ACETYLCHOLINESTERASE IN FROG SYMPATHETIC AND DORSAL ROOT GANGLIA

A Study by Electron Microscope Cytochemistry and Microgasometric Analysis with the Magnetic Diver

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ABSTRACT

The localization and chemical determination of acetylcholinesterase in the frog sympathetic and dorsal root ganglia were studied by a combination of the methods of electron microscopy, histochemistry, and microgasometric analysis with the magnetic diver. The Koelle-Friedenwald copper thiocholine histochemical method was modified by eliminating the sulfide conversion and by treatment of the tissue with potassium permanganate. In fixed tissue, enzymatic activity was demonstrated on the inner surface of the endoplasmic reticulum, nuclear envelope, subsurface cisternae, and agranular reticulum of the perikaryon and axon. In briefly fixed tissue, end product appeared also at the axon-sheath and the sheath-sheath interface. Activity at the synaptic junction was most readily obtained in unfixed tissue. Isolated neurons recovered from the diver following chemical analysis were studied with the electron microscope. Cells having a high enzyme activity showed a badly ruptured or absent neural plasmalemma and sheath. In this case the measured activity was apparently due to the enzyme present in the endoplasmic reticulum. Neurons having low activity exhibited an intact plasmalemma and sheath. This may reflect the effectiveness of the neural plasmalemma and sheath as a penetration barrier. The effects of fixation on enzyme activity are discussed. Electron microscopic examination of cells following microgasometric analysis is shown to be essential for the interpretation of the biochemical data.

INTRODUCTION

Electron microscope cytochemical studies of acetylcholinesterase in the nervous system can be used to advantage in conjunction with microgasometric analysis of this enzyme in individual neurons with the magnetic diver (9, 11, 13, 51). The cytochemical localization of end product in tissue blocks can suggest what components of the individual neurons contain the enzyme responsible

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for the quantitative biochemical data. At the same time, biochemical support for the cytochemical identification of the enzyme is obtained. Electron microscope examination of single neurons following microgasometric analysis can significantly influence the interpretation of the biochemical data by providing information on the morphological state of the cell.

Cholinesterase activity of isolated sympathetic and dorsal root neurons has been analyzed by use of the Cartesian diver (21, 22). The magnetic diver (13), however, offers a more sensitive method for quantitative determination of the activity of the enzyme. Isolated neurons have previously been examined with the electron microscope (28, 59, 60), but no microchemical analysis was done. In those studies extensive disruption or removal of the cell membrane was reported. Johnston and Roots (28) stated that the absence of a physical boundary from isolated neurons would undoubtedly alter rates of penetration of substrates or other components into the cell, and suggested that metabolic examination of these cells be monitored by electron microscope studies.

A number of techniques have been employed with varying degrees of success to the study of cholinesterase localization with the electron microscope, such as the azo dye method (16, 38), thiolacetic acid methods (3, 4, 6, 16, 32, 46, 67, 73), thiocholine methods (5, 7, 29, 39, 40, 44, 65, 72), and the reduced OsO_4 method (24). So far, thiolacetic acid methods have been used most extensively, but the substrate is hydrolyzed by various types of cholinesterases and esterases. The specificity of this method, therefore, relies solely on the use of selective inhibitors. A more specific substrate, such as a thiocholine salt, in addition to selective inhibitors, would be more desirable.

The cytochemical technique employed in this study is based on the copper thiocholine method devised by Koelle and Friedenwald (33). Modifications of this method, which have been used to study the nervous system of a variety of species at the light microscope level, in general have shown that acetylcholinesterase is localized predominantly within neurons, and that nonspecific cholinesterases are in satellite cells, glia, and connective tissue elements. The extensive bibliography is reviewed by Koelle (31).

A number of investigators (5, 7, 29, 44) have encountered difficulties in obtaining satisfactory localization when the Koelle-Friedenwald method

was applied to electron microscope techniques. An attempt was made to modify the procedure by using silver as the capturing agent (5, 7), but the size of the end product and its localization did not seem to be significantly improved. Karnovsky (29) developed an indirect thiocholine method utilizing copper ferrocyanide as the end product. This method appeared to give satisfactory localization of cholinesterase activity in rat sciatic nerve, but a randomly dispersed nonenzymatic precipitate was reported (65). Miledi (44) reported that the copper thiocholine complex, untreated with sulfide, could readily be washed away from its initial site by solutions, such as phosphate buffer, thereby leading to false localization of end product. Contrary to the results of others (5, 7, 29, 44), Lewis and Shute (39) claimed satisfactory localization of acetylcholinesterase in the nervous system by use of the copper thiocholine method, which included conversion of the complex to copper sulfide with sodium sulfide and postfixation with osmium tetroxide. Zajicek et al. (79), have emphasized, however, the possibility of diffusion of the end product during conversion, and Real and Luciano (56) have pointed out that certain sulfides are removed from tissue after prolonged fixation in osmium tetroxide.

The Koelle-Friedenwald method does have the advantage of utilizing a relatively specific substrate (33) in a medium that is stable for many hours. The present investigation was undertaken to determine whether further modifications could make this method more usable for the localization of cholinesterases at the ultrastructural level. In preliminary reports of this study (12, 72), it was shown that elimination of the sulfide conversion, as suggested for light microscopy (26, 79), and postfixation of the tissue with potassium permanganate (41), provides a reproducible method for electron microscopy in which the end product is well localized. It was planned to study the cytochemical localization of acetylcholinesterase in cells from the sympathetic and dorsal root ganglia and also to perform quantitative measurements of the enzyme in isolated cells from these ganglia. An examination was also done of the fine structure of isolated cells following microgasometric measurements, in order to verify the presence or absence of the cell membrane and satellite sheath, since the condition of these structures might be expected to influence the interpretation of the quantitative

data. Attention was also paid to the morphological preservation of cell organelles.

The frog was used for this study, since a previous histochemical and manometric study had reported that the cholinesterase in the frog central nervous system was predominantly of the specific type (66). Nonspecific cholinesterase, if present, was not detected by direct chemical assay or histochemical characterization. The use of this species, therefore, reduces or eliminates this variable. Nevertheless, precautions were taken to use appropriate inhibitors and substrates for further specifying the enzyme. A number of studies on the fine structure of sympathetic and dorsal root ganglia have appeared (2, 14, 18, 45, 53–55, 61–63, 70, 71, 78) which provide a morphological basis for the present study.

MATERIALS AND METHODS

Animals and Fixation

Adult frogs, *Rana pipiens*, were decerebrated and the cervical sympathetic and lumbar dorsal root ganglia were fixed either by perfusion through the heart or by immersion in fixative. The fixative was 2 or 4% formaldehyde, prepared from paraformaldehyde (58), or 2% glutaraldehyde (64), buffered to pH 7.4 with 0.05 M or 0.1 M phosphate buffer. For some specimens 120 mM sodium chloride and 3 mM magnesium sulfate were added to the fixative. The ganglia were cut into 0.2- to 0.3-mm blocks or else teased into small cell clusters in the fixative. The specimens were fixed for 5 to 60 min at 4°C or room temperature and then washed for 1 to 16 hr in several changes of frog Ringer's solution (120 mM sodium chloride and 3 mM magnesium sulfate). Some specimen blocks were not fixed prior to incubation, in order to determine the effect of fixation on enzyme activity and on barriers to cellular penetration of the substrate.

Incubation Medium

The tissue blocks were incubated with constant agitation for 5 to 30 min at 4°C in a freshly prepared medium similar to that recommended by Koelle and Friedenwald (33). For the demonstration of sites of acetylcholinesterase (AChE) activity, acetylthiocholine iodide (AThCh) 3.46 × 10⁻³ M (used as the iodide), and iso-OMPA (tetraisopropyl pyrophosphortetramide) 2 × 10⁻⁵ M or 2 × 10⁻⁴ M, which is considered to be an irreversible inhibitor of nonspecific or butyrylcholinesterase (BuChE) (52), were added to the following medium: 0.02 M glycine, 0.004 M copper sulfate, 0.03 M magnesium sulfate, and 0.02 M sodium hydrogen maleate buffer at pH 6.

In earlier studies 0.12 M sodium chloride was added, but was not found necessary in later studies. The medium was adjusted to pH 6 with 0.02 M sodium hydroxide. Prior to incubation, the tissue was preincubated for 30 min in the copper glycinate medium lacking the substrate but containing the appropriate inhibitor.

Controls

To determine the effect of inhibitors on AChE activity, the following substances were used in the preincubation and incubation medium: BW 284 C 51, 2 × 10⁻⁴ M, or eserine (physostigmine sulfate) 1 × 10⁻⁴ M, which is considered to be a reversible inhibitor of cholinesterases (ChE) in general.

To determine whether sites of nonspecific cholinesterase activity were present, butyrylthiocholine iodide (BuThCh) 3.1 × 10⁻³ M (used as the iodide) was employed as the substrate in the above medium with or without the inhibitor BW 284 C 51 (1,5-bis-[4-allyldimethylammoniumphenyl] pentan-3-one dibromide) 1 × 10⁻⁵ M, 2 × 10⁻⁵ M, 2 × 10⁻⁴ M, which is considered to be a reversible inhibitor of specific cholinesterase, or acetylcholinesterase (20, 30).

Following incubation, the tissue was washed briefly in cold distilled water and then treated with a freshly prepared solution of 1% potassium permanganate (41) for 10 min. Longer fixation periods may result in brittle tissue blocks.

Conversion

The effect of conversion of the copper thiocholine end product to copper sulfide was tested by use of ammonium sulfide (33), sodium sulfide buffered to pH 5 with acetic acid (39), and hydrogen sulfide (26) saturated solutions. Conversion was carried out in this study by immersing blocks of tissue, containing the copper thiocholine end product, for 15 to 30 min in distilled water or in 0.02 M sodium hydrogen maleate buffer at pH 6, freshly saturated with hydrogen sulfide. The tissue was then washed thoroughly in the corresponding solution to remove the sulfide reagent, and fixed for 10 to 15 min in cold 1 or 2% osmium tetroxide (50) buffered to pH 7.4 with Veronal-acetate or phosphate buffer.

Electron Microscopy

Following fixation in either potassium permanganate or osmium tetroxide, the tissue was dehydrated in a graded series of cold ethanol or acetone (50, 70, 95 and 100%). Dehydration was continued in several changes of absolute alcohol or acetone for 60 to 90 min. The specimens were embedded in either Epon 812 (42) or Durcupan (Fluka A. G., Buchs, SG, Switzerland) according to the manufacturer's directions.

Thick sections for phase microscopy and thin sections for electron microscopy were cut with glass knives on a Porter-Blum (Sorvall) microtome or on an LKB ultratome. Thin sections were stained with uranyl acetate for 5 min (77), and then with lead citrate for 30 min (57). Electron micrographs were taken with a Siemens Elmiskop I.

Tests of the Method

Since factors other than substrate and inhibitor may affect the results of the method, the following experiments were done: (1) blocks of fixed and unfixed tissues were incubated in the medium lacking substrate for 2 hr to determine whether nonspecific binding occurs between the tissue and the components of the medium; (2) blocks of fixed tissue were incubated in the medium containing AThCh, which was prepared 4 hr and 24 hr prior to incubation to determine the stability of the medium; (3) blocks of fixed tissue were completely cut by alternating thick and thin sections to determine whether the components of the medium penetrated into the interior of the blocks; (4) blocks of fixed tissue containing the copper thiocholine end product were dehydrated and embedded with no further postfixation. The end product was examined unstained in thin sections. The following procedures were then performed in an attempt to determine which of the common reagents used for electron microscope technique readily solubilize or cause diffusion of the copper thiocholine end product. Thin sections of cytochemically reacted blocks were supported on Formvar-coated copper-mesh grids and were placed section side down on the meniscus of the following solutions at room temperature and 4°C for 15 min: distilled water; frog Ringer's solution; saline; saturated sodium sulfate; sodium acetate; graded concentrations of alcohol or acetone; sodium hydrogen maleate buffer at pH 6; sodium acetate buffer at pH 5; various buffers at pH 7.4 (veronal-acetate, phosphate, cacodylate, s-collidine), 1 and 2% osmium tetroxide in water and with the various buffers at pH 7.4; 1% potassium permanganate; and routine staining solutions (uranyl acetate, uranyl nitrate, lead acetate, lead hydroxide, and lead citrate). Reagents, such as distilled water, sodium sulfate, alcohol, acetone, and potassium permanganate, were tested for an additional 15-min period. (5) Blocks of fixed tissue containing the end product which had been converted to copper sulfide were dehydrated and embedded with no further postfixation. Thin sections of this tissue were examined before and after flotation for 30 min on a 1% buffered osmium tetroxide solution at 4°C.

Microgasometric Analysis of Isolated Cells

The following procedures were done by freehand technique with the aid of a dissecting microscope.

Dorsal root or sympathetic ganglia were removed from *Rana pipiens* or *Rana temporaria* and placed in cold frog Ringer's solution in a small dish coated with a thin layer of agar. The connective tissue capsule was pulled away with fine forceps, exposing the underlying cells and nerve processes. The cells or fibers were teased free with a very fine glass needle. They were transferred into the usual bicarbonate reaction medium containing substrate and, in some cases, an inhibitor and introduced into the ampulla (80). The same procedure was then followed as described previously (9). The substrates used were acetylcholine iodide (ACh), 3×10^{-3} M, and butyrylcholine iodide (BuCh), 1×10^{-2} M, used as iodides. The inhibitors were BW 284 C 51, 2×10^{-5} M, iso-OMPA, 2×10^{-5} M, and eserine, 1×10^{-7} M, 1×10^{-6} M, 1×10^{-5} M.

In some experiments two different substrates in succession or one substrate followed by an inhibitor were used on the same sample. In these cases the ampulla was removed from the flotation vessel after the first measurement, then opened, and the reaction fluid around the sample exchanged for the one containing the other substrate or inhibitor (10, 80).

