THE USE OF SPECIFIC ANTIBODY IN ELECTRON MICROSCOPY

II. The Visualization of Mercury-Labeled Antibody in the Electron Microscope

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ABSTRACT

Glycerinated chicken muscle was stained with antimyosin antibody conjugated with mercury and fluorescein. The antibody was visualized in both the electron and the fluorescence microscope by using adjacent thin and thick sections. In order to make this possible, Araldite was used as the embedding medium. The mercury was reduced to metallic mercury in the electron beam and either migrated in the section or was sublimated in the vacuum. Therefore special techniques of carbon filming had to be used to prevent this. Some non-specific staining occurred because of the binding of mercury to available sulfhydryl groups in the tissue. The available sulfhydryl groups were blocked by pretreating the tissue with iodoacetic acid and formaldehyde. The non-specific staining which occurred after this treatment was easily removed by brief washing with a buffered solution of thioglycolic acid.

INTRODUCTION

The preparation of an antimyosin antibody conjugate containing both mercury and fluorescein was described in the preceding paper (1). The mercury was indirectly linked to the protein through diazo coupling of tetraacetoxymercuri-arsanilic acid and the fluorescein was directly coupled using the fluorescein isocyanate. After conjugation the antibody retained its specificity as was shown by its staining properties under fluorescence microscopy. The purpose of the mercury atoms in the conjugate was to increase the electron-scattering power of the antibody molecules. The visualization of such an antibody conjugate in sections by electron microscopy depends on its electron-scattering power relative to the rest of the tissue proteins. Therefore the localization of the antibody conjugate in combination with the specific tissue antigen is ultimately possible regardless of how the antigen is distributed in the cytoplasm. Its distribution may be along definite membrane structures or filaments, or it may be diffusely localized in the cytoplasm.

As will become apparent later, the technical difficulties involved in visualizing alternate sections of the antibody-stained tissue by electron and fluorescence microscopy, at present, do not permit resolution of individual antibody molecules. However, an increase in density due to the presence of the antibody conjugate can be observed in the electron microscope, and this density increase can be compared with the corresponding fluorescent areas observed by fluorescence microscopy, using alternate thin and thick sections. The ultimate precision of localization of the antigen by means of the antibody conjugate

is limited by the size of the antibody molecule itself, because the substituted organic mercurial and fluorescein are small molecules in comparison with the size of the antibody molecule.

MATERIALS

An RCA EMU-3C electron microscope was used. All work was done at 50 kv with a 50 μ objective aperture and a 0.25 mm. condenser aperture.

METHODS

Glycerinated chicken neck muscle was used throughout this investigation. The animal was sacrificed by decapitation, eviscerated, skinned, and immediately plunged into chopped ice and allowed to cool to 0°C for approximately ½ hour. The whole neck was then removed and placed in 50 per cent glycerol at 0°C and agitated slowly with several changes of 50 per cent glycerol for approximately 48 hours. It was then stored for at least 2 weeks at −24°C. Small pieces of muscle were excised and small fibers of less than 0.5 mm in diameter were dissected out and cut into lengths of approximately 1 to 2 mm in 25 per cent glycerol, M/60 PO_4 buffer pH 7.4, and were kept at 0–5°C. Some of the muscle fibers (muscle I) received no additional treatment before staining with the conjugated γ-globulins. Others (muscle II) were fixed in 1 per cent formaldehyde, 0.2 per cent iodoacetic acid, in 25 per cent glycerol, M/60 PO_4 buffer pH 7.4, at 0–5°C for approximately 3 hours, in order to block available SH groups and prevent non-specific staining of the tissue through the mercury (see Fig. 1). Each muscle preparation was separated into two tubes, washed with solvent (25 per cent glycerol, M/60 PO_4 buffer pH 7.4), and stained respectively with doubly conjugated normal γ-globulin and doubly conjugated antibody γ-globulin. Staining time was from 12

