ELECTRON MICROSCOPE LOCALIZATION OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE IN THE SUPERIOR CERVICAL GANGLION OF THE CAT

I. Normal Ganglion

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ABSTRACT

The distributions of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in the superior cervical ganglion (SCG) of the cat were determined by electron microscopy (EM) with the bis-(thioacetoxy)aurate (I), or Au(TA)₂, method. Before the infusion of fixative, one of the enzymes was selectively, irreversibly inactivated in vivo, as confirmed by light microscope (LM) examination of sections of the stellate ganglion stained by the more specific copper thiocholine method. Physostigmine-treated controls, for inhibition of AChE or BuChE, were stained concomitantly with tissue for enzyme localization by the Au(TA)₂ method for EM examination in each experiment. It was concluded that most of the AChE of the cat SCG is present in the plasma membranes of the preganglionic axons and their terminals, and in the dendritic and perikaryonal plasma membranes of the postsynaptic ganglion cells. BuChE is confined largely to the postsynaptic neuronal plasma membranes. Reasons for the discrepancies between the localizations found by the present direct EM observations and those deduced earlier from LM comparisons of normal and denervated SCG are discussed. It is proposed that a trophic factor released by the preganglionic terminals is probably required for the synthesis of postsynaptic neuronal AChE, and that BuChE may serve as a precursor of AChE at that site.
enzymes was observed throughout the neuropil, and intense staining for AChE was noted also in the perikarya of occasional (<1%, see reference 23) cholinergic ganglion cells. Within a few days after preganglionic denervation, there was a nearly total disappearance of AChE-staining from the neuropil but only a relatively small decrease in the intensity of staining for BuChE. This pattern remained essentially unchanged for several weeks, and was consistent with the results of quantitative studies (35, 62). From this and other evidence it was concluded that, in the normal ganglion, AChE is confined largely to the preganglionic fibers and their terminals, and BuChE to the capsular glial or Schwann cells.

A decade ago it was possible with the gold-thiolacetic acid (37) and gold-thiocholine (38) methods to demonstrate by electron microscopic (EM) the discrete localization of AChE and pseudo-ChE at the pre- and postjunctional membranes of the mouse motor endplate, with a minimum of end product in the junctional cleft (10). However, neither of these methods provided satisfactory EM localization of the enzymes in the SCG, where the synaptic clefts and other intermembranous distances are considerably narrower, and AChE is present at lower concentration and in a more soluble, possibly different isoenzymatic form (41). These obstacles have since been largely overcome. The bis-(thioacetoxy) aurate (I), or Au(TA)2, method, in which both the substrate and capturing agent are present in a readily penetrating single complex, (Au(TA)2)−, permits extremely precise localization of the immediately formed final reaction product, aurous sulfide; artifacts due to diffusion or translocation of the finely granular, electron-opaque precipitate from sites of enzyme activity are thus minimized (33, 36). In addition, procedures have been developed for the selective, irreversible inactivation of either AChE or BuChE in vivo (34, 43), and conditions of prefixation with buffered formaldehyde have been established that afford an acceptable compromise between degree of inactivation of the enzymes and limitation of their diffusion with a reasonable degree of morphological preservation (35).

The conclusions from the present, direct EM observations of the cytological localizations of AChE and BuChE in the cat SCG are at considerable variance with those of the earlier LM studies, which were based largely on the aforementioned indirect evidence. The most likely reasons for the discrepancies and their implications are considered in the Discussion.

MATERIALS AND METHODS

Denervation

In all EM investigations, the right, normal SCG was compared with the left SCG which had been denervated preganglionically 6–35 days previously. Findings in the denervated ganglia will be described in a subsequent paper. Cats were anesthetized with sodium pentobarbital, 35 mg/kg, intraperitoneally, and under semisterile conditions the contents of the left carotid sheath were exposed just caudal to the larynx; the common carotid artery was separated and a 1.5–2-cm segment of the remainder (vagosympathetic trunk and internal jugular vein) was excised between silk ligatures. After the skin incision was sutured, the cat was given an intramuscular injection of 1.0 ml of Combiotic (procaine penicillin G plus dihydrostreptomycin sulfate, Pfizer Chemicals Div., Pfizer, Inc., New York).