A similar procedure was followed in experiments in which the effect of fixation on cholinesterase activity was measured. The enzyme activity of unfixed tissue was determined, then the ampulla was opened, and the sample was exposed to 2% glutaraldehyde buffered to pH 7.4 with phosphate buffer for 30 min at 4°C. The sample was washed with frog Ringer's solution, transferred to fresh reaction mixture, and the cholinesterase activity measured in the usual way.

The length and diameter of the nerve fiber samples were measured with a calibrated micrometer eyepiece, and the fresh weight was calculated assuming that 1 mm^3 corresponds to 1 mg of tissue.

Electron Microscopy of Isolated Cells

Following microgasometric analysis, a number of samples were removed from the diver and fixed in 1% potassium permanganate for 5 to 10 min. They were then dehydrated and embedded in the same reagents previously described for blocks of tissue. Certain precautions were taken to minimize the possibility of losing the specimens. Most of the exchanges of reagents through the specimen were done in a micropipette. It was usually necessary to add a small amount of embedding medium to either the propylene oxide or acetone exchanges. The specimen was embedded in a drop of either Epon or Durcupan placed on the surface of the flattened pyramid of a blank, previously made in a B.E.E.M. capsule (Better Equipment for Electron Microscopy, Inc., Bronx, New York). The specimens were serially thick-sectioned until the tissue could be identified by phase

microscopy, then serially thin-sectioned and examined with the electron microscope.

OBSERVATIONS

Neurons of both the sympathetic and dorsal root ganglia fixed in either glutaraldehyde or formaldehyde contain the end product of the cytochemical reaction in association with the cisternae of the endoplasmic reticulum (Figs. 1, 2, ER; Figs. 3, 4, arrows), including the nuclear envelope (Figs. 1, 2, NE, and subsurface cisternae (Figs. 5, 6, SSC). Positive subsurface cisternae were often located at the postsynaptic surface in sympathetic neurons (Fig. 6, SSC). Most of the tubuli and canaliculi of the Golgi complex (Figs. 1, 7, G) do not show any reaction, although occasionally portions of tubules may exhibit end product (Figs. 1, 7, arrows). When present, the end product is usually located in what appears to be areas of transition between the Golgi complex and the endoplasmic reticulum.

The smallest end product obtainable so far is a 250- to 300-A particle (Fig. 3, arrow), which appears with a short incubation period of 5 to 10 min. In favorable sites, such as in dilated portions of the endoplasmic reticulum, the first deposition of end product is found adherent to the internal surface of the cisternal wall (Fig. 4, arrow). With longer incubation periods the size of the copper thiocholine end product increases, particularly in length, filling the cisternae. The larger end product, therefore, indicates that the endoplasmic reticulum in that location has enzyme activity, but the more precise original site of deposition of the product within the cisterna is obscured.

The distribution of end product within the cisternae is sporadic, but the number of active sites increases with longer incubation periods. A large proportion of the cisternae of the endoplasmic reticulum (Figs. 1, 19, ER) of most of the sympathetic neurons show a positive reaction after a 20- to 30-min incubation period. There is a greater variation in the number of positively reacting sites in the endoplasmic reticulum of neurons in the dorsal root ganglion, even between closely grouped cells. A correlation can be made between morphology and the cytochemical activity of two cell types identified in the dorsal root ganglion. One type, a small neuron which has few filaments but a large amount of endoplasmic reticulum (Fig. 2, ER), shows a large number of positively reacting sites. Another type, a moderate to large, densely filamented neuron which has a relatively small amount of endoplasmic reticulum, contains fewer positively reacting sites (Fig. 22, arrows). The endoplasmic reticulum is randomly scattered among large bundles of filaments (F) in this type of neuron, and is equally sparse among the aggregates of ribosomes of the peripherally located Nissl bodies. Filaments are demonstrated better in OsO4-fixed preparations than in permanganate-treated tissue (62). The morphology of other medium- to large-size neurons cannot be correlated with their cytochemistry. The endoplasmic reticulum and filaments are roughly equal in proportion, and they are randomly dispersed throughout the neural perikaryon. Some of these neurons have a large number of positively reacting cisternae; in other cases, relatively few are positive.

The end product is not present in the nucleoplasm (Figs. 1, 2, N), mitochondria (Figs. 2, 3, 7, M), dense bodies (Fig. 2, B), or in the endoplasmic reticulum of satellite cells, Schwann cells, endothelial cells, or fibroblasts in either the sympathetic or dorsal root ganglion, under the same conditions of fixation and incubation. Although random deposits may be found from time to time, in general very little precipitate is seen throughout the connective tissue areas (Fig. 1, CT) in properly fixed and incubated tissues.

The agranular reticulum of the proximal portion of the axons of sympathetic neurons (Fig. 1, double-shafted arrow), as well as of other unmyelinated (Fig. 8, arrows) or myelinated axons (Fig. 9, arrows) within the ganglion, often contains end product. The identification of large processes lacking myelin sheaths as "axons" (Fig. 8) is supported by previous studies which reported the absence of dendrites on frog sympathetic neurons (53, 68, 70). Moreover, this process closely resmebles the broad axon of the sympathetic neuron (Fig. 1). The membrane and the interface of the axon and Schwann cell of many myelinated (Fig. 10, arrow) and unmyelinated fibers (Figs. 11, 12, arrows) in the sympathetic ganglion often contain dense linear end product, particularly after brief periods of fixation. A similar reaction may be found at the neural plasmalemma and Schwann sheath and at wrappings of Schwann cell cytoplasm (Fig. 12, double-shafted arrow) and satellite cell cytoplasm. A positive reaction in the same sites has been seen in the dorsal root ganglion. The end product has been seen only occasionaly in association with synapses of fixed sympathetic ganglia. When present, the end product is on both opposing membranes

and fills the cleft between the nerve ending and the surface of its sheath (Fig. 13, arrow), or of the neural cytoplasm (Fig. 14, arrow).

Unfixed Tissue

The application of the present cytochemical method to unfixed tissue gives somewhat different results than with fixed tissue. A positive reaction is much more readily obtained around synaptic endings in tissue unfixed prior to incubation (Fig. 15, *SY, SY'*). The end product is present at neural (arrows) and sheath (double-shafted arrow) surfaces of the synaptic ending. The end product is usually somewhat longer and crystalline in unfixed tissue, and tends to be less discretely localized to specific sites. Unlike the findings in fixed tissue (Fig. 1, *ER*), very little end product is found associated with the endoplasmic reticulum as long as the sheath and neural plasmalemma remain intact. End product may be found, however, in the endoplasmic reticulum of cells showing obvious damage and disruption of their surfaces. A crystalline precipitate is often randomly distributed throughout the connective tissue (*CT*) of unfixed tissue even after short incubation periods, thereby making it difficult to properly evaluate the cytochemical reaction in nerves and sheath cells.

Inhibitors and Other Tests

The end product of the cytochemical reaction using AThCh as substrate is present in fixed or unfixed tissue, with (Fig. 1) or without (Fig. 19) the use of the inhibitor, iso-OMPA. Iso-OMPA at a concentration of 2×10^{-4} M was routinely used in these preparations to be certain that the end product being demonstrated is not due to butyrylcholinesterase activity. At this concentration some of the acetylcholinesterase activity may be par-

tially inhibited, since fewer sites are positive than in uninhibited tissue and longer incubation periods may be used.

BW 284 C 51 effectively inhibits enzyme activity in fixed (Fig. 17) and unfixed tissues. Only rarely is end product found after the use of this inhibitor.

Eserine inhibits the surface reaction found in unfixed tissue (Fig. 16), that is, the activity at the synaptic junction, the axon–Schwann cell interface, and between folds of sheath cell cytoplasm. There is a significant decrease in the amount of end product present in the endoplasmic reticulum of fixed tissue in eserine-treated preparations, as compared to uninhibited preparations, particularly after short incubation periods. With longer incubation periods (Fig. 18), there is an increase in the number of active sites in the eserine preparation, but the reaction is never so marked as in the uninhibited specimen (Fig. 19).

No activity was found in this tissue when butyrylthiocholine was used as substrate.

There is no precipitate in either fixed or unfixed tissue when the substrate is omitted from the medium, even after 2 hr of incubation. Tissue incubated in the medium prepared with the substrate either 4 or 24 hr prior to incubation showed very little difference from that incubated in medium prepared just before use. Blocks of tissue which were completely sectioned by alternating several thick sections with thin ones showed that the central portion of the blocks exhibited the same variations in activity, from one cell to another, as was found closer to the surface.

The copper thiocholine end product in tissue previously fixed in glutaraldehyde, but not treated with potassium permanganate prior to dehydration and embedding, can be seen as an electron-opaque crystallike material (Fig. 20, arrow) in

FIGURE 1 Electron micrograph of a typical glutaraldehyde-fixed sympathetic neuron, treated with reagents for AChE localization. The end product is associated with the endoplasmic reticulum (*ER*) scattered throughout the cytoplasm, as well as with the nuclear envelope (*NE*) and the agranular reticulum of the axon (double-shafted arrow). No precipitate is present in the nucleus (*N*). Most of the components of the Golgi complex (*G*) are negative, although areas of transition between the Golgi complex and the endoplasmic reticulum are positive (arrows). The thin layers of satellite cell cytoplasm, which invest the perikaryon closely over most of its surface, form a loose, thin-walled sheath over the axon hillock, the incoming nerves (*AX*), and their synaptic terminals (*SY*). A material of moderate density (*) is enclosed by the sheath. The connective tissue area (*CT*) is free of random precipitate. (AThCh + iso-OMPA, 30 min; postfixed in KMnO₄, stained section). × 13,000.
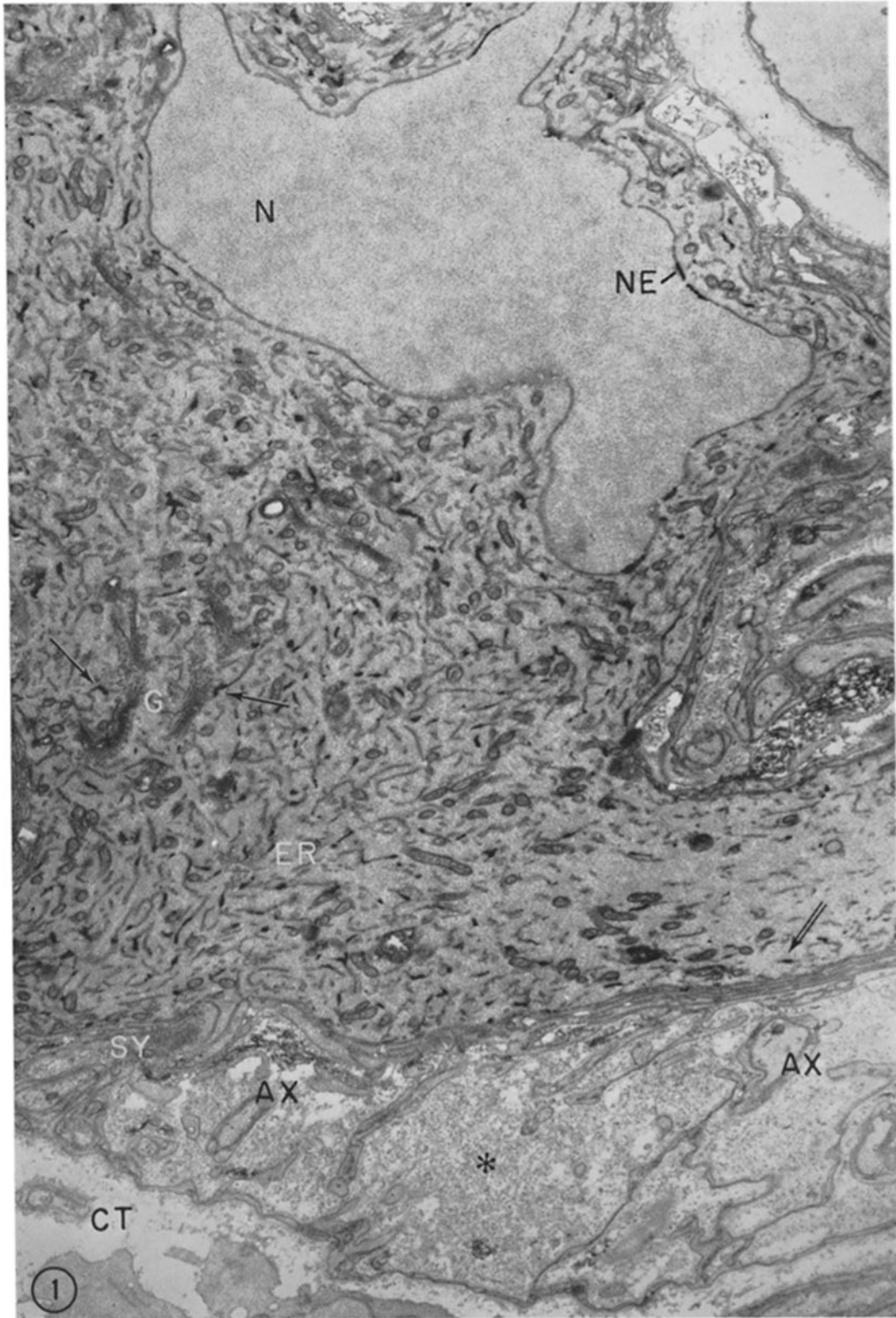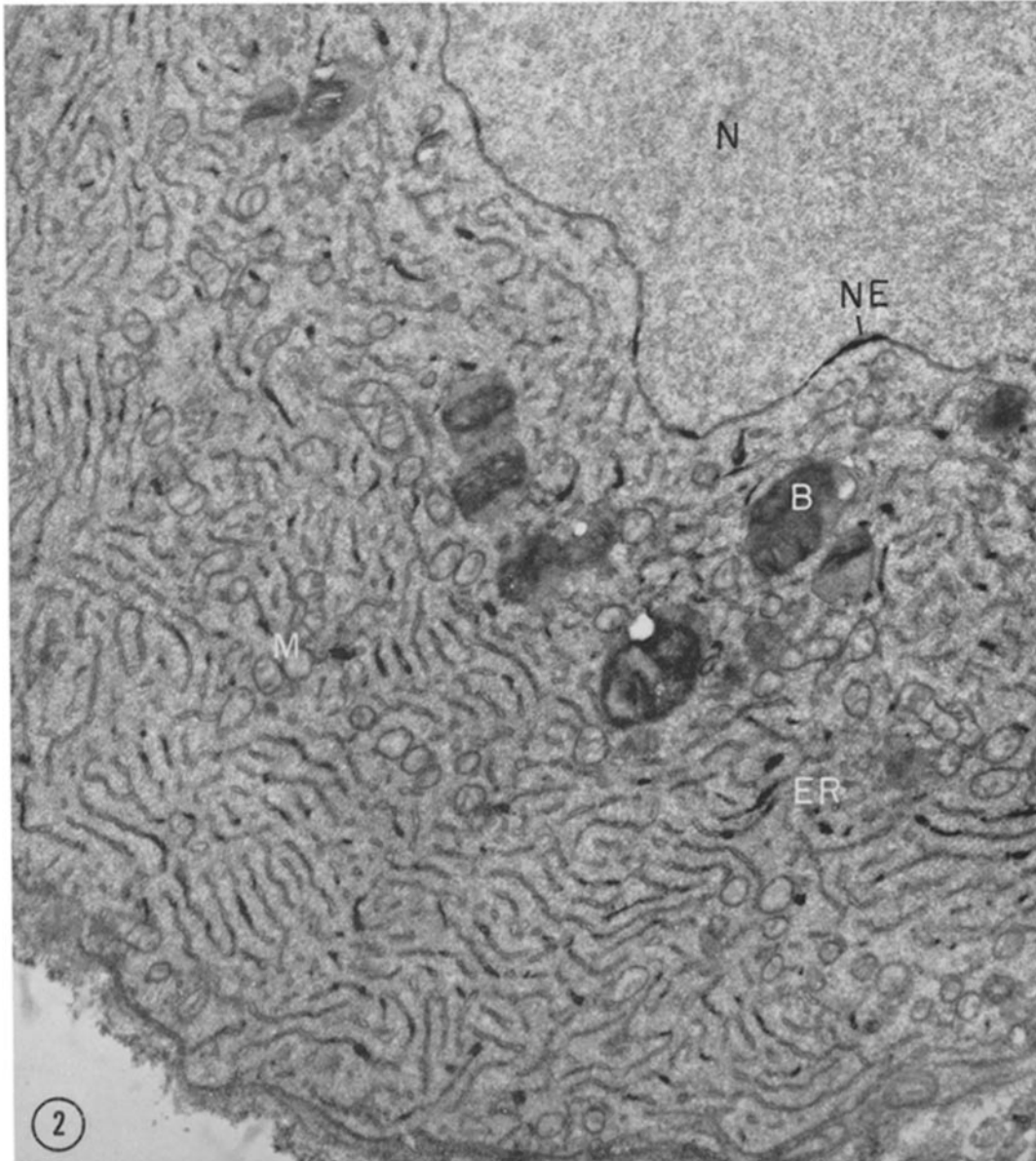
FIGURE 2 An electron micrograph of a small glutaraldehyde-fixed neuron from the dorsal root ganglion, containing a large proportion of cisternae of the endoplasmic reticulum (*ER*). The nuclear envelope (*NE*) and many cisternae contain the end product of enzymatic activity, whereas the nucleus (*N*), mitochondria (*M*), and large heterogeneous bodies (*B*) are negative. (AThCh + iso-OMPA, 20 min; postfixed in KMnO₄, stained section). × 19,500.

