FINE STRUCTURAL LOCALIZATION OF ACETYLCHOLINESTERASE IN ELECTROPLAQUE OF THE ELECTRIC EEL

FLOYD E. BLOOM and RUSSELL J. BARRNETT

From the Department of Anatomy, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

The electroplaques composing the electric organ of the eel, *Electrophorus electricus*, have been utilized for the dual purpose of demonstrating the subcellular sites of acetylcholinesterase activity and as a model for comparison of the several cytochemical methods available. Fresh tissue and tissue fixed by immersion in formalin, hydroxyadipaldehyde, or glutaraldehyde was reacted with the Cu-thiocholine method, the Cu-ferrocyanide thiocholine method, or the thiolacetic acid (TAA) method using Pb, Ag, or Au as capture reagents. Controls were obtained by omission of substrate, or by addition to complete media of varying concentrations of different cholinesterase inhibitors. Reactions were run at 0–5°C at a pH range of 5.0–7.1 for 0.25 to 120 min. Regardless of the capture metal, the localization obtained with TAA as substrate was identical with that observed with acetylthiocholine, the majority of precipitate being deposited on or near the external innervated surface of the plaque and within the tubulovesicular organelles opening onto the innervated surface. Both of the thiocholine methods and the Pb-TAA method showed reaction product in synaptic vesicles of the nerve endings innervating the plaque which was uninhibitable by 10^{-4} M physostigmine. All methods also showed some inhibitor-sensitive deposition of reaction product in the mucoid material forming the immediate extracellular environment of the innervated surface.

The enzyme acetylcholinesterase (AChE) has been the subject of numerous biochemical and pharmacological experiments probing the relationship of hydrolytic activity to the junctional and nonjunctional aspects of bioelectrogenic phenomena (1). The cytochemical localizations of this enzyme activity at a fine structural level in other tissues have been performed by methods which vary in their selective substrate specificity, in the succinctness of localization, and in consequent interpretability (3–11). The electric organ of the electric eel would appear to be an unusually favorable tissue for a comparative analysis of available cytochemical methods, since previous biochemical work by Nachmansohn (12) has shown that the enzyme, having considerable substrate specificity, is present in extraordinarily high concentration within a small portion of the mass of the electroplaques composing the electric organ. Moreover, because of the intensive electrophysiological interest already devoted to these preparations (13–16), the results of cytochemical observations could be expected to have broad biological interest. The following experiments, briefly announced elsewhere (17), were undertaken towards this purpose.

MATERIALS AND METHODS

Portions of the main organ and the Sachs Organ of specimens of the electric eel, *Electrophorus electricus*, were obtained. Manually sliced transverse sections

(1 to 2 cm in diameter) of the entire eel at the level of the electric organ were immediately immersed into solutions of buffered aldehydes and, during fixation, were trimmed of outer skin, muscle, and surrounding tissue (see Fig. 1) into large blocks consisting only of columns of electroplaques. Similar blocks were frozen and stored on dry ice until used for cytochemical reactions either as unfixed tissue or after fixation prior to incubation. In addition, single electroplaques (2.0 × 0.5 × 0.01 cm) were dissected to the stage used in electrophysiological experiments.[1] and then fixed by immersion. Tissues were immersed in fixative for 0.5–4.0 hr, followed by "storage" in isotonic buffer for varying periods. The fixatives consisted of 10% formalin, 4 to 6% glutaraldehyde, or 6% hydroxyadipaldehyde, buffered to pH 7.2–7.4 with 0.05 M cacodylate or 0.1 M Tris-maleate, made isotonic with sucrose. Initially, relatively crude glutaraldehyde (Union Carbide Co., New York) was partially purified by several passages through an Amberlite MB-1 ion exchange resin which routinely increased the pH from less than 3.5 to more than 5.0, and which also removed the yellowish discoloration. Biological grade glutaraldehyde (50%, Fisher Chemicals, Pittsburgh) was subsequently used routinely.

CYTOCHEMICAL REACTIONS: Sections were cut on the freezing microtome (40 to 60 μ) or freehand sections (100 to 200 μ) were made on unfrozen tissue. Reaction mixtures of Gerebtzoff (18–19) and of Karnovsky (20), using acetylthiocholine as substrate, were followed as originally described, except that incubation periods were from 5 to 60 min at pH 6.8–7.1, at 0–4°C. Controls for these reactions consisted of elimination of the substrate, substitution of butyrylthiocholine for acetylthiocholine or addition of physostigmine salicylate (10^{-4} to 10^{-6} M) to the acetylthiocholine medium. In tissues reacted with the Gerebtzoff medium, conversion of the reaction product to copper sulfide by exposure to $(NH_4)_2S$ was omitted in the majority of instances, since it was determined that copper thiocholine sulfate (21) was sufficiently dense to be adequately visualized in the electron microscope.

The incubation media, using thiolacetic acid (TAA) (22, 23) as substrate, were prepared essentially as described in previous publications (6). However, the TAA was relatively pure and virtually colorless.[2] Various capture reagents (0.005 M) were assayed in addition to $Pb(NO_3)_2$, these included $CdCH_3COOH$, $CuSO_4$, $FeCl_3$, $AgNO_3$, $AuCl_4$, and $NaAu(S_2O_3)_2$ (11). When $Pb(NO_3)_2$ was the capture metal, the concentration was varied from 0.005 to 0.01 M and the amount of TAA was varied from 0.25 to 0.125 cc. Additional reactions were performed in which the tissue slices were preincubated in a cacodylate-buffered Pb^{++} solution for 15 to 60 min prior to incubation in the complete media. Reactions using Pb-TAA combination were performed over a wide pH range (5.0–7.2), using a 0.05 M Na cacodylate-HNO_3 buffer. Tissue slices were pre-chilled to 5°C and all reactions were run in an ice bath (1–4°C) for periods of 0.25 to 120 min. Controls for the TAA reaction consisted of omission of the substrate from the medium, or the addition to the complete media of one of the following cholinesterase inhibitors: physostigmine (10^{-4} to 10^{-6} M), prostigmine (10^{-5} M), diisopropylfluorophosphate (10^{-3} to 10^{-6} M) and edrophonium (10^{-5} and 10^{-6} M). These controls were performed simultaneously with the regular cytochemical reactions, utilizing tissue slices taken from the same block of electric organ. The conditions of incubation were identical, except that control tissues were preincubated in buffered solutions containing the cholinesterase inhibitors for 15 to 50 min at 37°C prior to chilling and incubation in the regular medium containing an inhibitor.

At the conclusion of all cytochemical and control reactions, the slices were washed in frequent changes of cold buffer or de-ionized distilled water for 30 to 60 min and refixed with Veronal-buffered 1% osmium tetroxide for 1 hr (24). Increasing concentrations of ethanol were used for dehydration, and the tissues were embedded in Maraglas with DER 732 (25) as plasticizing agent. The flat cap ends of polyethylene capsules (BEEM) were utilized as individual embedding dishes to permit orientation of the tissue. Sections having interference colors of pale gold to gray were cut with an LKB microtome, and viewed in an RCA EMU-3F electron microscope. Initial micrographs were made on unstained thin sections. Subsequently, thin sections were counterstained on the grids with saturated aqueous uranyl acetate when it was determined that this reaction, which enhances the contrast of morphological elements, did not interfere with and could be differentiated from the final products of the cytochemical reactions.

RESULTS

Fine Structure

Aspects of fine structure not readily discernible in previously published micrographs of *Electrophorus* electroplaques will be detailed from observations of thin sections of tissues fixed by primary immersion in buffered glutaraldehyde, hydroxy-

[1] The authors are indebted to Dr. D. Nachmansohn and his colleagues for providing the tissues. Indeed, Dr. Nachmansohn and coworkers have been most helpful throughout this work, providing stimulus, discussion, and criticism.

[2] TAA (98% pure) was obtained by special arrangement with Eastman Organic Chemicals, Rochester, New York.

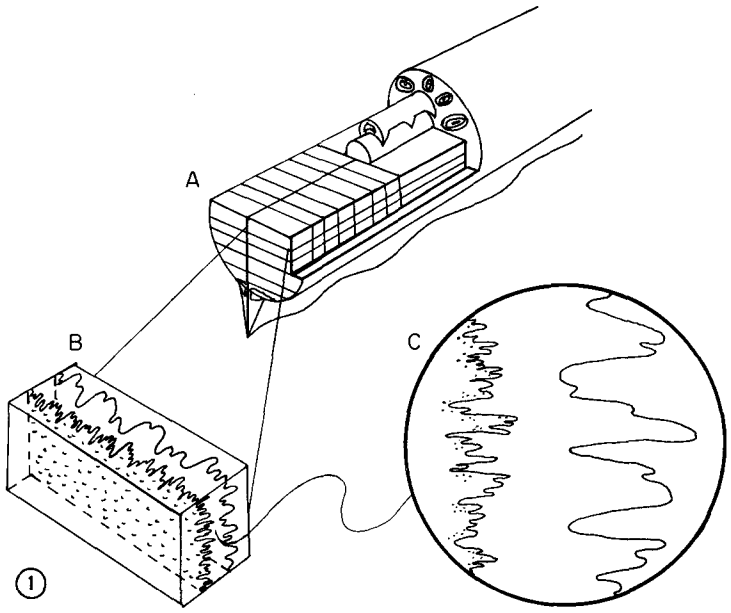


FIGURE 1 Diagrammatic sketch of *Electrophorus* cut away dorsally to reveal electric organ (*A*) in situ. *B* indicates posterolateral view of one electroplaque within its mucoid-filled compartment, and *C* shows edge of plaque with innervated (left) and noninnervated (right) sides.

adipaldehyde, or formalin and refixation in osmium tetroxide. Each multinucleated electroplaque is enclosed within a dense collagenous rectangular compartment whose long axis runs mediolaterally. Across the caudal third of each compartment, the electroplaque extends as a plate or band, measuring 1 to 3 mm dorsoventrally and 0.1 to 0.3 mm anteroposteriorly, with large anterior papillae extending from the noninnervated surface. Finer, shorter, but more numerous papillae extend from the posterior, innervated surface (Fig. 1 a). The narrow extracellular space caudal to the innervated side and the anterior $\frac{2}{3}$ of the plaque compartment rostral to the noninnervated surface are filled with a mucoid gel (26).

