

The Cytological Localization of Intracellular Neuronal Acetylcholinesterase*

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ABSTRACT

Sections of cat ciliary ganglia were stained for acetylcholinesterase activity by several modifications of the acetylthiocholine method in order to achieve optimal accuracy of cytological localization of the enzyme. These were compared by ordinary light and phase contrast microscopy with similar sections stained by standard techniques for Nissl substance, the Golgi apparatus, and the neurofibrillae, and by intravital methylene blue. The pattern of cytoplasmic distribution of acetylcholinesterase corresponded most closely with that of the Nissl substance. Following total inactivation of the ganglionic acetylcholinesterase by intravenously administered di-isopropyl fluorophosphate, the reappearance of the enzyme *in vivo* occurred at the same cytoplasmic sites prior to its reappearance at the cell membrane or preganglionic axonal terminations. These observations, and reports cited from the literature, provide support for the hypothesis that acetylcholinesterase is synthesized within the endoplasmic reticulum, then transported via its canaliculi to the surface of the cell and its processes, where its functional sites are oriented externally to the lipoidal membrane.

It has been shown histochemically that acetylcholinesterase (AChE) is present in relatively high concentrations throughout the entire lengths of cholinergic neurons; non-cholinergic neurons contain generally much lower concentrations (1, 2). Presently available histochemical techniques for AChE do not permit assuredly accurate, direct localization of the enzyme beyond the limits of resolution of light microscopy. However, studies of the distributions and sites of action of certain anticholinesterase (anti-ChE) agents have made possible the formulation of an hypothesis concerning the cytological localization of the enzyme. When the slowly reversible bis-quater-

nary anti-ChE agent, ambenonium chloride (*N,N'*-bis (2-diethylaminoethyl) oxamide bis-2-chlorobenzyl chloride; mytelase chloride; WIN-8077), was injected intravenously into cats over a wide dosage range, inhibition of the AChE of autonomic ganglia was produced only in the preganglionic fibers and their terminations, and at the periphery of the cholinergic ganglion cells. This portion, readily accessible to the lipid-insoluble bis-quaternary agent, was designated the external, or functional, AChE. The uninhibited portion, within the cytoplasm of the cholinergic neurons, was termed the internal, or reserve, AChE (3). In an earlier study (4), a quaternary irreversible anti-ChE agent, 2-diethoxyphosphorylthioethyltrimethylammonium iodide (phospholine iodide, 217-MI), did not affect the cerebral AChE of rabbits when injected intravenously, although its tertiary analog readily did so. When the quaternary compound was injected in relatively high concentrations into a lateral cerebral ventricle, thereby circumventing the blood-brain

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barrier, there was marked inactivation of cerebral AChE. Subsequently, regeneration of AChE activity occurred at the same rate as following equivalent inactivation by the systemically administered tertiary analog, despite evidence for the persistence intracellularly of the free quaternary compound but not of the tertiary analog. To account for these observations, it was suggested that within the cytoplasm, recently synthesized enzyme and the uncombined quaternary inhibitor might have been separated by the membranes of the endoplasmic reticulum, which others have proposed to be the site of protein synthesis (5), (6).

The endoplasmic reticulum has been described as an elaborate system of fenestrated, interconnected canaliculi and vesicles which extends throughout the cytoplasm between the nuclear and cellular membranes of all types of neurons and other cells (6, 7). In the perikaryon, it is further characterized by the presence of aggregates of basophilic, ribonucleic acid (RNA)-rich granules, adhering to its membranes and scattered within the interreticular spaces. The dimensions of these various components have been shown by electron microscopy to range up to only a few hundred millimicrons. However, it has been demonstrated fairly convincingly that the granular endoplasmic reticulum, as revealed by the electron microscope, corresponds with the classical Nissl bodies which are seen with the light microscope after certain selective stains (see Figs. 1 and 2 of reference 6). Furthermore, structures identical with the Nissl bodies have been found in living neurons by ultraviolet (8) as well as phase contrast (9, 10) microscopy, in refutation of claims that they are artifacts of fixation.

The aims of the present study were to determine whether the distribution of cytoplasmic AChE could be shown to correspond with the Nissl substance (presumably the granular endoplasmic reticulum), or with any other previously described cellular component, and whether the Nissl substance might represent the site of synthesis of AChE for the entire neuron. Sections of cat ciliary ganglia, which contain essentially only cholinergic neurons, were stained by several standard histological techniques. Similar sections were stained for AChE by various modifications of the acetylthiocholine (AThCh) method in attempting to obtain optimal cytological ac-

curacy of localization. The slides were then examined by ordinary light and by phase contrast microscopy. The site of initial reappearance of AChE following its inactivation by diisopropyl fluorophosphate (DFP) was investigated similarly. A preliminary report of the findings has been published in abstract form (11).

Material and Methods

Cats were anesthetized with sodium pentobarbital, 30 mg. per kg., given intraperitoneally, and were sacrificed by the intravenous injection of air. The ciliary ganglia were removed as rapidly as possible and placed in chilled physiological saline solution. Frozen sections were cut in the longitudinal plane at thicknesses ranging from 5 to 30 microns, and were transferred immediately, prior to thawing, to clean slides.

In the standard acetylthiocholine method for cat tissues (1), the slides are distributed among four preincubation solutions containing Na_2SO_4 , with or without diisopropyl fluorophosphate (DFP), in which they are kept for 30 minutes at 30–35°C. (Table I). Diffusion of AChE is practically eliminated in 24 per cent Na_2SO_4 , whereas non-specific ChE requires 28 per cent Na_2SO_4 for this purpose. Under these conditions, 10^{-7} M DFP produces nearly complete, selective inactivation of non-specific ChE's. The slides are then transferred to incubation solutions, which contain the same concentrations of Na_2SO_4 , in addition to substrate (AThCh or butyryl thiocholine (BuThCh)), copper glycinate, magnesium chloride, and sodium maleate buffer; the final pH is approximately 6.0. Acetylthiocholine is hydrolyzed by both AChE and non-specific ChE's, whereas BuThCh is hydrolyzed more rapidly by non-specific ChE's, but not at a significant velocity by AChE. During the 30 to 60 minute incubation period, as the substrates are hydrolyzed, the liberated thiocholine is precipitated as a copper mercaptide salt, which subsequently is converted to a brownish amorphous deposit of CuS by immersion in $(\text{NH}_4)_2\text{S}$ solution. The slides are then gold-toned, dehydrated, and mounted. As indicated in the accompanying table, AChE and non-specific ChE's are localized selectively by procedures B and C, respectively, and both types of enzyme are stained by procedure A. Procedure D serves as a control for the detection of possible staining through the action of non-cholinesterase enzymes, adsorption of copper ion, or other non-enzymatic factors. Limitations of the specificity of the method and their control have been demonstrated by the use of additional selective inhibitors (2). Its application to tissues from other species requires preliminary determination of the appropriate concentrations of Na_2SO_4 and DFP (2).