association with the endoplasmic reticulum of neurons. This end product is removed from thin sections (Fig. 21, arrow) which have been floated for 15 min on the meniscus of the following sub-stances: (1) dilute salt solutions including sodium acetate; (2) osmium tetroxide in water or in buffers; (3) all buffers tested; and (4) all of the staining solutions tested. The copper thiocholine end

TABLE I

ChE Activity of Cells Isolated from the Dorsal Root Ganglion

Activity is expressed in μl $CO_2 \times 10^{-5}$/hr/sample.

No. of cells in sample	ACh 3×10^{-3} M Without inhibitor	Iso-OMPA* 2×10^{-5} M	BW 284 C 51* 2×10^{-5} M	BuCh 1×10^{-2} M Without inhibitor	Iso-OMPA 2×10^{-5} M	BW 284 C 51 2×10^{-5} M
1	1.8					
1	3.3					
1	4.8	5.0				
1	7.5		0.06			
1	12.3		Not measurable			
1	38.4					
1	59.4					
3	22.0	22.1				
6				Not measurable		
8	13.3			0.80		
11	8.6			0.77		
14				0.70	0.53	
18				0.65		Not measurable

* Iso-OMPA is considered to be a specific inhibitor of nonspecific cholinesterases, and BW 284 C 51 of acetylcholinesterase.

product is retained when the section is floated for 30 min on distilled water, various concentrations of alcohol or acetone, saturated sodium sulfate, or potassium permanganate. The end product is retained for even longer periods of time if the reagents are used at 4°C. Dense particles of copper sulfide in glutaraldehyde-fixed tissue, which has not been treated with additional fixatives, remain in thin sections floated for 30 min on a 1% buffered osmium tetroxide solution at 4°C. It is recognized that these procedures do not necessarily predict the effect of the reagent on the copper thiocholine end product in the tissue prior to dehydration and embedding. Nevertheless, the resulting information was useful in developing the techniques used here by determining which reagents are most likely to permit retention of the end product.

Conversion

Conversion of the copper thiocholine end product to copper sulfide was accomplished with the least evidence of displacement of end product by immersion of briefly reacted specimens in distilled water, freshly saturated with hydrogen sulfide. The 100- to 200-A copper sulfide end product appears in the same sites as in control tissue, i.e. tissue containing the copper thiocholine end product treated with potassium permanganate. The use of sodium sulfide buffered to pH 5 with acetic

acid (39) or of maleate buffer saturated with hydrogen sulfide results in the same dense particles (Fig. 22, arrows), and often better preservation of the tissue. Somewhat more displacement of end product may be found in these cases, however, probably due to the solubility of copper thiocholine in dilute salt solutions. Evidence of displacement of end product was much greater with the use of ammonium sulfide (33). Although copper sulfide particles may be found in the same sites as in the control, the results are not consistent. In some specimens, large stellate precipitates occur and the fine particles are randomly distributed. In others, the end product appears to be completely removed.

Measurement of Cholinesterase Activity and Examination of Isolated Cells

Tables I and II present the results of tests on cells from the dorsal root ganglion and on sympathetic nerve fibers to determine the specificity of the enzyme activity measured in these experiments. The hydrolysis of butyrylcholine is very low compared to that of acetylcholine. The hydrolysis of both substrates is only slightly affected, if at all, by iso-OMPA (2×10^{-5} M), an inhibitor of nonspecific cholinesterase, but is significantly inhibited by BW 284 C 51, an inhibitor of acetylcholinesterase.

Table III shows the effect of eserine inhibition

TABLE II

ChE Activity of Sympathetic Nerve Fibers

Activity expressed in μl CO_2 $\times$ 10^{-5}/mg fresh tissue/hr

ACh 3 $\times$ 10^{-3}			BuCh 1 $\times$ 10^{-2} M		
Without inhibitor	Iso-AOMP 2 $\times$ 10^{-5} M	BW 284C 51 2 $\times$ 10^{-5} M	Without inhibitor	IsoOMPA 2 $\times$ 10^{-5} M	BW 284 C 51 2 $\times$ 10^{-6} M
9.42			0.76		
9.97			0.88		
9.20	9.04				
6.64	6.71				
10.50		0.32			
			0.61	0.60	
			0.47		0.08
			0.42		0.05

TABLE III

Inhibition of ChE Activity by Eserine of Cells Isolated from the Dorsal Root Ganglion

The activity is expressed in μl CO_2 $\times$ 10^{-5}/hr/ sample

No. of cells in sample	ACh 3 $\times$ 10^{-3} M			
	Without inhibitor	1 $\times$ 10^{-5} M	1 $\times$ 10^{-6} M	1 $\times$ 10^{-7} M
7	14.5	0.9		
4	18.7	1.6		
5	18.5	1.9		
4	13.2		5.1	
6	21.0		10.7	
11	24.2		9.3	
4	9.1			8.8
9	17.8			14.6

on the enzyme activity in clusters of dorsal root ganglion cells. Eserine significantly inhibits the hydrolysis of acetylcholine at a concentration of 1×10^{-5} M, but even at this concentration the inhibition is less effective than that produced by BW 284 C 51.

Tables IV and V show the effect of fixation on cholinesterase activity in cells from the dorsal root

and sympathetic ganglia. In both cases, the measured enzyme activity is reduced, but the loss of activity is greater in specimens from the sympathetic ganglion.

The data obtained from the measurement of cholinesterase activity, as well as the results of electron microscope examination of the same samples following microgasometric analysis, are summarized in Table VI. The separation of the

FIGURE 3 Electron micrograph of the endoplasmic reticulum of a glutaraldehyde-fixed sympathetic neuron, reacted for AChE activity. The smallest end product obtainable, so far, has a particle size of about 250 to 300 A (arrow). Mitochondra (M) are negative. Since this tissue was postfixed in $KMnO_4$, the ribosomes are not apparent. (AThCh + iso-OMPA, 7 min). $\times$ 85,000.

FIGURE 4 An electron micrograph of a preparation similar to that in Fig. 3. The end product can clearly be seen adhering to the internal wall of a dilated cisterna (arrow). $\times$ 85,000.

FIGURE 5 An electron micrograph illustrating the end product of enzymatic activity in a subsurface cisterna (SSC) of the proximal portion of a glutaraldehyde-fixed sympathetic axon (AX). A single layer of sheath cell cytoplasm surrounds the axon. (AThCh + iso-OMPA, 30 min). $\times$ 84,000.

FIGURE 6 An electron micrograph of a specimen treated like that in Fig. 5. A subsurface cisterna (SSC) is located on the postsynaptic side of an axosomatic synapse. Another portion of the endoplasmic reticulum (ER) of the sympathetic neuron contains end product. (AThCh + iso-OMPA, 30 min). $\times$ 72,000.