The surface of both the innervated and noninnervated portions of the electroplaque are typically found to possess multiple tubulovesicular channels or "caveolae" (29) extending into the most peripheral 100- to 1000-mμ of plaque cytoplasm. These channels are more extensively developed on the papillary projections than at the base of the electroplaque. The channels of the innervated surface (Figs. 2 and 3) are shorter, broader, and more tortuous than those of the noninnervated surface (Figs. 9 and 10) which are more numerous and regular. All channels are lined internally with a fine filamentous coating, seen particularly well after staining with uranyl acetate (Fig. 15). Typical Golgi apparatus, nuclei with regular envelopes, irregularly shaped empty saccules and small empty vesicles often situated in relation to the saccules were frequently noted within the cytoplasm of the papillae (Figs. 2, 4, and 10). The mitochondria, which are long and narrow with transversely arrayed cristae and internal dense granules, were most frequently found in a perinuclear position, although they were also accumulated near the surface of the innervated side (Figs. 2, 4). The remainder of the plaque cytoplasm contains fine short filaments having no apparent orientation (Figs. 2, 4, and 25). There is uninterrupted cytoplasmic homogeneity from the innervated to the noninnervated side.

Junctions between nerve terminals and plaque are more common on the distal portions of the innervated papillae than at their bases. Myelinated axons approach the innervated surface enclosed within a perineural sheath composed of extended "glial" membranes and cytoplasm (Fig. 2); the inner sheath (periaxonal) space contains filamentous mucoid material as well as collagen fibrils. As the nerve fibers come into immediate apposition to the electroplaque, the outer "glial" sheath and the myelin are replaced by a thin layer of "glial" cytoplasm, usually covering that surface

of the nerve ending not opposed to the plaque (Figs. 4, 16). One nerve fiber may have several adjacent zones of apposition; each zone shows accumulation of synaptic vesicles and junctional clefts on the order of 150 to 300 A (Figs. 2 and 4). Well fixed nerve endings are quite closely packed with electron-lucent synaptic vesicles which are 4 to 600 A in diameter (Figs. 11 to 14).

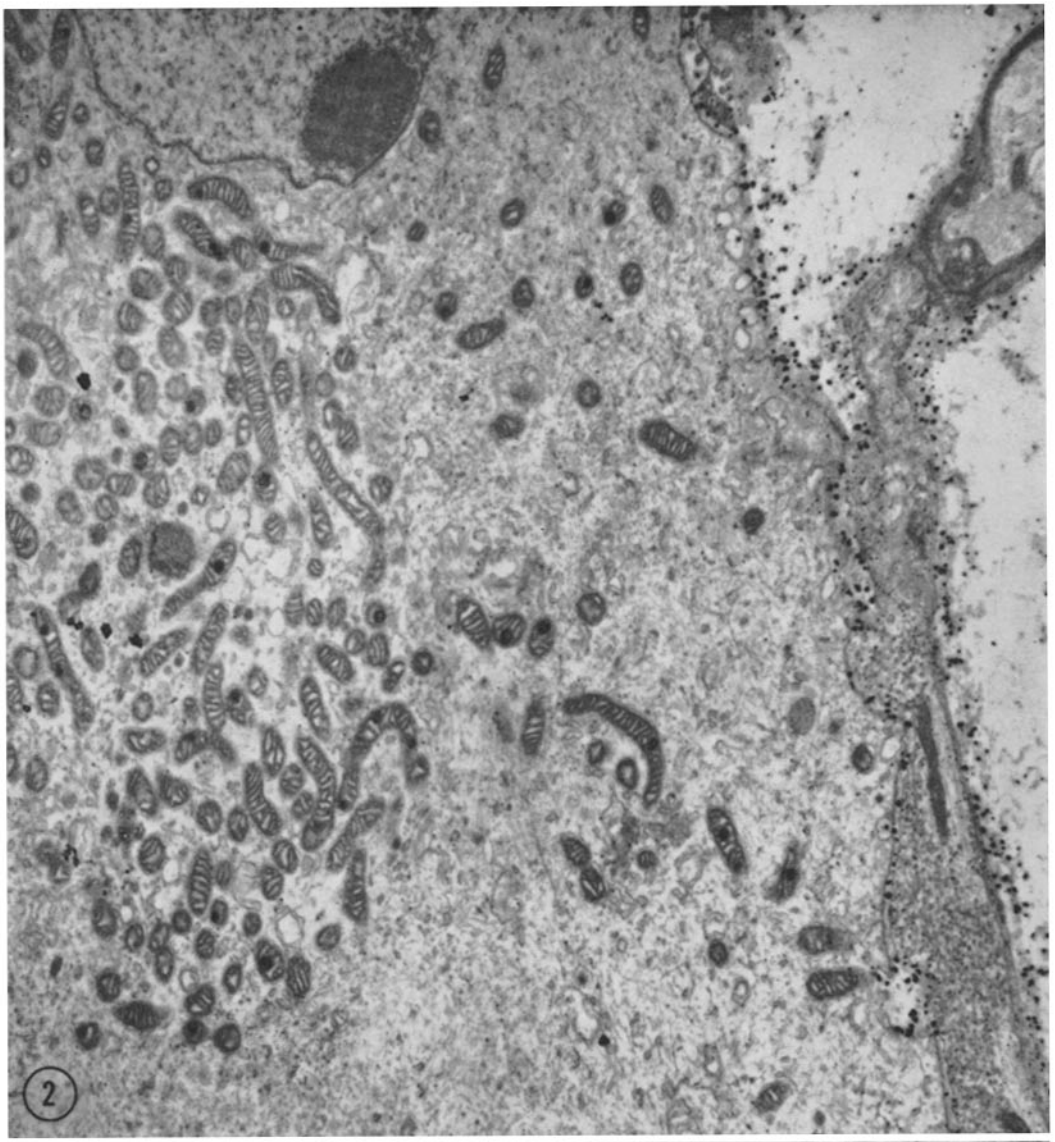
Cross-sections through preterminal axoplasm reveal both neurotubules and neurofilaments, in addition to mitochondria and occasional synaptic vesicles with electron-lucent contents (Figs. 3, 21). No subsynaptic specialization was observed, but a fine dense line was frequently found through the synaptic cleft at the interfaces of the mucoid coats of pre- and postjunctional elements (Fig. 22). Concavities of the postjunctional plaque surface at areas subjacent to nerve endings were most commonly observed at the tips of innervated papillae (Fig. 4), while at junctional sites on the base and more proximal portions of the papillae the electroplaque surface was not adapted to the presence of the nerve ending (Fig. 2). At all junctional areas, the opening of tubulovesicular cytoplasmic channels into a junctional cleft was a frequent finding (Figs. 4, 5, and 16).

Two types of supportive cells were observed. One type, which contributes to the sheath surrounding the myelinated axons, has a large irregularly shaped nucleus exhibiting peripherally clumped chromatin (Fig. 19). The dense cytoplasm of this cell contains both smooth and rough endoplasmic reticulum, the former often appearing to be dilated. The second type of supportive cell was seen on both surfaces of the plaque; its nucleus is smaller with less dense accumulations of chromatin, while the cytoplasm exhibits dense mitochondria with dense matrices, partially filled cytoplasmic vacuoles, and very little endoplasmic reticulum. Its most prominent feature is the long and narrow cellular processes extending toward the innervated surface, and often lying in intimate contact with this surface (Figs. 4, 18).

With the use of uranyl acetate staining of thin sections, a filamentous, collagen-laced, mucoid extracellular space substance (M.E.S.S.) was revealed within the extracellular space of both the innervated and noninnervated surfaces of the electroplaque (Figs. 3, 16, 21). In the optimally preserved specimens, this material (M.E.S.S.) was found within the tubular extensions of the surface (Figs. 3, 21) and in a layer, several hundred millimicra in thickness, adherent to many of the cellular elements ("glia," nerve endings, blood vessels) normally found in the furrows between the papillary projections of both surfaces of the electroplaque. The extracellular substance was particularly electron-opaque when incubation with a heavy metal salt at pH 6 or less preceded the refixation in osmium tetroxide and subsequent staining of sections with uranyl acetate (Fig. 16). Staining 1-μ-thick Maraglas sections of the tissue with 0.5% toluidine blue demonstrated metachromatic reactivity of this substance. These latter results, to-

FIGURE 2 Low power view of innervated surface and contents of plaque, fixed in glutaraldehyde, reacted with Pb-TAA method, pH 7, for 15 min and refixed in osmium tetroxide. At right, terminal portion of nerve containing many vesicles makes broad contact with surface of plaque. Filaments and mitochondria also occur in axoplasm. At upper right, termination of myelin sheath is shown. Numerous large tubulovesicles occur in cytoplasm of plaque, especially in relation to surface membrane. Cytoplasm of plaque contains filaments, smooth membrane-bounded profiles, granules, numerous mitochondria containing dense granules, and nucleus (upper left). Final product of enzyme reaction is localized to entire surface of plaque including junctional cleft, as well as to peripheral tubulovesicles and surface of unmyelinated axon. × 24,000.