In attempts to obtain optimal cytological localization of AChE, the standard AThCh method was modified as follows:

TABLE I
Variable Components of Preincubation and Incubation Solutions for Selective Localization of AChE and Non-Specific (Pseudo-, Butyro-) ChE in Cat Tissues

Procedure	Na ₂ SO ₄	DFP (pre-incubation)	Substrate (incubation)	Enzyme localized
A	per cent 24	—	AThCh	AChE plus non-specified ChE
B	24	10 ⁻⁷ M	AThCh	AChE
C	28	—	BuThCh	Non-specific ChE
D	24	10 ⁻⁷ M	BuThCh	Control

a. Minimal exposure to air (2 to 5 seconds) between the time of placing the unfrozen sections on slides and introducing them into the preincubation solutions. This precaution was followed throughout the present study, and appeared to be one of the most critical factors for maintaining normal structural integrity.

b. Reduction of the standard 30-minute period in the preincubation solutions to 5 to 10 minutes. When the solution contained DFP for the selective inactivation of non-specific ChE (procedure B), its concentration would have had to be increased to produce the same degree of inactivation as obtained with 30 minutes' incubation, since the reaction is a pseudomonomolecular one. However, the subsequent step, incubation with AThCh (see below), was usually limited to 3 to 15 minutes. Under these conditions staining for non-specific ChE was scarcely detectable, hence DFP was usually omitted from the preincubation solution (procedure A).

c. Variation of the period in the incubation solution (containing AThCh) from 3 to 60 minutes.

d. Omission of gold-toning following development with (NH₄)₂S, or omission of the latter step altogether. The latter modification has been reported by Zajicek *et al.* (12) and by Holmstedt (13), and is said to result in greater accuracy of localization.

Sections from approximately eight ciliary ganglia were stained with the aforementioned modifications.

Approximately forty ganglia were fixed either in 10 per cent neutral formalin or in concentrations of ethyl alcohol ranging from 70 to 100 per cent, for varying periods prior to freezing and sectioning. Both fixatives have been used with the AThCh method previously (14-17), and are known to cause partial inactivation of AChE (18, 19).

Prior to sacrifice, ten cats were given intra-arterial injections of methylene blue for intravital staining of neurons by a modification of the Ehrlich method (20, 21). In some cases, alternate sections were stained additionally for AChE.

Approximately twenty cats were given 4.0 mg. DFP/kg., intravenously, following 3.0 mg. atropine

sulfate/kg., intraperitoneally. This dose of DFP causes practically complete, irreversible inactivation of the ganglionic AChE (3, 22). The animals were sacrificed at intervals ranging from 15 to 300 minutes after DFP, and ganglia were stained by the various modifications described above in order to study sites of initial re-appearance of AChE.

In addition to the foregoing procedures for localizing AChE activity, a large number of sections was stained by the following standard techniques: cresyl violet staining for Nissl substance (23); Elftman's direct method for the Golgi apparatus (24, 25); Bielschowsky's (26) and Cajal's (27) silver impregnation for neurofibrils.

Sections were examined and photographed both by ordinary light and by phase contrast microscopy.

RESULTS

Characteristic neurons of the ciliary ganglion stained by standard histological methods are illustrated in Figs. 1 to 4. The section stained for Nissl substance by the cresyl violet method (Fig. 1) shows the typical irregular bodies throughout the cytoplasm, in addition to the nucleolus and the moderately heavily stained nuclei of the capsular glial cells. At this magnification it is not possible to distinguish the specific elements of the endoplasmic reticulum and its individual RNA-rich granules; the latter are probably responsible for the selective staining (28). The Golgi apparatus (Fig. 2) is represented by coarser strands and darkly stained granules which occupy predominantly the intermediary zone of the cytoplasm. The neurofibrils coursing throughout the cytoplasm are seen in the Cajal's silver-stained section in Fig. 3. The intravital methylene blue preparation (Fig. 4) demonstrates the boundaries of the perikaryon and its axonal and dendritic processes, and the degree of preservation of the structural integrity of the neurons obtained with the sectioning technique used.

When fresh frozen sections 5 microns in thickness were treated by the standard AThCh method with the aforementioned modifications (*a* to *c*), staining was evident after periods of incubation as short as 3 minutes, and was quite distinct at 5 minutes (Fig. 5). The more intense staining at the periphery of the perikaryon has been shown to be associated with both the neuronal membrane and the terminations of the preganglionic fibers (29). Throughout the cytoplasmic region, between the cell membrane and the nucleus, the pattern of staining consists of extremely fine striations and dots; although the orientation

of the former is irregular, it appears predominantly concentric. When thicker sections were employed, or when the period of incubation with substrate was prolonged, the picture obtained was similar but the elements were heavier and coarser (Fig. 6). Omission of development with $(\text{NH}_4)_2\text{S}$ resulted in a similar but considerably lighter pattern of staining, consisting of fine crystalline deposits (Fig. 7). In all cases, the distribution resembled most closely that of the Nissl substance in the sections stained by the various standard methods (Figs. 1 to 4). The photomicrograph of a control section which was not incubated with AThCh, but was taken under identical conditions of exposure is virtually blank (Fig. 8).