Selective Inactivation of BuChE or AChE

The cat was anesthetized as before, and either BuChE or AChE was irreversibly, selectively inactivated by minor modifications of procedures described previously (34, 43). BuChE was inactivated by the intravenous injection of 3.0 μmol of tetramonoisopropyl pyrophosphotetramide (iso-OMPA)/kg, which causes inactivation of over 98% of the ganglionic BuChE with no detectable loss of AChE. For the selective inactivation of AChE, the femoral artery was first catheterized to record blood pressure by means of a transducer coupled with a Physiograph, type DMP-4A (Narco-Bio-Systems, Inc., Houston, Texas), and the femoral vein was catheterized for injections; artificial respiration was administered by means of a tracheal catheter attached to a Palmer pump. After the injection of atropine sulfate (1.0 mg/kg, intraperitoneally, and 1.0 mg/kg, intravenously) and 3.0 mg mephentermine sulfate/kg, intravenously, to insure a patent airway and elevate the blood pressure, respectively, an intravenous infusion of the reversible, selective BuChE-inhibitor, 10-(α-diethylamino)propionyl) phenothiazine HCl (Astra 1397), was given in a total dose of 100–200 μmol/kg over approx. 15 min, as rapidly as possible without causing a lethal fall in blood pressure; after an interval of 3 min, this was followed by the intravenous injection of 2.0 μmol/kg of the irreversible inactivator of AChE and BuChE, isopropyl methylphosphonofluoridate (sarin). With this procedure, approx. 98% of the ganglionic AChE is inactivated irreversibly while approx. 50% of the BuChE is preserved (43). In the earlier experiments of this series, 2-diethoxyphosphinylthioethylidemethylamine acid oxalate (217 AO), 1.0 μmol/kg, was given in place of sarin.
ously to iso-OMPA, 10^-3 M at 5~ for 1 h, were in-
its use here permitted all portions of the SCG to be
qualitatively identical with those in the SCG (31), and
were placed on slides and stained by the more specific
acetylthiocholine (AThCh) or butyrylthiocholine (Bu-
30, 60, and 120 min in the standard media containing
distributions of AChE and BuChE in this ganglion are
were cut from a portion of the steUate ganglion that had
processed for the EM studies. Slides were incubated for
in veronal acetate buffer (56) for 2 h at 4~ dehydrated
order to insure adequate removal of the unreacted gold
and sectioned at 150 [m with a Smith-Farquhar Sorvall
sections exhibiting yellow-to-gold interference colors
were cut with a diamond knife on a Sorvall Porter-Blum
ethanol, and finally embedded in Epon 812 (50). Thin
sections were cut with a razor blade into chunks of approx. 1 mm^3. In
some experiments, one-half was embedded in 7% agar and sectioned at 150 [m with a Smith-Farquhar Sorvall
TC-2 tissue sectioner (DuPont Co., Instrument Products
Div., Wilmington, Del.). However, after it was found
that all reagents in the incubation medium apparently
penetrate to a considerable depth in formalin-fixed tis-
sue, only chunks were employed. The chunks and sec-
tions were left in the fixative at 2^C, with constant
shaking at 120 cycles/min on a Fisher Rotator (Fisher
Scientific Co., Pittsburgh, Pa.), for a total of 6 h from
the time of perfusion with fixative in situ. They were
then transferred to cold (2^C) Krebs-Ringer-calcium
solution in which they were kept overnight. This type of
fixation produces inactivation of approx. half the remain-
ing AChE and BuChE activities of the ganglia (35), but
appears to restrict effectively enzymatic diffusion (18)
and produce acceptable anatomical preservation, as de-
scribed below.

Copper Thiocholine Controls
In order to insure that the foregoing treatments in
vivo produced essentially total inactivation of ganglionic
BuChE or AChE, with preservation of most of the
activity of the other enzyme, 10-[mu]m cryostat sections
were cut from a portion of the stellate ganglion that had
been removed from each cat and fixed, and sections
were placed on slides and stained by the more specific
CuThCh method (31, 35) for examination by LM. The
distributions of AChE and BuChE in this ganglion are
qualitatively identical with those in the SCG (31), and
its use here permitted all portions of the SCG to be
processed for the EM studies. Slides were incubated for
30, 60, and 120 min in the standard media containing
acetylthiocholine (AThCh) or butyrylthiocholine (Bu-
ThCh) as substrate. In addition, slides exposed previ-
ously to iso-OMPA, 10^-3 M at 5^C for 1 h, were in-
cluded; this produces essentially total inactivation of
BuChE without detectable loss of AChE (34). Staining
with ATCh but not with BuThCh, with or without iso-
OMPA-treatment, was considered confirmation of selec-
tive inactivation of BuChE and preservation of AChE.
Staining with BuThCh and lighter staining with ATCh, both of which were blocked by prior treatment with iso-
OMPA, was accepted as evidence of AChE-inactivation
and retention of BuChE. In all EM observations de-
scribed below, these criteria of specificity were met.

Physostigmine Controls
Since the Au(TA)_2 method is not highly specific, it is
necessary also to employ controls in which the enzyme
being studied by EM is inhibited selectively in order to
rule out staining due to enzymes other than AChE or
BuChE, or to adsorption of the (Au(TA)_2)^+ complex
or its breakdown products. For this purpose physostigmine,
a highly selective inhibitor of AChE and BuChE, was
employed. Only at sites where staining was blocked in
remaining AChE or BuChE activity be identified with certainty. Control
chunks and Smith-Farquhar sections were kept in Krebs-
Ringer-calcium solution containing 10^-3 M physostig-
mine overnight to insure its adequate penetration; in the
preincubation and incubation solutions, the concentra-
tion was reduced to 3 x 10^-8 M.

Histochemical Reaction
The day after removal and fixation of tissue, chunks
and sections were stained for AChE or BuChE by the
Au(TA)_2 method described previously (36). During 1 h
in the preincubation solution, 3-6 h in the incubation
solution (changed hourly), and 1 h in the postincubation
solution, the temperature was maintained at 5 ± 1^C
and the vessels were shaken at 120 cycles/min on a Fisher Rotator. (All findings reported here were ob-
tained with 6-h incubation.) The tissues were then
transferred to the rinse solution (4% formaldehyde in
maleate buffer, pH 7.0) and stored overnight at 4^C in
order to insure adequate removal of the unreacted gold
complex. Subsequently, they were postfixed in 1% OsO_4
in veronal acetate buffer (56) for 2 h at 4^C, dehydrated
in a series of increasingly concentrated solutions of
ethanol, and finally embedded in Epon 812 (50). Thin
sections exhibiting yellow-to-gold interference colors
were cut with a diamond knife on a Sorvall Porter-Blum
MT-2 ultramicrotome (Du Pont Co., Instrument Prod-
ucts Div., Wilmington, Del.) and mounted on unfilmed
copper grids; no further treatment to enhance contrast
was employed. Yellow-to-gold sections were selected
over thinner (silver) ones because the latter proved to be
very fragile in the electron beam when not supported by
a carbon film; also, the background intracellular detail
such as unstained membranous elements, cytoplasm,
and nucleoplasm appear in better contrast in the final

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electron micrographs when moderately thicker sections are used. Sections were examined and micrographs were taken with a Philips EM-300 electron microscope, operated at 60 kV.

Many preliminary experiments were performed in the course of developing the foregoing procedures. For the final series, conducted as described above, nine normal (plus nine denervated) SCGs were examined for the localization of AChE, and six normal (plus five denervated) SCGs for BuChE.