FIGURE 3 Portion of innervated surface of plaque prepared as above and incubated in Pb-TAA medium for 1 min at pH 5 after preincubation in Pb-containing solution without substrate for 30 min. This section is heavily stained (30 min) with uranyl acetate to show mucoid extracellular space substance (M.E.S.S.) (upper) abutting on surface of plaque and filling surface tubulovesicles. Final reaction product is distributed in dense mucoid substances in relation to surface of the plaque. Portions of axon (arrows) occur in M.E.S.S. × 32,000.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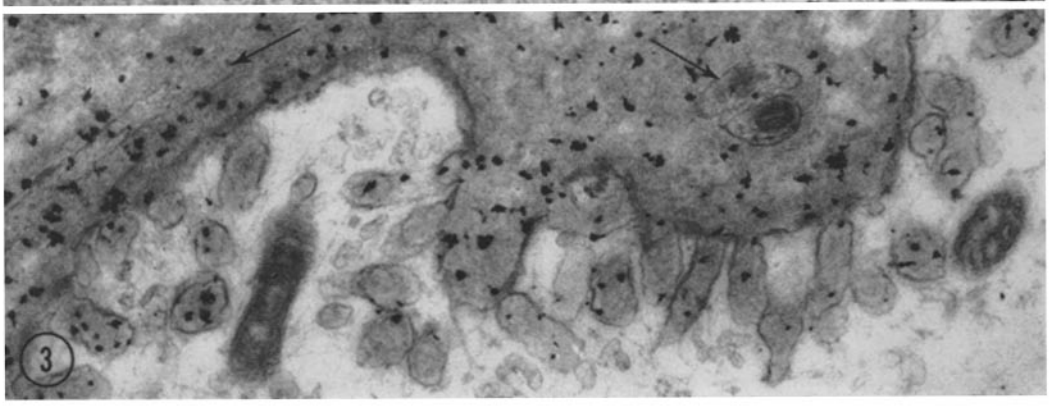
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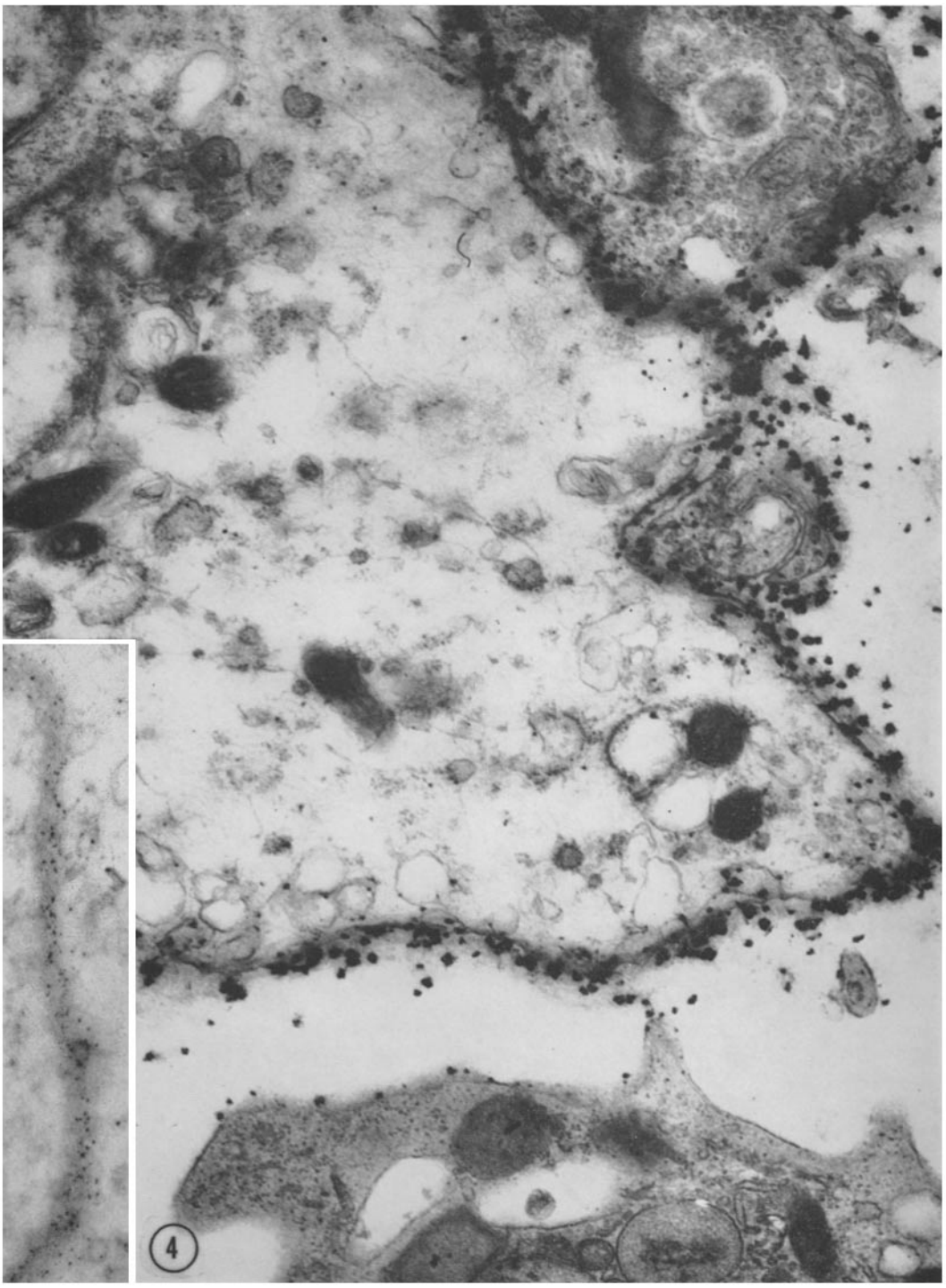


FIGURE 4 Surface of glutaraldehyde-fixed electroplaque incubated in the Pb-TAA media at pH 5 for 1 min prior to fixation in osmium tetroxide. Final products of reaction occur in relation to innervated surface of plaque and its peripheral tubulovesicles, and on surface of two axons and within their synaptic vesicles. Cytoplasmic contents including nuclear envelope are unreactive. Insert, however, shows activity in relation to nuclear envelope that was obtained by incubation of similar material at pH 7. × 34,000.

gether with the previous descriptions of electroplaque histology (27–30), would seem to confirm that the extracellular "space" is filled with a gelatinous acid mucopolysaccharide.

Tissue Preparation

Best preservation was obtained in those glutaraldehyde-fixed blocks which were removed from tissue slabs less than 2 electroplaque columns thick. Thicker blocks tended to show much less adequate uniformity in preservation of fine structural detail, particularly after several weeks' storage in buffer. Formalin-fixed tissue and tissue blocks frozen on dry ice with subsequent refixation in glutaraldehyde exhibited generally less acceptable retention of intracellular morphology (Figs. 2 and 24). In those electroplaques which were dissected free of external connective tissue elements prior to the immersion fixation, broken membranes, dilated mitochondria, and endoplasmic reticulum, and swollen nerve endings with a sparse content of synaptic vesicles were frequently observable. Preservation of enzymatic activity was surveyed light microscopically by comparing the intensity of reaction product deposited in 40-μ frozen sections, using either of the acetylthiocholine methods (18, 20) or the Pb-TAA method performed at pH's 6.5 to 7.0. Incubations were performed in an ice bath and observed with a 60-power dissecting microscope. Direct coloration of tissue fixed with either glutaraldehyde or hydroxyadipaldehyde was detectable by the deposition of the reddish brown copper ferrocyanide (9) or black lead sulfide after almost identical intervals of 12 to 14 min, on repeated assays. The deposition of copper sulfide with the Cu-acetylthiocholine method corresponded temporally. Unfixed tissue and tissue blocks briefly (2 to 3 min) immersed in cold 0.25% buffered osmium tetroxide before incubation were not noticeably more reactive with the copper-thiocholine method, when compared at 10 min of incubation, than glutaraldehyde- or hydroxyadipaldehyde-fixed tissue. The almost complete disorganization of subcellular detail made the unfixed and the briefly osmium tetroxide–treated tissues unsuitable for electron microscope localization of the enzyme activity. Tissue fixed in formalin also showed demonstrable enzyme activity but of less intensity for the same duration of incubation than tissues fixed in either glutaraldehyde or hydroxyadipaldehyde. No difficulty with excess background reaction was noted when the copper ferrocyanide method was used on eel tissue fixed in either of the dialdehydes (Figs. 24, 25). Similarly, spurious background reaction was not encountered in any of the fixed tissues with the TAA (Figs. 2, 4) or thiocholine methods (Figs. 26, 27) when certain precautions described below were observed.

Regardless of the aldehyde fixative used, enzyme activity was most easily demonstrable during the 2- to 24-day period following excision and fixation of the electroplaques (*See* Figs. 4 and 5). With increased storage, more prolonged incubations were required to yield cytochemical reactions equivalent to those of briefly washed tissue. However, enzymatic activity at both light and electron microscope levels was still demonstrable after 5 months of postfixation storage (24) in cold buffer. This result was not considered unusual, since some enzyme activities were found to be cytochemically demonstrable after 1 yr of buffer storage (unpublished results).

Deposition of Reaction Product

A small electron-opaque final reaction product was obtained without ammonium sulfide conversion of copper-thiocholine (Figs. 26, 27). As in the case of the copper ferrocyanide deposits, which were several times larger in size (Figs. 24, 25), the amount of reactivity demonstrable after set periods of incubation was less than with TAA (compare Fig. 4 with Figs. 24 to 27). However, because of the repeated observation, with all methods, of reaction product localized to the M.E.S.S. abutting the plasma membrane of the innervated side (Figs. 4, 8, 23, 24, 26), adjustments of the fixation (concentration and duration) and of the concentration of ingredients in the incubation mixture were undertaken to reduce possible diffusion of the final product. Since the TAA method is a direct one-step reaction from substrate to captured precipitate with relatively less chance of diffusion, incubation adjustments were attempted with the Pb-TAA reaction.

Both decreasing the substrate concentration by half and/or doubling the Pb^{++} concentration decreased the total amount of final reaction product detectable after a 10- or 20-min incubation. These modifications did not prevent the appearance of some final product in the M.E.S.S. occurring in close proximity to the surface of the innervated side of electroplaques. Incubations of 1 min or less, even when the tissue slices had been chilled prior to the reaction, also did not prevent

this extracellular deposition (Figs. 4 and 5). Furthermore, similar localizations of end product were still obtained when either silver (Fig. 19) or gold (Fig. 23) was substituted for the lead, or when the capture salts were preincubated in buffer with the tissue slices for as long as 90 min to assure adequate penetration of the metal reagent prior to the addition of substrate (Figs. 4 to 6, 10).

In order to obtain more information as to whether the enzyme activity demonstrated within the M.E.S.S., especially that close to the innervated surface, is artifactitious, the incubation media were reexamined to eliminate any other sources of false precipitation. Substitution of Tris-maleate buffer for cacodylate buffer or substitution of nitric acid for hydrochloric acid in the preparation of the cacodylate buffer was of less obvious benefit than the use of relatively pure TAA and the titration of the TAA to the reaction pH with carbonate-free sodium hydroxide. This step markedly reduced the occurrence of the occasional large deposits which were unrelated to structural elements.