A similar series of sections, photographed with the phase contrast microscope at slightly higher magnification, is illustrated in Figs. 9 to 12. Here the unstained control (Fig. 12) shows the general outline of the neuron, its nucleus, and certain surrounding structures. Against this background can be seen the patterns of staining for AChE with 5- and 15-minute periods of incubation with substrate, followed by development with $(\text{NH}_4)_2\text{S}$ (Figs. 9 and 10), and with 5 minutes' incubation without development (Fig. 11). In general, the distribution of staining is similar to that observed with ordinary light, but, while more intense, the limits are less clearly defined. In some of the sections (Figs. 9 and 11), stained material can be noted within the nuclear area. This was observed occasionally, and is most likely the result either of an artifact of sectioning (*i.e.*, the dragging of cytoplasmic elements across the nuclear space by the knife-edge) or of the inclusion of a cytoplasmic layer above or below the nuclear membrane. In most neurons, the nuclei were unstained.

Fixation by immersion of whole ganglia for 24 hours in 10 per cent neutral formalin or in aqueous alcoholic solutions ranging from 70 to 100 per cent resulted in practically complete inactivation of AChE activity, as indicated by the absence of significant staining. The absence of AChE was confirmed manometrically by assays of homogenates of ganglia, conducted as described previously (22). The difference between these results and those obtained with the motor end-plates of skeletal muscle, where staining persisted after similar fixation (14, 30), is probably due primarily to the extremely high concentrations of AChE present at the latter sites. Considerable amounts of AChE were detectable in the ciliary ganglia after fixation with either 10 per cent neutral

formalin or 70 to 95 per cent alcohol for 1 to 5 hours. Enzymatic activity decreased progressively with increasing concentrations of alcohol and times of fixation; after 3 hours in 100 per cent alcohol, practically no activity was noted. Both fixatives caused cytoplasmic shrinkage as indicated by the distortion of the cellular and nuclear boundaries (Figs. 13 to 16). This feature, and the degree of reduction of AChE activity were uniform throughout the ganglia, indicating thorough penetration by both formalin and alcohol. The pattern of staining for AChE did not differ greatly from that noted in unfixated sections. After either fixative, staining was greatest at the region of the neuronal membrane. Throughout the cytoplasm, it presented the appearance of a reticulum, interspersed with aggregate denser clumps of stained material. These features are more clearly apparent in the phase contrast photomicrographs (Figs. 15 and 16) than in those taken with ordinary light (Figs. 13 and 14).

The stability of the enzyme and its approximate pattern of distribution in the absence of fixation were investigated by storing a series of ganglia in physiological saline solution at 4°C., and preparing and staining sections at varying intervals. After 9 days, there was still a reasonably similar appearance to that noted in fresh material (Fig. 17); essentially the same pattern was noted at 21 days.

When cats were sacrificed 15 minutes after the administration of DFP, 4.0 mg./kg., *i.v.*, no AChE was detectable in the ganglia (Fig. 18), as reported previously (3). At approximately 30 minutes, faint staining was noted sometimes, and this increased progressively with longer intervals between the times of administration of DFP and sacrifice. At approximately 300 minutes, staining was quite marked (Figs. 19 and 20). However, even at this time, it was confined almost exclusively to the cytoplasm, and virtually absent from the cell membrane or the surrounding preganglionic terminations. The pattern of cytoplasmic staining was lighter, but otherwise appeared identical with that seen in controls. Since the alkyl phosphorylation of AChE by DFP is practically irreversible (31), it can be assumed that staining in these sections was due to newly synthesized enzyme.

DISCUSSION

The enzymatic histochemical method for localizing AChE here employed consists essentially

in incubating sections in a solution containing AThCh and copper glycinate (32); as the substrate is hydrolyzed, it is trapped as the mercaptide, which was shown by Malmgren and Sylvén (33) to be actually copper thiocholine sulfate. The whitish crystalline deposit is then converted to amorphous, or crystalline, brownish black CuS by development with $(\text{NH}_4)_2\text{S}$. Diffusion of the enzyme is minimized by the incorporation of high concentrations of Na_2SO_4 in the preincubation and incubation media (1). Specificity is assured by the use of controls containing a series of selective inhibitors (34, 2). The accuracy of localization is limited by several factors, which recently have been analyzed mathematically for enzymatic histochemical methods in general by Holt and O'Sullivan (35).

Of the various modifications employed in the present study to minimize artifacts of localization, the most important appeared to be the shortening of the period of exposure of sections to air, and of the preincubation and incubation periods. Under these circumstances, diffusion of the enzyme from its initial sites, and distortion of all cellular structures would be expected to be minimized. With increasing periods of incubation with substrate, the accuracy of localization is impaired further by the growth and accretion of more copper thiocholine sulfate crystals, so that staining spreads to areas adjacent to the actual zones of enzymatic activity. This was noted by comparison of sections of normal and denervated ganglia which were incubated for periods progressing from 10 to 80 minutes (29). The shortest incubation period producing visible precipitate will, of course, depend upon the concentration of the enzyme. With motor end-plates, where activity is especially high (1), most accurate results would probably be obtained with periods shorter than the 3- to 5-minute intervals used here for ciliary ganglia.

Neither the use of fixatives, nor the omission of development with $(\text{NH}_4)_2\text{S}$ appeared to add significantly to the accuracy of localization. In the previously mentioned reports in which the former modification was introduced, the incubation periods were generally considerably longer than those used here, and Na_2SO_4 was usually omitted. Consequently, structural damage and enzymatic diffusion were probably of greater importance. One of the primary objections to the use of fixatives is the uncertainty as to the degree of enzymatic inactivation which occurs

at different sites in the tissue (30). Misleading results which can result from excessive inactivation of AChE by formalin fixation have been pointed out by Bergner (36). The same objection applies to prior treatment of tissues with acetone in order to dissolve lipid barriers to penetration of the substrate (37). The combination of freezing, sectioning at 5 microns, and thawing should allow its ready access to all enzymatic sites regardless of membranous relationships *in situ* (3).