FIGURE 7 An electron micrograph of a typical Golgi area from a preparation similar to that in Fig. 1. Most of the tubuli of the Golgi complex (G) are negative although some areas, which may be transitions between the Golgi complex and the endoplasmic reticulum (arrow), contain end product. No end product is present in mitochondria (M). $\times$ 72,000.
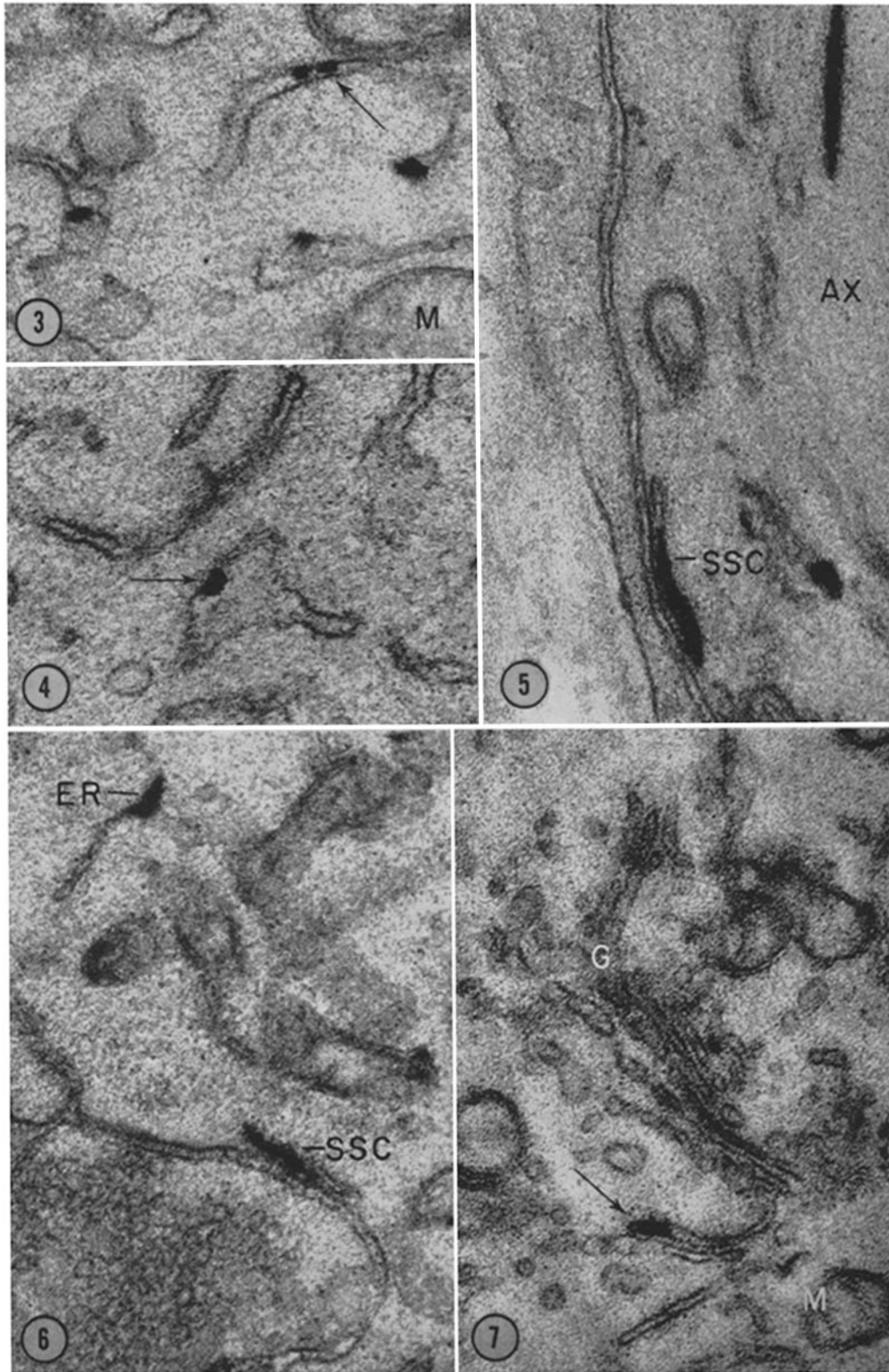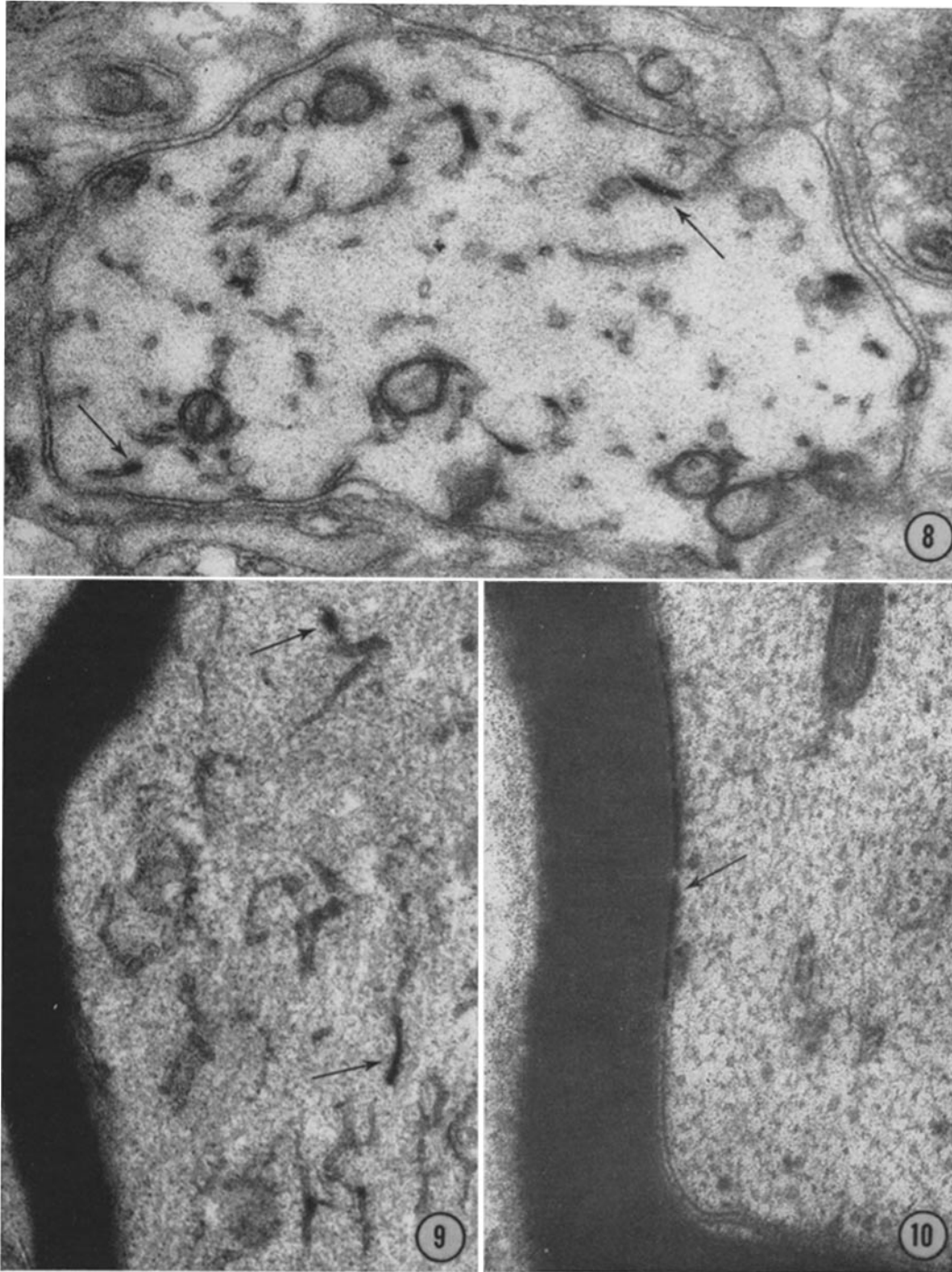
FIGURE 8   An electron micrograph of a sympathetic axon fixed briefly in glutaraldehyde and reacted for enzyme activity. End product is present in the agranular reticulum (arrows). AThCh + iso-OMPA, 5 min; postfixed in KMnO₄, stained section). × 52,500.

FIGURE 9   Electron micrograph of a myelinated sympathetic axon fixed in glutaraldehyde and reacted for enzyme activity. End product is present in the agranular reticulum (arrows). AThCh + iso-OMPA, 30 min; postfixed in KMnO₄, stained section. × 42,000.

FIGURE 10   An electron micrograph of a myelinated axon treated like that in Fig. 9. End product is present on the membranes and in the cleft between the axon and Schwann cell (arrow). × 62,000.
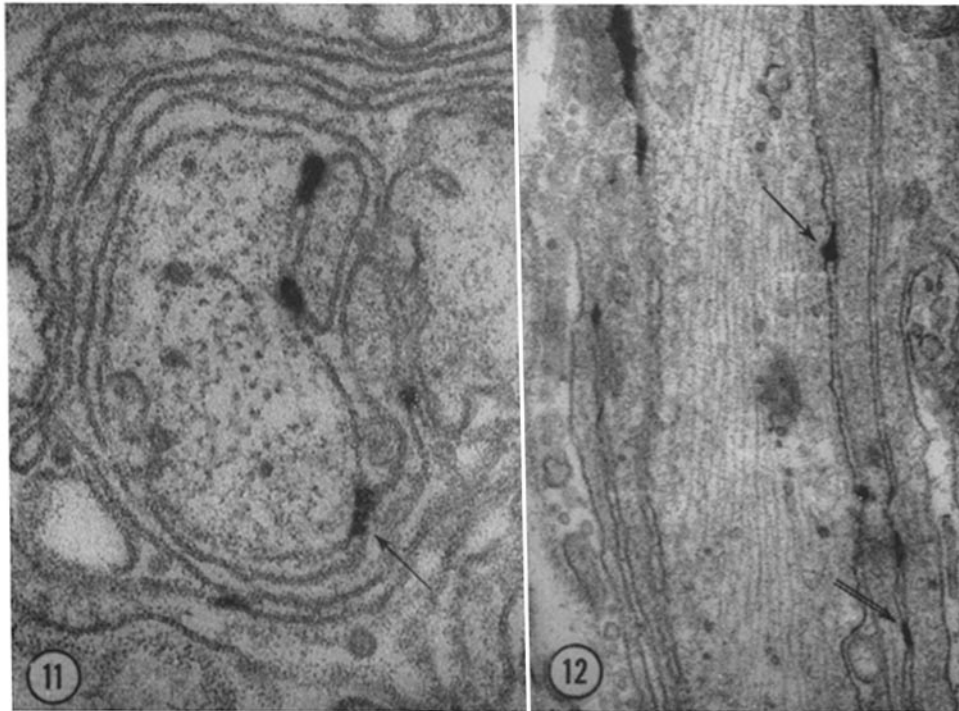
FIGURE 11 Electron micrograph of a small unmyelinated sympathetic nerve fiber fixed briefly in formaldehyde and reacted for enzyme activity. End product is present on the membranes and in the cleft between the axon and Schwann cell (arrow). (AThCh + iso-OMPA, 10 min; postfixed in $KMnO_4$, stained section). × 80,000.

FIGURE 12 An electron micrograph of an unmyelinated axon fixed in formaldehyde and reacted for enzyme activity. End product is present on the membrane and in the cleft between the axon and Schwann cell (arrow) and also between layers of the sheath cell (double-shafted arrow). (AThCh + iso-OMPA, 10 min; postfixed in $KMnO_4$, stained section. × 37,000.

samples into high and low activity groups can be correlated with the degree of preservation of cell structure in each group. Damaged neurons exhibited considerably higher enzymatic activity than neurons having intact plasma membranes and satellite sheaths.

Phase microscopy is useful for general orientation in studies of isolated cells, but can give only limited information concerning the condition of the sheath, the neural plasmalemma, and the internal organelles. Although a sheath is visible in the phase micrograph of the sample having a high activity of 63.3 × 10^{-5} μl CO_2/hr (Fig. 23, S), examination of the vacuolated regions (arrow) and other surface areas of the neuron with the electron microscope revealed that the sheath and neural plasmalemma were ruptured and detached (Fig.

24). The neural plasmalemma often appears to be firmly adherent to the cell membrane of the sheath. Rupture seems to occur commonly at sites of swollen subsurface cisternae.

Many of the neurons having high activity lacked a satellite sheath and plasmalemma entirely. The absence of the sheath and the damage at the base of the cell were apparent in the phase micrograph of the sample having a high activity of 54.6 × 10^{-5} μl CO_2/hr (Fig. 26), but examination with the electron microscope was necessary to confirm the absence of the neural plasmalemma (Fig. 27). The nucleus (Fig. 25, N), nuclear envelope, Golgi complex, filaments, and dense bodies (B) exhibit far fewer morphological alterations in ruptured cells than do mitochondria and the endoplasmic reticulum. Mitochondria (M) are

TABLE IV

ChE Activity of Cells Isolated from the Dorsal Root Ganglion before and after Fixation in 2% Glutaraldehyde for 30 Min

Activity expressed in μl CO_2 $\times$ $10^{-5}/hr/sample$

No. of cells in sample	Before fixation	After fixation	Amt. of inhibition
			%
6	15.8	11.5	27.2
8	12.3	9.0	26.7
11	15.2	12.1	20.5
13	10.8	7.1	34.3

usually swollen. Cisternal components of Nissl bodies (Figs. 24, 27, *NB*) are less likely to swell as much as subsurface cisternae or randomly oriented cisternae.

A phase micrograph of a section through the sample having a low activity of 3.6 $\times$ 10^{-5} μl CO_2/hr (Fig. 28) shows that the section consists of a neuron having granular cytoplasm and a short axon segment (*AX*), surrounded by a satellite sheath (*S*), and possibly some connective tissue. When examined with the electron microscope, the sheath cell and neural plasmalemma were intact. Although there was a peripheral zone of little density (arrow) in the neural cytoplasm, this area contained neurofilaments and, therefore, did not indicate cell damage as in Fig. 23.

Although the satellite sheath may be thick enough to be visible in phase micrographs, as in Figs. 23 and 28, often the sheath is so thin that it is below the limit of resolution of the phase microscope. In some places, the sheath cytoplasm sur-

rounding one of the two neurons in the sample having a low activity of 5.4 $\times$ 10^{-5} μl CO_2/hr is approximately 500 A thick (Fig. 30, arrow). The neural plasmalemma and sheath were intact around both of the neurons. The internal organelles are usually better preserved in the neurons having an intact cell membrane and satellite sheath. Mitochondria (Fig. 29, *M*) may show reasonably good preservation in some cells, but may exhibit swelling in others. The endoplasmic reticulum usually shows a moderate degree of swelling (Figs. 29, 30, *ER*), which is usually less marked in Nissl bodies (*NB*). Subsurface cisternae may be markedly swollen (Fig. 30, *SSC*).

DISCUSSION

Specificity and Reliability of the Cytochemical Method

The sites of acetylcholinesterase activity have been shown cytochemically in the sympathetic and dorsal root ganglia of the frog by use of the substrate, acetylthiocholine, in conjunction with iso-OMPA, which is considered to be an inhibitor of nonspecific cholinesterase (52). The identity of the enzyme was further confirmed by the inhibition of enzyme activity after the use of BW 284 C 51, which is assumed to be a potent selective inhibitor of acetylcholinesterase (20, 30). Moreover, no activity was found when butyrylthiocholine was used as a substrate in either fixed or unfixed tissue. The results of biochemical studies of cells from similar tissue (Tables I and II) support the conclusion that the enzyme being demonstrated is predominantly acetylcholinesterase.

FIGURE 13 An electron micrograph of a preterminal portion of a synapse, fixed briefly in formaldehyde, illustrating the end product (arrow) on the membranes and in the cleft between the synaptic end bulb and its sheath. Typical synaptic vesicles (*V*) and glycogen (*GL*) are present in the terminal. (AThCh + iso-OMPA, 10 min). $\times$ 47,000.

FIGURE 14 An electron micrograph of an axo-axonic synapse fixed in formaldehyde and reacted for enzyme activity. End product (arrow) is present on the membranes and in the cleft of the synaptic terminal (*SY*) and axon (*AX*). (AThCh + iso-OMPA, 10 min; postfixed in KMnO$_4$, stained section). $\times$ 88,000.