These conditions having been met, comparison of electron micrographs substantiated the light microscope findings on the effects of the various fixatives upon the reaction intensity. After similar incubations in identical media, tissues of the same storage longevity exhibited smaller and fewer deposits of reaction precipitate in formalin-fixed slices than in slices fixed in either hydroxyadipaldehyde or glutaraldehyde. Similarly, among glutaraldehyde-fixed slices of the same storage longevity, which were reacted with TAA mixtures at pH 5.0, the resulting precipitate was more rapidly accumulated and less widely distributed (Fig. 4) than with any of the other incubation media. This pH was then utilized for most of the cytochemical tests. In regard to the size of reaction precipitate obtained, a 2- to 3-fold variation was observed in the particle sizes at any one site of localization. This was to some extent related to the fixative used (Figs. 4, 24, and 25), the precipitate formed (Figs. 4, 19, 23, 24, and 26) and, as described below, the subcellular source of the enzyme activity demonstrated.

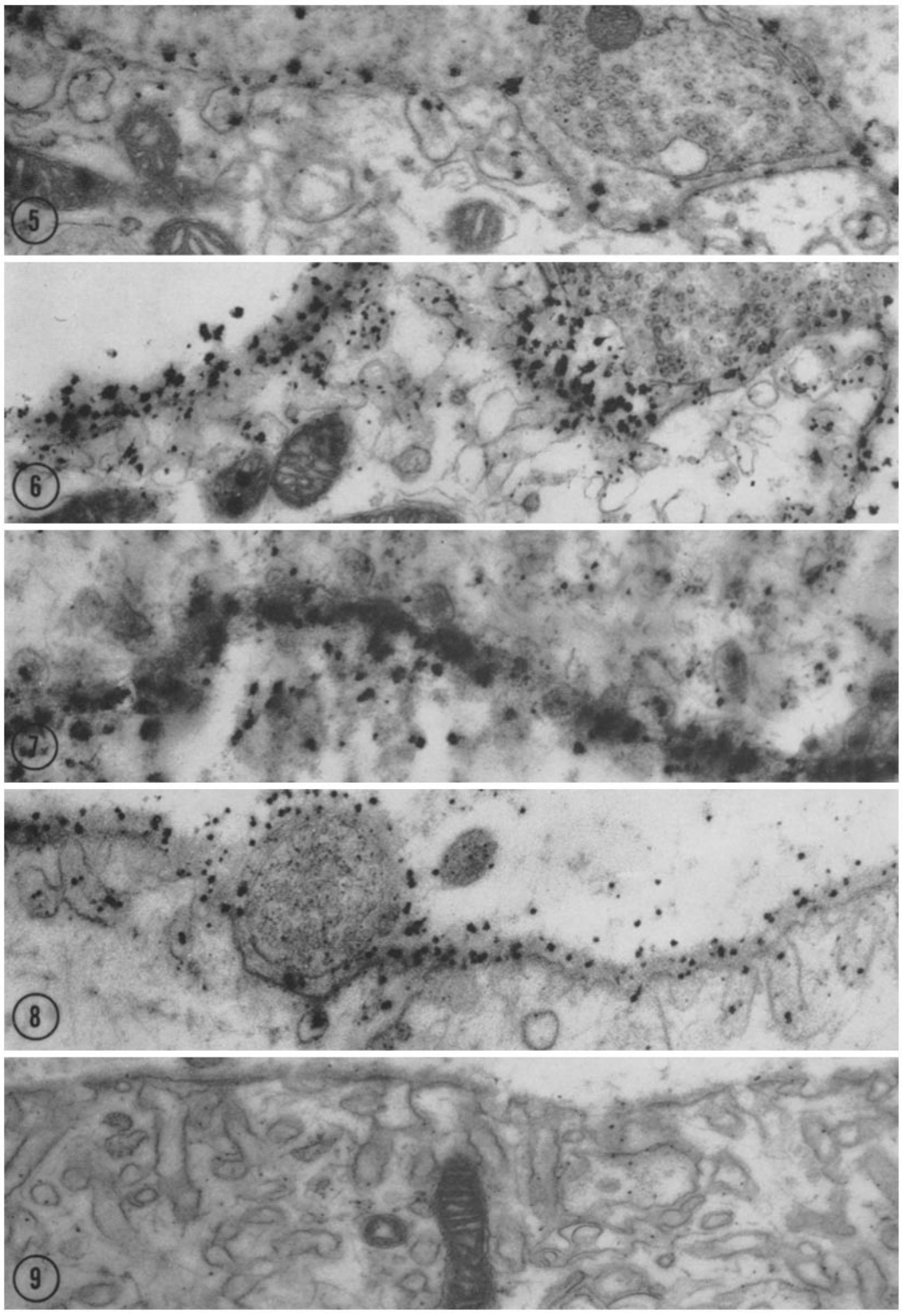
Fine Structural Localization

The overwhelming preponderance of reaction product, with each of the methods as finally used, was deposited on or near the outer surface of the plasma membranes of the innervated side of the electroplaque and within the adjacent 100-mμ layer of M.E.S.S. (Figs. 2, 4, 5 to 8, 18, 19, 23, 24 and 26). With Pb-TAA, final products of similar size and of only slightly lesser number were found within the mucoid-filled tubulovesicles at the periphery of the innervated surface (Figs. 3, 6, 8). Deposits of final product of the same size, number, and distribution were also found surrounding the terminal nonmyelinated portions of the nerve endings (Fig. 4). Fewer deposits occurred on the axonal membrane, on its thin "glial" covering, and in the layer of M.E.S.S. which surrounded the nerve endings at their junctions with the electroplaque (Figs. 8 and 12). It should be noted that the deposition of reaction product was not limited to junctional sites but was confluent over the whole of the innervated surface of the electroplaque (Figs. 2, 4, 5 and 8). Merging sites of innervated surface localization could frequently be seen: in relation to the outer membrane, the membranes of the tubulovesicles (surface extensions) and to the mucoid material adjacent to the membranes (Figs. 4, 6, and 8). Moreover, it should be emphasized that the distribution of final product after 1 min of incubation was the same as after 30 or 60 min (Figs. 5 to 7).

Reaction with the Pb-TAA mixture also resulted

FIGURES 5 to 8 Innervated surfaces of glutaraldehyde-fixed electroplaques incubated in Pb-TAA media. All show similar localization of final product confined to surface of plaque, tubulovesicles, and synaptic vesicles of terminal axon. Fig. 5 (× 29,000) is taken from specimen incubated for 1 min at pH 5; Fig. 6 (× 31,000) is from a 15-min incubation, and Fig. 7 (× 33,000) is from a 60-min incubation. Fig. 8 (× 28,000) shows tissue which was incubated at pH 7 for 15 min.

FIGURE 9 Noninnervated surface of electroplaque fixed in glutaraldehyde and incubated for 20 min in Pb-TAA media at pH 7. Note numerous and tortuous channels continuous with the surface. Little activity is associated with the surface or the content of the channels. × 25,000.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in several sites of localization at which the lead sulfide particles formed were extremely small (50 A or less) and also fewer in number. These sites, which often required prolonged incubations (more than 20 min) for their demonstration, were observed mainly with reactions performed at pH 6.8–7.0, and include the following sites: the small and large nonmucoid-filled vacuoles and vesicles of the innervated half of the plaque, the nuclear envelope (Fig. 4, insert), neurofilaments within axon terminals and in relation to "glial" processes and at nodes of Ranvier. Details of reactive sites in axons will be covered in a subsequent communication. Additional sites of the small reaction product with this method were confined to the synaptic vesicles (Figs. 5, 6, 11 and 12) and the longer and narrower tubulovesicles of the noninnervated portion of the electroplaque (Fig. 20). Capillaries, erythrocytes, and the collagenous outer walls of the electroplaques showed no enzyme reactivity.

Substitution of a silver salt for lead produced identical localization of the large reaction product at the innervated surface (Figs. 18 and 19), but no activity was demonstrable within the "glia" (Fig. 18), the axoplasmic tubules or nodes, and nuclear envelopes of the electroplaque. The amount of reaction product which was deposited on the synaptic vesicles was much less than that with the Pb-TAA method and was confined to the outer surface of the membranes limiting the vesicles, rather than within the vesicle contents as observed with the Pb-TAA. When the Ag-TAA reaction was performed at the more alkaline end of the pH range (Fig. 22), all the scant precipitate formed on the innervated surface was of the fine particle type, and none was detectable on the synaptic vesicles or on the noninnervated surface membranes.

The use of gold sodium thiosulfate as recently suggested for the capture reagent in the TAA reaction (11) was also found to produce a very small yet distinctly particulate reaction product in those sites which exhibited the large reaction particle with both Pb and Ag media. Thus, the reaction localization with Au-TAA at pH 5.0 resembled that with Ag-TAA at pH 6.8, with final products limited to the innervated surface and the adjacent M.E.S.S., and to mucoid-filled portions of the adjoining tubulovesicles (Fig. 23). There was no final product within neural elements or at the noninnervated portion of the plaque.

Both of the acetylthiocholine methods (18, 20) reproduced the results obtained with the TAA method, in that those sites of large particle deposition were identical (Figs. 24 to 27). Reaction product was also found, with these media, at nodes of Ranvier, within the layer of mucoid material surrounding myelinated axons, around terminal portions of nerve endings, and within the synaptic vesicles (Figs. 25, 27). This last reaction (i.e. on the synaptic vesicles) was never as obvious as that seen with Pb-TAA, however. Examination of tissue reacted with butyrylthiocholine revealed no deposition of reaction product in any of the above sites, but only in relation to the empty tubulovesicles of the innervated side of the electroplaques.

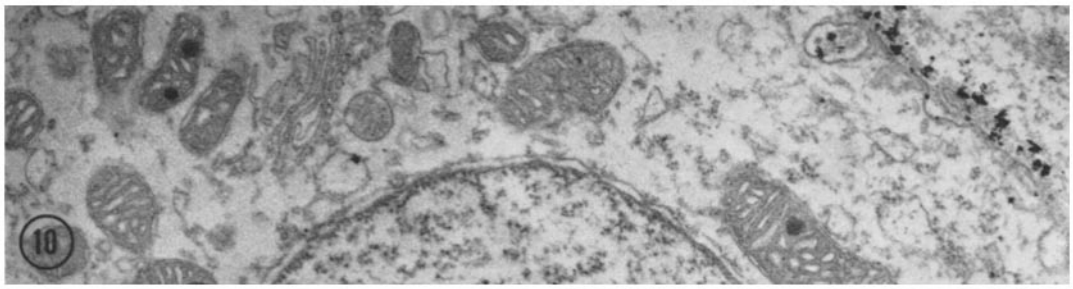
Control Experiments

Particular attention was paid to the tissue distribution of the capture reagent in the absence of substrate. When buffer rinses of at least 15-min

FIGURE 10 Portion of electroplaque reacted (Pb-TAA) at pH 5 for 1 min, showing activity in relation to surface (upper right) and details of the plaque contents including nucleus, mitochondria, Golgi apparatus, dilated vesicles, and small granules and filaments. × 25,000.

