The use of Cholinesterase Techniques for the Demonstration of Peripheral Nervous Structures

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With two plates (figs. 1 and 2)

SUMMARY

An account is given of a modification of the method of Koelle and Friedenwald for the demonstration of cholinesterase activity. When this method is employed, diffusion artifacts are minimal, and good histological pictures of central and peripheral nervous structures can be obtained. Certain advantages of this method over metallic impregnation and methylene blue techniques for demonstration of nervous elements are discussed, and its application to a variety of tissues described.

INTRODUCTION

Investigations into the fine structure of the peripheral nervous system have depended to a great extent on metallic impregnation techniques, especially those involving the use of silver. A large number of variations have been described (see Addison, 1950). Although these methods are extremely valuable, they have several disadvantages, of which the worst are inconsistency of results and a tendency for the impregnation of non-nervous elements. Bielschowsky techniques are especially prone to the latter fault, and results may be particularly misleading when the tissue is rich in reticular fibres, which are easily confused with nerves. Silver impregnation techniques also suffer from the disadvantage that relatively thin sections are essential; this enables impregnated structures to be examined at high magnifications, but hinders the easy perception of the inter-relationship of various elements.

Ehrlich’s methylene blue method and its later modifications have also been widely used for nerve staining. Recently Hillarp (1946), Meyling (1953), and Mitchell (1956) have made wide use of this dye in investigations into the fine structure of the peripheral extensions of the autonomic nervous system. Tissue used for this method of staining must be fresh and unfixed. It is preferable, indeed, to inject the dye while the animal is still living (Mitchell, 1953). Although satisfactory demonstration of nerve-fibres in many organs may be obtained by the use of this dye, the difficulty of obtaining adequate sections, while at the same time retaining the stain, limits the use of the method. Whole mounts of suitable tissues, such as heart-wall (Holmes, 1957) may, however, be made.

METHODS AND APPLICATIONS

The cholinesterase technique was introduced by Koelle and Friedenwald (1949) and improved by Koelle (1951). It has subsequently been modified by Coërs (1953), Snell and McIntyre (1956), and by Coupland and Holmes [Quarterly Journal of Microscopical Science, Vol. 98, part 3, pp. 327–330, Sept. 1957.]
The method has been found to be of value in the demonstration of nervous elements in many different situations. We have found that the technique which gives the best results with mammals is as follows. Tissues are removed from the animal as soon after death as possible, although adequate preparations have been obtained with post-mortem material received 8 h after death. The tissues are fixed in 10% formol-saline for 14–24 h at about 4°C. Frozen sections of the required thickness are then cut and washed in isotonic saline for 30 min or longer. These are mounted on clean slides and allowed to dry in air for about 20 min to ensure adherence. The slides are then incubated in the following solution:

- Copper glycine 0.6 ml (glycine, 3.75 g; CuSO₄·5H₂O, 2.5 g; distilled water to 100 ml)
- Magnesium chloride solution, 0.6 ml (MgCl₂·6H₂O, 9.52 g; distilled water to 100 ml)
- M/5 acetic acid-sodium acetate buffer at required pH, 5 ml
- Sodium sulphate solution, 7.6 ml (Na₂SO₄, anhydrous, 40 g; distilled water to 100 ml)
- Acetyl thiocholine or butyryl thiocholine solution, 1.2 ml (acetyl thiocholine or butyryl thiocholine iodide, 23 mg; distilled water, 1.2 ml; CuSO₄ (0.1 M solution), 0.4 ml; centrifuged, and supernatant decanted for use).

After incubation the tissues are washed for 2 min in water, treated with dilute ammonium sulphide, washed again in water for 2 min, dehydrated, and mounted in Canada balsam. Slides should be allowed to dry after mounting at room temperature. A fine brown deposit is formed at sites of cholinesterase activity. If this method is followed, diffusion artifacts are minimal.

The pH of the solution is of vital importance in the demonstration of nerve-fibres. The optimum pH varies with species, while the incubation time is directly dependent upon it. The use of a pH of 6 and above results in a heavy background deposit due to the presence of pseudocholinesterase and non-specific esterases. As a result of this, nerve-fibres, although giving a positive reaction, are not readily observed. It is therefore necessary to use a pH which is compatible with a satisfactory reaction in the nervous elements, but which gives minimal general tissue reaction. This has been found to be pH 5.6 in the rabbit, pH 5 in the rat, pH 4.6–5 in man, cat, and dog. The use of a lower

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**Fig. 1 (plate).**

A, part of nerve network in atrial wall of rabbit heart. 200-μ section, acetyl thiocholine substrate, pH 5.6, incubation time 16 h.

B, thick nerve-fibres giving off fine branches which form a net around a small vessel running across the lower part of the figure. 200-μ section rabbit atrial wall, butyryl thiocholine substrate, pH 5.6, incubated 16 h.

C, Pacinian corpuscle in cat pancreas. A positive reaction occurs over the central core. A nerve-net may be seen in the surrounding acinar tissue. Acetyl thiocholine substrate, pH 5, incubation time 5 h.

D, nerve-net in cornea of rat. Whole mount. Acetyl thiocholine substrate, pH 5, incubation time 16 h.

E, Auerbach's plexus in cat ileum. Acetyl thiocholine substrate, pH 5, incubation time 6 h.

