A Diazo Coupling Method for the Electron Microscopic Localization of Cholinesterase*

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PLATES 188 TO 194

(Received for publication, June 26, 1959)

ABSTRACT

The presence of cholinesterase at the myoneural junction of intercostal muscle has been demonstrated in both light and electron microscopic preparations. A new simultaneous diazo coupling technique using α-naphthyl acetate as substrate and "hexazonium pararosanilin" as coupler has been applied to cold formalin-fixed tissues. After postfixation in buffered osmium tetroxide the sites of esterase activity are faithfully demonstrated at a high level of resolution. The details of cholinesterase distribution and some technical aspects of the procedure are discussed.

INTRODUCTION

Since knowledge of the intracellular distribution of enzyme systems should offer considerable aid to the understanding of cellular metabolic systems, the development of histochemical methods for electron microscopy has been a major concern of many cytochemists. Some of the general principles for the development of such techniques have recently been stated by Barrnett and Palade (2) and methods have been published for the demonstration of several enzyme systems (1, 3, 4, 13, 29).

The most important prerequisites for electron microscopic histochemical methods are that they (1) provide critical localization and (2) allow reasonable preservation of morphological detail. The theoretical criteria which must be met by a histochemical "capture reaction" in order to provide critical localization have recently been restated by Holt (16, 17) and also by Nachlas *et al.* (23). In brief, the enzyme must be fixed in the position which it occupies *in vivo* without being inactivated, the tissue must be permeable to both the substrate and the "capturing" reagent, the reaction between the product of the enzymatic reaction and the "capturing" reagent should produce a product which is insoluble, and the rate of the enzyme reaction should not be so rapid as to deplete locally the supply of "capturing" reagent. It has been stated that, in order for a histochemical reaction to be applicable to electron microscopy, its product must be electron opaque in addition to being insoluble in the reagents used in the preparation of tissues for electron microscopy (2). In addition, it must be relatively stable to the electron beam (22, 24).

Since electron contrast is roughly proportional to density (mass/volume) (14, 15, 32), a minimum requirement is that the product have a density greater than the average density of the material of a section of fixed tissue. (The maximum density of fixed tissue elements is approximately 1.6 gm./cc. (24).) It is then clear that if a sufficient concentration of product of a histochemical reaction can be deposited in a tissue, and if that product has a density equal to or exceeding approximately 1.6 gm./cc., it should not, in principle, be difficult to detect the material by the contrast it would produce in an electron image.

Preliminary experiments in our laboratory with a new diazonium reagent have shown that this compound, when coupled with α-naphthol, produces an azo dye which is essentially insoluble in all aqueous and organic solvents so far tested. The utilization of naphthyl acetate and its derivatives as substrates for the demonstration of esterases

* Supported, in part, by a grant from Myasthenia Gravis, Inc. to the Lewisohn Fund of the Mount Sinai Hospital.

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with simultaneous diazo coupling has recently been reviewed by Burstone (5). It has been shown by Koelle (18), Couteaux (6, 7) and Denz (11) that the esterase activity demonstrated histochemically in the neuromuscular junction of rat diaphragm is due to "acetyl cholinesterase (12).," Denz also offered evidence that the enzymatic hydrolysis of α-naphthyl acetate in the neuromuscular junction was due to acetyl cholinesterase. It was, therefore, decided to attempt the application of a diazo coupling method for cholinesterase to electron microscopy, using α-naphthyl acetate as a substrate and the new reagent as the coupler.

Material and Methods

Adult albino mice were anaesthetized with ether and chilled in an ice bath to a pre-terminal state. The shoulder girdle and neck musculature were then rapidly dissected from the thorax and the hemithoraces were separated. The intercostal muscles were placed under moderate stretch by inserting a steel or teflon spring between the first and last ribs in each hemithorax, and the entire preparation was immersed in fixative at 0–4°C. Under these conditions the thickness of the intercostal musculature was 0.5 to 1 mm. Morphological controls were fixed in 1 per cent buffered osmium tetroxide (25) for 2 to 4 hours, rinsed briefly in distilled water, cut from the ribs in small blocks, approximately 0.5 mm. square, dehydrated in graded alcohols and embedded in 5 per cent methyl/95 per cent butyl methacrylate. For enzyme studies the tissue was fixed for 1 to 2 hours in 10 per cent formalin buffered with 1/15 M phosphate at pH 7.4 at 0–4°C. It was then transferred directly to the incubating medium which is prepared as follows:

Diazo reagent, "hexazonium pararosanilin": *stock solution A:* 1 gm. of pararosanilin (basic fuchsin) is dissolved in 30 ml. of hot 2 N hydrochloric acid, cooled, and filtered. *Stock solution B:* 1 gm. of sodium nitrite is dissolved in 30 ml. of distilled water. These solutions are routinely stored in dropping bottles and are stable at room temperature for at least 2 months. The incubating medium is prepared directly before use. 3 to 5 drops each of solutions *A* and *B* are mixed in a small test tube. The diazotization is complete in 30 to 60 seconds, producing a clear, straw yellow to light amber solution. The reagent is then added to 30 ml. of the phosphate-buffered 10 per cent formalin solution (the fixative) at 0°C. and the pH is readjusted to 7.0 by the dropwise addition of 1 M dibasic sodium phosphate. The solution is then saturated with α-naphthyl acetate which is added in the form of an emulsion prepared by shaking 30 to 60 mg. of this substance in 2 to 3 ml. of hot (50–60°C.) water (9).

The tissue is transferred into this medium at or near 0°C. and held at this temperature in an ice bath for 4 to 6 hours. If the incubating medium becomes cloudy, it should be replaced by fresh solution. At the end of the incubation period, the tissue is returned to the buffered 10 per cent formol at 0°C. for washing.

For this study, the tissue was then treated in a number of different ways: (1) Some tissue was fixed in formalin for an additional 24 to 48 hours. Small, well "stained" portions were then selected, cut out, dehydrated in graded alcohols, and either embedded in 10 per cent methyl/90 per cent butyl methacrylate or paraffin, or teased and mounted directly in permount. (2) Other tissue was transferred directly to two changes, 2 to 3 minutes each, of buffered 1 per cent osmium tetroxide at 0°C., and then fixed in this reagent for 2 to 4 hours. It was then rinsed in distilled ice water; selected small blocks of intercostal muscle were cut from the ribs, dehydrated in graded alcohols, and embedded in methacrylate. 1 μ sections were cut from blocks oriented so that the long axis of the muscle fibers was perpendicular to edge of the glass knife on a Porter-Blum microtome. These were serially examined in the light or phase microscope, usually without removing the methacrylate. "Stained" neuromuscular junctions were easily located. In control material, a more careful examination under oil-immersion in the phase microscope was usually required. When a suitable neuromuscular junction was found, the block was further trimmed and silver to platinum-gray sections were cut and examined in a Philips EM100A electron microscope equipped with a 20 μ aperture and compensated objective.