FIGURES 11 and 12 Reactions obtained in synaptic vesicles of terminal axon. Fig. 11 (× 45,000) shows the small particulate deposit in vesicles after incubation in Pb-TAA for 15 min at pH 7. Fig. 12 (× 47,000) shows similar deposits, obtained by incubation with Pb-TAA for 1 min at pH 5, occurring mainly in relation to the inner surface of the vesicle membrane. Both figures show large deposits in relation to surface of the ending and in junctional clefts.

FIGURES 13 and 14 Control preparations incubated in buffered solution of $Pb(NO_3)_2$ for 30 min. The preparation in Fig. 13 (× 40,000) was incubated at pH 7 and shows many dense deposits within the vesicles, whereas the preparation in Fig. 14 (× 40,000) was incubated at pH 5 and shows fewer and smaller deposits within the vesicles.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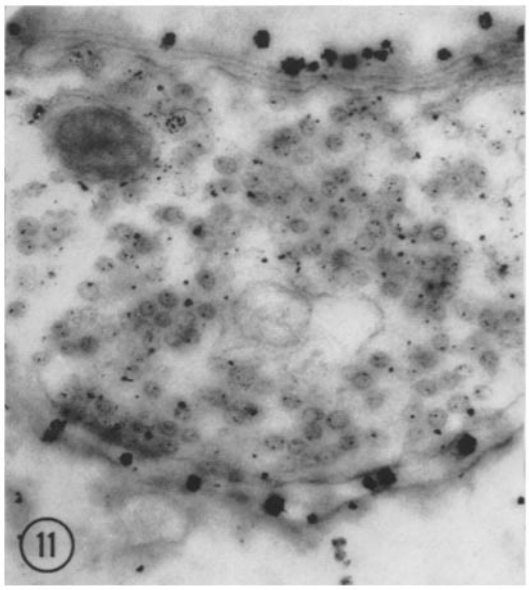
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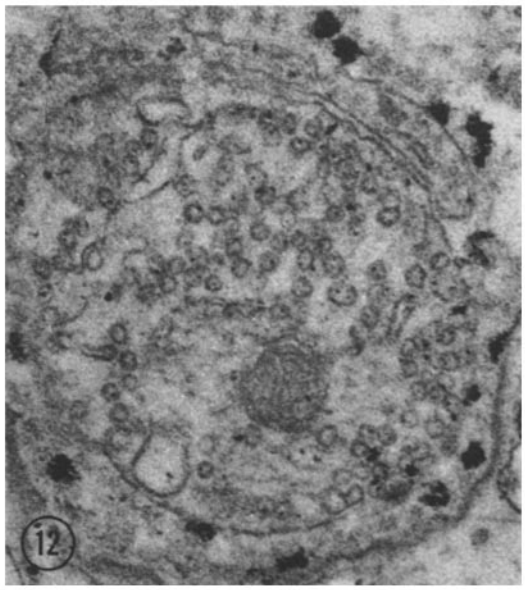
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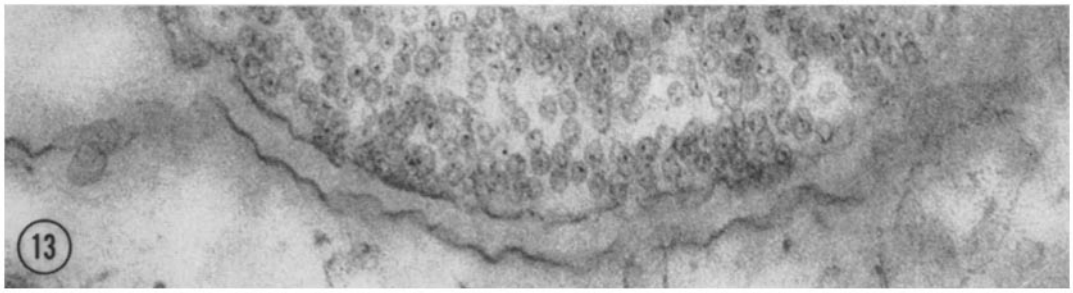
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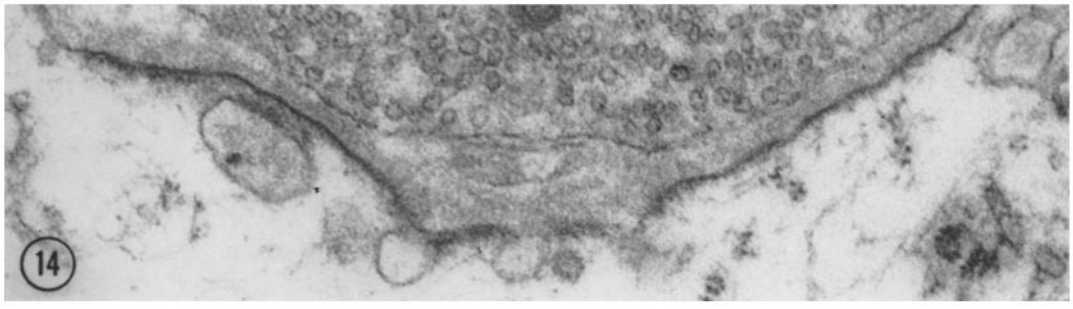
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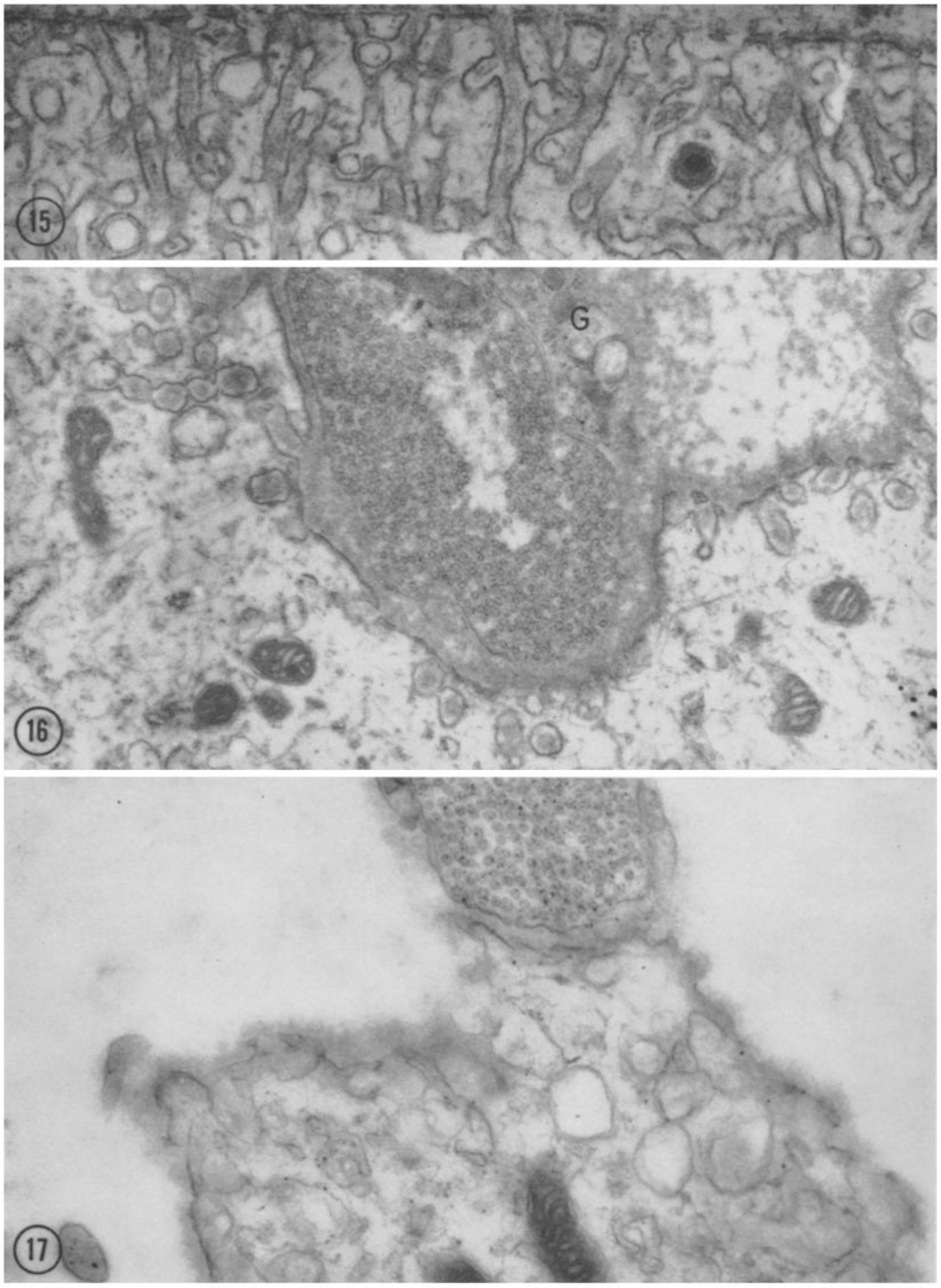
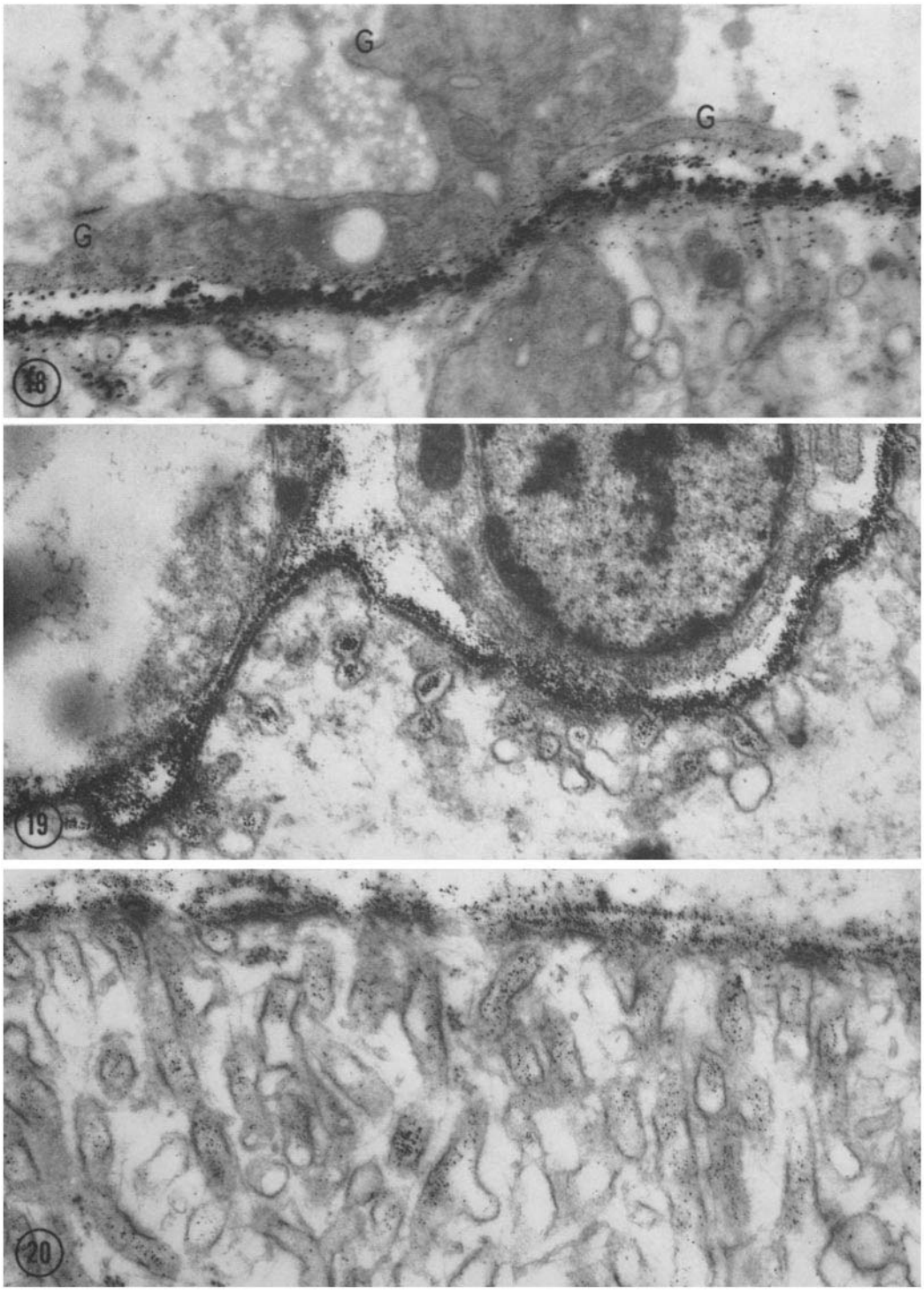