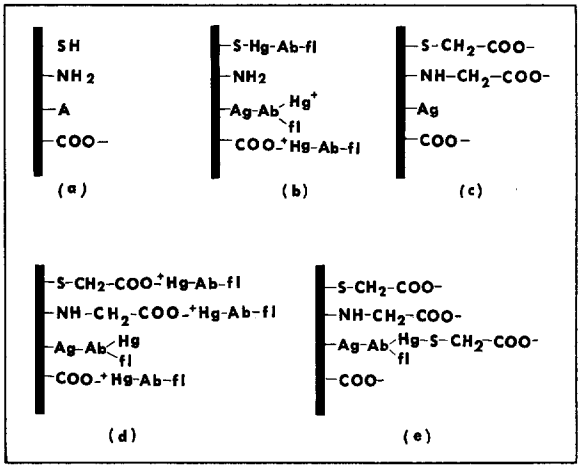


FIGURE 1

Reactions with the functional groups of tissue during the staining procedures: *a*, muscle I before staining with antibody conjugate; *b*, muscle I after staining with antibody conjugate; *c*, muscle II before staining with antibody conjugate; *d*, muscle II after staining with antibody conjugate; *e*, muscle II after staining with antibody conjugate followed by a buffered thioglycolic acid wash. *Ag*, antigenic site; *Hg—Ab—fl*, antibody doubly conjugated with mercury (*Hg*) and fluorescein (*fl*).

For fluorescence microscopy a Reichert Zetopan research microscope was used. Dark-field illumination was obtained using a Zeiss cardioid condenser and a 100 × achromatic oil immersion objective containing a built-in ultraviolet filter. The light source was an Osram HBO-200 lamp. The combination of Corning 5840 and Schott UG-1 filters was used for the incident light. Exposures were of the order of 5 minutes using Kodak type IIa-G spectroscopic plates and Kodak D-19 developer. Printing was done almost entirely on Kodak F-3 paper using Kodak Dektol developer.

The antibody and normal γ-globulin conjugates were prepared as described previously (1).

Araldite epoxy resin was used throughout as an embedding medium. The Araldite mixture found by Finck (2) to give optimal results was used.

to 15 hours with frequent agitation at 0–5°C. All fibers were then washed with solvent for 24 to 30 hours at 0–5°C with twelve changes and frequent agitation. The material in each tube was again separated into two tubes. One group received a 2 hour wash with a solution of 1 ml thioglycolic acid (purified, Fischer) + 50 ml solvent adjusted to a final pH of 7.5 with 6 N NaOH. Two changes of this solution were used with occasional agitation. The other group was left in the wash solution. All samples were then brought through a graded series of ethanol solutions (20, 40, 60, 80, 95, 100 per cent) and allowed to stand in 100 per cent ethanol at room temperature approximately 1 hour with two changes of 100 per cent ethanol. The pieces of tissue were then removed to capsules, the ethanol was drained, and the Araldite resin with accelerator was added. It was difficult to change the Araldite resin since the refractive index of the tissue is so close to that of the Araldite that it was not easily visible. The capsules were allowed to stand 1 hour at room temperature, then 1 hour at 40°C, and were then transferred to a 55°C oven for polymerization for 2 days. In many of the capsules the permeation of the tissue by the resin was not adequate and sectioning was impossible. These were discarded. It was not possible to use methacrylate as an embedding material since it quenched the fluorescence of the flourescein.

Two techniques of carbon sandwiching were used for observation of the sections in the electron microscope. All carbon films were made by evaporating a 2 mm length of carbon 1 mm in diameter onto grids at a distance of 13 cm from the source. Evaporation was carried out when the vacuum reached a pressure of 0.03 μ of mercury. The first technique of carbon sandwiching (method A) consisted in evaporating a film of carbon onto a grid previously coated with a Formvar film. The section was then placed on this carbon film and another film of carbon was evaporated directly onto the section in intimate contact with it. The second technique (method B) consisted in placing the section on the grid without a supporting film (the grid spaces being small enough to support the section). A carbon film was then evaporated directly onto one side of the section, the grid was turned over, and another carbon film was evaporated directly onto the other side of the section. This gave very intimate contact between the section and the carbon on both sides.

Direct observation of sections prepared as above for electron microscopy was possible by fluorescence microscopy if thin athene grids were used. The grid containing the sections was immersed in immersion oil between a slide and coverslip. After observation in the fluorescence microscope the immersion oil could be removed with ethylene dichloride and the specimen could be observed in the electron microscope. In the thin sections needed for electron microscopy, rapid quenching of the fluorescence occurred on irradiation with ultraviolet light and it was impossible to take photographs. Therefore alternate thin and thick sections were used for obtaining electron micrographs and fluorescence micrographs respectively. No observations could be made of sections by phase microscopy because of the similarity in refractive index between the tissue and the embedding medium.