RESULTS

Prelude

Cat SCG cells are multipolar (60), 25–32 μm in diameter (11), and >99% are probably adrenergic (23). Early LM cytological studies (3, 11, 60) described myelinated presynaptic axons from upper thoracic rami entering the ganglion via the cervical sympathetic trunk, losing their myelin, and branching extensively to synapse with one or several of the numerous (up to 10 or 12), long, extensively branched postsynaptic dendrites. No presynaptic axons were found to pass through the ganglion without synapsing (60). Axons of the ganglion cells were more difficult to identify, but those that could be traced did not appear to arise from the cell body, except in rare instances, but rather issued in nearly all cases from a dendrite, usually at some distance from the cell body (11). Furthermore, they did not appear to give off collateral branches, leading to the conclusion that postganglionic axons always end in the periphery and never in the ganglion of their origin (11, 60). On the basis of combined histological and physiological studies, it was concluded that a considerable number of the postganglionic axons acquire myelin sheaths before exiting via a postganglionic branch, and that the cervical sympathetic trunk and SCG contain few if any afferent fibers (3, 60). It should also be noted that small intensely fluorescent (SIF) or chromaffin cells, which are numerous in the rat SCG (17) and have been considered to function there as interneurons, are extremely rare in the cat SCG and consist almost exclusively of the type (II) devoid of efferent processes (69). Since EM studies of the cat SCG after preganglionic denervation failed to reveal the presence of any typical synaptic junctions (21, 45), it is unlikely that the normal ganglion contains significant numbers of interneurons or recurrent collaterals. The foregoing studies therefore are for the most part consistent with a simplified view of the cat SCG as a fairly uniform population of adrenergic cell bodies innervated by cholinergic preganglionic fibers; evidence for the presence of recurrent axon collaterals, interneurons, or afferent fibers is generally lacking.

Several EM studies (4, 14, 15, 21, 45, 57, 58) and reviews (20, 52) have dealt with the morphology of the cat SCG. For interpretation of the present findings, we are particularly indebted to the painstaking studies by Elfvin (14, 15) made on three-dimensional reconstructions from extensive numbers of serial sections. Elfvin described presynaptic unmyelinated axons of small diameter (0.1–0.3 μm) paralleling and winding around postsynaptic dendritic branches for considerable distances; at the repeated sites of axodendritic synaptic contact, the former widen into varicosities containing mitochondria and clusters of synaptic vesicles, and the postsynaptic membranes are usually thickened. The overwhelming majority of synaptic contacts found were axodendritic, occurring in reasonably close proximity to the perikarya, although rare axosomatic synapses were also present. Occasionally, two presynaptic axon profiles were found in close mutual contact without any Schwann cell cytoplasm intervening between their contiguous plasma membranes (15). Of much more frequent occurrence, however, were dendrodendritic contacts (14); one type consisted of a simple close association of plasma membranes, but a second, more complex type, sometimes with a length of 80–200 nm, was characterized by additional faintly opaque material on the cytoplasmic sides of the associated membranes. This latter type appeared structurally highly organized and might serve the function of adhesion (20), whereas any of the above contacts might be sites of metabolic interaction (14, 15). It was not determined whether any of these contacts are between neurites of a single or of separate neurons. Since essentially all of the processes issuing from the perikaryon must be dendrites, based on deCastro’s descriptions cited above (11), Elfvin (14, 15) concentrated his study on these processes arising from the cell body, and the small presumably presynaptic axonal varicosities and terminals impinging upon them. The identification of these latter elements (or profiles) as presynaptic has since been confirmed by their disappearance after preganglionic denervation (21, 45). Unfortunately, Elfvin was not able to identify and
describe axons of the postsynaptic cells, even using serial sections. These processes had been described in LM silver-impregnated preparations by their length and lack of collateral branching (11), but they probably do not possess any fine structural features to distinguish them from the dendrites from which they originate. Schwann cells ensheathed or encapsulated almost all axons, dendrites, and cell bodies.

Our purpose in the present study was to elucidate as precisely as possible the location of both AChE and BuChE in or on the various cellular elements of the cat SCG, while using information gleaned from Elfvin’s studies to identify correctly the profiles as portions of presynaptic axon, postsynaptic dendrite, cell body, or satellite cell.

The outermost 10-15 μm of a tissue chunk was usually less than optimally preserved, and it stained coarsely. Immediately beneath this layer was generally a region extending inward for at least 200-300 μm that was fairly well preserved and stained more or less consistently; our study was concentrated around the numerous dendritic branches and other structures in this portion. The core of the tissue chunk, 200-300 μm in diameter, was considered to be less reliably stained and was therefore avoided.

AChE Staining: Cats Treated with iso-OMPA in vivo for Selective Inactivation of BuChE

Myelinated axons are numerous throughout the ganglion, and nearly all exhibit intensely stained axolemmas (Figs. 1 and 2); as noted below, staining here and in all more distal regions of the axolemma was blocked by physostigmine. The aurous sulfide endproduct is seen as a finely granular deposit at intermediate or high magnification, and, even though the axolemma is heavily stained, the deposit does not appear to extend more than slightly into the intercellular space between it and the first layer of myelin (Fig. 1). The deposit is distributed over the entire axolemma, and is not arranged in a patchy or discontinuous manner. Myelin membranes are unstained as is the plasmalemma of the Schwann cell surrounding them. An occasional myelinated axon is present that is devoid of staining at the axolemma (Fig. 2); this is probably not due to inadequate penetration of reagents through the thick myelin covering since intra-axoplasmic structures such as mitochondria and smooth endoplasmic reticulum (ER) membranes exhibit physostigmine-resistant (vide infra) staining of variable intensity. On the basis of the reports cited above, it is likely that these AChE-negative myelinated fibers are postsynaptic adrenergic axons, or possibly primary afferent fibers whose cells of origin lie cranial to the SCG. No staining is detectable at the neurofilaments or microtubules here or in the subsequent micrographs.