FIGURE 15 Control noninnervated side of plaque incubated in Pb-TAA containing physostigmine (10^{-4} M) at pH 5 for 30 min. Little or no activity present. Stained with uranyl acetate. × 29,000.

FIGURE 16 Control preparation of innervated surface of plaque incubated in Pb-TAA containing physostigmine (10^{-4} M) at pH 5 for 30 min. No activity occurs in relation to surface or tubulovesicles of the plaque. Synaptic vesicles, however, still show Pb deposits. Note unreactive glial process (*G*) covering one surface of nerve ending. Stained with uranyl acetate. × 25,000.

FIGURE 17 Control preparation of innervated surface of plaque incubated in Pb-TAA containing edrophonium (10^{-4} M) at pH 7 for 30 min. Deposits of final product are confined to vesicles of nerve ending and tubulovesicles of plaques. × 29,000.



FIGURES 18 to 20 Preparations incubated 30 min in Ag-TAA media at pH 5; sections stained with uranyl acetate. Fig. 18 (× 28,000) shows reaction confined to surface and tubulovesicles of innervated side of plaque. "Glial" processes (*G*) are unreactive. Fig. 19 (× 29,000) is taken from sections incubated in media containing a less than effective amount of physostigmine (10^{-6} M). Note that, though activity occurs in the tubulovesicles and in relation to the surface of the plaque, the plasma membrane of the plaque is clearly unreacted and is separated by a clear zone, approximately 200 A, from the small particles of final product in the M.E.S.S. Surfaces of supportive cell are unreactive. Fig. 20 (× 33,000), a 60-min incubation, shows final product in relation to surface and channels on noninnervated surface.

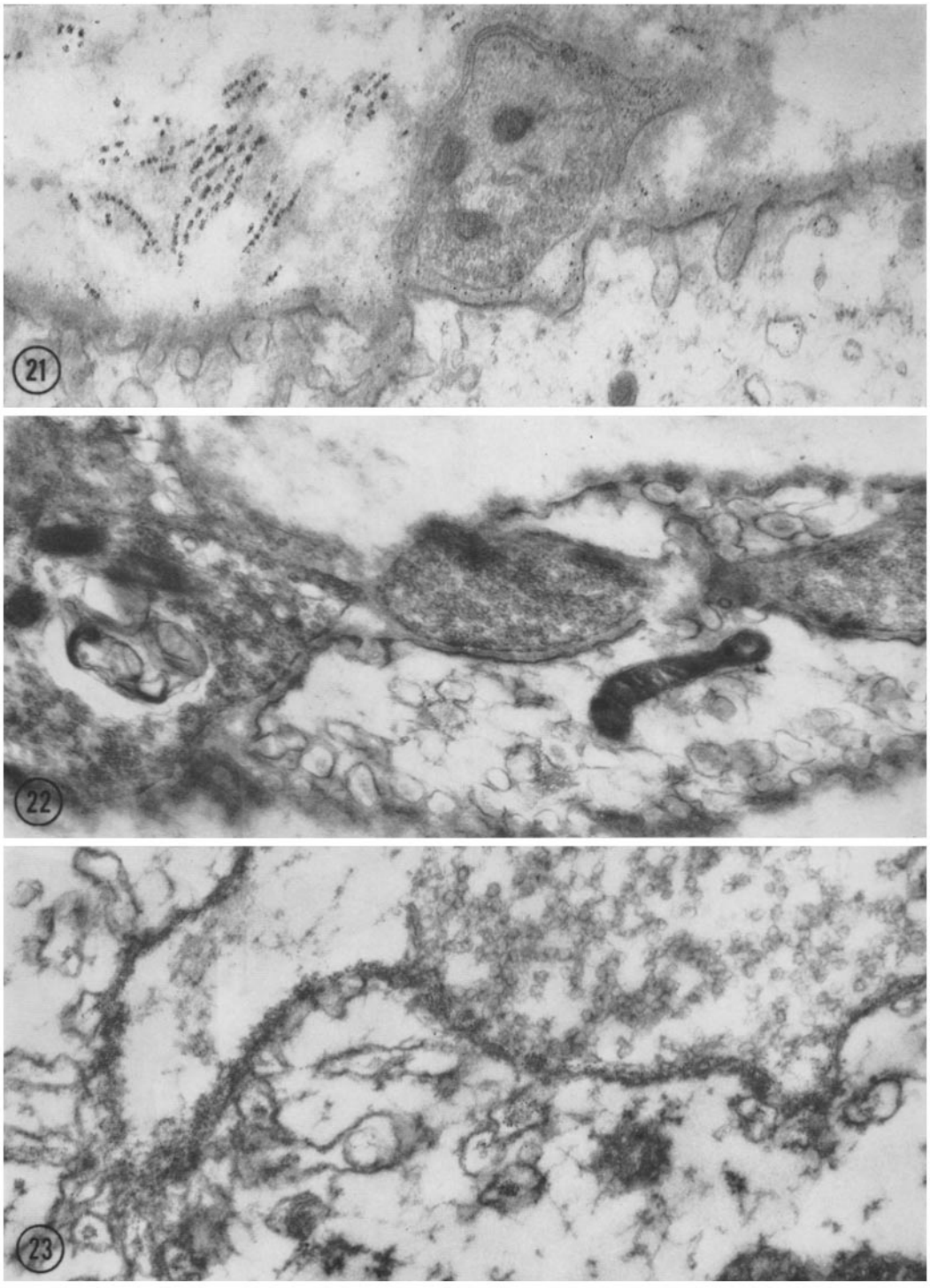
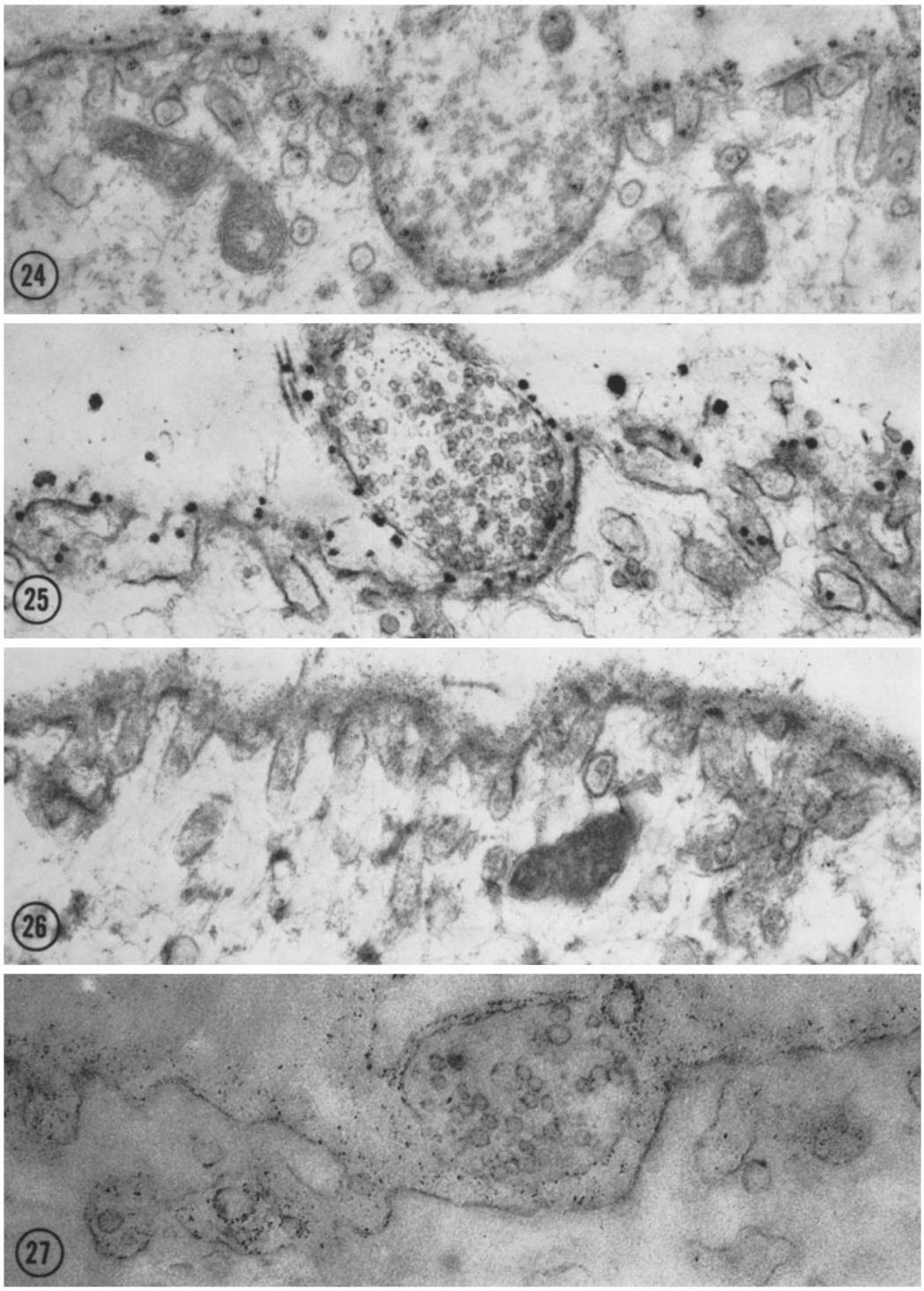