For each hemithorax treated as above, the opposite hemithorax was handled as a reagent blank (enzyme control), receiving identical treatment, except that the α-naphthyl acetate substrate was omitted from the incubating medium. All electron micrographs were taken at 100 kv. Enzyme controls and treated sections were exposed to as nearly identical "histories" in the electron microscope as possible and were photographed under virtually identical conditions.

In preliminary experiments fixation in 0°C., 10 per cent buffered formalin for 24 hours was followed by paraffin embedding. Sections were carried through the enzyme incubation after removal of paraffin. After periods of fixation of up to 1 hour at 0°C., in buffered 1 per cent osmium tetroxide, the tissue was found to retain patches of enzyme activity; however, this was too weak to allow electron microscopic localization (in well fixed areas). Fixation in ice cold, 0.6 per cent buffered potassium permanganate (21) for periods up to 4 hours yielded good preservation of enzyme activity; however, the tissue became extremely brittle and morphological preservation was, in general, poor.

RESULTS

The product of the histochemical reaction is a dark, red-brown precipitate which is sharply

localized in the neuromuscular junction in the region which Couteaux calls the "subneural apparatus." The apearance of the formol-fixed preparations in whole mount is illustrated in Figs. 1 and 2. Figs. 3 *A* and *B* are brightfield and phase contrast micrographs of a 1 μ methacrylate section of a formalin-fixed end-plate. Fig. 3 *C* is an electron micrograph of an adjacent thin section of the same end-plate. Although formalin fixation alone does not result in good contrast of tissue detail in the electron microscope, the azo dye deposit appears in higher contrast than the surrounding tissue. Comparison of the three pictures reveals that the density of the two (teloglial) nuclei is not due to deposition of dye. It should be noted that, in the case of formol-fixed tissue, an increase in contrast between the dye deposit and the surrounding areas occurs as the beam intensity is increased. However, even low beam (22) pictures reveal reasonable contrast. Formol-fixed material which has been treated with the diazo reagent without substrate, suffers less loss of tissue contrast when exposed to the electron beam than material which has been treated with formalin alone. (There is, of course, no enzyme specific dye deposition.)

The appearance of histochemically treated, formol-fixed material, in whole mounts and in sections, in the light and electron microscopes shows localization of esterase activity in the region of the "subneural apparatus." Histochemical treatment of 1 μ paraffin sections of formalin-fixed material revealed essentially the same localization in the light microscope. However, cytomorphological detail was not sufficiently well preserved by formalin fixation *alone* to permit localization in the electron microscope with appreciably greater precision than that allowed by light microscopy.

Figs. 4, 5 *B*, 6 *B*, and 7 are electron micrographs of post-osmicated, histochemically treated end-plates, and 5 *A* and 6 *A* represent enzyme controls. These preparations demonstrate enzyme localization with excellent resolution and good preservation of morphological detail. Figs. 5 *B* and 6 *B* are progressively higher magnifications of the area enclosed by the smaller rectangle in Fig. 4. Figs. 5 *A*, and 6 *A* represent a corresponding area in an enzyme control section, each at a magnification identical to that of its mate. Comparison of the enzyme and control sections reveals an almost homogeneous deposit (presumably of azo dye) occupying the synaptic space between the axonal and muscle plasma membranes and between the (subneural) folds of the muscle plasma membrane. These spaces have actually been distended to approximately twice the width which they occupy in the control sections, and the membranes bordering them have acquired a smoothed appearance. Fig. 7 demonstrates the high resolution of which the method is capable. The single unlabelled arrow points to a space enclosed by two membranes which is approximately 100 A wide and contains a deposit of enzyme product. This space may represent an infolding of the axonal plasma membrane on the surface which is apposed to the teloglial plasma membrane (see Text-fig. 1). Enzyme product was also found in the space between these apposed membranes. In contrast to this, the double membrane structure indicated by the barred arrow shows no deposit of enzyme product. (This latter structure represents a special type of inclusion found in the cytoplasm of the axon terminal and has been described elsewhere (19).)

Figs. 8 *A* and *B* are electron micrographs taken of a section of neuromuscular junction from tissue which was handled as an enzyme control. Both pictures were exposed, developed, and printed under identical conditions. *A* was taken immediately after the beam had been brought to full intensity at 100 kv., and *B* after the section had been exposed to the full beam at 100 kv. for about 10 minutes. There has been a considerable decrease in density throughout the section, while nucleoprotein granules (Palade granules) and membrane structures stand out in greater contrast relative to the rest of the section. The changes demonstrated in no way compare with the differences observed between the histochemically treated and control sections. It is, therefore, extremely unlikely that these latter differences are attributable to differential "sublimation" of synaptic structures (22, 24).