Unmyelinated axons can be identified positively as preganglionic only where they are near-terminal or comprise a varicosity possessing numerous 40-60 nm synaptic vesicles. In favorable profiles of these (Figs. 3 and 4), the axolemma appears to be completely and discretely stained, usually without the deposit extending into the adjacent intercellular space or appearing on apposed Schwann cell membranes. Within the axoplasm, staining is noted at vesicular structures and at mitochondrial membranes; however, the only stained structures that were rendered totally blank by physostigmine are occasional synaptic vesicles (Figs. 4–6). If lightly stained, the deposit appears to outline the vesicle membrane (Fig. 4); in other instances, it tends to fill the vesicle interior as well (Figs. 5 and 6). Adjacent Schwann cell plasma membranes are unstained, but the ER is stained (Figs. 3–6).

At axodendritic synapses the entireties of the plasma membranes of both axon and dendrite are stained, perhaps slightly more heavily at the site of junctional contact than in their adjacent continuations (Figs. 5 and 6). The synaptic clefts as well as other intercellular spaces are essentially devoid of deposit. The relative intensities of staining of the junctional membranes vary; these membranes are sometimes about equally stained as in Fig. 5, and in other instances either the pre- or the postsynaptic membrane (as in Fig. 6) may be more heavily stained. Such variations cannot necessarily be assumed to indicate quantitative differences in enzyme activity, as discussed below.

Plasma membranes of cell processes that can be identified as dendrites at areas other than synaptic sites are also well stained, whether they occur alone (Figs. 3 and 4), at dendrodendritic contacts (Fig. 7), or where they are seen arising from a perikaryon (Fig. 8); the perikaryonal membrane at these latter sites is also usually well stained. Staining at these sites was largely or totally blocked by physostigmine, in contrast to that at the ER and mitochondria of the same regions.
Two interesting, contrasting patterns of physostigmine-resistant staining were noted in mitochondrial membranes, for which no explanation can be offered. In near-terminal axonal varicosities (Figs. 3) and the portions involved in synaptic junctions (Figs. 5 and 6), both the inner folded and the outer mitochondrial membranes are characteristically stained. In definitely identifiable dendrites, either immediately postsynaptic (Figs. 5 and 6) or extending from a cell body (Fig. 8), and in neuronal perikarya (Fig. 8) and in Schwann cells (Figs. 3 and 6), only the outer mitochondrial membrane is generally stained. Early in the present study, it was considered that this difference is sufficiently consistent to provide a means for the tentative distinction between small axonal and dendritic cell processes found alone. On this basis, the very small neurite profile in the upper left of Fig. 3 is identified as an axon, whereas the neurites in which only the outer mitochondrial membranes are stained and which occur singly (Figs. 3 and 4) or in close contact with another similar neurite (Fig. 7) are classified as dendrites. Both patterns of mitochondrial staining persisted in the presence of physostigmine and after inactivation of AChE by sarin, as well as in sections stained for BuChE (vide infra).

The dendrodendritic contact illustrated in Fig. 7, both membranes of which are stained, is of the second, more highly organized type described by Elfvin (14); it shows additional faintly electron-opaque material on the cytoplasmic sides of the respective membranes (Fig. 7). These regions of specialized contact, perhaps serving as attachment plaques, are as much as 1,000 nm in length, and the contiguous plasma membranes appear more nearly straight or only gently curved as compared to membranes facing Schwann cells, and are separated by a uniform, approx. 10-nm intercellular space. As in Elfvin’s report (14), it could not be distinguished whether these contacts are between cell processes of a single or of separate neurons.

Figs. 9-12 illustrate sections from iso-OMPA-treated cats that were stained in the presence of physostigmine, \(3 \times 10^{-8} \text{ M}\). The AuC deposit, therefore, represents nonspecific esterases, although minimal residual AChE or even nonenzymatic adsorption of the heavy metal ion might

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The following symbols are the various identifying letters used in succeeding electron micrographs (Figs. 1–19):

- **A**: axon
- **D**: dendrite
- **ERa**: endoplasmic reticulum in an axon
- **ERd**: endoplasmic reticulum in a dendrite
- **ERs**: endoplasmic reticulum in a Schwann cell
- **es**: extracellular space
- **L**: lysosome
- **Ma**: mitochondrion in an axon
- **Md**: mitochondrion in a dendrite
- **Mp**: mitochondrion in a perikaryon
- **Ms**: mitochondrion in a Schwann cell
- **mt**: microtubules
- **My**: myelin
- **N**: nucleus
- **nf**: neurofilaments
- **P**: perikaryon
- **rER**: rough endoplasmic reticulum
- **Sc**: Schwann cell
- **sv**: synaptic vesicles

**FIGURES 1–8** Figs. 1–8 were stained by the Au(TA)₂ method, after administration of iso-OMPA in vivo to inactivate BuChE, and therefore demonstrate AChE plus nonspecific esterases.

**FIGURE 1** A myelinated axon (A). The axolemma is intensely stained; and in favorable views (arrows) the stain is seen confined to the axolemma and not filling the intercellular space between the axolemma and the first layer of myelin (My). The axoplasm contains stained mitochondria (Ma) and membranes of vesicles and tubules of smooth ER (ERa), as well as unstained, faintly distinguishable neurofilaments (nf) and microtubules (mt). Schwann cell (Sc) cytoplasm and plasma membrane are unstained, but stain is present in Schwann cell mitochondria (Ms) and ER membranes (ERs). Extracellular space, (es). \(\times 50,500\). Bar, 0.5 \(\mu\text{m}\).