FIGURE 21 Section of electroplaque incubated in Ag-TAA for 60 min at pH 7. Little final product occurs at surface of plaque or in tubulovesicles. Synaptic vesicles show very little or no reaction. Collagen fibers are nonspecifically and periodically stained. × 32,000.

FIGURE 22 Control preparation of surface of electroplaque incubated in buffered solution (pH 7) of $AgNO_3$ for 30 min. No binding of Ag has occurred. × 42,000.

FIGURE 23 Section of electroplaque incubated in Au-TAA at pH 5 for 30 min. Reaction product, seen as small particles, is confined to innervated surface of plaque as well as to tubulovesicles. No activity occurs in synaptic vesicles. × 42,000.



FIGURES 24 to 27 Illustrates the use of acetylthiocholine as substrate. Fig. 24 (× 28,000) is from formalin-fixed plaque, and Fig. 25 (× 28,000) is from hydroxyadipaldehyde-fixed plaque; both plaques were incubated with the Karnovsky modification, pH 6.8, for 15 min. Activity occurs in relation to innervated surface of plaque and within tubulovesicles. Fig. 25 shows slight activity occurring in synaptic vesicles of nerve ending, and also prominently displays the filaments of the plaque.

Figs. 26 (× 35,000) and 27 (× 28,000) are from specimens fixed in glutaraldehyde and hydroxyadipaldehyde, respectively; both specimens were incubated in the Gerebtzoff modification (pH 6.8; 15 min) in which the final product is Cu-thiocholine. Note that particles of final product are exceedingly small and that, as before, the localization is confined to the innervated surface, tubulovesicles, and synaptic vesicles. Stained with uranyl acetate.

duration were used after incubation, the only significant sites of lead and copper binding detectable at pH 6.5–7.2 were within the contents of synaptic vesicles (Fig. 13). However, the less densely deposited product which occurred in these controls could only be distinguished subjectively from the reaction obtained in synaptic vesicles with regular incubation. Although not immediately obvious, the control deposits usually showed decreased electron opacity and less clearly definable shape (*See* Figs. 11, 13). Tissue incubated in $AgNO_3$ at pH's above 6.5 showed some argentophilia of collagen fibers (Fig. 22). When tissue was incubated in solutions of silver, gold, or lead salts at the more acid pH's, very little or no binding of metal in any structures was detectable (Figs. 14, 22). Examination of tissues incubated in a buffer solution containing lead salts without substrate for 15 min followed by fixation in osmium tetroxide without any intervening wash step indicated that, although the tissue was liberally and irregularly spotted by electron-opaque particles (presumably lead ligands) bound to the cytoplasmic elements of supportive cells, plaque, and neurons, the synaptic vesicles were free of precipitate at pH's below 6.0.

When cholinesterase inhibitors were added to the incubation media, all sites of deposition of large particle final product were eliminated regardless of substrate or capture reagent used in the methods (Figs. 15 to 17). Complete inhibition of these sites was obtained with 10^{-5} M physostigmine (Fig. 16), edrophonium (Fig. 7), and prostigmine. Diisopropylfluorophosphate (10^{-6} M) added to the media produced almost complete inhibition at these sites. However, no concentration (up to 10^{-3} M) of any of the inhibitors, even with prolonged preincubations, was sufficient to prevent the Pb-TAA reaction on synaptic vesicles at either end of the pH spectrum (Figs. 15 and 16). Equally resistant was the reaction deposited from the Pb-TAA media on the nuclear envelopes (Fig. 4, insert) and "empty" tubulovesicles (Fig. 17) at pH 6.8-7.0. The smaller-sized reaction deposits, obtained with both the Ag-TAA and Pb-TAA media, on the noninnervated side were also inhibitable with 10^{-4} M physostigmine (Fig. 15) and 10^{-5} M diisopropylfluorophosphate, as was that in the M.E.S.S. surrounding the nerve terminals and myelinated fibers. When less than a completely inhibiting quantity of physostigmine (10^{-6} M) was used, the deposits of final product in inhibited slices were smaller than those in uninhibited slices incubated simultaneously, but the distribution of reaction in the M.E.S.S. was the same. However, the final product deposited at the plasma membrane of the innervated side of the plaque was now far less apparent (Fig. 19).

DISCUSSION

A rigorous interpretation of the cytochemical results would be that the electron-opaque precipitates accurately represent the sites of deposition of those insoluble salts produced by reaction of the capture reagent and the product of the hydrolysis of the substrate by the tissue enzyme. The equation of this binding site with the site of the enzymatic activity assumes that the final product deposits at or near the site of substrate hydrolysis and is not displaced from that site by the subsequent steps required for visualization of the tissue with the electron microscope. In the present experiments, the cytochemical demonstration of acetylcholinesterase (AChE) activity resulted in the deposition of electron-opaque final products of three different techniques at identical fine structural sites: along the innervated surface of the electroplaque and around the nerve endings abutting it. Since these techniques vary in the chemical reactions required to produce the final product, the probability of similar displacement of the reaction product in each case would seem low.

However, when the final product localizations are inspected closely, differences are apparent. In regard to both the size and number of the deposits of final product produced within a given incubation period, the acetylthiocholine ferrocyanide method yielded the largest and fewest electron-opaque deposits. Other methodological variations in results are more worthy of note. Two types of final product were revealed with the low pH Ag-TAA media and both Pb-TAA media. Coarse irregular particles were observed in relation to to the membranes of the innervated electroplaque surface, the tubulovesicles contiguous with the innervated surface, the abutting nerve endings, and the mucoid extracellular space substance (M.E.S.S.) adherent to these structures. A finer final product was observed within the tubulovesicles of the noninnervated side, on synaptic vesicles within the nerve endings, at multiple cytoplasmic sites within the plaque, and on the surfaces of supportive cells nearest the innervated surface. With the exception of the synaptic vesicle

reaction, discussed separately below, none of the other fine particle sites could be demonstrated with either of the acetylthiocholine methods, which also showed much less activity at the large particle sites.

Before further considering the subtle divergences among the results obtained by various techniques, several features of the TAA reaction should be examined. As indicated in the above results, the size and amount of the coarse TAA final product were related to the substrate concentration, the media pH, the capture metal, and, to some extent, to the longevity of postfixation storage of the tissue. This longevity may be correlated with the effects of incomplete inhibition of the enzyme with 10^{-6} M physostigmine, which also reduced the particle size at sites of activity. For such reasons, sharper localization of final products may be associated with decreased enzymatic activity. This is also borne out by the fine particle deposits on the noninnervated side. Although these sites were equally as sensitive to the inhibitors of AChE as the innervated surface reaction, they required more prolonged incubation for cytochemical demonstration. Other sites of intracytoplasmic fine particle localization (e.g. nuclear envelope) were not inhibitable by the highest concentrations of AChE inhibitors. This suggests the possibility that the activities of enzymes other than AChE are demonstrated at these sites with this media. Finally, while some TAA final product could occasionally be seen on the surface of supportive cells abutting on the innervated surface of the plaque, it was not seen at the opposite surface of the same cells. This result suggests that, regardless of precautions taken, some diffusion of reaction product occurred at the sites of high activity.

A comparison of the results of the thiocholine incubations with those of the TAA method suggests that some small amount of diffusion of reaction product occurred. The smaller total amount of reaction precipitates obtained with these thiocholine methods and the inability of these methods to demonstrate the enzyme of the noninnervated side may be related to the decreased quantity of substrate but may further indicate their decreased efficiency in the demonstration of enzyme activity. However, a relatively poorer permeability of the substrate into the tissue may also be of importance. On the other hand, the thiocholine methods do manifest higher enzyme selectivity, since only sites which are sensitive to inhibitors of AChE were demonstrated.

