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A Histochemical Method for Localizing Cholinesterase Activity.*

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The exact site of cholinesterase (ChE) activity in relation to the structural elements of various tissues has been a matter of uncertainty. Information on this subject up to the present time has been derived indirectly from studies correlating the over-all ChE activities of different samples of tissues with the relative proportions of certain cells or structures present.[1,2] Gomori[3] has published a histochemical method for the direct visualization of the location of ChE activity, in which the substrates used are long-chain fatty acid esters of choline. However, his protocols indicate that the rate of splitting of these compounds by various tissues is extremely low in comparison with that of acetylcholine (ACh); with such enzyme sources as rat brain, which is said to contain only specific ChE,[4] and purified preparations of erythrocyte and electric organ ChE, little or no hydrolysis of these compounds occurred. It seems likely, therefore, that this technic localizes only nonspecific esterases, many of which hydrolyze ACh, but not specific ChE which is considered of physiological significance in the transmission of nerve impulses. In the method reported here, the substrate employed is acetylthiocholine (AThCh) which, as shown below, is hydrolyzed at a more rapid rate than ACh by both specific ChE and nonspecific esterases, presumably because of the weaker

$$\text{—C(=O)—S—} \quad \text{and} \quad \text{—C(=O)—O—}$$

linkage of the —C—S— than of the —C—O— bond. The present technic appears to indicate the location of all enzymes classified under the general term "cholinesterase", and does not permit differentiation between the several types of ACh-splitting enzymes. Such a distinction is now being attempted by the use of the sulfur homologues of acetyl-beta-methylcholine and benzoylcholine as substrates, and by means of selective inhibitors.

Acetylthiocholine Iodide ($CH_3CSCH_2CH_2N(CH_3)_3I$) was first synthesized by Renshaw *et al.*,[5] who noted that its pharmacological actions were similar to those of ACh but of briefer duration. We have employed their method of preparation with minor modifications.† Glick[6] reported that this ester was hydrolyzed more rapidly than ACh by horse serum ChE. The same relationship was found to hold for all sources of ChE tested in the present study, as shown in Table I. ChE activity was determined manometrically by a

* This work was supported by the National Institute of Health, John and Mary Markle Foundation, and the Chalfant Fund.

1 Marnay, A., and Nachmansohn, D., *J. Physiol.*, 1938, **92**, 37.

2 Sawyer, C. H., and Hollinshead, W. H., *J. Neurophysiol.*, 1945, **8**, 137.

3 Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 354.

4 Mendel, B., and Rudney, H., *Science*, 1943, **98**, 201.

5 Renshaw, R. R., Dreisbach, P. F., Ziff, M., and Green, D., *J. Am. Chem. Soc.*, 1938, **60**, 1765.

† We are grateful to Dr. W. Gump of Givaudan-Delawanna, Inc., Dr. Max Tishler of Merck & Co., and Mr. P. W. Blume of Michigan Chemical Corp., for generous supplies of the initial compound, beta-chloro-ethyl dimethyl ammonium chloride. The latter organization supplies this substance commercially. Dr. Eric W. Martin, Philadelphia, Pa., suggested modifications of the synthesis. It has been brought to our attention that AThCh can be obtained from the Bios Laboratories, Inc., New York City.

6 Glick, D., *J. Biol. Chem.*, 1939, **130**, 527.

TABLE I.
Rates of Hydrolysis of Choline Esters by Enzymes from Various Sources.