On observation of sections prepared for fluorescence microscopy, it was found that Araldite showed autofluorescence with increased time of exposure to the ultraviolet source. However, during the 5 minute exposure needed, the intense initial fluorescence of the tissue and the slow build-up of fluorescence in the embedding medium permitted photography.

Serial sections of all the material were also investigated after staining with phosphotungstic acid as follows: Sections were placed on grids and the whole grid was immersed in a 1 per cent solution of phosphotungstic acid in 100 per cent ethanol for 1 minute followed by 1 minute in 100 per cent ethanol.

RESULTS AND DISCUSSION

Several difficulties were encountered in the visualization of the doubly conjugated antibody. The relatively small number of mercury atoms added in comparison with the total molecular weight of the antibody molecule resulted in a small increase in density relative to the density of the surrounding tissue protein (1). Osmium fixation could not be used because it destroyed the fluorescence of the antibody and thus prevented comparison of alternate sections for fluorescence and electron microscopy. Another difficulty was that under the electron beam, mercury vaporized out of the section, or it formed dense globules which migrated in the section. To control this, the special technique of carbon filming described above as "method B" was used.

Watson (3) has reported observations of increases in contrast of material after sandwiching sections between Formvar films. This is probably due to prevention of sublimation of metal or other material. Hall (4) was able to observe colloidal droplets of mercury in the electron microscope by sandwiching them between silica films. The colloidal suspension was placed on a previously prepared silica film and another film was evaporated over it. Single carbon films have been used primarily for specimen support to prevent distortions in the electron beam (5–7). We attempted to prevent the loss of mercury by sandwiching the sections between carbon films.

All sections were spread with xylol (8) in an

attempt to remove ripples, since carbon filming stabilizes any sectioning distortions present (6). Fig. 2 *a* is a micrograph of a section of muscle I stained with antibody conjugated with fluorescein but no mercury, and Fig. 2 *b* shows the same muscle preparation stained with antibody conjugated with fluorescein and mercury. These sections were carbon-sandwiched using method A as previously described. For confirmation of antibody staining in both cases, serial sections of each were checked by fluorescence microscopy and are shown in Figs. 3 *a* and 3 *b* respectively. Note the dense droplets of material present in Fig. 2 *b*. The electron beam appears to have caused reduction of the mercury to metallic mercury which has migrated and collected in little droplets at points on the structure. The carbon sandwich method A seems to have prevented loss of mercury through sublimation off the section and into the microscope column. It fails, however, to prevent migration of mercury within the specimen.

Carbon sandwich method B, by which the carbon film is deposited in more intimate contact with both sides of the specimen, was found to prevent the migration of mercury within the specimen. Fig. 2 *c* is a micrograph of a section treated by method B. The section was cut from the same specimen as was the section treated by method A (Fig. 2 *b*). Fig. 4 shows a larger field of the same, at lower magnification. No dense droplets of mercury are present; however, the density of the Z lines in the micrographs is much higher than was expected, in comparison with the control material of Fig. 2 *a*.

Careful observation of the corresponding fluorescence micrograph (Fig. 3 *b*) shows some faint, non-specific staining of Z lines. The Z line density observed in the electron microscope may therefore be due to binding of the antibody-mercury conjugate to available SH groups in the tissue through the Hg—S bond. An unsuccessful attempt was made to reverse this binding in muscle I by washing the stained tissue with a buffered thioglycolic acid solution. In muscle II, the tissue was treated with iodoacetic acid and formaldehyde before staining in an attempt to block the available SH groups. Staining of this tissue with the doubly conjugated antibody or with the doubly conjugated normal γ-globulin resulted in an increase in the degree of non-specific staining. However, it was found that this non-specific staining could be easily washed off with a short wash with buffered thioglycolic acid. The most plausible explanation of these observations is as follows (see Fig. 1): The Hg—S bond

FIGURE 2

Antibody-stained, glycerinated chicken neck muscle. All material was embedded in Araldite. *A*, A band.

Fig. 2*a*, electron micrograph of muscle I (see text) stained with anti-L-meromyosin antibody. Antibody was conjugated with fluorescein but not with mercury. Sections were sandwiched between carbon films by method A described in text. × 29,000.

Fig. 2*b*, electron micrograph of muscle I stained with antimyosin antibody. Antibody was conjugated with fluorescein and mercury. Sections were sandwiched between carbon films by method A. × 59,000.