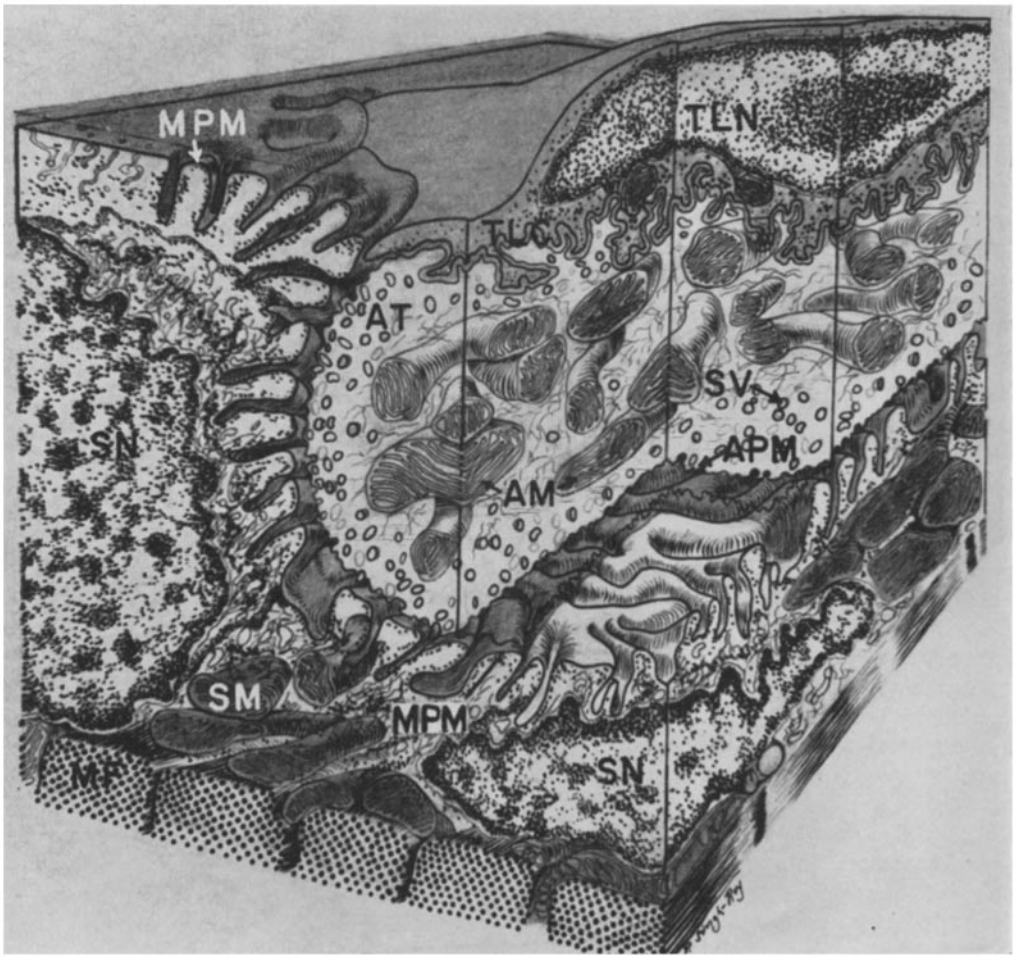
Mouse and rat muscle retains adequate enzyme activity in the neuromuscular junctions even after having been stored in 10 per cent buffered formol at 8°C. for (at least) 3 weeks.[1]

[1] By comparison, enzyme in the neuromuscular junctions of the garter snake was found to be almost completely inactive after 3 days of storage under the same conditions, and the enzymes in single electroplaque of the rays, *Narcine* and *Torpedo*, were inactive after only 12 hours. Similar species differences in resistance of cholinesterase to inactivation by formalin was reported by Taxi (30).

DISCUSSION

In searching for a method for the localization of cholinesterase which would be suitable for electron microscopic applications, the most obvious choice which presents itself is the Koelle method (18) or Couteaux's modification thereof (7), since the histochemical product of such a reaction is a dense compound. Unfortunately, as Couteaux has pointed out, the reaction must be carried out at pH 5 if serious diffusion artifacts are to be avoided. This condition for the reaction has so far prevented the preservation of adequate morphological detail for electron microscopy.

The light microscopic localization of esterase activity in the neuromuscluar junction by the method which has been described agrees entirely with that which has been demonstrated for acetyl



TEXT-FIG. 1. Diagrammatic reconstruction of a portion of a neuromuscular junction. Part of the axon terminal has been cut away to reveal the pattern of the subneural (junctional) folds in the sarcoplasmic membrane lining the synaptic gutter. Magnification: approximately × 35,000.

Key to Symbols

AM, Mitochondrion of axon terminal
APM, Axonal plasma membrane
AT, Axon terminal
MF, Myofibril
MPM, Muscle plasma membrane
SM, Muscle mitochondrion or sarcosome
SN, Sole plate nucleus
SV, Synaptic vesicle
TLC, Teloglial cell (cytoplasm)
TLN, Teloglial nucleus

cholinesterase by Couteaux and others using acetyl thiocholine substrates (6, 18), and by Holt, using indoxyl acetate derivatives (16). For precision of localization in the light microscope, the pictures obtained with the method described compare favorably with the very best published pictures obtained by other methods.

Text-fig. 1 is a picture of a three dimensional, conceptual reconstruction of a portion of a neuromuscular junction surrounding a branch of the axon terminal. The axon terminal, lying in a gutter in the sarcoplasm of the "sole plate" which is lined by an intricately folded plasma membrane, is separated from this membrane by a relatively constant space of 250 to 350 A (19, 26, 28). The portion of the axon terminal which is not surrounded by sarcoplasm is covered by a thin layer of cytoplasm belonging to a teloglial cell (19) (terminal Schwann cell—see also Figs. 4, 7, and 8). In this region, the greatly folded plasma membrane of the axon terminal is separated by a constant spacing of about 100 A from the plasma membrane of the teloglial cell, and the two membranes closely parallel one another. It is in these spaces, between the axon terminal and its two adjacent cells, that we find our enzyme product accumulating.

Concise localization, entirely outside of cell boundaries, raises the question whether such localization is an artifact. If the cell membrane were to bar selectively the diffusion of the diazo reagent while permitting free passage of the substrate and its hydrolytic products, and the enzyme were located at some intracellular site close to the cell membrane, the described distribution of product might be expected. There are several reasons for believing that the diazo reagent does freely diffuse *into* the cell under the conditions of the histochemical reaction: (1) At the electron microscopic level: formalin-fixed muscle which has been treated with the diazo reagent under the conditions of the enzyme incubation, shows less diminution of contrast of both *sarcoplasmic* and *axoplasmic* components during exposure to the electron beam than tissue which has been treated with formalin alone. This is probably due to reaction of the diazo reagent with phenolic aromatic rings and imidazole groups in tissue proteins. (2) At the light microscope level: if a piece of intercostal muscle is treated with diazo reagent followed by several rinses in cold, buffered formalin, and is then immersed in an α-naphthol solution, thin sections of such muscle show *all* tissue elements (protein) to be stained deep red. Since each molecule of the reagent has three diazonium groups (10), some of these are left free to react with the naphthol after the reaction with tissue (8). (3) The method described here, when applied to some other intact tissues (31) (as opposed to sections) or whole protozoans such as *Tetrahymena* reveals clear-cut localizations of enzyme activity in *intracellular* structures by light microscopy. (4) When the method is applied to paraffin sections, it reveals light-microscopic localization in the neuromuscular junction which is indistinguishable from that obtained under the conditions of treatment used for electron microscopic preparations.