Theoretically, the omission of conversion of the mercaptide salt to CuS should eliminate one possible source of artifactual localization. When the former crystals are in aqueous suspension, or in smear preparations, treatment with $(\text{NH}_4)_2\text{S}$ can cause their dissolution and replacement by amorphous CuS, but the two sites may not be identical (12, 33). This modification does appear to have definite advantages when the method is used for isolated intact blood cells (12) and neurons (38). Under the circumstances of the present study, the sites of localization of AChE appeared identical with and without $(\text{NH}_4)_2\text{S}$ development, and the latter step permitted more convenient observation with the ordinary light microscope. Adaptation of the present technique for electron microscopy will require the extremely rapid formation of a precipitate, crystalline or otherwise, with dimensions far smaller than those obtained at present. Until this and the other factors which limit the accuracy of localization are improved, further increase in magnification of the cellular structures cannot be expected to provide more direct evidence of the sites of AChE.

The study (4) cited in the introduction indicated that both synthesis and hydrolytic reactivation of neuronal AChE could occur in the presence of an intracellularly distributed, uncombined, quaternary anticholinesterase agent. To explain this phenomenon, it was suggested that the agent and the cytoplasmic AChE might be separated by intracellular membranes, such as those of the endoplasmic reticulum. The present investigation was designed to explore this possibility. The tentative conclusions from the indirect evidence at hand may now be considered. The similarity between the pattern of staining of cytoplasmic AChE and that of the Nissl substance has been noted. In contrast, the Golgi apparatus of similar neurons, as stained by the Elftman direct silver method, consisted of more discretely stained, coarser elements concentrated

in the intermediary zone of the cytoplasm. Although the association of AChE with the latter structure cannot be ruled out by the present evidence, it seems unlikely that it represents a major cytoplasmic site of the enzyme. Treatment with alcohol prior to silver impregnation and reduction by this technique prevent the appearance of the Golgi apparatus, presumably because of the alcohol solubility of its components (25). Prolonged fixation of the ganglia with either alcohol or formalin resulted in inactivation of the AChE, but its distribution after 3 to 5 hours in either fixative was found to be identical with that in fresh, unfixated ganglia. The controversial questions concerning the structure and significance of the Golgi apparatus have been reviewed extensively (39-42). It has been suggested that it may represent an agranular reticulum which resembles the endoplasmic reticulum in certain respects (6).

The existence of neurofibrillae as a true component of neurons no longer seems in doubt. Electron micrographs have revealed structures similar in general appearance and distribution to those long known from silver impregnation studies (6, 43, 44). Their appearance in the present series bore no resemblance to the AChE-staining pattern, hence it is unlikely that they represent a site of the enzyme.

In addition to the present observations, there is evidence from the literature which indicates the endoplasmic reticulum as an important site of localization of the cytoplasmic AChE. Assays of cell fractions isolated by differential centrifugation from homogenates of liver (45) have shown that most of the AChE is concentrated in the microsomes. These particles are by no means homogeneous, but they probably represent chiefly fragments of the endoplasmic reticulum (46). The results of an earlier study (47), in which the AChE of cat liver was reported to be confined to the mitochondria, were interpreted (46) as due to the microsomes having been included in the mitochondrial fraction under the conditions of centrifugation employed. Although a predominantly microsomal location of AChE was found also in the adrenal medulla (48), in that tissue the enzyme appears to be confined almost exclusively to the preganglionic fibers and their terminations (1, 49); hence, some portion of these structures must have contributed significantly to the microsomal fraction. In the above studies the mito-

chondria of the liver and adrenal medulla were found to be practically devoid of the enzyme.

The initial reappearance of AChE, following its inactivation by DFP, in the same Nissl-like cytoplasmic pattern as prior to inactivation is consistent with the concept that this is the site of synthesis of the enzyme. The evidence that protein synthesis is one of the primary functions of the endoplasmic reticulum and its associated RNA-rich granules has been reviewed in the references noted above (6; 28, pages 36 to 47).

The likelihood that AChE is synthesized in the nerve cell and transported down the axon has been suggested by Dale (50). Hebb and Waites (51) have presented evidence that this occurs with choline acetylase. From the present results and the reports quoted, the following sequence of events might be postulated concerning the origin, distribution, and function of AChE. The potentiality for the synthesis of the enzyme may be conferred on a specific fraction of the RNA of the granules of the endoplasmic reticulum by a corresponding, relatively stable deoxyribonucleic acid fraction of the nucleus (28, pages 226 to 295). Under the influence of the specific RNA, AChE may be synthesized within the endoplasmic reticulum, then transported through its canalicular system throughout the perikaryon, and along the lengths of the axonal and dendritic terminations. In this connection it might be noted that although the Nissl substance, or RNA, is confined to the perikaryon and the immediately adjacent portions of the processes, the reticulum continues to their terminations (52). At the surface of the neuron and its processes, the enzyme is then so oriented that its active centers are external to the lipoidal membrane; most of the AChE at the axonal terminations seems to be so situated (29). This "external," or "functional" AChE is most favorably disposed for the rapid hydrolysis of acetylcholine liberated either during the resting stage or in the course of synaptic or neuro-effector transmission (53).

It is obvious that considerably more evidence is required to establish the validity of the foregoing hypothesis. Furthermore, it neither explains the function of AChE at non-neuronal sites, nor encompasses the possibility that the enzyme may have other than a passive role within the endoplasmic reticulum (54, 55). The solutions to these problems will require the combined application of more refined histochemical and neurophysiological techniques.

Addendum: Subsequent to submission of the foregoing manuscript, a study (Toschi, G., *Exp. Cell Research*, 1959, **16**, 232, and Hanzon, V., and Toschi, G., *Exp. Cell Research*, 1959, **16**, 256) appeared in which it was shown that the microsomal fraction of homogenates of rat brain consists chiefly of two components: small, dense granules and vesicles composed of thin membranes. Partial separation of the two components revealed the former to be rich in RNA, and that most of the total AChE was firmly attached to the vesicular membranes. It was concluded that the AChE-rich membranes probably were derived chiefly from the neuronal α -cytomembranes or endoplasmic reticulum.

