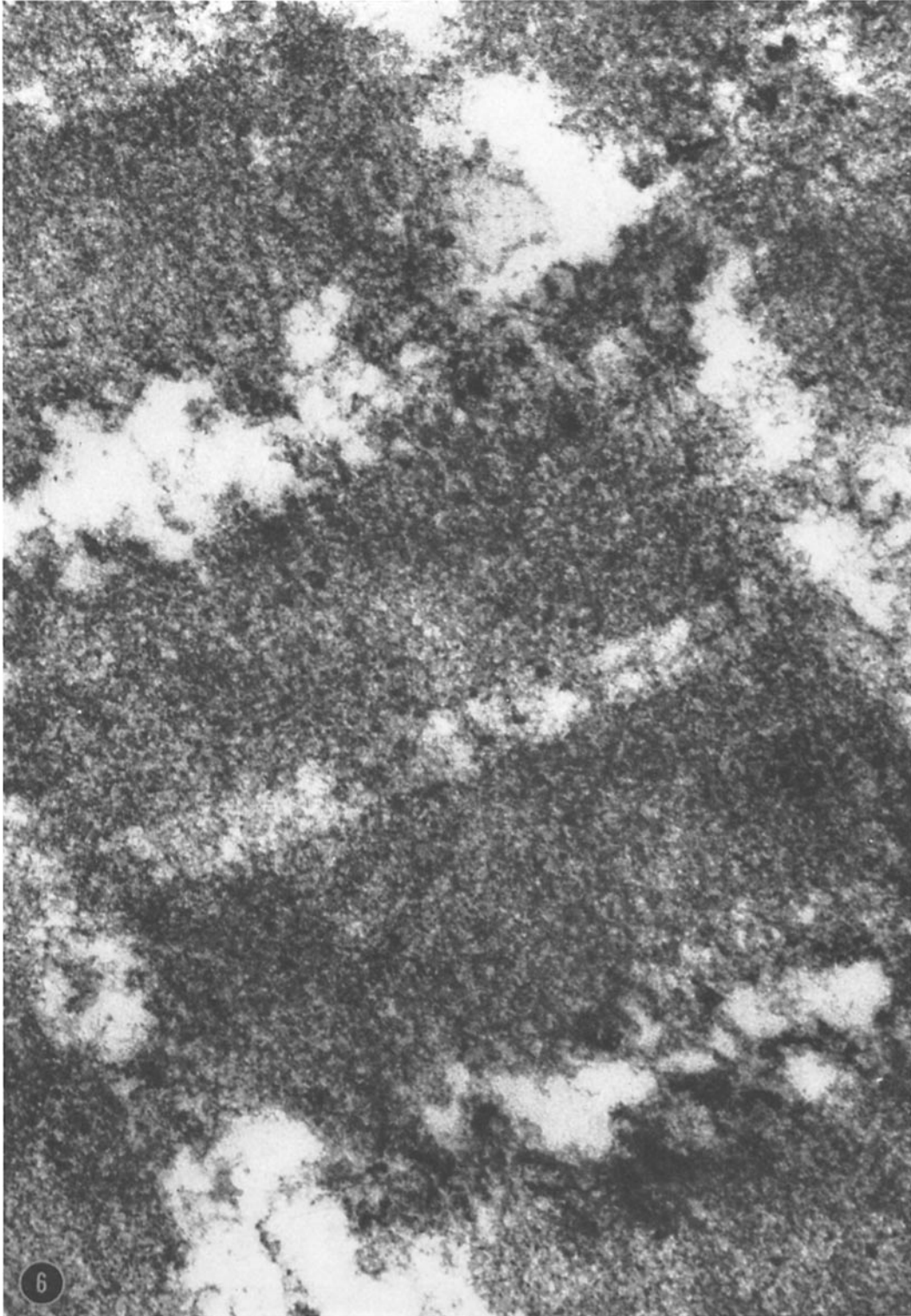


FIGURE 7

Glycerinated chicken breast muscle stained with anti-H-meromyosin or antiactin and then extracted with 0.6 M KI-ATP. It was then fixed in 1 per cent OsO₄ "Palade fixative" pH 7.4. Araldite was used as an embedding medium. The sections were stained with 1 per cent phosphotungstic acid in alcohol. Heavy staining of the central region of the A band with anti-H-meromyosin or antiactin prevents extraction of this region with 0.6 M KI-ATP owing to the insolubility of the antigen-antibody complex in the extracting solution. This figure is to be compared with Fig. 5 and Fig. 4. Note that the distance between the insoluble regions in the extracted muscle of Fig. 7 is smaller than the distance between the stained regions of the unextracted muscle of Fig. 4. Extraction of protein from the unstained region of the sarcomere seems to have permitted the resulting structure to shorten. $\times 80,000$.