The question must now arise as to whether the final reaction products within the M.E.S.S. are, in fact, sites of enzymatic activity or are possibly indicative of diffusion. All methods exhibited final products in the M.E.S.S. close to the innervated surface of the plaque. No modification of the TAA procedure was capable of preventing the simultaneous appearance of the sites within the M.E.S.S. sensitive to AChE inhibitors. However, from the preceding discussion, these experimental observations may not be entirely satisfactory arguments against the possibility of diffusion. The alternatives are that the enzyme activity is on the plasma membrane of the innervated side, in the M.E.S.S., or at both sites. Consequently, either the plasma membrane site or the reaction in the M.E.S.S. may be considered an artifact of the method. That the reaction in the M.E.S.S. may be due to enzyme actually located there cannot be completely excluded. The observation that this reaction site alone survived 10^{-6} M physostigmine treatment may be interpreted in support of this assumption. If a membrane-bound enzyme does exist, as repeatedly observed here, it may be more clearly separated on the basis of differences in sensitivity to pharmacological inhibition. Karlin (31), studying centrifugal fractions of *Electrophorus* homogenates, has confirmed the association of AChE activity with membrane remnants, although no pharmacological results were reported. However, it may be pertinent that the fine structural localization of the reaction on the noninnervated surface was always found in the M.E.S.S.

Since the possibility exists that the reaction in the M.E.S.S. is due to enzyme located there, and since more activity was seen there than at other sites, the origin of the M.E.S.S. becomes an important question. Tubulovesicular structures apparently continuous with the plasma membrane of both the innervated and noninnervated side contained AChE-reactive M.E.S.S. The amount of M.E.S.S. appeared proportional to the distance of the tubulovesicle from the surface. According to these observations, the tubulovesicles could be micropinocytotic channels which incorporate M.E.S.S. of extra-plaque origin (32). Alternatively, the same morphological observations could be interpreted as evidence of plaque production of AChE-reactive M.E.S.S. In support of this

latter interpretation is a possible morphological and functional continuum: large saccules → empty small vesicles (? saccule buds) → vesicles containing dense material → tubulovesicles containing M.E.S.S. With the appearance of the M.E.S.S. - filled cytoplasmic vesicles, physostigmine-sensitive enzymatic activity is demonstrable. The membranes of these cytoplasmic tubulovesicular channels eventually become continuous with the surface while their contents are free to diffuse into the extracellular space. The fact that only the M.E.S.S. associated with the immediate surface of the plaque showed enzymatic activity favors this possibility. Similarly appearing M.E.S.S. occurred distant from the surface within other cellular structures (supporting cells, blood vessels, axons, and sheath cells), but was frequently unreactive. The possibility of AChE secretion by a postjunctional structure has been raised previously (32) but is still, admittedly, only a possibility.

Currently also unresolvable is the significance of the final product localized to the synaptic vesicles with the Pb-TAA, Ag-TAA, and thiocholine methods, since Pb^{++} and Cu^{++} were bound to these vesicles in the absence of substrate at pH's above 6.5. At pH 5, the substrate-free control preparations exhibited little or no binding of metal to the vesicles. In complete media (Pb-TAA or Ag-TAA) discrete fine electron-opaque particles were observed in the interior of the vesicles. A localization of final product similar to this was reported by Miledi using the Cu-acetylthiocholine media on frog neuromuscular junctions (8). Although Smith and Treherne (10) reported no nerve ending vesicle reaction in their investigation of sites of TAA hydrolysis in insect abdominal ganglia, their Figs. 8 and 9 indicate the same type of dense product in synaptic vesicles described here. Because the localization on eel nerve ending vesicles could not be prevented with any of the AChE inhibitors, it is not possible to refer to this activity as a cholinesterase. Indeed, in view of the rapidity with which the deposits from Pb-TAA media were found to occur here and of the lack of any significant further deposition with more prolonged incubations, it may be questioned whether the vesicle deposits actually represent enzyme activity or, rather, some chemical reaction of a vesicle component with the Pb^{++} or Cu^{++} ions.

Prior to the advent of dialdehyde fixation (24), investigations of the fine structural cytochemistry of the Pb-TAA reactive sites in neuromuscular junctions (6) suggested that an enzyme capable of splitting TAA was present in the synaptic vesicles of reactive nerve endings. Because of differences in the inhibitory response to the anticholinesterases at different junctional sites, this vesicle reaction was interpreted as being different from the junctional acetylcholinesterase reaction. Although the present results in *Electrophorus* do not support the previous studies in rat, the discrepancy may rest on other than species differences. For example, the Pb^{++} deposits on the vesicles could serve as a nidus for the accretion of a larger precipitate, particularly if very active hydrolysis of the TAA resulted in relative depletion of capture metal with consequent diffusion of reaction product only to the interior of synaptic vesicles. However, this does not explain the differential anticholinesterase sensitivity previously reported (6); and in light of the present results, reinvestigation of the myoneural junction has indicated vesicle activity to TAA (unpublished results).

It is also of interest, however, that a Pb^{++} binding which is resistant to inhibitors of AChE has been observed within the vesicles of certain nerve endings in mammalian adrenal medulla, cervical ganglion, iris, caudate nucleus, and cerebellar cortex (33), whereas other synaptic vesicles in the brain showed no such reaction. Since TAA may be hydrolyzed by many enzymes other than AChE (34, 35), it is also possible that one of these (e.g. acetyltransferase) could be the source of the final deposit within the vesicles.

From a cytochemical viewpoint, any of the methods surveyed were sufficient to demonstrate the physostigmine-sensitive sites of final deposit localization. The known enzyme composition of the electroplaque (12) has made the distinction between "specific" cholinesterase and "nonspecific" cholinesterase unnecessary in this case. Thus, while the TAA method clearly does demonstrate AChE in the eel electroplaque, the possible interpretive interference of other enzymes which can split this substrate in tissues of less restricted enzyme composition must be controlled by the proper use of pharmacological inhibitors.

The cytochemical results are also of pertinence from a neurobiological viewpoint. Nachmansohn and his collaborators have emphasized the constant relationship between bioelectric activity of excitable membranes and the presence of the

"Acetylcholine System" (1). The enzymes cholinacetylase (choline acetyl transferase) and cholinesterase are regarded as essential for nerve activity. Locally stored acetylcholine (ACh) and the acetylcholine receptors in the membrane are the other fundamental components of the system. When the membrane is stimulated, bound ACh is released and combines with its receptors, producing a local configurational change in the membrane. This alteration of membrane brought about by ACh is the trigger for the reactions leading to a subsequent increase in sodium permeability, proceeding to depolarization. The AChE which is thought to be structurally very close to the ACh receptor rapidly hydrolyzes the ACh and permits the receptor to return to its resting state, resulting in decreased sodium permeability. Intracellular current flow from the depolarized site releases ACh at points distal to the initially stimulated area, propagating the impulse. Nachmansohn further views the acetylcholine system as the mediator of transmission across junctional sites. Prejunctional or presynaptic ionic current flow, but not actual ACh, is thought to stimulate the postjunctional membrane. In this connection, synaptic vesicles as a potential storage site of transmitter substance have no obvious functional role, but all excitable membranes would be expected to have demonstrable cholinesterase.

The more classical concepts of junctional transmission (36–38) have held that the transmitter flows across the junctional cleft, stimulating the postsynaptic membrane. In this latter view, cholinesterase would necessarily be present only at those junctional sites where ACh was the transmitter, as, for example, autonomic ganglia (2) and neuromuscular junctions (7), although it might also be present at other sites within the neuron during biosynthesis of the enzyme (11). Views which are intermediate of these extremely polarized interpretations (see 2) have appeal but await substantiation.

Earlier electrophysiological studies have suggested that neuroplaque transmission presents many of the results required of a "cholinergic" synapse. The appropriate enzymes and substrate are present (1), and the pharmacological response to ACh, its analogues, and inhibitors are all in the appropriate direction (14, 15). Such experiments are not capable, however, of distinguishing this classically conceived "cholinergic" synapse from internal cholinergic transmission of the Nachmansohn type. In an attempt to solve this problem, a previous electron microscope cytochemical investigation of AChE in electric tissue (39) appeared to show AChE reactivity limited to sites of neuroplaque junctions. This latter investigation (39) was based upon an azo dye method utilizing alpha-naphthylacetate as substrate and was performed on electric tissue of a different species of electric fish. Variations in the results of the azo dye method of Lehrer and Ornstein (3) and the TAA method used in the present study have been discussed (2). The azo dye method was not used in the present study because, in addition to poor reproducibility, the long incubation periods required, and poor visualization of the end product, the investigation of the enzyme selectivity of the substrate and the applicability of cholinesterase pharmacology to this cytochemical reaction would have enlarged the scope of this already lengthy study to undesirable proportions. However, it is pertinent that the electrophysiological results in the species studied by Wachtel et al. (39) differ from those with *Electrophorus* (see below) by showing restricted areas of electrical responsiveness compatible with the more restricted sites of esterase demonstrability (5).

The present results with *Electrophorus* electroplaques only approach a fine resolution of the enzyme's cytological locus. The innervated surface plasma membrane and the extracellular space adjacent to this membrane are, except for the possibility of different sensitivities to physostigmine, virtually indistinguishable sites of acetylcholinesterase activity. A more accurate localization will require further technical refinement.

From a more general viewpoint, however, this degree of cytochemical resolution does demonstrate AChE activity at both junctional and nonjunctional sites of the innervated electroplaque surface and around the nerve terminals in contact with this surface.

The *Electrophorus* electroplaques would then seem to serve as an adequate example of a bioelectric system compatible with the broad concept of cholinesterase function. The uniformity of the enzyme activity over the entire surface of the innervated side of the plaque would be quite compatible with the physiological observations (13, 15, 16) that the entire innervated surface of each plaque depolarizes in response to a nerve volley. If the reactivity of the electroplaque nerve endings can also be seen at nodes of Ranvier (18)

within these axons, the electroplaque would be a model still more closely fitted to this general concept of the role of AChE. Indeed, preliminary fine structural results (33) indicate that eel nodal areas have AChE activity demonstrable by the above procedures.

At any rate, it would seem profitable to reinvestigate the previous localizations of AChE at other junctional sites (6, 39–42). Whether the apparently good correlation of bioelectric membrane and AChE can also be substantiated in higher forms of neural tissue remains to be seen. Recent results (33, 43) indicate that the AChE within mammalian brain is much more restricted in distribution. Finally, the precise functional role of the synaptic vesicle in relation to the distribution of AChE or, indeed, to synaptic transmission generally needs clarification. The Pb^{++} binding reaction described here may provide a productive appproach to that problem.

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