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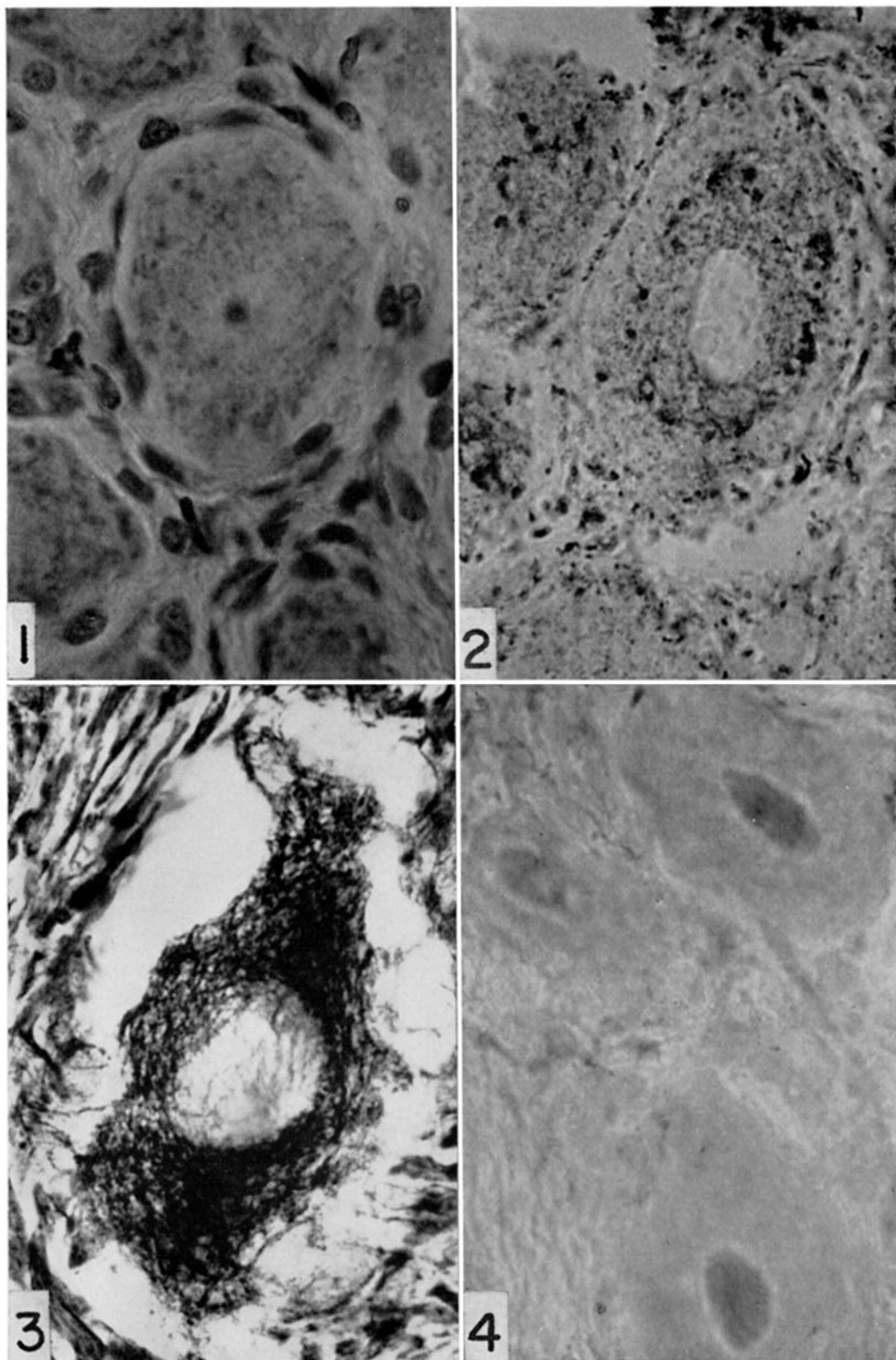
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EXPLANATION OF PLATES

PLATE 187

Ciliary Ganglion, Cat
Standard Histological Procedures

- FIG. 1. Cresyl violet stain for Nissl substance. $\times 1160$.
- FIG. 2. Elftman's direct silver method for Golgi material. Dark stained canaliculi or strands occupy the intermediary zone of cytoplasm. $\times 1450$.
- FIG. 3. Cajal's reduced silver for intracellular neurofibrils. Note the reticular appearance and dense distribution throughout the cytoplasm. $\times 1450$.
- FIG. 4. Intraarterial methylene blue for neural processes. Smooth outline of cell body, nucleus, and dendritic processes are seen without apparent morphological distortion. Terminal axons are also revealed. $\times 1160$.



(Fukuda and Koelle: Localization of intracellular AChE)