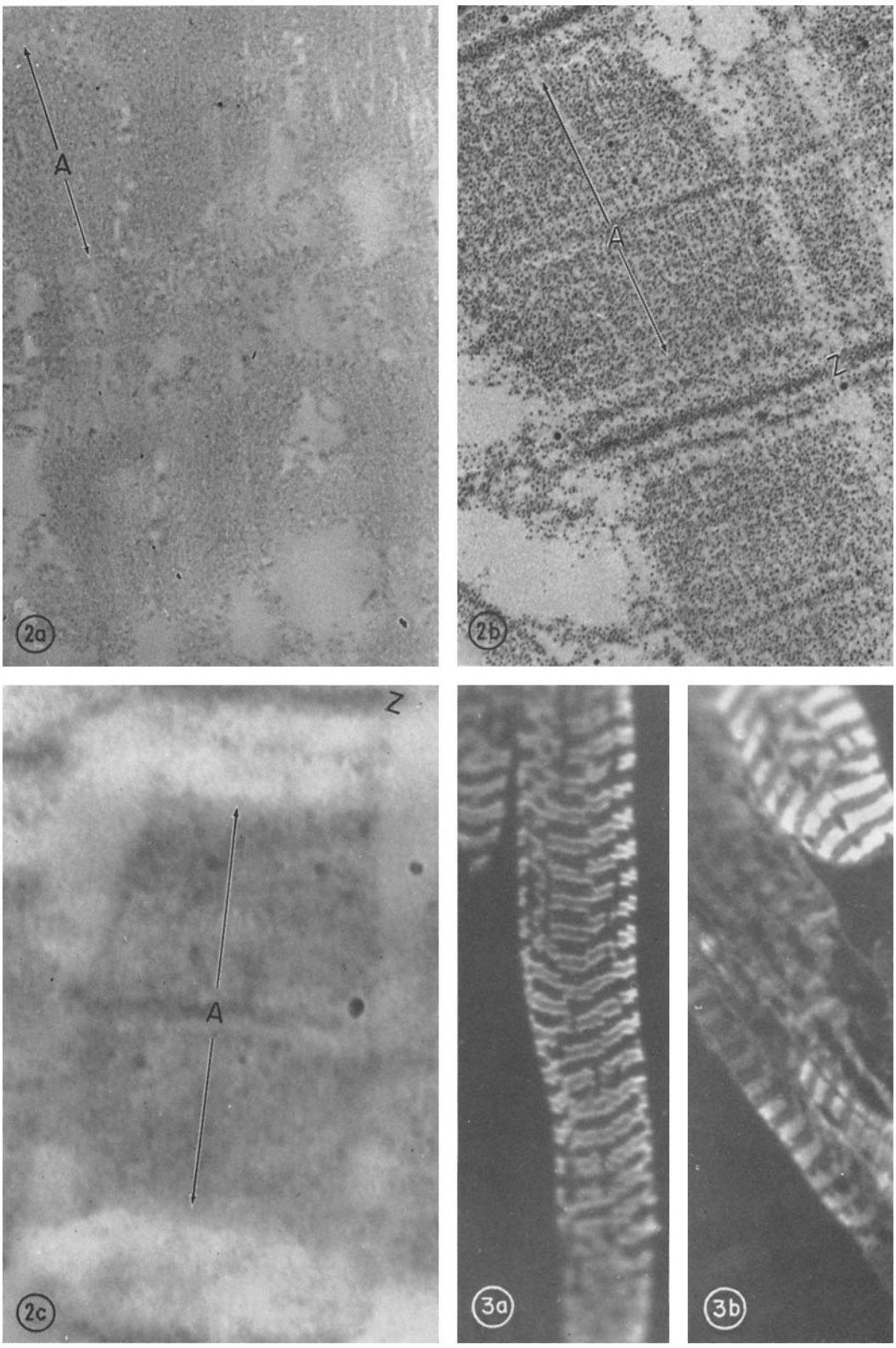
Fig. 2*c*, electron micrograph of an alternate section of material shown in Fig. 2*b* sandwiched between carbon films by method B (see text). *A*, A band; *Z*, Z line. × 59,000.

FIGURE 3

Fluorescence micrographs of alternate sections of material shown in Fig. 2.