**FIGURE 2** Two myelinated axons. The axolemma (arrow) of one (A₂) is intensely stained; the axolemma (arrow) of the other (A₁) is unstained. Mitochondria (Ma) and elements of the ER (ERa) are stained within the axoplasm; these organelles (Ms and ERs) also stain within Schwann cell cytoplasm (Sc). Unstained are myelin sheaths, Schwann cell (Sc) plasma membrane and extracellular space (es). \(\times 31,500\). Bar, 0.5 \(\mu\text{m}\).
also be present. It was not possible to employ controls from which the substrate was omitted, since the substrate and capturing agent are present as a single complex; the free aurous ion is not stable in aqueous solution. As mentioned above, physostigmine blocked all staining at the axolemmas of the presynaptic element wherever it could be positively identified, that is, where it is myelinated (Fig. 9), unmyelinated in near-terminal areas (Fig. 11), and at synaptic junctions (Figs. 10 and 12), indicating that essentially all of the enzymatic activity on the axolemma described above is AChE. Activity of the occasionally stained synaptic vesicles in axonal terminals was likewise prevented by physostigmine (Figs. 10-12). Dendritic (Figs. 10-12) and perikaryonal plasma membranes are usually unstained; however, an occasional dendritic (Figs. 11 and 12) or perikaryonal (Fig. 12) plasma membrane appears to retain at least a portion of its activity.

The foregoing sites at which staining is concluded to be due exclusively or predominantly to AChE are indicated diagrammatically in Fig. 20A.

The irregular staining of relatively light but variable intensity noted above at the membranes of the vesicular and tubular profiles of the ER in axons, Schwann cells, dendrites, and perikarya is present also in the physostigmine-treated controls (Figs. 9-12). Physostigmine-resistant staining was noted also at the perikaryonal Golgi membranes, although these structures are not included in the present figures.

Lysonomes at all sites show intense staining, both in the absence of physostigmine (Figs. 4, 6, 7, and 8) and in its presence (Figs. 10 and 11).

**BuChE Staining: Cats Treated with Astral 1397 Plus Sarin in vivo for Selective Inactivation of AChE**

On the basis of the criteria used here, the localization of definitely identifiable BuChE is confined to the plasma membranes of postsynaptic neuronal elements. Staining is most intense at the immediate postsynaptic sites of dendritic plasma membranes (Figs. 14-16), but extends to the remainder of the dendritic profiles and to the plasma membranes of the perikarya (Fig. 16). The pattern is also prominent at sites of dendrodendritic contact (Fig. 14). In the presence of physostigmine (Figs. 17-19), staining at all these sites, but not at those noted below, was blocked or markedly reduced (Fig. 17).

In contrast with the distribution of AChE, no staining for BuChE was noted in any of the presynaptic elements, including the axolemmas of any myelinated fibers (Fig. 13), small neurites identified as terminal or near-terminal axons by their contents of synaptic vesicles (Figs. 14-16) or by the characteristic staining of both outer and inner mitochondrial membranes (Figs. 14 and 16), or in any synaptic vesicles (Figs. 14-16). Staining identifiable as BuChE is indicated diagrammatically in Fig. 20B.
As with tissues stained for AChE, ER membranes of axons, dendrites, ganglion cell perikarya, and Schwann cells were stained irregularly both in the absence (Figs. 13-16) and in the presence (Figs. 17-19) of physostigmine. The same two contrasting patterns of physostigmine-resistant mitochondrial staining (i.e., inner as well as outer mitochondrial membranes stained only at presynaptic axonal sites) were noted here as in tissues from iso-OMPA-treated cats, as was intense staining of lysosomes. Again, no staining was detected in this series at axonal neurofilaments or microtubules or at Schwann cell plasma membranes.

DISCUSSION

The Au(TA)$_2$ method has been demonstrated in the accompanying electron micrographs to localize enzymatic activity by a finely granular, electron-opaque precipitate of Au$_2$S. At membranous sites, the deposit is generally even and continuous rather than patchy in distribution, with little extension into intercellular spaces, suggesting that diffusion of the reaction product is minimal. The crisp staining of the axolemmas of myelinated fibers as well as numerous intracellular structures indicates that the [Au(TA)$_2$]$^+$ complex penetrates formalin-fixed tissue satisfactorily to a distance beyond 200 μm from the surface. The principal limitation of the method is its lack of specificity, but for AChE and BuChE this has been overcome to a considerable extent by the employment of appropriate combinations of selective inhibitors in vivo and in vitro, and by the concomitant staining of sections for LM examination by the CuThCh method.

The now voluminous literature on the fine structural localization of cholinesterases has been partially reviewed recently (9, 46, 63, 68), and only those aspects that are most pertinent to the present findings will be cited here.

Most of the AChE in the cat SCG appeared to be confined to neuronal plasma membranes, both pre- and postsynaptic, whereas the localization of BuChE was restricted chiefly to the latter site, as depicted in Fig. 20. The AChE-stained presynaptic axon could be identified positively only where it was myelinated, approaching a synaptic contact, or actually involved in a synaptic junction. It is inferred from these findings that the axolemma of the unknown or unidentified segment between the myelinated portion and the synaptic endings is probably also endowed with AChE. Likewise, although our account of dendritic branches of various sizes and in various relationships is more completely illustrated, we still are inferring from these isolated views that probably the entirety of dendritic membranes and also those of the perikaryon from which they arise possess both AChE and BuChE. Further, since the enzyme activity on the axolemma was resistant to iso-OMPA but inactivated completely by physostigmine, the axolemma does not appear to possess a significant concentration of either BuChE or nonspecific esterase. On the other hand, the activity on the plasma membrane of the postsynaptic cell and its dendrites is comprised primarily of AChE and BuChE, but may include also some nonspecific

Figure 5 An axodendritic synapse with plasma membranes (large arrows) of both the axon (A) and dendrite (D) entirely stained, perhaps slightly more heavily at the site of axodendritic contact (double large arrows). The intervening synaptic cleft, however, as well as other intercellular spaces between the neurites and adjacent Schwann cells (Sc), are unstained. Among the numerous unstained synaptic vesicles in the axoplasm, a few vesicles (small arrows) appear to be filled with reaction product. Axonal mitochondria (Ma) are stained on both outer and inner folded membranes, whereas mitochondria of the dendrite (Md) and Schwann cells (Ms) are stained almost exclusively on the outer membrane. A few stained tubules of ER (ERs) are also present in the Schwann cells. × 50,500. Bar, 0.5 μm.