We are, therefore, led to conclude that the esterase activity demonstrated in the neuromuscular junction which, from all the evidence available at this time, is probably due to acetyl cholinesterase, is closely associated with the plasma membranes of the component structures. Since activity is present in the depths of the "subneural folds" of the muscle plasma membrane, this membrane undoubtedly contributes a major portion of the total cholinesterase activity of the neuromuscular junction. However, the finding of activity in the space between the axonal and teloglial plasma membranes requires the additional conclusion that at least some of the esterase activity is associated with one or both of these structures. Since no enzyme product has been detected on the cytoplasmic sides of the muscle, axonal, or teloglial plasma membranes, we are forced to conclude that either the active sites of the enzyme are exclusively located on the extracellular sides of the plasma membranes or the enzyme is located entirely within the spaces between these membranes. The physiological significance of these findings has been discussed elsewhere (20).

A brief discussion of some of the technical variables will now be presented.

It was found that tissue fixed in ice cold, 10 per cent formalin, buffered at pH 7.0 for 4 to 24 hours prior to postfixation in buffered 1 per cent osmium tetroxide showed morphological detail which was, in some respects, superior to that seen in tissue fixed in buffered osmium tetroxide alone (31). Prolonged washing of the tissue in water after the formalin and prior to osmium fixation, or omission of formalin from the incubating medium produced poorer morphological preservation. At no time

during either prefixation or the histochemical processing should the pH of the formalin be permitted to drop below 6.5. Unbuffered formalin rapidly drops in pH during fixation and results in poorer preservation.[2]

The particular combination of pH 7.0 and 0°–5°C. incubation temperature were chosen for the following additional reasons: (1) Diazonium salts inactivate cholinesterase. The rate of inactivation decreases rapidly with decreasing pH in the vicinity of neutrality. Below pH 7.0, the coupling rate has been found to diminish sufficiently to result in lowered cytochemical resolution. (2) The Q_{10} of enzymatic hydrolysis has been found to be lower than that of the inactivation reaction. By incubating near 0°C., the hydrolytic reaction is favored relative to inactivation. (3) Rates of diffusion are less temperature-dependent than chemical reactions. Incubation near 0°C. minimizes artifacts which can result from the local depletion of substrate and coupler at the enzyme site.

Despite the relatively unfavorable conditions for the enzymatic reaction, the possibility of deposition of massive quantities of product at the enzyme sites remains because of the enormous magnitude of enzyme turnover numbers. These have been found to range from 10^4 to 10^7 per active molecular site per minute (12). An acetylcholinesterase has been estimated to have a turnover number of 2×10^6 (27).

For the further development of histochemical reactions for electron microscopy, it should be re-emphasized that the atomic density of the histochemical product is not necessarily a decisive factor in methodology. It has been demonstrated here that useful contrast can be achieved with purely organic reagents.

The authors are grateful to Dr. B. J. Davis and Mr. Allan Wachtel for their freely given advice and discussions, and to Miss Ruby Tamura and Mr. John Weinstock for technical assistance.

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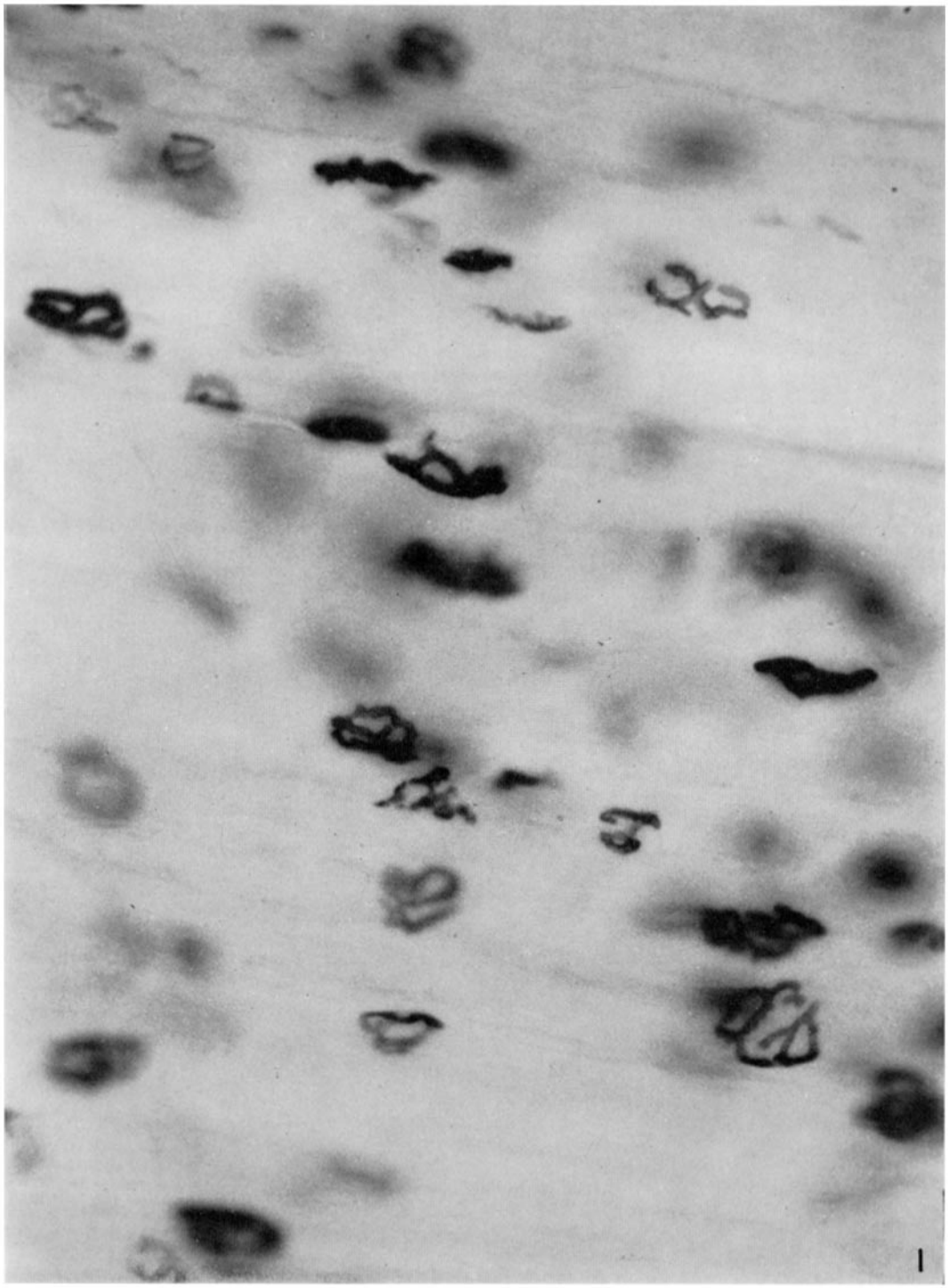
[2] Ornstein, L. and Davis, B. J., unpublished observations.