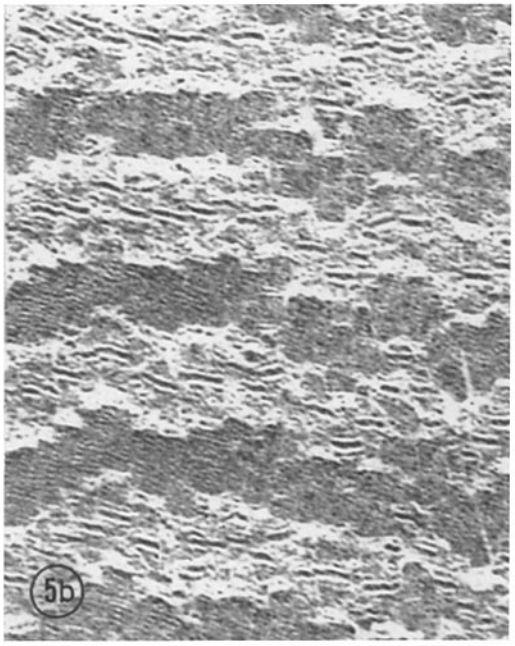
Fig. 3*a*, alternate thick section of material shown in the electron micrograph Fig. 2*a*. The bright areas of localization of the fluorescent antibody correspond to the lateral edges of the A band.

Fig. 3*b*, alternate thick section of material shown in the electron micrographs Figs. 2*b* and 2*c*. The bright areas of localization of the fluorescent antibody correspond to the A band. × 1700.


A
2a
A
Z
2b
Z
A
2c
3a
3b


4


5a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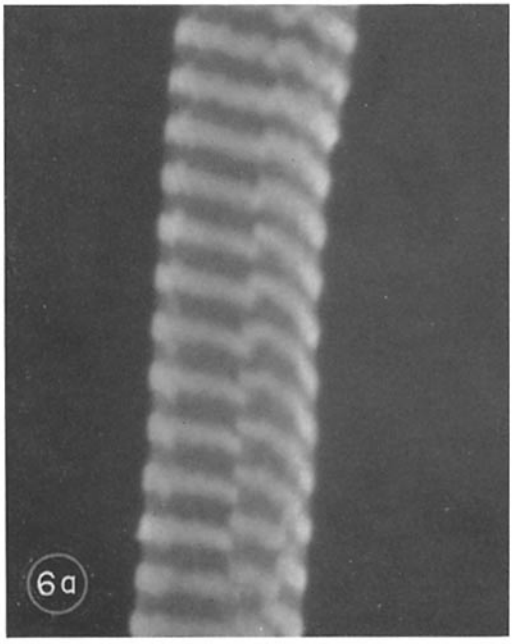
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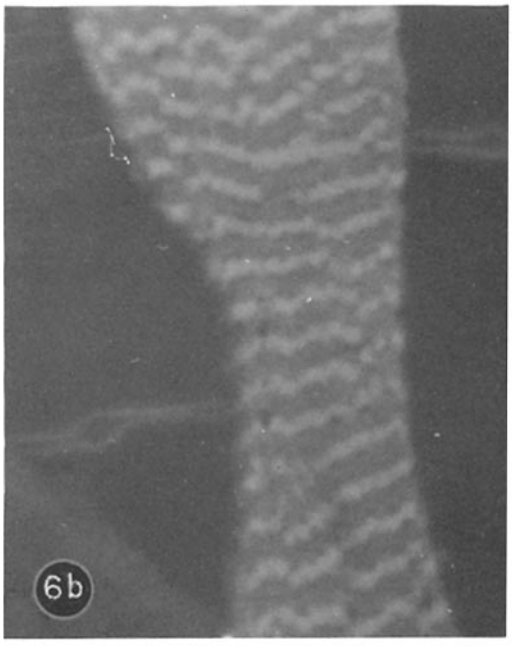
6b

has a very small dissociation constant in comparison with the dissociation constant of the Hg—carboxyl. Thioglycolic acid provides excess available SH group. In using thioglycolic acid to wash off non-specific stain in muscle I (Fig. 1 *b*) there would have to be a breaking of a tissue Hg—S bond to form a thioglycolic acid Hg—S bond. This does not occur easily, that is, over a short period of time. However, washing off non-specific stain from muscle II (Fig. 1 *d*) would require breaking a tissue Hg—carboxyl bond to form a thioglycolic acid Hg—S bond. The tissue Hg—carboxyl bonds of muscle II are therefore easily converted to thioglycolic acid Hg—S bonds which have a much smaller dissociation constant. The result is that only the conjugate bound to the tissue through the antigen-antibody bond remains and all other non-specifically bound conjugate is removed. If the tissue was pretreated with iodoacetic acid alone, the non-specific stain could not be completely washed off, presumably because not all available tissue SH groups were blocked.