Figure 6 An axodendritic synapse in which the dendrite (D) plasma membrane appears more densely stained than that of the axon (A) both at the area of synaptic contact (double large arrows) and at areas facing unstained Schwann cell (Sc) plasma membranes (single large arrows). The synaptic cleft and other intercellular spaces are unstained. One small vesicle (small arrow) and a mitochondrion (Ma) are stained within the axoplasm. Mitochondria of the dendrite (Md) and Schwann cell (Ms) are stained on their outer membranes. Other structures stained include ER membranes of the dendrite (ERd) and Schwann cells (ERs), and lysosomes (L) within the dendrite. × 31,500. Bar, 0.5 μm.
esterase, since it was not always entirely inactivated by physostigmine. In previous EM demonstrations of AChe in the SCG of the rat (24, 27) and frog (6, 67), in which modifications of the CuThCh method were employed, most of the enzymatic activity was localized within the clefts between the pre- and postsynaptic membranes. The difference in localization with the latter methods is probably due chiefly to the coarser dimensions and lower electron opacity of the reaction product, and possibly its diffusion or translocation before precipitation. This is reflected also in most previous descriptions of AChe-staining of axonal membranes, where the precipitate was generally found to fill the axolemmal-Schwann cell interspace (6, 47, 67, 68).

The only additional site where staining could be definitely attributed to AChe, since it was blocked by physostigmine, was in occasional synaptic vesicles. This pattern differs from that noted previously with the lead-thiolacetic acid method after brief OsO4 prefixation at the neuromuscular junction, where nearly all of the vesicles were stained but probably not due to AChe, since staining persisted after treatment with paraoxon (1). The present finding suggests the interesting possibility that in the recycling of the vesicular membrane components by endocytosis, as proposed by Heuser and Reese (22), the underlying mechanism makes an occasional error in membrane recognition and includes a portion of the AChe-containing axolemmal component. However, if this should occur to a significant extent, the AChe-content of the vesicular and axolemmal membranes should eventually be randomized. Alternatively, the AChe-stained vesicles may represent transport vesicles derived from the ER, as discussed below.

At sites where staining persisted in physostigmine-treated controls, indicating the presence of other esterases or possibly nonenzymatic adsorption of [Au(TA)₄]⁻ or a breakdown product, the additional presence of AChe or BuChE can not be excluded. Comparative intensities of staining at various sites afford only a rough approximation of relative enzyme concentrations. Consequently, there might be no discernible difference between staining produced by a nonspecific esterase plus a relatively low concentration of AChe or BuChE in one section and that observed in a comparable physostigmine-treated control in which the activity of AChe or BuChE had been suppressed. Thus, for example, the present results are not inconsistent with reports of staining for AChe at elements of the smooth ER of cholinergic axons by means of modifications of the more specific CuThCh method (9, 26, 28). The presence of AChe at such sites might be expected, in keeping with early proposals that most of the enzyme is synthesized in the rough ER of the perikaryon and then transported to the axonal terminals via the smooth ER (40, 44, 48). More recent evidence has indicated that approx. 15% of the total axonal AChe is in the process of rapid transport, two-thirds in the forward and one-third in the retrograde direction, and that the remainder is divided between the fixed and slowly transported fractions (49, 54, 59, 64). The site of the rapid transport fraction is uncertain; it has been assigned to the smooth ER (12) and, alternatively, to the axonal microtubules and/or neurofilaments in association with hypothetical transport filaments to which the rapidly

**Figure 7** A dendrodendritic contact of the complex type where the stained contiguous plasma membranes appear as rigidly parallel lines about 9 nm thick separated by an unstained intercellular space of about 10 nm, with additional faintly opaque material (small arrows) on the cytoplasmic surfaces of the associated membranes. The length of complex membrane apposition between the pairs of large arrows is nearly 1000 nm; dendrite plasma membranes continuous with this segment, and in simple apposition, are also stained. Other structures stained include dendrite mitochondria membranes (MD), ER membranes (ERd) and lysosomes (L), and some Schwann cell ER membranes (ERs). A Schwann cell nucleus (N) is also labeled. × 50,500. Bar, 0.5 μm.

**Figure 8** A well stained plasma membrane is present both on the perikaryon (P) and on a small dendritic branch (D) issuing from it. Mitochondria of the dendrite (MD), perikaryon (MP), and Schwann cell (Ms) exhibit staining on the outer membrane only. Some staining is present on membranes of the rough ER (rER) of the perikaryon; it can not be distinguished whether the stained ER membranes in the dendrite (ERd) are rough or smooth. Other structures labeled include Schwann cell cytoplasm (Sc) and lysosomes (L). × 31,500. Bar, 0.5 μm.

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Figure 12: An axon terminal (A) in synaptic contact with a dendrite (D₁), both having unstained plasma membranes. However, another dendrite (D₂) and a perikaryon (P) exhibit some residual staining on their plasma membranes (arrows). Mitochondria (Mp) and other membranous structures in the perikaryon are also moderately stained. In one area of Schwann cell cytoplasm, ER membranes (ERs) and mitochondria (Ms) are markedly stained; in other areas, they are unstained. × 31,500. Bar, 0.5 μm.

Figures 9–12: Figs. 9–12 were stained by the Au(TA)₄ method after administration of iso-OMPA in vivo, followed by physostigmine in vitro; they therefore demonstrate only nonspecific esterases, minimal residual AChE, and any metal ion adsorption.