FIGURE 15 An electron micrograph of a sympathetic neuron, which was unfixed prior to incubation for enzyme activity. The end product surrounds the large calyxlike ending (*SY*), and the smaller boutons (*SY'*) at the neural (arrows) and sheath surfaces (double-shafted arrow). Very little end product is present in the endoplasmic reticulum (*ER*). Some random precipitate is present in the connective tissue area (*CT*). (AThCh + iso-OMPA, 30 min; fixed in KMnO$_4$, stained section). $\times$ 19,000.
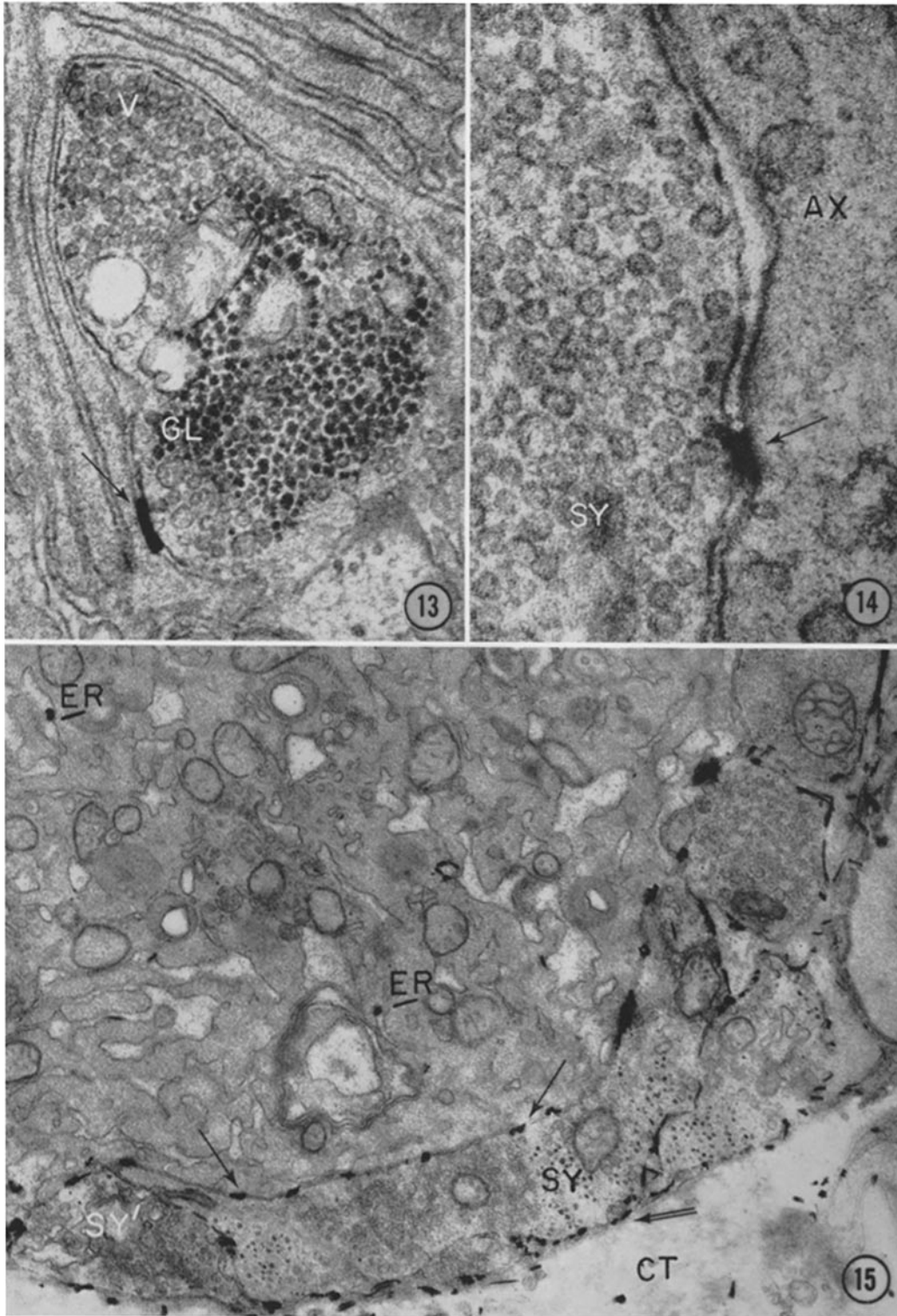
TABLE V

ChE Activity of Cells Isolated from the Sympathetic Ganglion before and after Fixation in 2% Glutaraldehyde for 30 Min

Activity expressed in μl CO_2 $\times$ 10^{-5}/hr/sample

No. of cells in sample	Before fixation	After fixation	Amt. of inhibition
			%
1	23.5		
1	68.8	41.0	40.5
1	2.1		
1	51.8		
1	8.8		
1	16.0	9.2	42.6
2	12.3	5.7	53.7
6	46.3	28.2	39.2

Inhibition studies using eserine have given results which require further clarification. Eserine inhibited enzymatic activity in unfixed tissue at the surface of the cell, that is, at the synapse, and at the axolemmal and sheath surfaces. This is in agreement with studies of the motor end plate (3) and of eel electroplaques (6). Eserine also inhibits a considerable amount of activity in the endoplasmic reticulum of frog neurons in fixed tissue

when short incubation periods are used. With longer incubation periods, eserine is much less effective than BW 284 C 51. A similar result was found in biochemical studies of eserine inhibition (Table III). Although eserine at a concentration of 1×10^{-5} M has a significant inhibitory effect on acetylcholinesterase activity of isolated dorsal root ganglion cells, the inhibition is less complete than that obtained with BW 284 C 51 (Table I). Some degree of resistance of the enzyme in the endoplasmic reticulum to eserine inhibition was previously noted by Torack and Barrnett (73). Hawkins and Mendel (25) have reported that the displacement of eserine from the enzyme by the substrate proceeds more rapidly in frog brain homogenates than with mammalian brain homogenates. Further studies are needed to elucidate the effect of eserine on the nervous system of the frog.

To distinguish between specific cholinesterase, or acetylcholinesterase, and nonspecific cholinesterase, sometimes referred to as butyrylcholinesterase, by the use of selective inhibitors requires considerable caution, as was explicity stressed by Pearse (52). The active groups in the "esteratic site" are generally considered to be similar in all ester-splitting enzymes (47). All inhibitors are,

FIGURE 16 An electron micrograph of the synaptic region of a sympathetic neuron, which was unfixed prior to incubation in the medium containing the inhibitor eserine. No end product was present at the synapse (SY) or at neural or sheath surfaces, although the uninhibited specimen showed a considerable amount of activity at these sites. (AThCh + eserine, 15 min; postfixed in KMnO4, stained section). $\times$ 26,000.

FIGURE 17 An electron micrograph of a sympathetic neuron, which had been fixed in glutaraldehyde prior to incubation in the medium containing the inhibitor BW 284 C 51. No end product is present in the cisternae of the endoplasmic reticulum (ER), although it is prominent in uninhibited specimens (compare with Fig. 19). The Golgi complex (G) is also negative. (AThCh + BW 284 C 51, 30 min; postfixed in KMnO4, stained section). $\times$ 26,000.

FIGURE 18 An electron micrograph of a sympathetic neuron which had been fixed in glutaraldehyde prior to incubation in the medium including the inhibitor eserine. This neuron contained a number of positive cisternae (ER), whereas many other neurons had much less activity. In general the amount of activity found in specimens treated with eserine is considerably less than that found in the uninhibited specimen (compare with Fig. 19). The Golgi complex (G) is negative. (AThCh + eserine, 30 min; postfixed in KMnO4, stained section). $\times$ 26,000.

FIGURE 19 An electron micrograph of a sympathetic neuron, which had been fixed in glutaraldehyde prior to incubation in the medium lacking inhibitors. Most of the cisternae (ER) contain end product. A dilated cisterna at the periphery of the Golgi complex (G) contains end product, but most of the complex is negative. (AThCh, 30 min; postfixed in KMnO4, stained section). $\times$ 26,000.
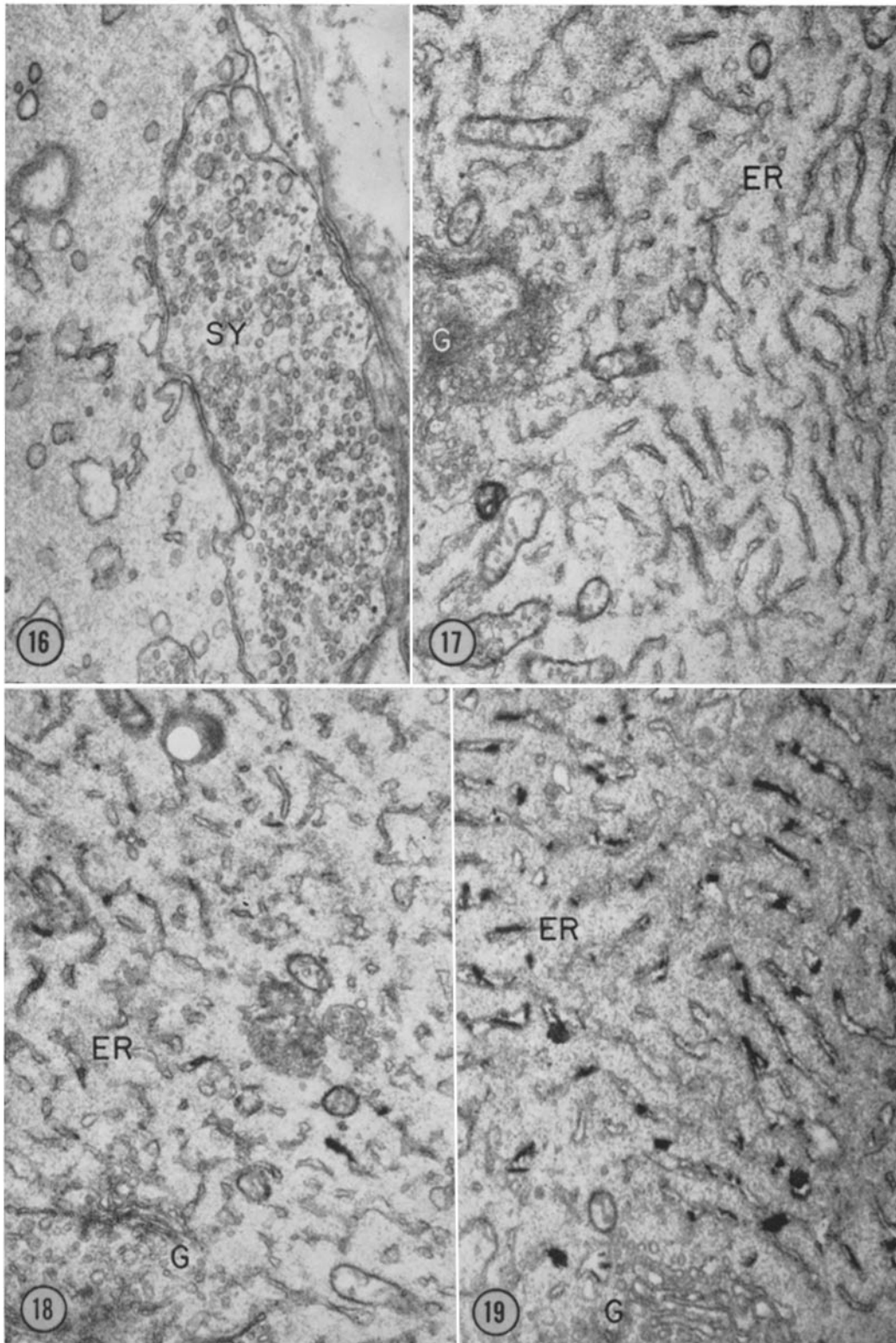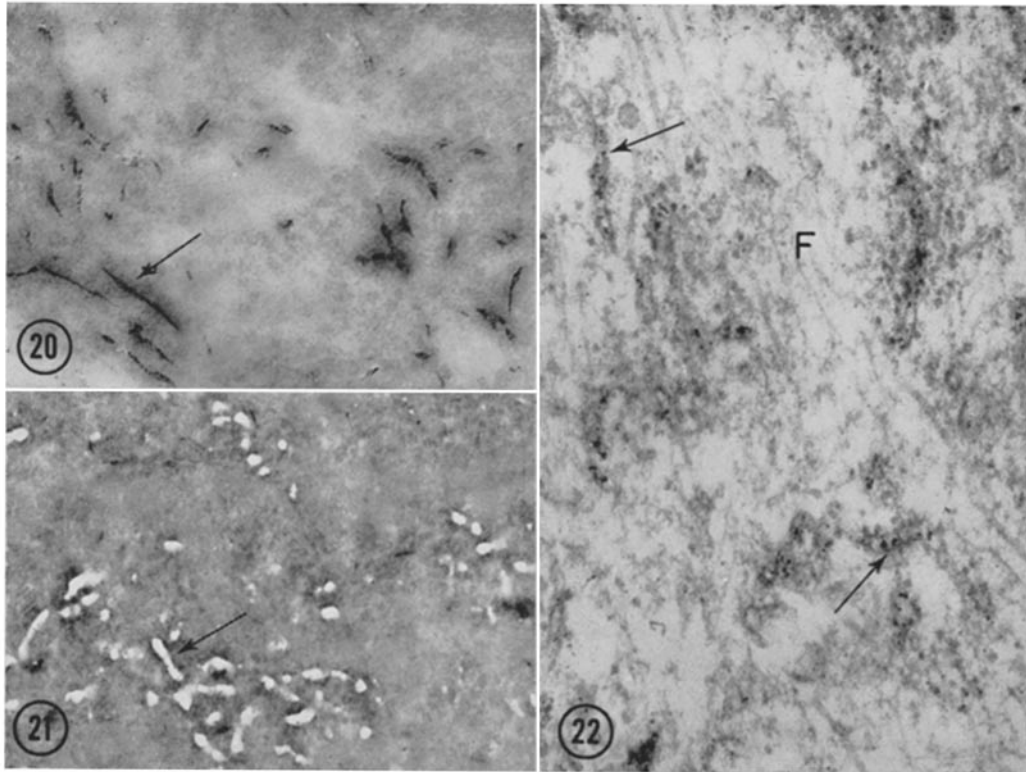
FIGURE 20   An electron micrograph illustrating the copper thiocholine end product in the cisternae of the endoplasmic reticulum (arrow). The tissue was not postfixed prior to dehydration. (AThCh + iso-OMPA, 30 min; unstained section). × 26,500.

FIGURE 21   An electron micrograph of a tissue section, similar to that in Fig. 20, which was treated with uranyl acetate. Holes (arrow) are present in the section at the former site of the copper thiocholine end product. × 19,000.