Samples of muscle II treated with the doubly conjugated antibody or normal γ-globulin without receiving the thioglycolic acid wash gave the electron micrographs shown in Figs. 5 *a* and 5 *b*. Alternate sections for fluorescence microscopy are shown in Figs. 6 *a* and 6 *b*. There is no difference between the micrographs of material treated with the normal γ-globulin conjugate and those of material treated with the antibody γ-globulin conjugate. Aliquots of this same material, when brought through the brief thioglycolic acid wash, gave the fluorescence photographs shown in Figs. 8 *a* and 8 *b*. The non-specific staining in fluorescence has been completely washed off. In Fig. 8 *c* is shown the additional control for this series stained with antibody with fluorescein but no mercury. Electron micrographs of serial sections of this material are shown in Figs. 7 *a*, 7 *b*, and 7 *c*, where it can be seen that the Z line density is no longer so prominent as it was in Figs. 4 and 5. Also the specific increase in density of the A band relative to the I band is striking for the mercury-labeled antibody stain as compared with the controls.

Careful comparison of all the micrographs presented makes it evident that the only possible explanation for the increase in contrast is that it is due to the added mercury. Theoretical treatments now available derived from work on large objects of uniform density would predict no

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FIGURE 4

Low magnification electron micrograph of the same area shown in Fig. 2*c*. × 9000.

FIGURE 5

Electron micrographs of muscle II (see text) stained with antimyosin, and normal globulin as control. Sections were carbon-sandwiched using method B (see text). These micrographs demonstrate the non-specific staining present, before removal with the buffered thioglycolic acid wash solution.

Fig. 5*a*, muscle II stained with antimyosin previously conjugated with mercury and fluorescein. This tissue was not treated with buffered thioglycolic acid wash solution.

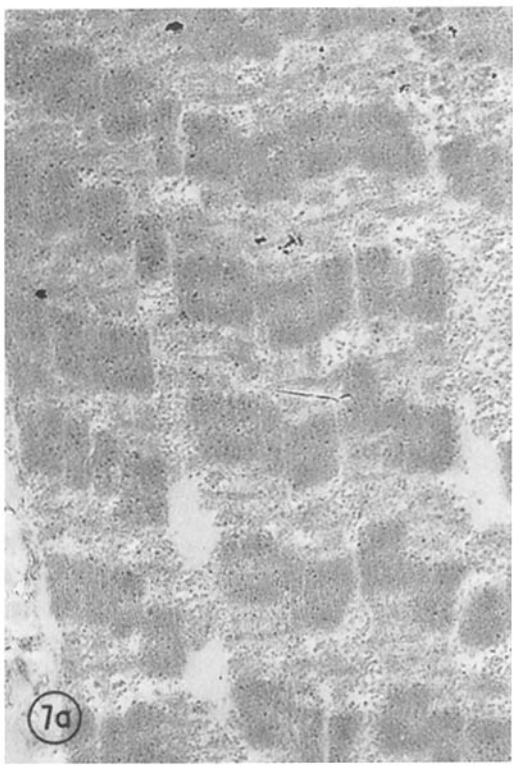
Fig. 5*b*, muscle II stained with normal globulin previously conjugated with mercury and fluorescein. This tissue was not treated with the buffered thioglycolic acid wash solution. × 9000.

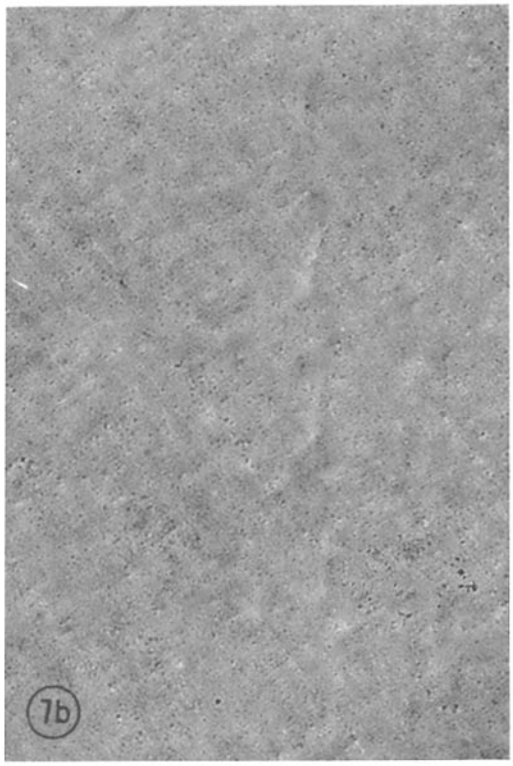
FIGURE 6

Fluorescence micrographs corresponding to the electron micrographs of Fig. 5. These were obtained from thick sections alternate to the thin sections used for the electron micrographs.