Enzyme source	Micromoles CO_2 per g or cc per hour		Q AThCh / Q ACh Ratio
	ACh	AThCh	
Rat Brain	224	367	1.64
" Erythrocytes	35	98	2.80
" Serum	23.4	62.3	2.67
Cat Erythrocytes	11.4	38.3	3.36
" Adrenal	50.6	104.5	2.06
Purified bovine Erythrocyte ChE (Winthrop-Stearns) 1.0 mg	1,820	2,700	1.49

modification of Ammon's[7] method. The initial concentration of each substrate was 5×10^{-3} M, which approaches the optimal range of ACh concentration for specific ChE,[8] and the pH of the solutions after gassing with 5% CO_2–95% N_2 was approximately 8.0. Parallel determinations with both substrates were run on all enzyme sources for periods of 10 to 30 minutes, depending on the rate of CO_2 production, and corrections were made for non-enzymatic hydrolysis. The concentration of Cu++ (0.002 M) employed in the histochemical procedure was found, under the same conditions, to produce no appreciable inhibition of rat brain homogenate ChE with either substrate, whereas 10^{-3} M di-isopropyl fluorophosphate (DFP) caused complete inhibition.

Histochemical Procedure. The method consists of incubating teased preparations or frozen tissue sections in a medium containing 4×10^{-3} M AThCh and 0.002 M copper glycinate at a pH of 8.06, saturated with copper thiocholine, for 10 to 60 minutes. The saturation of the substrate solution with the reaction product (in this case copper thiocholine) was first suggested in relation to enzymatic histochemical technics by Friedenwald and Becker.[9] It has the double advantage that diffusion of reaction product is greatly diminished or suppressed, thus enhancing the crispness of the histological localization, and, in addition, since the controls are incubated in the same solution and since the controls show no deposit, of demonstrating that the localization found in the test specimens is not due to mere staining or adsorption. Following this the sections are subjected to two brief rinsings in distilled water saturated with copper thiocholine, then immersed in ammonium sulfide solution, which converts the white precipitate of copper thiocholine to a dark brown amorphous deposit of copper sulfide. They are then affixed to slides with albumen, after which they are either mounted in glycerine or dehydrated through increasing concentrations of alcohol and counterstained as desired. Controls were run by allowing freshly-cut sections to stand in 10^{-3} M DFP in 0.85% saline for 30 minutes at room temperature, following which they were washed in distilled water and treated by exactly the same procedure as described above. In all preparations studied to date, the controls were completely free of the characteristic staining seen in the untreated preparations described below.

Reagents

Solution 1: 3.75 g Glycine, 18.0 cc N KOH, distilled water q.s. 100 cc.

Solution 2: 0.1 M copper sulfate.

Solution 3: 14.5 mg AThChI, 0.75 cc H_2O, 0.25 cc solution 2. Centrifuge. Decant supernatant solution from precipitated cupric iodide.

Copper Thiocholine prepared by alkalinizing a solution of AThCh in copper glycinate to pH 12.0 with KOH, allowing to stand overnight at room temperature, collecting the precipitate and washing with water.

[7] Ammon, R., *Arch. ges. Physiol.*, 1933, **233**, 484.

[8] Augustinsson, K.-B., *Acta Physiol. Scandinav.*, 1948, **15**: Supplementum 15, 1.

[9] Friedenwald, J. S., and Becker, B., *J. Cell. Comp. Physiol.*, 1948, **31**, 303.

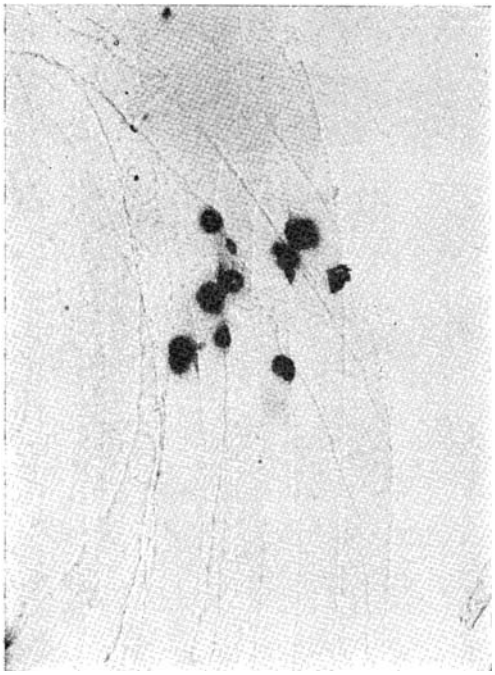

FIG. 1.
Rat intercostal muscle, × 125.