F, Meissner's plexus in rabbit ileum. Acetyl thiocholine substrate, pH 5.6, incubation time 16 h.
Fig. 1

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FIG. 2

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pH as employed by Snell and McIntyre (1956) and Snell and Garrett (1956), though of value in demonstrating sites of maximum cholinesterase activity and differentiating pre- and post-ganglionic sympathetic nerve-fibres, produces a very incomplete picture of the entire nerve-net in an organ, and as reported by Bergner (1957) fails to show small accumulations of cholinesterase in denervated motor end-plates.

The cholinesterase enzymes are usually divided into two distinct groups, true cholinesterases and pseudocholinesterases (Hawkins and Mendel, 1947). These may be distinguished histochemically by the use of di-isopropylfluorophosphate (DFP). After the tissue sections have been mounted on slides, they are immersed for 20 min in a $10^{-6}$ or $10^{-7}$ M solution of DFP in saline, rinsed for 5 min in two changes of saline, and finally placed in the incubating solution. Incubation of sections treated with DFP in a substrate containing acetyl thiocholine gives a positive reaction for true cholinesterase activity and usually gives a good demonstration of the nerve plexus; while incubation with butyryl thiocholine at the optimum pH for the species usually gives a negative result.

All nerve-fibres examined so far in man, cat, dog, rat, and rabbit have been found to contain both true and pseudocholinesterase. In foetal material most of the activity is due to pseudocholinesterase, and hence butyryl thiocholine is the most suitable substrate. In most post-natal tissue true and pseudocholinesterase are present in more or less equal amounts, and acetyl thiocholine is usually the most useful substrate, as the reaction due to tissue pseudocholinesterase is reduced to a minimum. Sensory nervous structures in the heart-wall of the cat and dog do, however, give a much stronger reaction with butyryl thiocholine.

Pseudocholinesterase activity is not confined to nervous elements, but may also be found, for example, in smooth muscle or capillary walls of the nervous system (Koelle, 1954). In the dog pancreas and adrenal the concentration of pseudocholinesterase in the capillary vessels and in acinar tissue has been found to be so great that in order to observe nerve-fibres tissues must be pre-treated with $10^{-6}$ M DFP even when acetyl thiocholine is used as a substrate.

By comparison with other methods the cholinesterase technique has several

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**Fig. 2 (plate).**

A, motor end-plate in rat diaphragm. Acetyl thiocholine substrate, pH 5, incubation time 20 min.

B, cervical region of rat uterus showing nerve-net in association with myometrium and vessels. Endometrium lies on the left. Acetyl thiocholine substrate, pH 5, incubation time 16 h.

C, thoracic region of rat spinal cord. A positive reaction occurs in nerve-cells of the dorsal, ventral, and lateral horns and in nerve-fibres. Section pre-treated with $10^{-6}$ M DFP, acetyl thiocholine substrate, pH 5, incubation time 16 h.

D, anterior horn of rat spinal cord shown in the previous figure (1). A positive reaction is given by all except the nuclear region of the anterior horn-cells.

E, dorsal root ganglion of rat. A positive reaction, due to true cholinesterase, is given by all except the nucleus of the cell-body, by satellite cells, and nerve-fibres. Acetyl thiocholine substrate, pH 5, incubation time 16 h.

F, dorsal root ganglion of rat. A positive reaction, due to pseudocholinesterase, is limited to the satellite cells and nerve-fibres. Butyryl thiocholine substrate, pH 5, incubation time 16 h.
advantages in the demonstration of nervous structures. The method is relatively simple to perform and has been found to be reliable. The combination of preliminary formalin fixation, buffering the substrate to an acid pH, and inclusion of sodium sulphate reduce diffusion artifacts. Also by ensuring a finely divided final precipitate, good microscopic appearances are obtained even with high magnifications (fig. 1, A). Although the method is not absolutely specific for nerve-fibres, the use of two substrates combined with suitable inhibitors can obviate any possible confusion between nerve-fibres and other structures. Appearances equal to those found in good methylene blue preparations have been obtained. The technique is particularly suitable for demonstrating the nerve-fibres which occur in the walls of hollow viscera or around blood-vessels. Nerve-fibres have been shown in thin sections of adrenal gland (Coupland and Holmes, 1957) and pancreas (Coupland, 1957), and the method has been found especially useful in the study of nerves of the mammalian heart (fig. 1, B). Receptor organs such as Pacinian and corpuscles and free nerve-fibres in the cornea (fig. 1, C, D) are well shown. Other structures which have been demonstrated include Auerbach’s and Meissner’s plexuses, and motor end-plates and nerves in the uterine wall (figs. 1, E, F; 2, A, B). Sections of spinal cord and ganglia also give good results (fig. 2, C–F).

The preliminary fixation in formalin, which is not possible with methylene blue techniques, allows easy cutting of satisfactory frozen sections. Nerves can be demonstrated readily in sections 10 to 200 μ in thickness, or in whole mounts of the walls of thin-walled viscera. The latter preparations present a complete picture in depth of the nervous elements in a single preparation. This is rarely possible with metallic impregnation methods. The sections appear to be permanent, although slight diffusion may occur in the balsam, particularly if the slides are dried in the oven.

REFERENCES

— and HOLMES, R. L., 1957. Ibid. (In press.)