Fig. 6*a*, fluorescence micrograph corresponding to the electron micrograph Fig. 5*a*.

Fig. 6*b*, fluorescence micrograph corresponding to the electron micrograph Fig. 5*b*. Bright areas are areas of fluorescence localization. × 2300.


7a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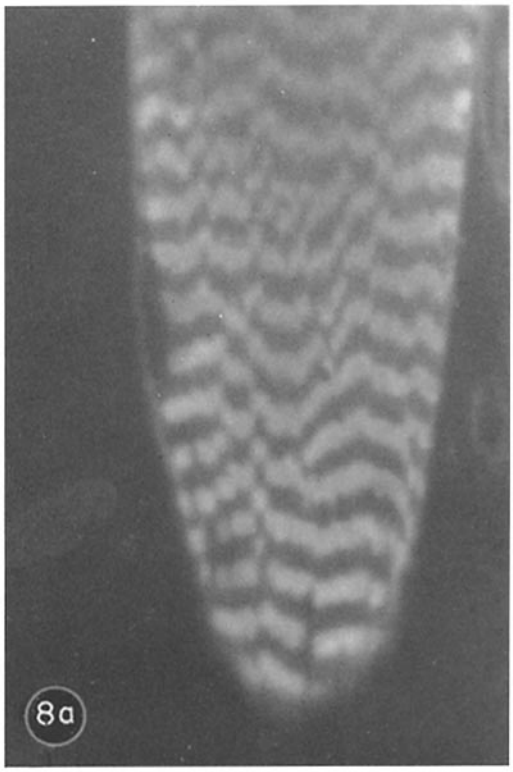
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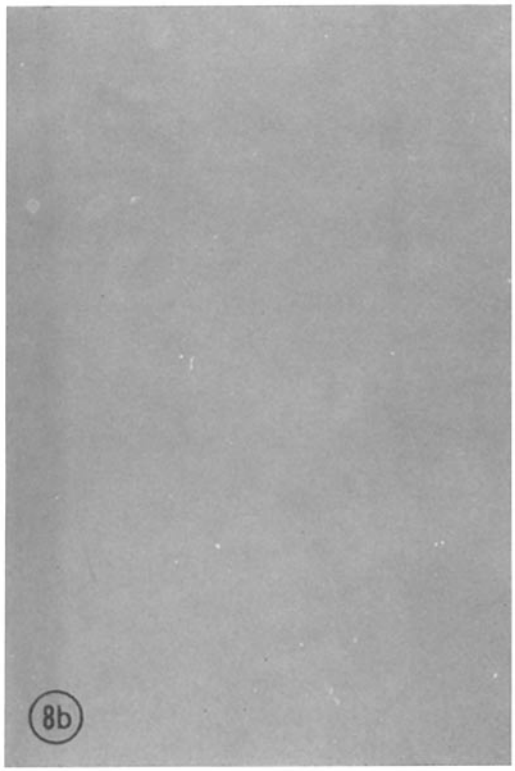
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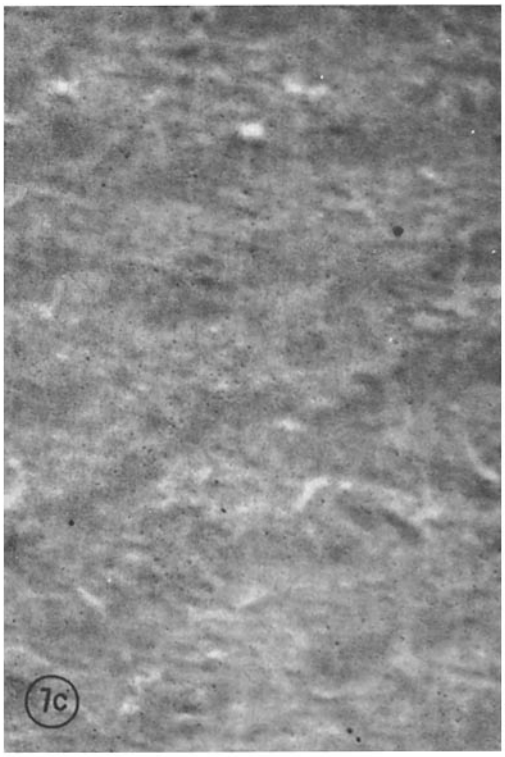