Explanation of Plates

(For key to symbols see Text-fig. 1, p. 402.)

PLATE 188

FIG. 1. Low power photomicrograph of a whole mount of mouse intercostal muscle, fixed for 1 hour in ice cold, buffered 10 per cent formalin, incubated for 4 hours at 0°C. in the α-naphthyl acetate, formol, "hexazonium pararosanilin" medium. Magnification: approximately × 250.

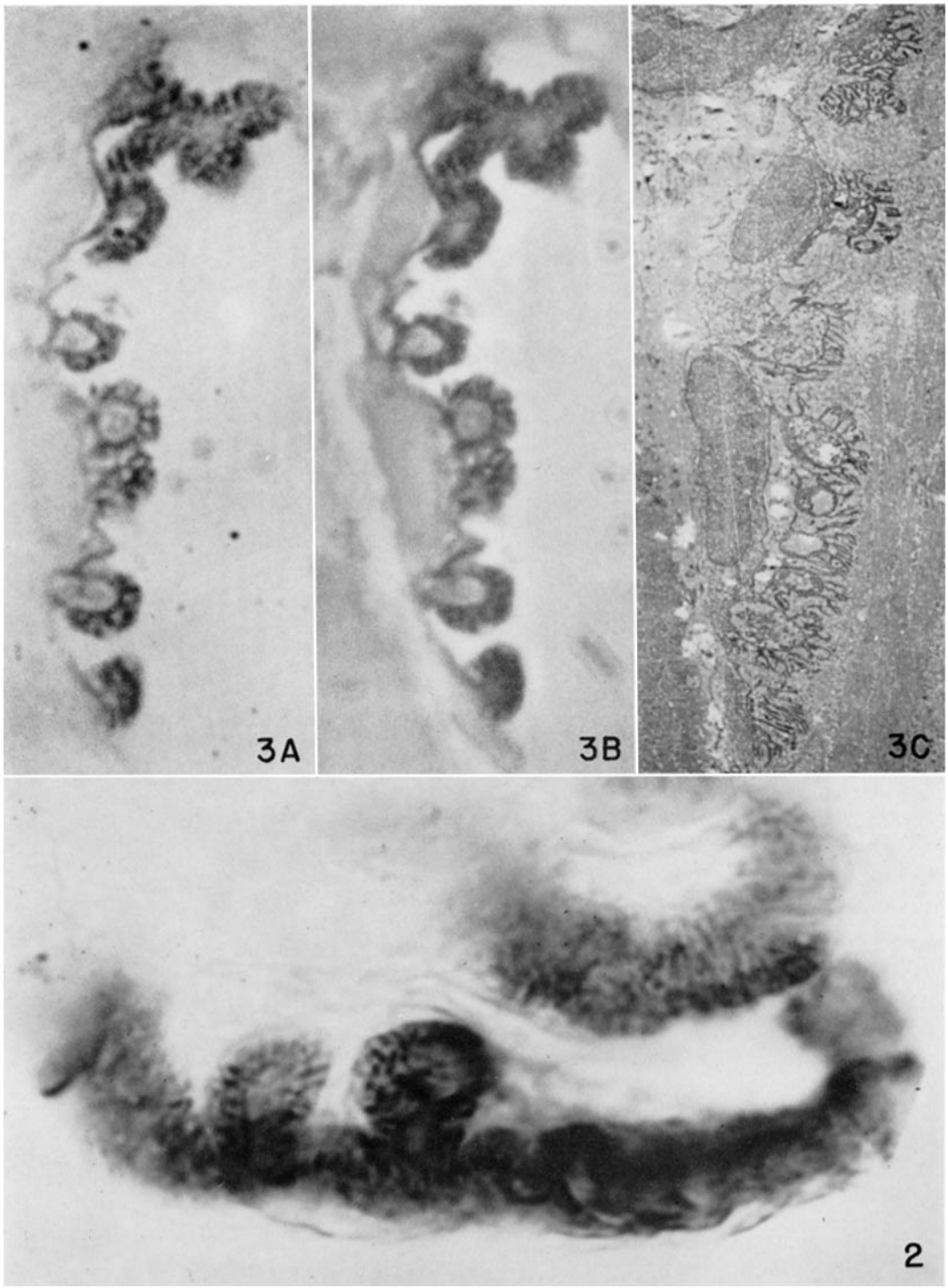


(Lehrer and Ornstein: Diazo coupling method)

PLATE 189

FIG. 2. Oil immersion photomicrograph of a whole mount of a single muscle fiber teased from a preparation similar to that shown in Fig. 1. The region of the neuromuscular junction is seen in "optical section." The pattern of folds of the "subneural apparatus" is discernible. Magnification: approximately × 2,200.

FIG. 3. Longitudinal methacrylate sections through a region of a neuromuscular junction similar to the one shown in Fig. 2. The main portion of the muscle fiber is at the right of each picture. Two teloglial nuclei may be seen to the left of center. *A*. One micron section 90× oil immersion apochromat objective. *B*. Same as *A* but with 90× oil immersion medium dark contrast phase objective. *C*. Ultrathin section (silver) adjacent to the section shown in *A* and *B*. Low power electron micrograph 100 kv. Comparison of the three pictures allows distinction between intrinsic tissue density and the density of the dye deposit. Magnification: approximately × 2,000.