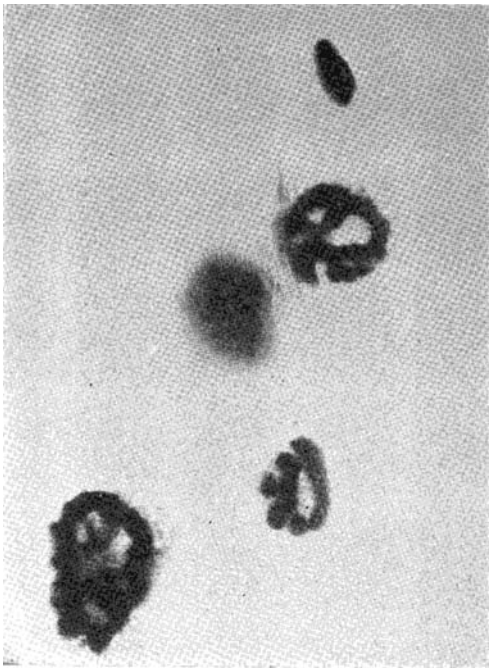

FIG. 2.
Rat intercostal muscle, × 600.

Incubation Solution. 0.4 cc solution 1, 0.2 cc solution 2, 8.6 cc H_2O; add small trace of copper thiocholine, disperse thoroughly, and place in water bath at 37° for at least 15 minutes, stirring occasionally. Immediately before using, add 0.8 cc solution 3, filter.

Results. The following descriptions and accompanying photomicrographs† are of preparations which were not counterstained. Other tissues studied to date, the interpretation of which has been less clear, include liver, spleen and heart.

Striated muscle. Teased preparations of intercostal muscle of the rat (Fig. 1, 2) following incubation for 10 minutes, revealed distinct structures identified as motor endplates. It is interesting to note that the picture bears a striking resemblance to that obtained of the same muscles of the mouse by Couteaux,[10] using the Janus green-ammonium

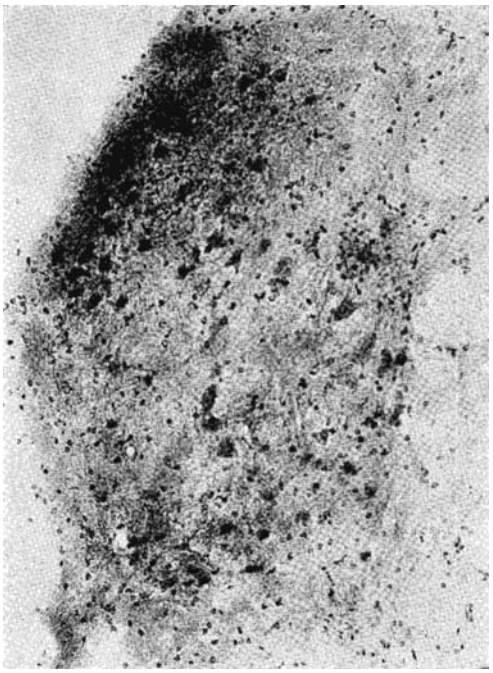

FIG. 3.
Rat medulla, region of gracile and cuneate nuclei, × 125.

† Photomicrographs taken by Mr. Delbert Parker.

[10] Couteaux, R., *Rev. Canadienne de Biol.*, 1947, **6**, 563.

molybdate stain which is considered selective for the subneural apparatus. Couteaux postulated that ChE is probably most concentrated in this region, a hypothesis with which the present findings are compatible. When preparations were allowed to incubate for longer periods, the sarcolemma and nuclei also showed varying degrees of staining.