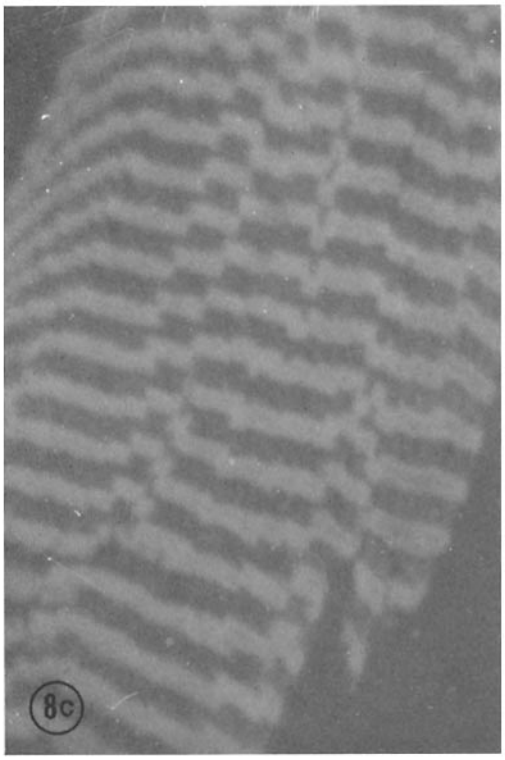

FIGURE 7

Electron micrographs of muscle II (see text) stained with antimyosin, normal globulin as control. The tissue was washed with a buffered solution of thioglycolic acid after staining, to remove the non-specific stain. Sections were carbon-sandwiched using method B (see text). × 9000.

Fig. 7*a*, muscle II stained with antimyosin previously conjugated with mercury and fluorescein. This is to be compared with the electron micrograph, Fig. 5*a*, of material which did not receive the buffered thioglycolic acid wash. The dense areas are the A bands, visible because of antibody localization.

Fig. 7*b*, muscle II stained with normal globulin previously conjugated with mercury and fluorescein. This is to be compared with the electron micrograph, Fig. 5*b*, of material which did not receive the buffered thioglycolic acid wash. Fig. 7*b* is also the control for the antibody staining observed in Fig. 7*a*.

Fig. 7*c*, muscle II stained with antimyosin previously conjugated with fluorescein but no mercury. This is an additional control for the antibody staining observed in Fig. 7*a*.

FIGURE 8

Fluorescence micrographs corresponding to the electron micrographs of Fig. 7. These were obtained from thick sections alternate to the thin sections used for the electron micrographs. × 2300.

Fig. 8*a*, fluorescence micrograph corresponding to the electron micrograph Fig. 7*a*. This is to be compared with the fluorescence micrograph, Fig. 6*a*, of material which did not receive the buffered thioglycolic acid wash.

Fig. 8*b*, fluorescence micrograph corresponding to the electron micrograph Fig. 7*b*. This is to be compared with the fluorescence micrograph, Fig. 6*b*, of material which did not receive the buffered thioglycolic acid wash. Fig. 8*b* is also the control for the antibody staining observed in Fig. 8*a*. The bright areas are the A bands, visible because of fluorescent antibody localization.

Fig. 8*c*, fluorescence micrograph corresponding to the electron micrograph Fig. 7*c*.


A
I
Z
9

visible contrast. Further experimental and theoretical work is needed to explain the discrepancy.

Alternate sections of all the material obtained for electron microscopy were stained by immersion of the grids in 1 per cent phosphotungstic acid in absolute alcohol. An example of this material is shown in Fig. 9. The stabilization and enhancement of contrast due to the heavy metal staining is striking. No differences were observed between antibody-stained and control materials when treated in this way.

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FIGURE 9

When sections were stained with phosphotungstic acid, a great deal of structure became visible. No differences in structure were observed with the variously treated material described in the text. *A*, A band; *I*, I band; *Z*, Z line. × 28,000.