FIGURE 22   An electron micrograph illustrating the end product after conversion to sulfide. Fine 100- to 200-A particles (arrows) and larger aggregates of this substance fill the cisternae of the endoplasmic reticulum of a filamented (F) neuron from a dorsal root ganglion. AThCh + iso-OMPA, 30 min; H₂S in maleate buffer, 30 min; postfixed in OsO₄, stained section. × 68,000.

therefore, only relatively specific as to the degree of inhibition. The reasons for these differences are still poorly understood. Few esterases have been analyzed in this respect, since pure or highly purified enzyme preparations are required for such information, and very few of these are available. The difficulty of a clear-cut distinction is greatly increased, when inhibitors are applied, in cytochemical studies, to enzymes within an organized structure where the ability of penetration, the pH, and the charges surrounding the enzyme, and a variety of factors may readily interfere with the reaction. On the other hand, in combination with

chemical studies on the rate of hydrolysis of acetylcholine and butyrylcholine (Tables I and II), the differences of the effects of inhibitors observed support the assumption that the enzyme in this tissue is an acetylcholinesterase. These results in peripheral ganglia are consistent with a histochemical and manometric study of the central nervous system of the frog by Shen et al. (66), who were able to detect only specific cholinesterase.

The cytochemical method employed in this study has the advantage of being essentially a one-step reaction in which the original reaction prod-

TABLE VI

Relationship between ChE Activity and Structural Condition of Cells Isolated from the Dorsal Root Ganglion

The activity is expressed in μl $CO_2 \times 10^{-5}$/hr/sample.

Cells with high activity	Cells with low activity
63.3 Partially ruptured sheath, neural plasmalemma only partially intact, short segment of axon	6.9 Two neurons. Sheaths surround both neurons, neural plasmalemmas intact
57.0 Sheath and neural plasmalemma absent	5.4 Two neurons. Sheaths surround both neurons, neural plasmalemmas intact
54.6 Sheath and neural plasmalemma absent	4.5 Sheath surrounds neuron, neural plasmalemma intact
49.5 Sheath and neural plasmalemma absent	3.6 Sheath surrounds neuron, neural plasmalemma intact, short segment of axon
49.5 Ruptured sheath, neural plasmalemma only partially intact, short segment of axon	3.0 Sheath surrounds neuron, neural plasmalemma intact, short segment of axon
47.1 Sheath and neural plasmalemma absent	2.4 Sheath surrounds neuron, neural plasmalemma intact, short segment of axon

uct may be visualized without further conversion. Postfixation by potassium permanganate probably oxidizes the copper thiocholine end product, thereby increasing its density and rendering it less susceptible to removal by other reagents used in routine electron microscope techniques. It is unlikely that treatment of the tissue with potassium permanganate for the length of time recommended in this study alters significantly the localization or the amount of end product present. This is indicated by the flotation experiments using thin sections of tissue containing the copper thiocholine end product untreated with the usual fixatives. After flotation on the surface of potassium permanganate for 30 min, the end product remains in the same sites as in the untreated section.

The preciseness with which enzymatic activity can be localized in any cytochemical method is dependent also on the size of the end product. Despite the advantages of this method, the smallest end product particle is about 250 to 300 A in diameter, which is somewhat larger than the diameter of cellular membranes and of the usual intercellular clefts.

As stated by Malmgren and Sylvén (43), the initial small deposits are the only part of the copper thiocholine end product which may reliably be ascribed to enzymatic activity. The presence of copper ion and copper thiocholine in the medium may cause growth of the original minute deposits into larger needle-shaped crystals to a degree, dependent upon the time of incubation and the

rate of diffusion of uncaptured thiocholine from the active site (43, 79).

Conversion of copper thiocholine to copper sulfide by use of sodium sulfide (39, 40), ammonium sulfide (33), or by hydrogen sulfide (26) as employed in this study, results in very fine dense particles about 100 to 200 A in diameter. These particles tend to be concentrated along membranes after short incubation periods, but fill the cisternae and the intercellular clefts after longer incubation. Although copper sulfide is a smaller particle, its localization is not more precise than the somewhat larger copper thiocholine crystal, since copper sulfide is derived from the latter.

Displacement of the end product may be seen when a weak salt or buffer is present in the converting medium, but this is much less pronounced than with ammonium sulfide. Since OsO_4 readily reacts with copper thiocholine, complete conversion to copper sulfide is necessary prior to postfixation. Other displacement artifacts could be due to removal of the unconverted end product by OsO_4 or staining solutions. The localization of copper sulfide does not appear to be affected by the short postfixation in OsO_4 used in this study. In spite of the disadvantages listed above, a converted OsO_4-treated specimen is desirable in certain instances since permanganate fixation does not demonstrate ribonucleoprotein particles satisfactorily. The visualization of these particles is useful for the identification of dendrites in the neuropil. These specimens should be compared

with unconverted, permanganate-treated tissue in which displacement of end product is less likely.

Localization

Short incubation periods produce a small end product which permits precise localization of enzymatic activity. Under these conditions, it was possible to show that the end product appears first on the internal surface of the membranes of the endoplasmic reticulum and projects into the cisternae. Longer incubation times demonstrate a greater number of reactive sites in a tissue, but the individual sites themselves are usually over-reacted, as judged by the larger end product. It should be emphasized that the presence of enzymatic activity in the nuclear envelope should not be confused with the diffusion artifact of nuclear staining, since there is no end product within the nucleoplasm. A similar reaction within the nuclear envelope was also reported in heart muscle (29) and neurons (40). Since the outer membrane of the nuclear envelope is often continuous with the endoplasmic reticulum (76), the presence of end product within the nuclear envelope is not surprising and may reflect the relationship between these structures.

At certain other intercellular areas in which cell membranes are more closely apposed, the end product cannot be so precisely localized as in the endoplasmic reticulum. In these sites, such as the synaptic regions, the interface of neural cytoplasm and sheath cytoplasm, and concentric sheath folds, the end product extends from one membrane to the other, filling the cleft. Thus, it cannot be determined whether the enzymatic activity is localized at one or the other membrane, or is within the cleft.

The use of thiolacetic acid as a substrate (6, 32, 46, 67, 73) is reported to be advantageous for the precise localization of the end product in association with cell membranes, but the same degree of specificity is not achieved. The thiolacetic acid method and the method used in this study show a similar localization of end product in certain sites, but also some discrepancies. The demonstration of enzymatic activity in association with membranes of the endoplasmic reticulum of neurons of the rat by the use of thiolacetic acid as substrate (32, 46, 73) is confirmed by the use of acetylthiocholine as substrate in the frog (72) and also in the rat (40). Enzymatic activity may also be obtained within the axon process (39, 46, 65, 72, 73), along the axonal surface (32, 39, 65, 67, 72), and along sheath folds (12, 32, 67, 72). It is likely that at these common sites of activity thiolacetic acid may indeed be hydrolyzed by a cholinesterase.

The localization of enzymatic activity at synaptic regions is more difficult to evaluate, particularly since the enzyme appears to be sensitive to fixatives at this site. At least some synapses show



FIGURE 23 Phase micrograph of the sample which showed a high enzymatic activity of 63.3 × 10⁻⁵ μl CO_2/hr. A granular neuron and a small segment of its axon (*AX*) are surrounded by a satellite sheath (*S*) and a small amount of connective tissue. Some vacuolated areas are present at the junction of the neuron and sheath cell (at arrow). Oil immersion. × 1250.

FIGURE 24 An electron micrograph illustrating the ruptured satellite sheath and neural plasmalemma of the cell in Fig. 23. The cisternae of the Nissl body (*NB*) beneath the rupture are not dilated. × 11,000.

FIGURE 25 An electron micrograph illustrating the swollen mitochondria (*M*) of the cell in Fig. 23. The nucleus (*N*), nuclear envelope, and dense bodies (*B*) are not significantly altered. × 22,000.

FIGURE 26 Phase micrograph of the sample which showed a high enzymatic activity of 54.6 × 10⁻⁵ μl CO_2/hr. Although this granular neuron retained its contour, the surface is slightly roughened and damage is evident at the base. No sheath cell is evident. Oil immersion. × 1400.

FIGURE 27 Electron micrograph of the surface of the cell in Fig. 26. The sheath and neural plasmalemma are absent. Cisternal components of the Nissl body (*NB*) show very little swelling. × 23,000.
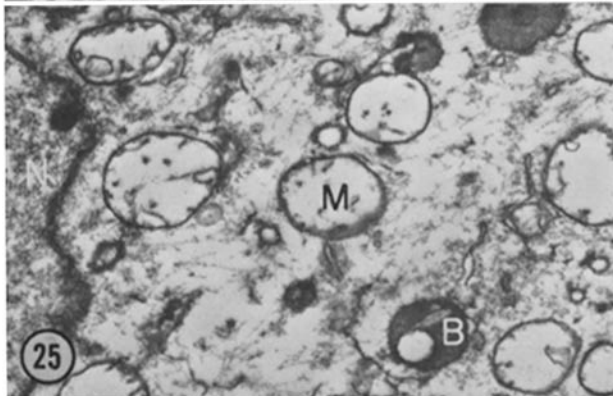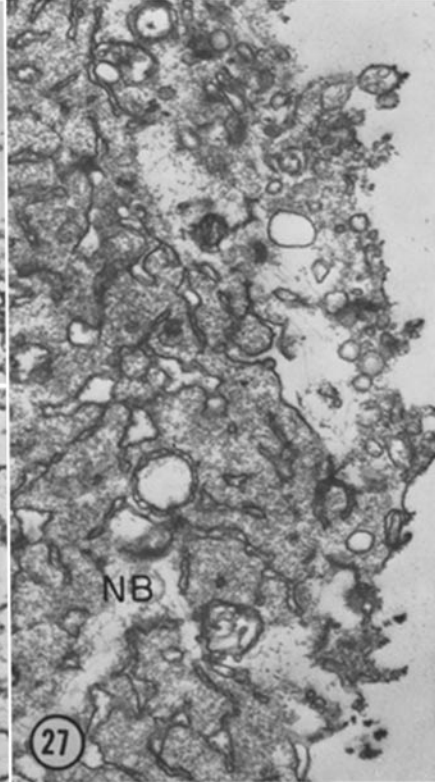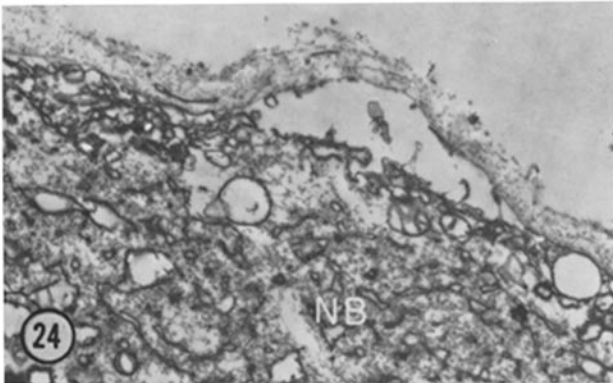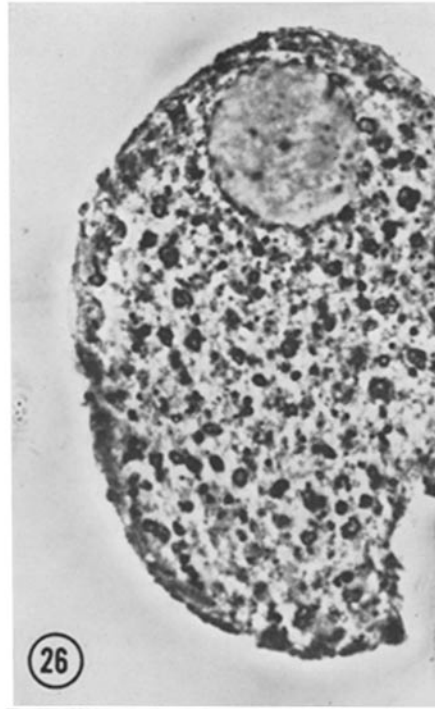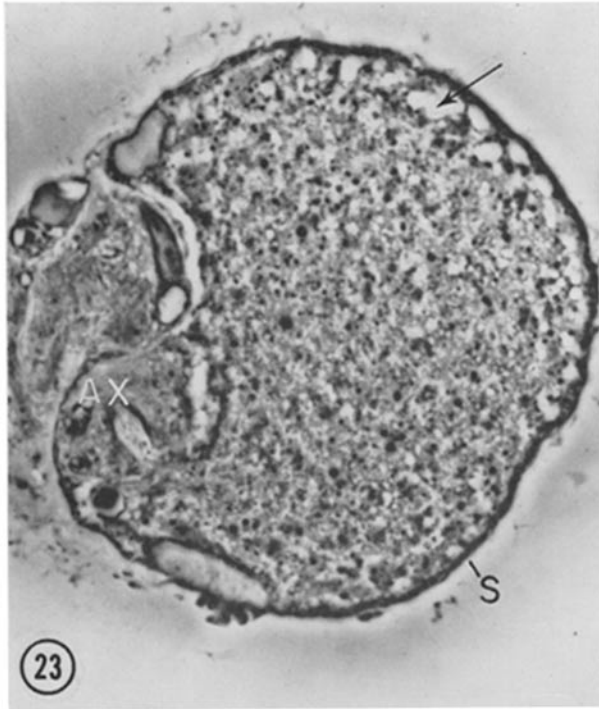
activity with one or the other substrate (12, 16, 32, 39, 46, 67, 72, 73), either on the opposing membranes, within the synaptic cleft, or on or within synaptic vesicles.

Organelles, such as lysosomes (46, 73), Golgi vacuoles (46, 73) and some mitochondria (73), show activity with thiolacetic acid as substrate. In the present study, using acetylthiocholine as substrate, these structures did not show activity. This discrepancy suggests that, at these sites, an enzyme other than cholinesterase is involved in the studies using thiolacetic acid. Torack and Barrnett (73) have suggested that the lysosomal enzyme might be cathepsin C, because of its resistance to the inhibitor E 600.