Figure 9: A myelinated axon after physostigmine inactivation of AChE exhibits stained axoplasmic mitochondria (Ma) and smooth ER membranes (ERa) and also Schwann cell ER membranes (ERs), about the same as in Figure 1. Unstained, however, is the axolemma (arrow). × 50,500. Bar, 0.5 μm.

Figure 10: The plasma membranes of a small axon terminal or varicosity (A) and a dendrite (D) in synaptic contact are completely devoid of stain, even at the postsynaptic membrane thickening (arrows). ER membranes of the dendrite (ERd) and Schwann cell (ERs) are stained, as are the outer mitochondrial membranes in both dendrite (Md) and Schwann cell (Ms), and a lysosome (L). Synaptic vesicles (sv) within the axoplasm are unstained, but a mitochondrion (Ma) shows some staining. × 50,500. Bar, 0.5 μm.

Figure 11: Profiles of three axon varicosities (A) exhibit unstained axolemmas and synaptic vesicles (sv), but characteristically stained mitochondria (Ma). The plasma membrane of one dendrite (D₁) shows some residual staining (arrow), while the plasma membranes of two other dendritic profiles (D₂ and D₃) are unstained. Some of the mitochondria of the dendrites (Md) and of a Schwann cell (Ms) show staining of the outer membrane only; others are practically unstained. Schwann cell cytoplasm (Sc) also contains some lightly stained ER membranes (ERs). × 31,500. Bar, 0.5 μm.
moved components are postulated to be bound (54, 55). In either case, the localization of this fraction may have been partially obscured here by the presence of other esterases, as mentioned, lost because of high diffusibility with inadequate fixation, or missed because its concentration is below the threshold detectable by the present method. The intense AChE staining of the axolemmas of most of the myelinated fibers focuses attention there as the probable major site of fixed AChE and perhaps of part of the slowly transported AChE as well. The concept of a continuously elongating sleeve of enzyme for eventual use at the axonal terminal (63) is not inconsistent with the original proposal of Weiss and Hiscoe (66), that axoplasmic flow represents the perpetual longitudinal growth of the entire axon. A substantial body of evidence (32, 63) weighs against the hypothesis that the AChE of the axolemma participates in impulse conduction (53).

Although it has not been demonstrated directly here that unmyelinated and myelinated postganglionic axonal membranes are devoid of AChE activity, it is reasonable to infer that this is so. The definite identification of postganglionic axons in the SCG is complicated by the fact that most of them probably arise from dendrites, and hence, in EM sections, cannot be related directly to their ganglion cells of origin (11, 14). The occasionally noted, unstained myelinated axons are probably in most cases postganglionic rather than afferent (3, 60); as will be described in a subsequent report, such fibers persisted after preganglionic denervation. In LM studies of the cat SCG stained by the more specific CuThCh method, the fibers of the preganglionic trunk are stained intensely, whereas those of the major postganglionic internal carotid trunk and the smaller branches are unstained (29, 31). This has been corroborated by EM observations of the AChE-stained cat nictitating membrane, in which less than 0.5% of the nearly exclusively adrenergic fibers that originate from the SCG was stained (19).

It is doubtful that the variable degrees of physostigmine-resistant staining noted at the mitochondria represent significant amounts of AChE or BuChE. Staining of these organelles by the lead thiolacetic acid method has been reported for a variety of tissues (2). While no reason can be offered for the difference noted consistently between the staining of mitochondria in identifiable preganglionic axons and their terminals, where the inner as well as the outer mitochondrial membranes were stained, and in dendrites and elsewhere (staining of outer membrane only), this feature appears to offer a useful if empiric means of distinguishing between isolated axonal and dendritic processes, at least in the cat SCG. Preganglionic denervation (as will be reported in detail in a succeeding publication) results in the loss of all myelinated axons with AChE-positive axolemmas, while allowing a number of AChE-negative axons to persist; hence, the AChE-positive axons are identified here as presynaptic, while the unstained axons, which remain after denervation, probably represent the myelinated postsynaptic axons referred to in Results. Denervation also removes all small neurites (with and without clus-

**Figures 13-16** Figs. 13-16 were stained by the Au(TA)₂₅ method, after administration of Astra 1397/sarin in vivo to inactivate AChE, and therefore demonstrate BuChE plus nonspecific esterases.

**Figure 13** A myelinated axon showing some staining of mitochondrial (Ma) and ER membranes (ERa), but an unstained axolemma (arrow). Within the Schwann cell some ER membranes (ERs) are also stained. × 50,500. Bar, 0.5 μm.

**Figure 14** An axon terminal (A₁), with unstained axolemma and synaptic vesicles (sv) but with characteristically stained inner and outer mitochondrial membranes (Ma), is in synaptic contact with a dendrite (D₁) exhibiting a completely stained plasma membrane, particularly at the postsynaptic thickening (arrow). A small neurite (A₂) at the lower right with unstained plasmalemma, but with lightly stained inner and outer mitochondrial membranes (Ma), is probably a near-terminal axon. A dendrite (D₄) is in simple, close appositional contact with two other dendrites (D₃ and D₄); all of their plasma membranes are stained. Still another isolated neurite profile (D₄) with a prominently stained plasmalemma is identified as a dendrite on the basis of the characteristic staining pattern of its mitochondria (Md), namely the prominent staining of the outer membrane but virtual absence of staining from the folded inner membrane. Other small neurites such as D₅, D₆, and D₇ with moderately stained plasmalemmas but without visibly stained mitochondria are also probably dendrites. Little is stained in the Schwann cells (Sc) except for outer mitochondrial membranes (Ms). × 31,500. Bar, 0.5 μm.
ters of synaptic vesicles) that contain mitochondria with stained outer and inner membranes. The small neurites that remain after denervation consistently exhibit mitochondria with only the outer mitochondrial membranes stained. For these reasons, the differential nonspecific staining of mitochondrial membranes appears consistent with our interpretation. Lysosomes likewise stained intensely in the presence of physostigmine; similar staining with the lead thiocetic acid method has been attributed to a C-esterase (65).