Brain. Sections of rat medulla (Fig. 3.4), incubated for 40 minutes, were stained very deeply in the regions of the gracile and cuneate nuclei. At these sites large neurons and the immediate portions of their processes were revealed distinctly, as well as numerous glial nuclei which appeared to be predominately microglial. Because of the thickness (30 μ) of the sections studied with the present technic, it could not be determined whether or not the irregular background staining was due entirely to intracellular material. Elsewhere, cell bodies, processes and nuclei of fibrous astrocytes and nuclei of microglia were visible. The general architecture of the former appeared to outline various fiber tracts.

Autonomic Ganglia. Incubation of sections

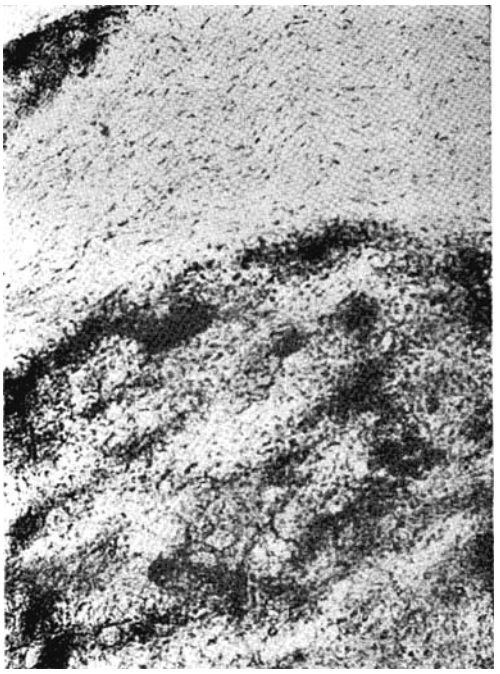

FIG. 5.
Cat superior cervical ganglion, × 125.

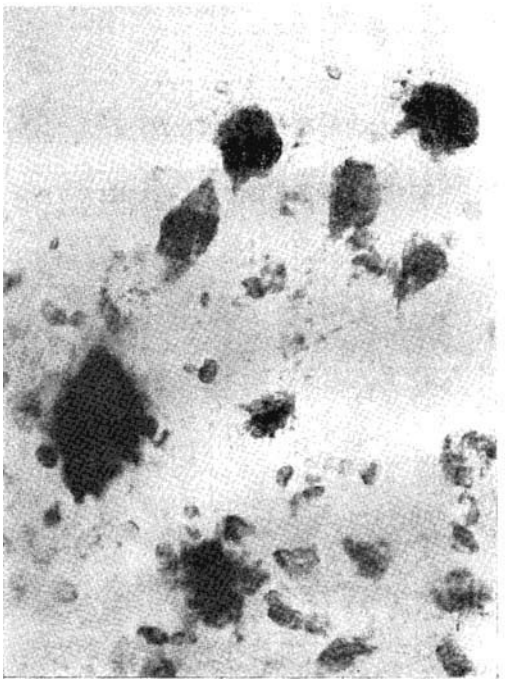

FIG. 4.
Rat medulla, region of gracile nucleus, × 600.

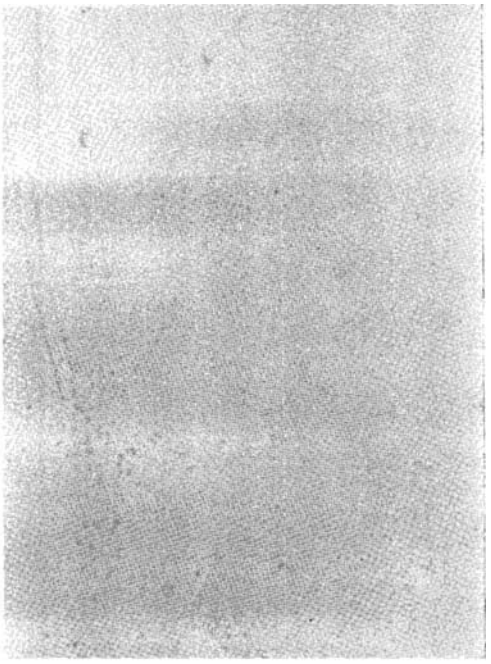

FIG. 6.
Cat superior cervical ganglion, previously incubated in 10^{-3} M DFP, × 125.