Factors Influencing the Demonstration of Cholinesterase Activity

The quantitative differences in enzymatic activity shown by microgasometric analysis (Tables I and VI) and the difference in number of cytochemically reactive sites observed from cell to cell in the dorsal root ganglion are probably due to a number of factors. Some neurons may actually have a higher intracellular enzymatic activity than others. This can be seen with the dissecting microscope during the procedure of sulfide conversion and is also suggested by the differences in morphology of the neurons when examined with the electron microscope. In some neurons, the endoplasmic reticulum fills the cytoplasm; in others, it is sparser and scattered among bundles of filaments. The number of histochemically reactive sites is usually higher in the former. Other factors, however, such as differences in the enzyme in various parts of the cell, the presence of cellular barriers, and fixation also influence the results obtained in this study.

One of these factors is the difference in sensitivity between the cholinesterase located intracellularly and that located at the surface of the cell. The enzyme in the elements of the endoplasmic reticulum is less sensitive to inhibition by eserine than that located at the interface of the neural and sheath cells and the synapse. A difference in the effect of inhibitors upon axonal and perikaryal enzyme activity was also noted by Torack and Barrnett (73).

The results shown in Table VI strongly support the assumption that the intact, unfixed neural plasmalemma and sheath form a permeability barrier to the penetration of the substrate into the cell. The cells with a disrupted satellite sheath and plasma membrane exhibit considerably higher enzyme activity when measured gasometrically than do the undamaged cells. The intracellular enzyme measured in the damaged cells was probably that associated with elements of the endoplasmic reticulum, since this organelle was the only structure remaining in the sample, which exhibited acetylcholinesterase activity when blocks of tissue were examined cytochemically. Fractionation studies showing the presence of cholinesterase, choline acetylase, and acetylcholine in the microsome fraction of brain tissue (1, 15, 17, 27, 37, 74) support this concept. Even taking into account the relative crudeness of the fractionation procedures which invariably give fractions composed of particles from structures other than the endoplasmic reticulum, such as the plasma membrane, it is,

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FIGURE 28 Phase micrograph of the sample which showed a low enzymatic activity of 3.6×10^{-5} μl CO_2/hr. A granular neuron and a small segment of its axon (AX) are surrounded by a satellite sheath (S) and a small amount of connective tissue. The cytoplasm in the axon and in some peripheral zones of the perikaryon (arrow) is less dense than centrally. $\times$ 1250.

FIGURE 29 Electron micrograph of a portion of one of the neurons in the sample which showed a low activity of 6.9×10^{-5} μl CO_2/hr. Most of the mitochondria (M) are not altered significantly. The endoplasmic reticulum (ER) shows some swelling. $\times$ 19,000.

FIGURE 30 Electron micrograph of a portion of one of the neurons in the sample which showed a low activity of 5.4×10^{-5} μl CO_2/hr. A thin satellite sheath, only 500 A thick in some areas (arrow), surrounds the neuron. Subsurface cisternae (SSC) and randomly oriented cisternae of the endoplasmic reticulum (ER) exhibit more swelling than those related to Nissl bodies (NB). Although some mitochondria are swollen, the majority are not altered morphologically. $\times$ 7300.
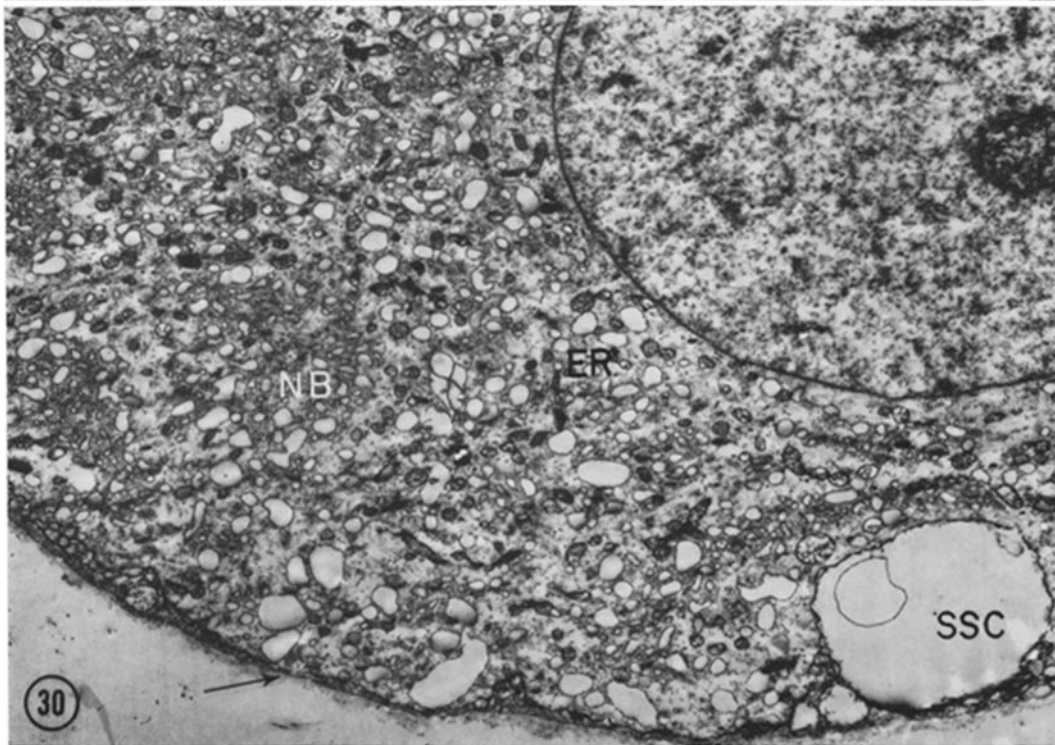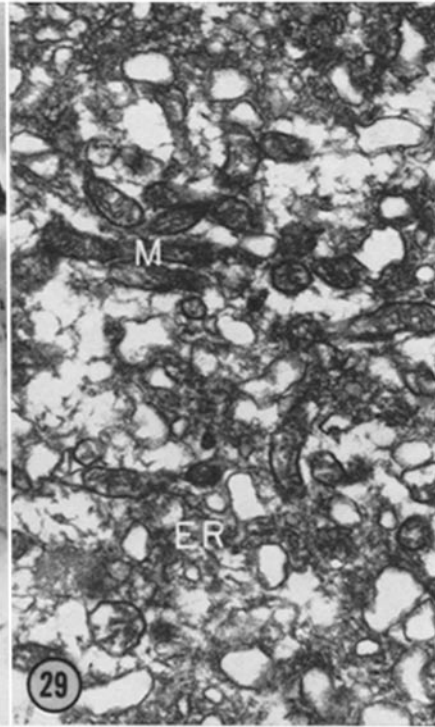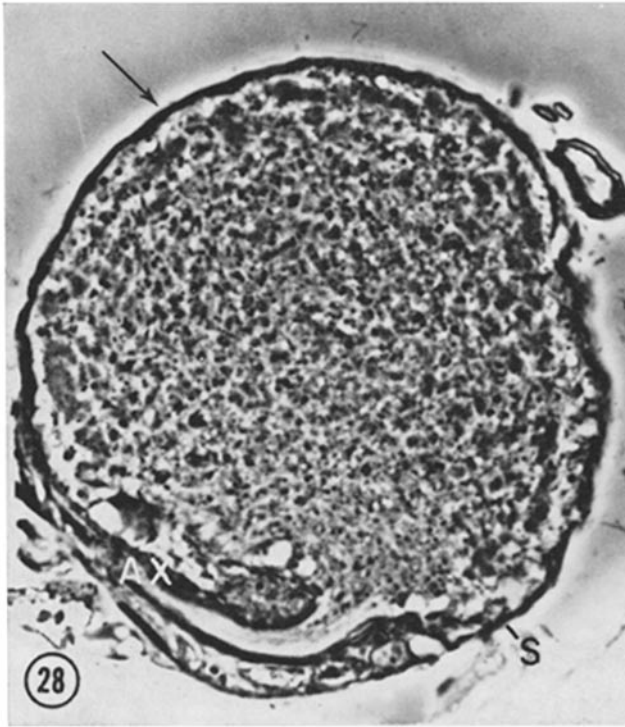
nonetheless, likely that a significant amount of the activity of the acetylcholine system is attributable to the endoplasmic reticulum.

Microgasometric measurements to determine the effect of fixation on cholinesterase activity (Tables IV and V) show a significant decrease after fixation. The cytochemical evidence presented in this study suggests that the enzyme is not affected uniformly throughout the cell, as was indicated by earlier biochemical studies of formaldehyde-fixed tissue (69). Fixation may have a number of simultaneous effects. The enzyme is at least partially inactivated by fixative, particularly at the surface of the cell. On the other hand, additional enzymatic sites are revealed by the fixative, possibly by precipitation or removal of the lipoprotein structures surrounding the enzyme. Small blocks of tissue rather than thin frozen sections were used in this study. The intact, unfixed sheath and plasmalemma of each neuron in the tissue block may impose a similar permeability barrier as seen in the combined microgasometric-electron microscopic studies (Table VI). Fixation of the tissue possibly diminishes the diffusion barrier to the incubation medium, permitting its components to reach the intracellular enzyme to a greater extent.

The data shown in Tables IV and V, therefore, probably reflect a composite of several factors. Most of the enzyme at the cell surface and at least a portion of the intracellular enzyme are probably inactivated. This could explain the spotty distribution of the end product throughout the endoplasmic reticulum. It cannot be determined, however, whether the absence of end product at any particular site is due to lack of the enzyme, inactivation by fixatives, or some other cause. Some of the intracellular enzyme appears to be at least partially "fixative resistant" in the cytochemical studies; therefore, some of the enzyme, which was not available for analysis in intact, unfixed tissue, may be measured following fixation. The higher percentage of inhibition observed in cells from the sympathetic ganglion (Table V) may reflect the inhibition of a greater concentration of surface enzyme which would be present at synapses adhering to the surface of these isolated neurons.

The observations of Giacobini (21) differ from the data presented here, in two respects. First, according to his report, Giacobini found sympathetic cells and nerve fibers in which cholinesterase was unmeasurable. In the present study in which the magnetic diver was used, all cells examined exhibited enzymatic activity with acetylcholine as substrate, in the absence of inhibition. This may be explained, at least partly, by the higher sensitivity of the technique employed here. There is a second difference, in which other factors are probably involved: the esterase activities of sympathetic cells reported here are generally lower than those found by Giacobini (21). This discrepancy is more difficult to explain, but perhaps the condition of the cell, as shown in Table VI, and the possibility of the adherence of synaptic knobs to sympathetic cells may have altered activity.

It is of interest that the acetylcholinesterase activity of sympathetic nerve fibers in the frog (Table II) is higher than that of sensory and motor fibers (8). Since enzymatic activity can be demonstrated in the Schwann sheath of the sympathetic fibers, it is not, at present, possible to determine what proportion of the activity is purely axonal.

It must be emphasized, however, that chemical determinations of isolated cells and fibers, whether with intact or disrupted sheaths or plasma membranes, may not indicate the total enzyme activity. Even in a relatively small structure, the enzymes may not be saturated, and some may not be reached at all. On the basis of previous experience, even in still smaller subcellular fragments, such as cleaned cell wall of squid giant axon (9), only a fraction of the enzyme activity present is measured. For an estimation of the total cholinesterase activity of a single cell, its disintegration should first be effected by sonication.

Significance

The question arises as to the role of acetylcholinesterase at the various sites shown in this study. The demonstration of enzymatic activity at the synapse and along the axonal surface is in agreement with physiological and biochemical data indicating a role of acetylcholinesterase in the permeability cycle of excitable membranes during transmission and conduction. This has been discussed in detail by Nachmansohn (47–49).

The significance of the finding of acetylcholesterase activity in the endoplasmic reticulum of neurons can only be speculated upon, but the absence of this activity from a similar location in sheath cells, fibroblasts, and endothelial cells in the fixed adult frog ganglion suggests that acetylcholinesterase has an important role in these neurons. Such activity at this site appears to be a

widespread characteristic of neurons in several species, although it is reported to be absent from neurons of the abdominal ganglion of the cockroach (67). Further investigation of this species with other techniques would be valuable.

The possibility that cholinesterase is synthesized in the endoplasmic reticulum of the normal adult perikaryon and is then transported via its canaliculi to the surface of the neuron and its processes, has been suggested by Koelle and co-workers (19, 32). Since acetylcholinesterase of the microsome fraction is reported to be firmly bound to membranes (74), one would question whether the enzyme is free to flow through the cisternae and tubuli of the endoplasmic reticulum. Another possible interpretation is that the organelle, itself, containing the enzyme bound to its external surface may move through the cytoplasm and axon, possibly by cytoplasmic streaming. Such a process could take place originally early in the development of the embryo. Evidence has been supplied (15, 34, 36) that acetylcholinesterase may be synthesized in the normal adult neuron, in the axon proper. Mechanisms for such a synthesis apparently do exist, even though the ribonucleic acid concentration is low (23, 35). The acetylcholinesterase in the agranular reticulum of axons, therefore, may have been synthesized at this site. The enzyme in the agranular reticulum may be unrelated functionally to that at the axolemmal surface and synapse, particularly since it is present in sympathetic postganglionic neurons not usually thought to be cholinergic.

The presence of end product within subsurface cisternae, which occur in direct apposition to the neural plasma membrane and often opposite synaptic terminals, raises interesting questions. Subsurface cisternae have been considered as a

possible functional component of the neuronal surface, as well as a link which may in some way coordinate excitation and intracellular metabolic processes (61).

The sarcoplasmic reticulum of muscle has also been shown to contain a cholinesterase (29, 75). Since evidence has accumulated in favor of the assumption that in excitable membranes the acetylcholine system is linked to the permeability cycle for ions during excitation (47-49), the question arises whether the membranes of the sarcoplasmic reticulum and endoplasmic reticulum may possess a reversible chemical mechanism controlling ion movements, similar to that in the plasma membrane. Such a possibility, still further extending the role of acetylcholine, should be examined.

Addendum

Since this manuscript was submitted, comparable cytochemical results have been reported by Novikoff, A. B., et al., *J. Cell Biol.* 1966, **29**, 525, concerning the dorsal root ganglia and peripheral nerve of the rat.