In view of the very small population of cholinergic ganglion cells in the cat SCG, where in accordance with LM observations intense staining of the rough ER would be expected, it is not surprising that none was noted. Similarly, no structures identifiable as SIF cells were found. On the other hand, the light, physostigmine-resistant staining for both AChE and BuChE noted consistently in the rough ER of the perikarya of ganglion cells, as at other sites, is consistent with the possibility discussed below that these enzymes as well as other esterases are synthesized postsynaptically for distribution to the dendritic and perikaryonal membranes.

As stated in the introduction, the cytological distributions of both enzymes noted here by direct EM observation are markedly different from those deduced from LM and quantitative studies in which normal and denervated ganglia were compared. The first of these is the extensive postsynaptic neuronal distribution of AChE. This indicates that the loss of practically all the AChE from the neuropil within a few days after preganglionic denervation (the confirmation of which by EM observation will be described in a subsequent publication) must be attributed to factors other than degeneration of its original site of localization. One possibility is that AChE is released continually by the presynaptic terminals, then migrates through the interneuronal and dendrite-Schwann cell interspaces to become incorporated on the dendritic and perikaryonal membranes at a relatively rapid turnover rate (9, 51). Two of the present observations seem inconsistent with this explanation: the extensive, uniform localization of postsynaptic AChE at sites far removed from contact with presynaptic terminals, and the absence of significant staining within the clefts. As at certain intraxonal sites, we can not exclude at present the possibility that AChE occurs within the clefts in a highly diffusible form or at a concentration below the threshold for staining. However, studies with radioactively labeled proteins have indicated that the transfer of macromolecules across synapses is a limited process (13). A more tenable hypothesis is that a trophic factor, released by the presynaptic terminals, is essential for the synthesis of AChE by the postsynaptic neurons. This possibility is now being explored.

The second unexpected finding is the localization of most of the ganglionic BuChE at postsynaptic neuronal membranes, where its distribution resembles that of AChE, rather than at the plasma membranes of Schwann cells as concluded previously. The earlier conclusion was based on the slow, limited decline in ganglionic BuChE after sectioning of the preganglionic trunk, cited above, and some related observations: the apparent restriction of BuChE in the dorsal root and ciliary ganglia (29) and in the brain (5, 61) of the cat.
Figures 17–19 Figs. 17–19 were stained by the Au(TA)$_2$ method after Astra 1397/sarin in vivo, followed by physostigmine in vitro; they therefore demonstrate only nonspecific esterases, minimal residual BuChE, and any metal ion adsorption.

Figure 17 To the right is a myelinated axon (A$_1$) with unstained axolemma (large arrow) but with stained mitochondria (Ma) and ER membranes (ERa). The adjacent Schwann cell also shows some staining in its mitochondria (Ms) and ER membranes (ERs). To the left a small unmyelinated axon (A$_2$), containing remnants of synaptic vesicles (sv), shows a faintly discernible unstained axolemma (small arrows) and characteristically stained inner and outer mitochondrial membranes (Ma). Two neurites (D) which show slight residual staining on the plasma membranes are probably dendrites. × 31,500. Bar, 0.5 µm.

Figure 18 A group of neurites that are practically unidentifiable. One containing stained lysosomes (L) and a lightly stained plasma membrane is probably a dendrite (D). Another process with unstained plasma membranes but with deeply stained mitochondria is probably an axon (A). Schwann cells (Sc) are unstained except for prominently stained mitochondria (Ms) and ER membranes (ERs). × 31,500. Bar, 0.5 µm.
glial and Schwann cells, and of most of the similar enzyme of the rat, propionylcholinesterase (PrChE), to glial and other non-neuronal cells in the CNS (30), along with reports of high concentrations of BuChE and minimal amounts of AChE in various types of gliomas (7, 8, 70). The physiological function of BuChE is unknown, for its chronic selective inhibition produces no detectable consistent effects (25, 32, 63). The observations made here, of the identical localization of BuChE and AChE at postsynaptic sites in the cat SCG, suggested that BuChE, either synthesized locally or taken up from the plasma, might serve there as a precursor in the synthesis of AChE. Evidence consistent with this hypothesis has been obtained with the SCG and other autonomic ganglia of the cat, where the persistent selective inactivation of BuChE by iso-OMPA was found to delay the regeneration of AChE after its inactivation by sarin (39). In the SCG of the rat, where both AChE and PrChE are present in the somata of high proportions of the adrenergic ganglion cells (16, 30), this relationship does not appear to apply. 1

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1 G. B. Koelle, and E. G. Smyrl. Manuscript in preparation.
Figure 20 A diagrammatic representation of the localization of AChE and BuChE demonstrated in the present study. In Figure 20A, AChE is shown as the bold line representing the axolemma of the innervating cholinergic axon (Ax) where it is myelinated (My) and where it is unmyelinated, or covered only by a sheath of Schwann cell (Sc) cytoplasm, down to and including the axodendritic contact. The enzyme is also localized on the plasma membrane of the postsynaptic dendrites (Den) and the perikaryon (P). BuChE (Fig. 20B) is localized only in the plasmalemma of the postsynaptic ganglion cell (P) and its dendrites (Den). Not included in the diagram are the numerous other dendrites issuing from the perikaryon which also possess AChE and BuChE, and the intracellular organelles in which we can not determine definitely whether or not cholinesterase enzymes are present due to the interference of nonspecific esterases, i.e., persistence of significant staining in the presence of physostigmine. The postganglionic axon, which probably arises from a dendrite at some distance from the cell body, is not depicted; however, on the basis of numerous reports of LM and EM histochemistry, it is likely that it is devoid of significant concentrations of AChE or BuChE.

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