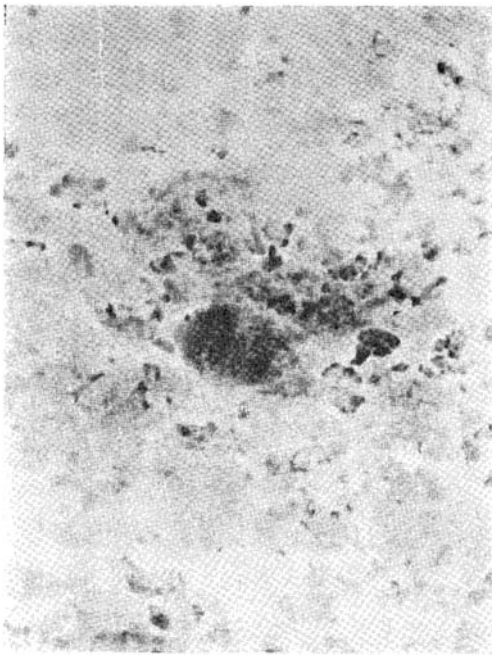

FIG. 7.
Cat superior cervical ganglion, × 600.

of cat superior cervical ganglion for one hour resulted in extremely dark staining (Fig. 5) which was completely lacking in the DFP-treated controls (Fig. 6). In the associated trunk, the nuclei of Schwann's cells were distinguishable, along with the general outline of the nerve fibers. Besides the heavily-staining ganglion cells and nuclei of satellite cells, both of which were seen better in sections incubated for one-half this period (Fig. 7), a fairly heavy background stain was present, forming an irregular pattern. No definite identification has been made of the structures represented by the discreet particles forming this background. It was much less dense in sections of the stellate ganglion of the same animal, where the ganglion cells, in consequence, stood out more sharply.

In sections of the rat ileum (Fig. 8), ganglion cells were stained in the areas of the submucous and myenteric plexi, and appeared to be far more numerous in the latter. The nuclei and general outlines of the muscle fibers were also stained.

Adrenal. The ganglion cells and nuclei of

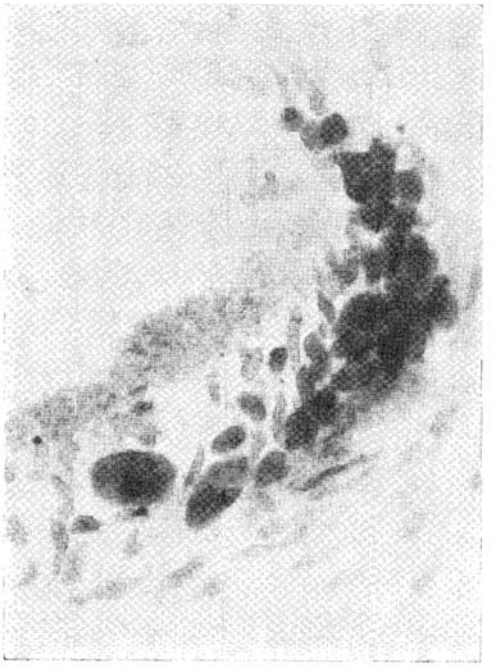

FIG. 8.
Rat ileum, region of myenteric plexus, × 600.