(Lehrer and Ornstein: Diazo coupling method)

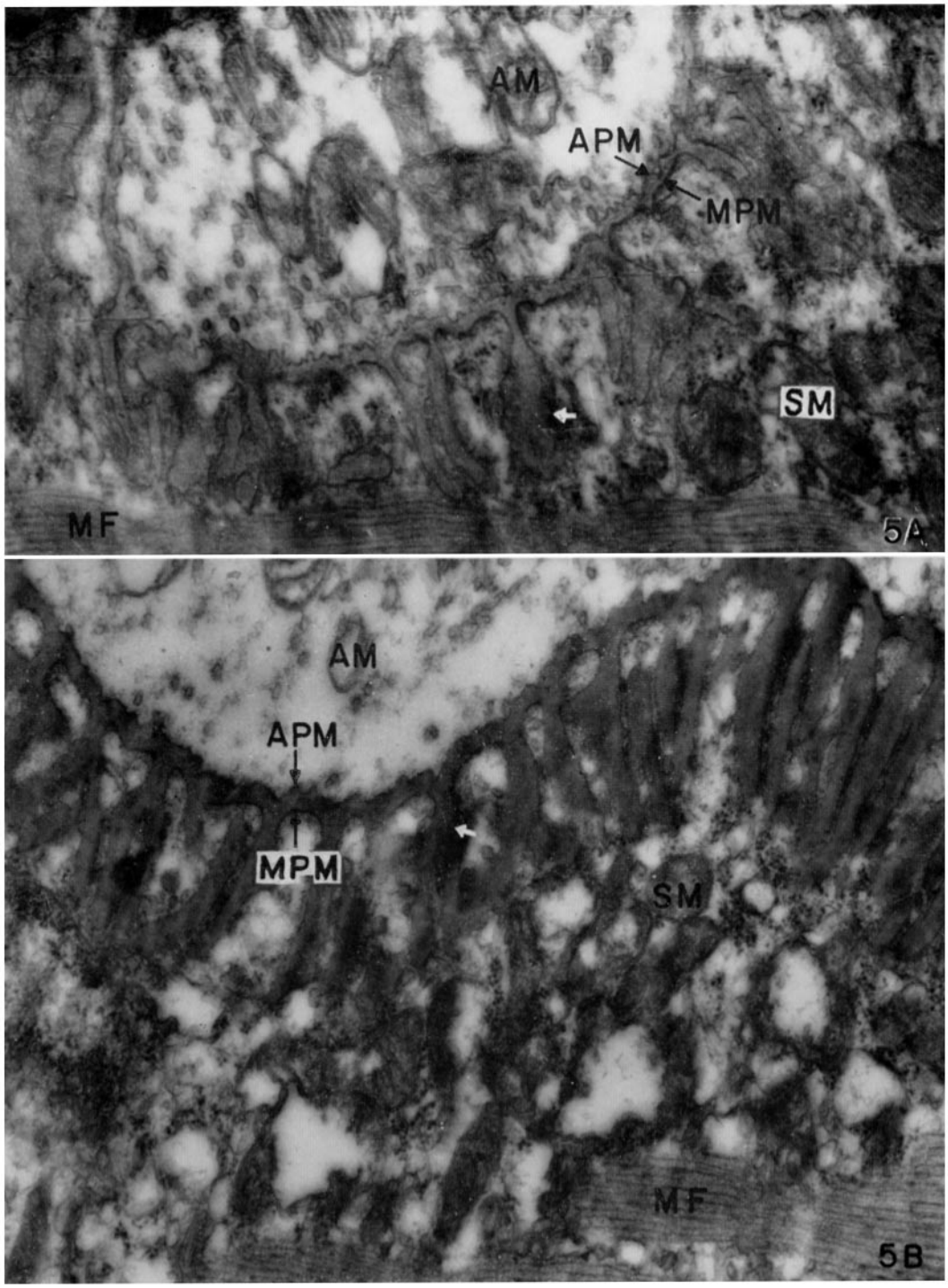
PLATE 190

FIG. 4. Section through a neuromuscular junction oriented much like that in Fig. 3. A teloglial nucleus is seen at the upper left and what is probably a fibroblast at the lower left. The tissue has been fixed in cold buffered 10 per cent formalin, incubated for 4 hours at 0°C. in the α-naphthyl acetate, formol, "hexazonium pararosanilin" medium, and postfixed in osmium tetroxide for 4 hours. The areas in the small and large rectangles are shown at higher magnification in Figs. 6 *B* and 7, respectively. An almost homogeneous deposit of azo dye is seen in the synaptic or junctional folds, the synaptic space, and in the space between the teloglia and the axon terminal. Magnification: × 17,500.

(Lehrer and Ornstein: Diazo coupling method)

PLATE 191

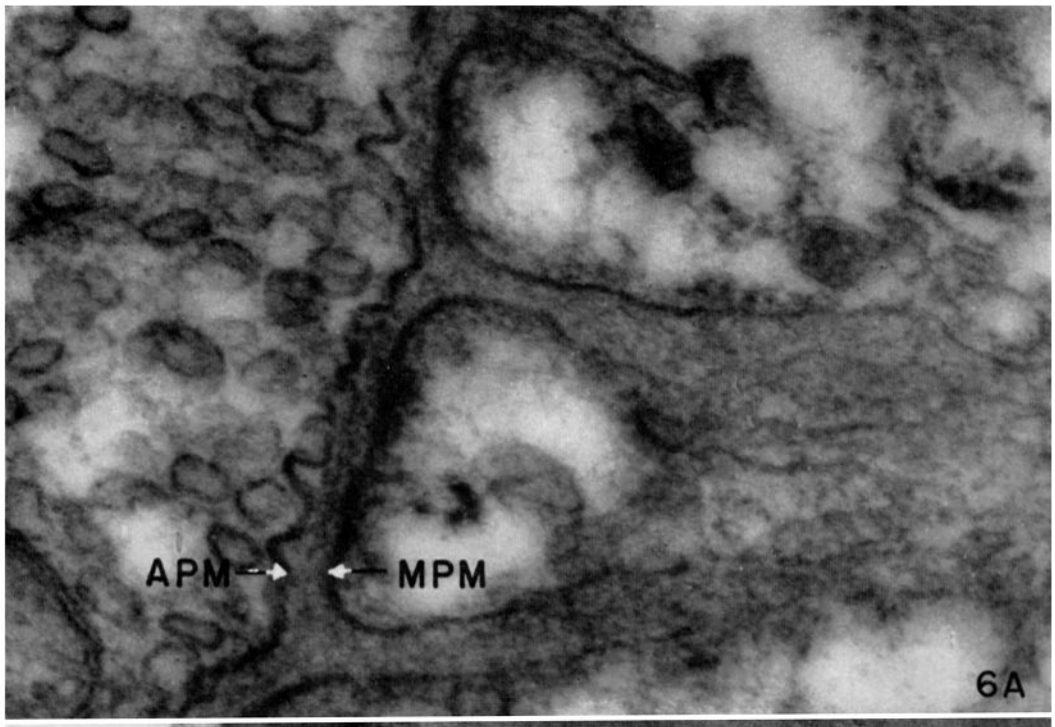
FIG. 5. *B* is from a section of tissue treated as that shown in Fig. 4. *A* is a comparable area from a section of tissue treated (as an enzyme control) in an identical fashion as the tissue in *B* except for the omission of the α-naphthyl acetate substrate from the incubation medium. Photographic parameters are nearly identical for the two pictures. The direction of sectioning was horizontal with respect to the orientation of the pictures. The spotty densities (unlabelled arrows) are probably due to cutting artifacts and appear in both types of material. The differences in density and texture of the material which fills the synaptic space (opposed arrows) and the space within the junctional folds are apparent. In *B* these spaces also appear widened and the membranes lining them appear smoothed as compared with *A*. Magnification: × 50,000.