PLATE 188

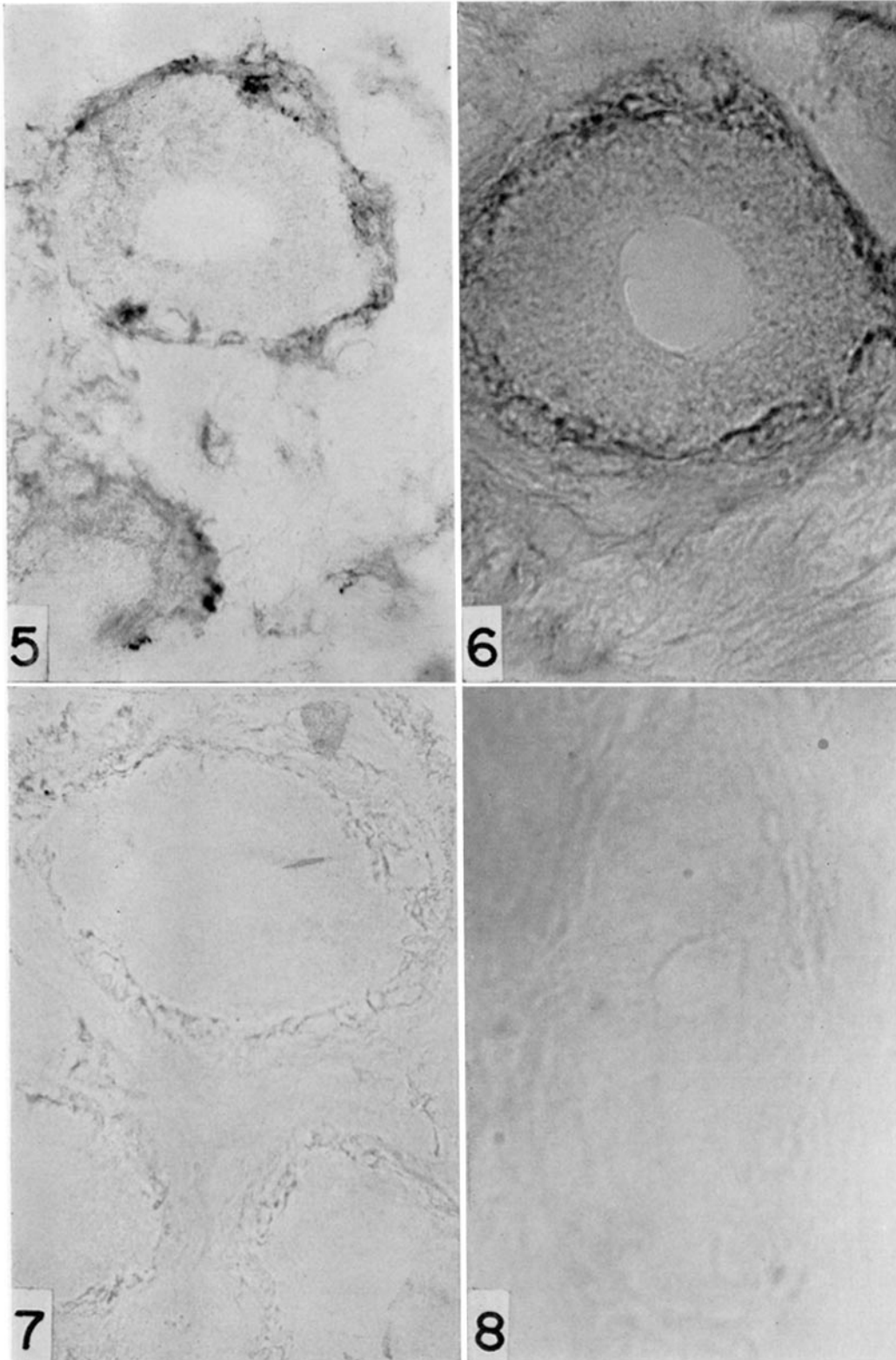
Ciliary Ganglion, Cat
AChE Localization

FIG. 5. Five μ section, 5 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. Light staining of cytoplasm surrounded by more heavily stained peripheral area. Note the resemblance of the pattern of cytoplasmic staining to that of the Nissl substance (Fig. 1). $\times 1160$.

FIG. 6. Ten μ section, 15 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. Note the increased intensity of staining at all sites. $\times 1160$.

FIG. 7. Five μ section, 5 minutes incubation, but without development with $(\text{NH}_4)_2\text{S}$. Distribution of the whitish precipitate of copper thiocholine is identical with that of the brownish precipitate of copper sulfide in Fig. 5. $\times 1160$.

FIG. 8. Control. Fresh frozen section, 5 μ section, fixed in formalin, dehydrated, and mounted. $\times 1160$.



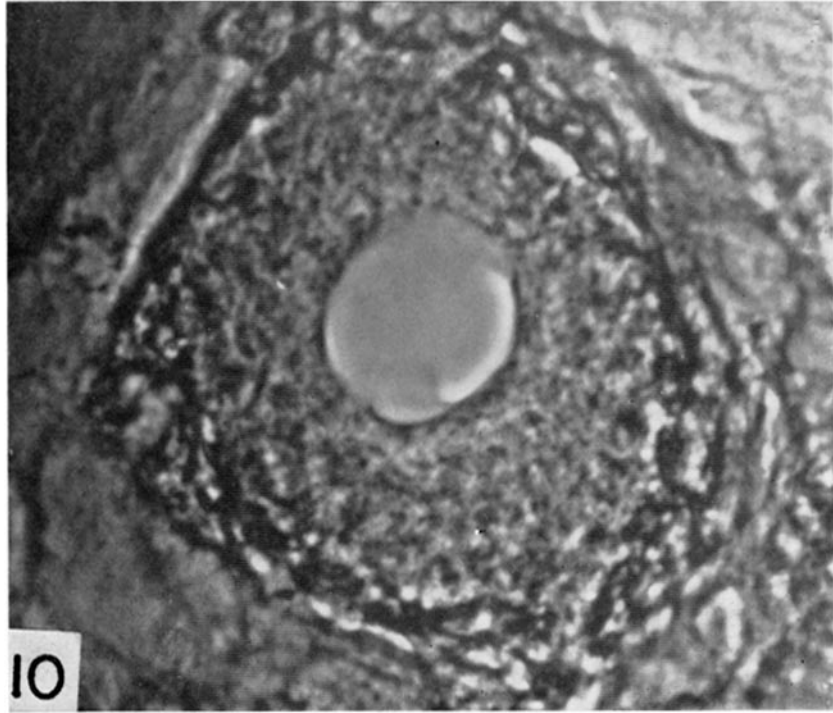
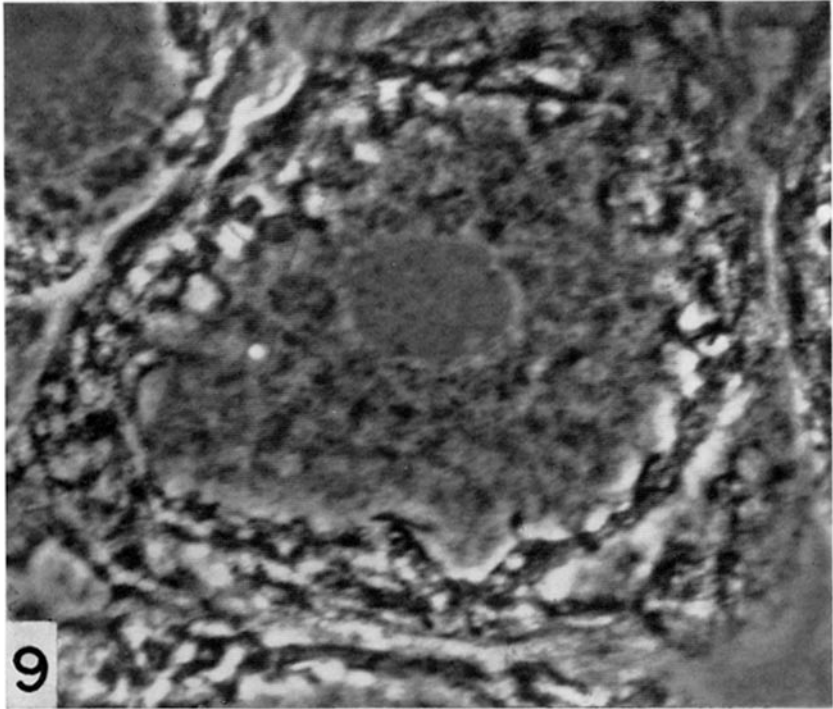
(Fukuda and Koelle: Localization of intracellular AChE)