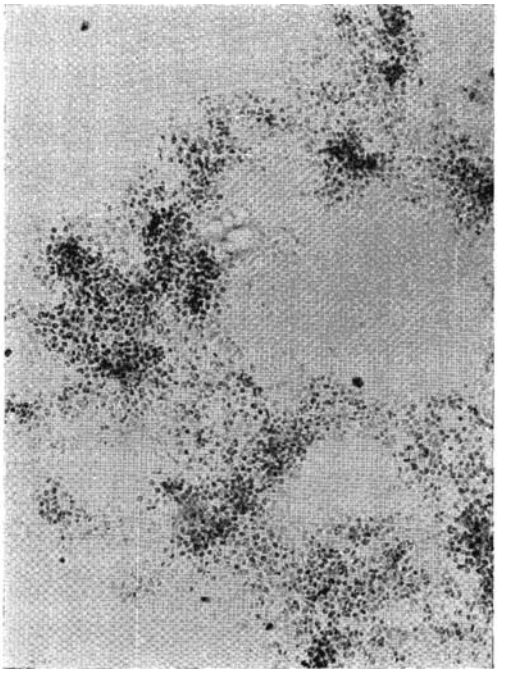

FIG. 9.
Cat adrenal medulla, × 125.

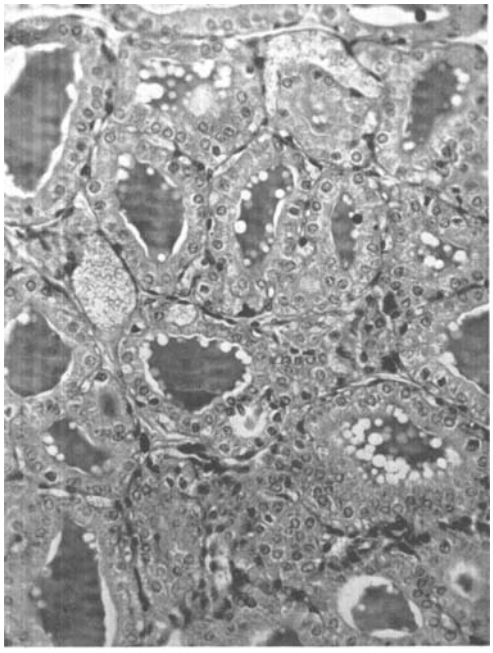

FIG. 10.
Cat adrenal medulla, × 600.

what appeared to be chromaffin cells of the adrenal medulla (Fig. 9, 10) of the cat were deeply stained, forming a pattern of irregular cords. No staining was seen in the cortex.

Comments. In any histochemical procedure the question arises as to whether or not the area where a precipitate is seen represents the actual site of enzymatic action. The alternative interpretation is that diffusion of the initial product of hydrolysis or of the subsequent reaction product has occurred, followed by a precipitation at an adjacent site. In the present study, the possibility of diffusion prior to precipitation was minimized by the previous saturation of the incubation medium with the final reaction product, copper thiocholine. The efficacy of this procedure in promoting immediate precipitation is of course dependent upon the adequate penetration of both copper thiocholine and copper glycinate from the surrounding medium to the sites of the enzyme in the tissues. That such occurred may be inferred from the fact that in preliminary tests in which saturation with copper thiocholine was omitted, precipitation occurred only after prolonged incubation (18-24 hours) and was manifested, after treatment with ammonium sulfide, by the appearance of clumps of acicular crystals of copper sulfide which bore no resemblance to cellular structures.

The intensity of the specific stain at any site is dependent upon several factors, foremost among which are probably the concentration of the enzyme and its reaction velocity or turnover number. The localization of specific ChE is favored in the present technic by a substrate concentration (4×10^{-3} M) in the optimal range for that enzyme; however, nonspecific esterases can also hydrolyze the substrate at this concentration, although at a slower rate. Methods which are being studied at present for localizing separately specific ChE and nonspecific esterases have been mentioned above.

Summary. A histochemical method is presented for localizing ChE activity by incubating tissue sections in a medium containing acetylthiocholine, copper glycinate and copper thiocholine. Results obtained with several tissues containing specific ChE are described and illustrated.