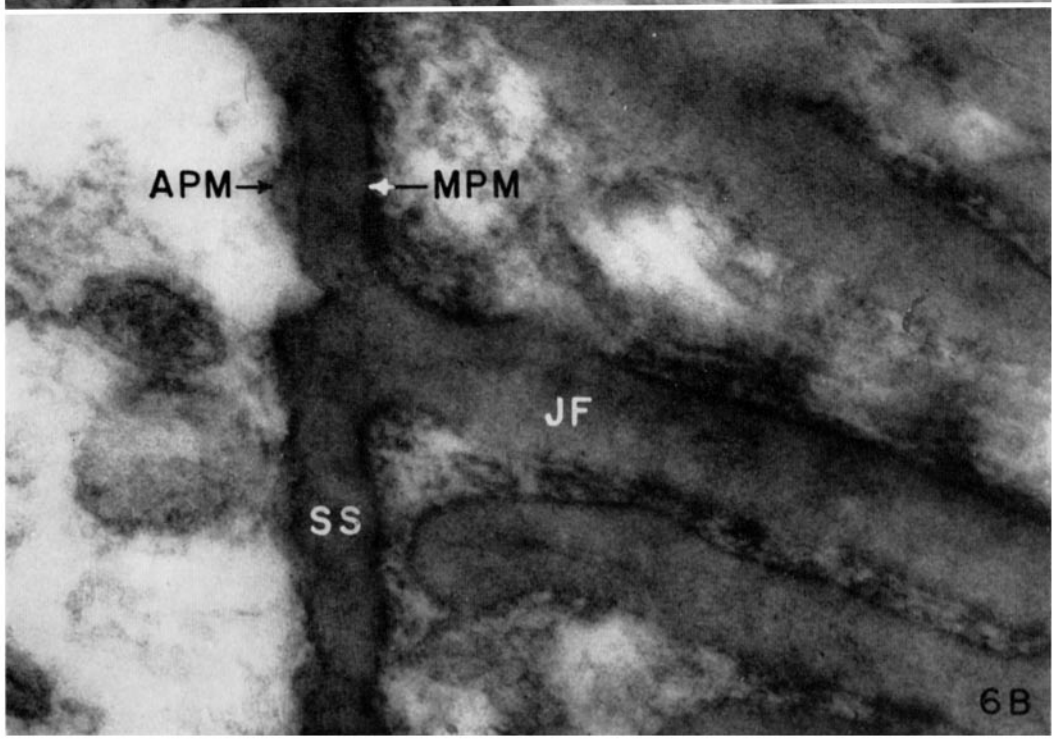


(Lehrer and Ornstein: Diazo coupling method)

PLATE 192

FIG. 6. High magnification views of a portion of the section in Fig. 4 (Fig. 6 *A*) and its enzyme control (Fig. 6 *B*). Photographic parameters again are nearly identical. The differences pointed out in Fig. 5 are here more obvious. It should again be noted that the densities of certain areas along the muscle plasma membrane are comparable in both pictures and are, therefore, not likely to be due to a deposit of azo dye resulting from enzyme activity. *JF*, junctional fold; *SS*, synaptic space. Magnification: × 200,000.