PLATE 189

Ciliary Ganglion, Cal
AChE Localization. Phase Contrast

FIG. 9. Five μ section, 5 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. Note the dense strands, with interconnections, distributed throughout the cytoplasm. $\times 1450$.

FIG. 10. Ten μ section, 15 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. $\times 1450$.



(Fukuda and Koelle: Localization of intracellular AChE)

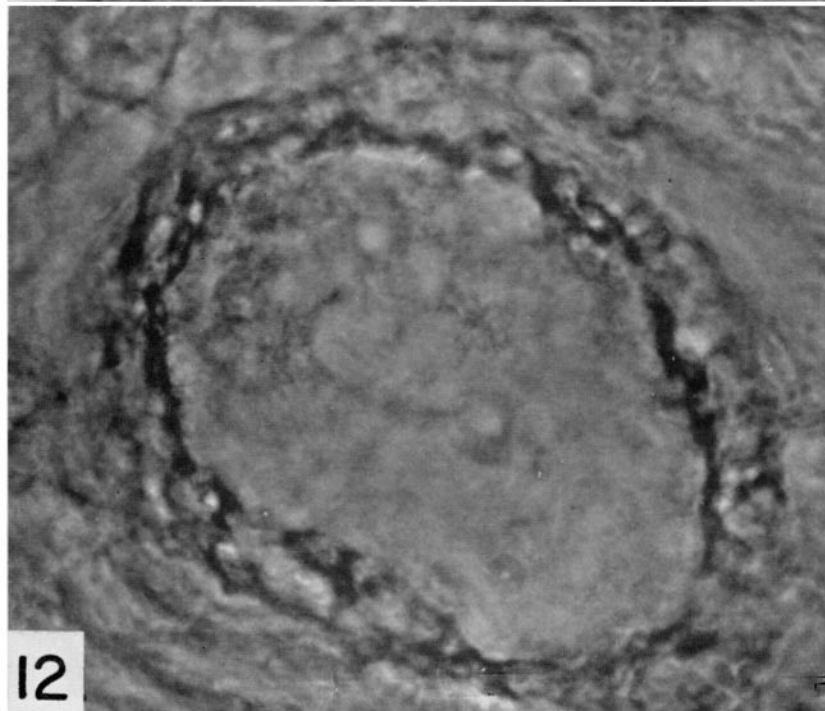
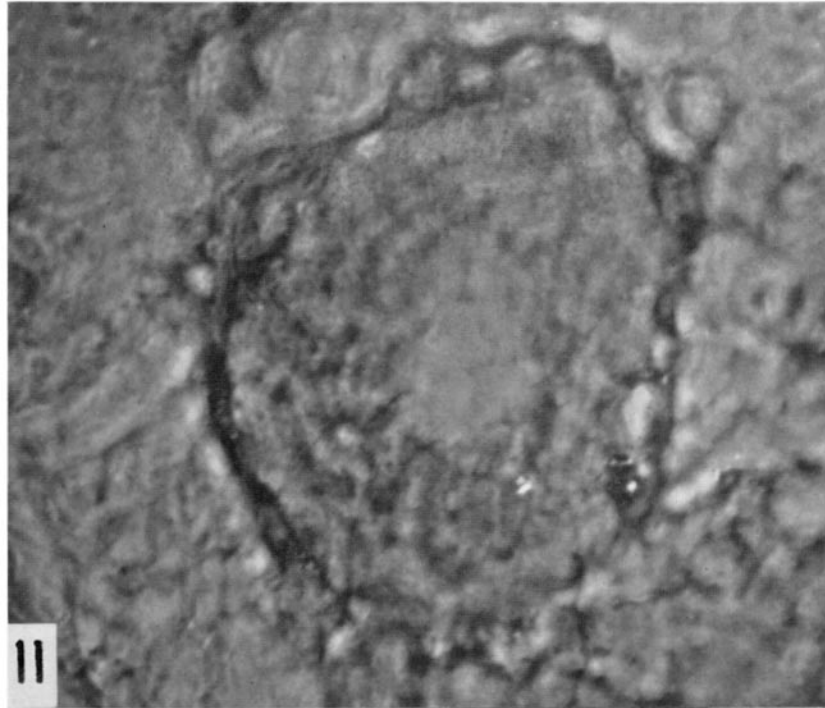
PLATE 190

Ciliary Ganglion, Cat

AChE Localization. Phase Contrast

FIG. 11. Five μ section, 5 minutes incubation as in Fig. 9, but without development with $(\text{NH}_4)_2\text{S}$. Nuclear region reveals artifactual deposits. $\times 1450$.

FIG. 12. Control. Fresh frozen section, 5 μ thick, kept in preincubation solution and developed with $(\text{NH}_4)_2\text{S}$. Cytoplasm appears virtually blank. $\times 1450$.