REFERENCES

1. ALDRIDGE, W. N., and JOHNSON, M. K., Cholinesterase, succinic dehydrogenase, nucleic acid, esterase and glutathione reductase in subcellular fractions from rat brain, *Biochem. J.*, 1959, **73**, 270.
2. ANDRES, K. H., Untersuchungen über den Feinbau von Spinalganglien, *Z. Zellforsch. u. Mikr. Anat.*, 1961, **55**, 1.
3. BARRNETT, R. J., The fine structural localization of acetylcholinesterase at the myoneural junction, *J. Cell Biol.*, 1962, **12**, 247.
4. BARRNETT, R. J., and PALADE, G. E., Enzymatic activity in the M band, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 163.
5. BIRKS, R. I., and BROWN, L. M., A method for locating the cholinesterase of a mammalian myoneural junction by electron microscopy, *J. Physiol.*, London, 1960, **152**, 5P(abstract).
6. BLOOM, F. E., and BARRNETT, R. J., Fine structural localization of acetylcholinesterase activity in eel electroplaques, *J. Cell Biol.*, 1965, **27**, 13A(abstract).
7. BROWN, L. M., A thiocholine method for locating cholinesterase activity by electron microscopy,

in Histochemistry of cholinesterase, Symposium, Basel, *Bibliot. Anat.*, 1961, **2**, 21.

8. Brzin, M., and Dettbarn, W.-D., Cholinesterase activity of nodal and internodal regions of myelineated nerve fibers of frog, *J. Cell Biol.*, in press.

9. Brzin, M., Dettbarn, W.-D., Rosenberg, P., and Nachmansohn, D., Cholinesterase activity per unit surface area of conducting membranes, *J. Cell Biol.*, 1965, **26**, 353.

10. Brzin, M., and Majcen-Tkačev, Ž., Cholinesterase in denervated end plates and muscle fibers, *J. Cell Biol.*, 1963, **19**, 349.

11. Brzin, M., Kovic, M., and Oman, S., The magnetic diver balance, *Compt. rend. trav. Lab. Carlsberg*, 1964, **34**, 407.

12. Brzin, M., Tennyson, V. M., and Duffy, P., Combined cytochemical-electron microscopic localization of cholinesterases in the nervous system, Symposium, *in* Biochemistry and Pharmacology of the Basal Ganglia, (E. Costa, L. J. Coté, and M. D. Yahr, editors), Hewlett, New York, Raven Press, 1966, 65.

13. Brzin, M., and Zeuthen, E., Notes on the possible use of the magnetic diver for respiration measurements (error 10^{-7} μl/hour), *Compt. rend. trav. Lab. Carlsberg*, 1964, **34**, 427.

14. Cervòs-Navarro, J., Elektronenmikroskopische Untersuchungen an Spinalganglien. I. Nervenzellen, *Arch. Psychiat. Nervenkh.*, 1959, **199**, 643.

15. Clouet, D. H., and Waelsch, H., Amino acid and protein metabolism of the brain. VIII. The recovery of cholinesterase in the nervous system of the frog after inhibition, *J. Neurochem.*, 1961, **8**, 201.

16. de Lorenzo, A. J. D., Electron microscopy of the cerebral cortex. I. The ultrastructure and histochemistry of synaptic junctions, *Bull. Johns Hopkins Hosp.*, 1961, **108**, 258.

17. De Robertis, E., Neurochemical studies in isolated nerve endings and synaptic vesicles of the CNS, *in* Histophysiology of Synapses and Neurosecretion, New York, Pergamon Press, Inc., 1964, 122.

18. Elfvin, L.-G., The ultrastructure of the superior cervical sympathetic ganglion of the cat, *J. Ultrastruct. Research*, 1963, **8**, 403.

19. Fukuda, T., and Koelle, G. B., The cytological localization of intracellular neuronal acetylcholinesterase, *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 433.

20. Fulton, M. P., and Mogey, G. A., Some selective inhibitors of true cholinesterases, *Brit. J. Pharmacol.*, 1954, **9**, 138.

21. Giacobini, E., Quantitative determination of cholinesterase in individual sympathetic cells, *J. Neurochem.*, 1957, **1**, 234.

22. Giacobini, E., Quantitative determination of cholinesterase in individual spinal ganglion cells, *Acta Physiol. Scand.*, 1959, **45**, 238.

23. Grampp, W., and Edström, J. E., The effect of nervous activity on ribonucleic acid of the crustacean receptor neuron, *J. Neurochem.*, 1963, **10**, 725.

24. Hanker, J. S., Seaman, A. R., Weiss, L. P., Ueno, H., Bergman, R. A., and Seligman, A. M., Osmiophilic reagents: New cytochemical principle for light and electron microscopy, *Science*, 1964, **146**, 1939.

25. Hawkins, R. D., and Mendel, B., True cholinesterase with pronounced resistance to eserine, *J. Cell. and Comp. Physiol.*, 1946, **27**, 69.

26. Holmstedt, B., A modification of the thiocholine method for the determination of cholinesterase. II. Histochemical application, *Acta Physiol. Scand.*, 1957, **40**, 331.

27. Holmstedt, B., and Toschi, G., Enzymatic properties of cholinesterase in subcellular fractions of rat brain, *Acta Physiol. Scand.*, 1959, **47**, 280.

28. Johnston, P. V., and Roots, B. I., The neurone surface, *Nature*, 1965, **205**, 778.

29. Karnovsky, M. J., The localization of cholinesterase activity in rat cardiac muscle by electron microscopy, *J. Cell Biol.*, 1964, **23**, 217.

30. Koelle, G. B., The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons, *J. Pharmacol. and Exp. Therap.*, 1955, **114**, 167.

31. Koelle, G. B., Cytological distributions and physiological functions of cholinesterases, *in* Handbuch der Experimentellen Pharmakologie, (G. B. Koelle, editor), Berlin, Springer-Verlag, 1963, **15**, 187.

32. Koelle, G. B., and Foroglou-Kerameos, C., Electron microscopic localization of cholinesterases in a sympathetic ganglion by a gold-thiolacetic acid method, *Life Sciences*, 1965, **4**, 417.

33. Koelle, G. B., and Friedenwald, J. S., A histochemical method for localizing cholinesterase activity, *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 617.

34. Koenig, E., Synthetic mechanisms in the axon. I. Local axonal synthesis of acetylcholinesterase, *J. Neurochem.*, 1965, **12**, 343.

35. Koenig, E., Synthetic mechanisms in the axon. II. RNA in myelin-free axons of the cat, *J. Neurochem.*, 1965, **12**, 357.

36. Koenig, E., and Koelle, G. B., Mode of regeneration of acetylcholinesterase in cholinergic neurons following irreversible inactivation, *J. Neurochem.*, 1961, **8**, 169.

37. Laverty, R., Michaelson, I. A., Sharman,

R. M., and WHITTAKER, V. P., The subcellular localization of dopamine and acetylcholine in the dog caudate nucleus, *Brit. J. Pharma.ol.*, 1963, **21**, 482.

38. LEHRER, G. M., and ORNSTEIN, L., A diazo coupling method for the electron microscopic localization of cholinesterase, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 399.

39. LEWIS, P. R., and SHUTE, C. C. D., Demonstration of cholinesterase activity with the electron microscope, *J. Physiol., London*, 1964, **175**, 5P(abstract).

40. LEWIS, P. R., PINE, A. B., and SHUTE, C. C. D., The electron microscopic distribution of cholinesterase in rat brain, *J. Physiol.*, 1965, **131**, 15P(abstract).

41. LUFT, J. H., Permanganate—a new fixative for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 799.

42. LUFT, J. H., Improvements in epoxy embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.

43. MALMGREN, H., and SYLVÉN, B., On the chemistry of the thiocholine method of Koelle, *J. Histochem. and Cytochem.*, 1955, **3**, 441.

44. MILEDI, R., Electron microscopical localization of products from histochemical reactions used to detect cholinesterase in muscle, *Nature*, 1964, **204**, 293.

45. METUZALS, J., Ultrastructure of myelinated nerve fibers in the central nervous system of the frog, *J. Ultrastruct. Research*, 1963, **8**, 30.

46. MORI, S., MAEDA, T., and SHIMIZU, N., Electron-microscopic histochemistry of cholinesterases in the rat brain, *Histochemie*, 1964, **4**, 65.

47. NACHMANSOHN, D., Chemical and Molecular Basis of Nerve Activity, New York, Academic Press Inc., 1959.

48. NACHMANSOHN, D., Chemical control of ion movements across conducting membranes, *in* New Perspectives in Biology, *Biochimica et Biophysica Acta*, Library, (M. Sela, editor), Amsterdam, Elsevier Publishing Company, 1964, **4**, 176.

49. NACHMANSOHN, D., Chemical control of bioelectric currents in membranes of conducting cells, *J. Mt. Sinai Hosp.*, 1964, **31**, 549.

50. PALADE, G. E., A study of fixation for electron microscopy, *J. Exp. Med.*, 1952, **95**, 285.

51. PAVLIN, R., Cholinesterases in reticular nerve cells, *J. Neurochem.*, 1965, **12**, 515.

52. PEARSE, A. G. E., Carboxylic esterases, *in* Histochemistry, Theoretical and Applied (A. G. E., Pearse, editor), Boston, Little, Brown and Company, 1960, 456.

53. PICK, J., The submicroscopic organization of the sympathetic ganglion in the frog (*Rana pipiens*), *J. Comp. Neurol.*, 1963, **120**, 409.

54. PICK, J., The fine structure of sympathetic neurons in X-irradiated frogs, *J. Cell Biol.*, 1965, **26**, 335.

55. PICK, J., GERDIN, C., and DeLEMOS, C., On the ultrastructure of spinal nerve roots in the frog (*Rana pipiens*), *Anat. Rec.*, 1963, **146**, 61.

56. REAL, E., and LUCIANO, L., A probable source of errors in electron-histochemistry, *J. Histochem. and Cytochem.*, 1964, **12**, 713.

57. REYNOLDS, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, 1963, **17**, 208.

58. ROBERTSON, J. D., BODENHEIMER, T. S., and STAGE, D. E., The ultrastructure of Mauthner cell synapses and nodes in goldfish brains, *J. Cell Biol.*, 1963, **19**, 159.

59. ROOTS, B. I., and JOHNSTON, P. V., Neurons of ox brain nuclei: their isolation and appearance by light and electron microscopy, *J. Ultrastruct. Research*, 1964, **10**, 350.

60. ROOTS, B. I., and JOHNSTON, P. V., Isolated rabbit neurons: electron microscopical observations, *Nature*, 1965, **207**, 315.

61. ROSENBLUTH, J., Subsurface cisterns and their relationship to the neuronal plasma membrane, *J. Cell Biol.*, 1962, **13**, 405.

62. ROSENBLUTH, J., Contrast between osmium-fixed and permanganate-fixed toad spinal ganglia, *J. Cell Biol.*, 1963, **16**, 143.

63. ROSENBLUTH, J., and PALAY, S. L., Electron microscopic observations on the interface between neurons and capsular cells in dorsal root ganglia of the rat, 1960, **136**, 268, (abstract).

64. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.

65. SCHLAEPFER, W. W., and TORACK, R. M., The ultrastructural localization of cholinesterase activity in the sciatic nerve of the rat, *J. Histochem. and Cytochem,*, 1966, **14**, 369.

66. SHEN, S. C., GREENFIELD, P., and BOELL, E. J., The distribution of cholinesterase in the frog brain, *J. Comp. Neurol.*, 1955, **102**, 717.

67. SMITH, D. S., and TREHERNE, J. E., The electron microscopic localization of cholinesterase activity in the central nervous system of an insect, *Periplaneta Americana L.*, *J. Cell Biol.*, 1965, **26**, 445.

68. SMIRNOW, A., Die Struktur der Nervenzellen im Sympatheticus der Amphibien, *Archiv mikr. Anat. u. Entwicklngsmech.*, 1890, **35**, 407.

69. TAXI, J., Action du formol sur l'activité de

diverses préparationes de cholinesterases, *J. physiol. et path. gén.*, 1952, **44**, 595.

70. TAXI, J., Étude de l'ultrastructure des zones synaptiques dans les ganglions sympathétiques de la grenouille, *Comp. rend. Acad. sc.*, 1961, **252**, 174.

71. TAXI, J., Contribution a l'ètude des connexions des neurones moteurs du systeme nerveux autonome, *Ann. Sc. Nat., Zool.*, 1965, **7**, 416.

72. TENNYSON, V., BRZIN, M., and DUFFY, P. E., Electron microscopic localization of acetylcholinesterase in the neurons of frog sympathetic ganglia, *J. Cell Biol.*, 1965, **27**, 105A (abstract).

73. TORACK, R. M., and BARRNETT, R. J., Fine structural localization of cholinesterase activity in the rat brain stem, *Exp. Neurol.*, 1962, **6**, 224.

74. TOSCHI, G., A biochemical study of brain microsomes, *Exp. Cell Research*, 1959, **16**, 232.

75. ULBRECHT, G., and KRUCHKENBERG, P., Acetyl-

cholinesterase in the sarcoplasmic reticulum of skeletal muscle, *Nature*, 1965, **206**, 305.

76. WATSON, M. L., The nuclear envelope. Its structure and relation to cytoplasmic membranes, *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 257.

77. WATSON, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 475.

78. YAMAMOTO, T., Some observations on the fine structure of the sympathetic ganglion of bullfrog, *J. Cell Biol.*, 1963, **16**, 159.

79. ZAJICEK, J., SYLVÉN, B., and DATA, N., Attempts to demonstrate acetylcholinesterase activity in blood and bone-marrow cells by a modified thiocholine technique, *J. Histochem. and Cytochem.*, 1954, **2**, 115.

80. ZAJICEK, J., and ZEUTHEN, E., Quantitative determination by a special "ampulla-diver" of cholinesterase activity in individual cells, with notes on other uses of the method, *in* General Cytochemical Methods, (J. F. Danielli, editor), New York, Academic Press Inc., 1961, **2**, 131.