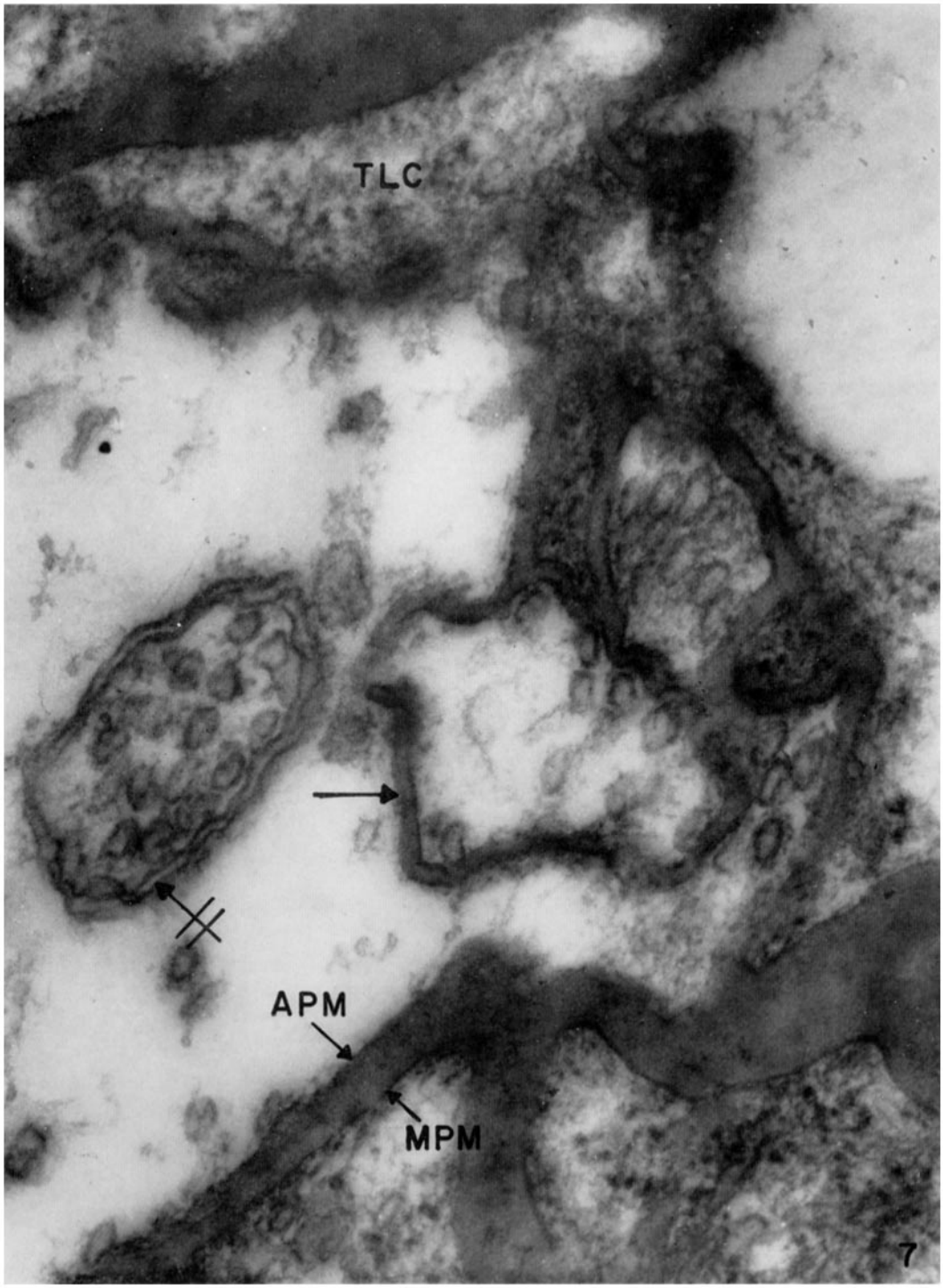




(Lehrer and Ornstein: Diazo coupling method)

PLATE 193

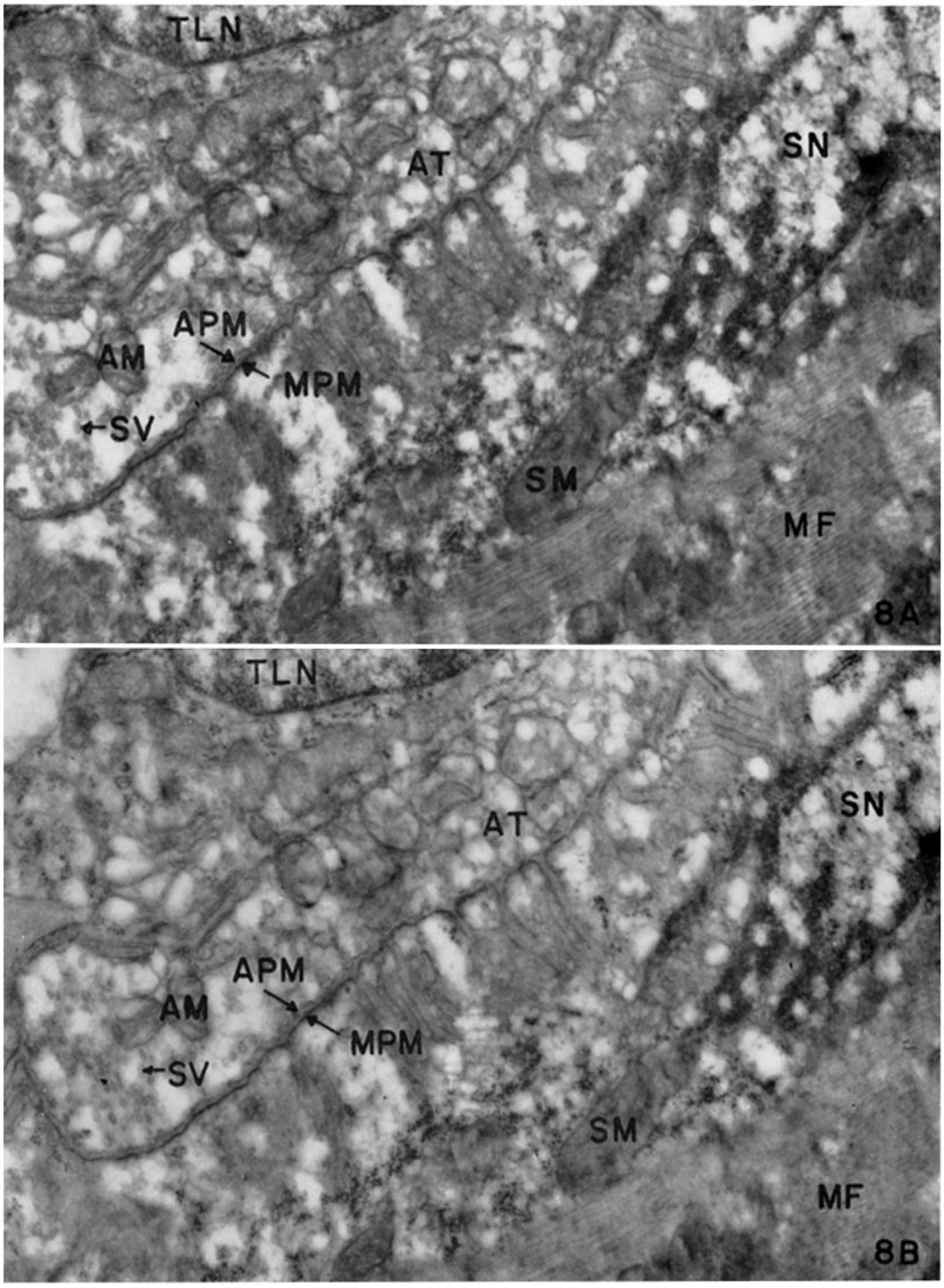
FIG. 7. High magnification of the area enclosed by the larger rectangle in Fig. 4. For control, compare Fig. 6 *A*. The homogeneous deposits of azo dye may be seen in the synaptic space (opposed arrows) and in the 100 *A* space which is continuous with the space between the teloglial cell and the axon (plain arrow). A space enclosed by two membranes of similar dimensions which has no density due to dye deposit is indicated by the barred arrow. Magnification: × 200,000.



(Lehrer and Ornstein: Diazo coupling method)

PLATE 194

FIG. 8. Longitudinal section through a neuromuscular junction, cut from tissue which was handled as an enzyme control. The two pictures were taken of the same area of the same section under identical conditions and were processed identically. *A* was taken immediately after the section had been exposed to "cross-over" at 100 kv. and *B*, after the section had been exposed to the beam for about 10 more minutes. Magnification: × 25,000.



(Lehrer and Ornstein: Diazo coupling method)