(Fukuda and Koelle: Localization of intracellular AChE)

PLATE 191

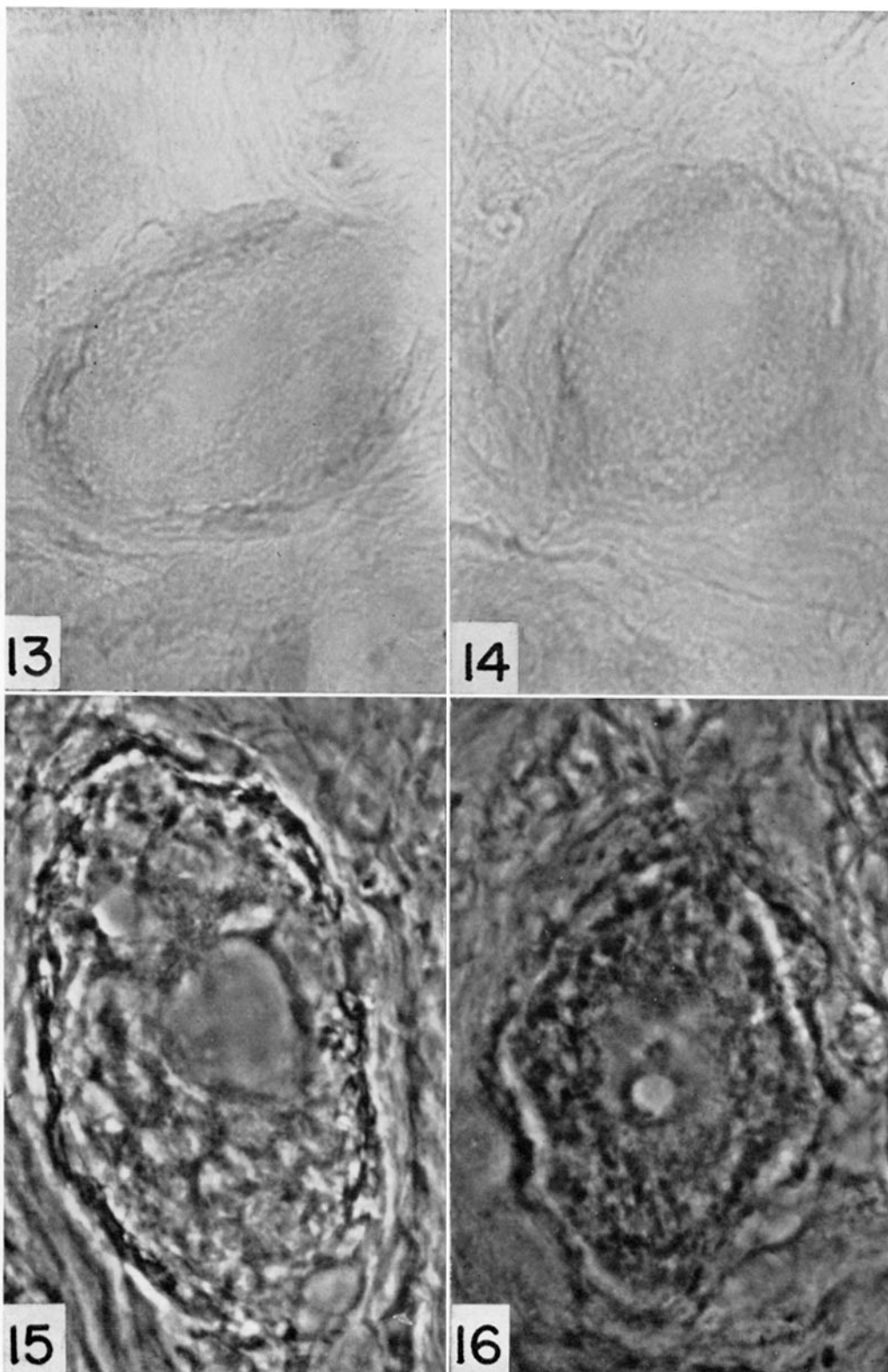
Ciliary Ganglion, Cat
AChE Localization

FIG. 13. Ganglion fixed in 10 per cent neutral formalin at 4°C. for 3 hours. Ten μ section incubated for 10 minutes and developed with $(\text{NH}_4)_2\text{S}$. The structure appears well preserved. Cytoplasmic features seem identical with those of Figs. 5 and 6; only the staining in this figure is somewhat reduced in intensity because of partial inactivation of the enzyme. \times 1160.

FIG. 14. Ganglion fixed in 85 per cent alcohol at 4°C. for 3 hours. Otherwise, treatment the same as in Fig. 13. \times 1160.

FIG. 15. Formalin fixation as in Fig. 13. Ten μ section, 15 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. Phase contrast photomicrograph. \times 1450.

FIG. 16. Alcohol fixation as in Fig. 14. Ten μ section, 15 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. Phase contrast photomicrograph. \times 1450.



(Fukuda and Koelle: Localization of intracellular AChE)

PLATE 192

Ciliary Ganglion, Cat
AChE Localization

FIG. 17. Ganglion stored at 4°C. in physiological saline solution for 9 days. Ten μ section, 20 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. Note prominent enlargement of nuclear space, detachment of peripheral cytoplasm from cell membrane, and fine precipitate within the nuclear space. Otherwise, the remainder of the perikaryon appears quite similar to those of the preceding figures. Phase contrast photomicrograph. $\times 1450$.

FIG. 18. Ganglion removed 15 minutes following intravenous injection of DFP, 4.0 mg./kg. Ten μ section, 15 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. Note complete inactivation of AChE. $\times 1160$.

FIG. 19. Ganglion removed 300 minutes following the same dose of DFP as in Fig. 18. Ten μ section, 10 minutes incubation, developed with $(\text{NH}_4)_2\text{S}$. With reappearance of AChE activity, the enzyme appears confined entirely within the cytoplasm. Even at this stage of reappearance, cytoplasmic features show a close similarity to those of the earlier figures. $\times 1160$.

FIG. 20. The same reaction as in Fig. 19, phase contrast photomicrograph. $\times 1450$.

