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G. DEMONSTRATION OF CATECHOLAMINES AND CHOLINESTERASES IN THE SAME SECTION

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Catecholamines form intensely fluorescent compounds when treated with formaldehyde. A method for the histochemical demonstration of norepinephrine (NE) in the adrenal medulla based on this principle was reported over 10 years ago (1, 3). In this method, fresh tissue was fixed in an aqueous solution of formaldehyde and sections from it were examined in the fluorescence microscope; the specificity of the method was controlled by direct chemical analysis of microdissected pieces of tissue (3).

Lagunoff *et al.* (20) were the first to employ formaldehyde in vapor form for histochemical localization of monoamines. About the same time, the high sensitivity of the formaldehyde vapor technique was observed by Falck and Torp (12) and by the present writer (5–7). Application of this method to nervous tissues (11) has since then proved fruitful in the microscopic study of monoaminergic neurones, as will be evident from other papers of the present symposium.

While the above techniques make it possible to localize catecholamines in tissue sections, histochemical demonstration of other transmitter substances is not yet possible. However, it is possible to demonstrate histochemically an enzyme closely associated with cholinergic transmission, *i.e.*, acetylcholinesterase (16, 19). Studies carried out with this method have provided valuable information, and have led to a new hypothesis concerning cholinergic transmission (17, 18).

In view of the close interaction between monoaminergic and cholinergic systems it appeared of interest to try to demonstrate in the same tissue section both catecholamines and cholinesterases. If an aqueous solution of formaldehyde is used for the localization of NE, which is possible only in organs with high concentrations of firmly bound catecholamine, such as the adrenal medulla, little difficulty is encountered in subsequent demonstration of cholinesterases. It was thus found, for example, that a nerve net with a high activity of nonspecific cholinesterase is selectively associated with the NE-containing cell groups of the adrenal medulla, while the fine fibers exhibiting acetylcholinesterase activity were evenly distributed through the whole adrenal medulla (4).

Combination of the formaldehyde vapor technique with the acetylcholinesterase method obviously provides possibilities with a wider scope in the correlative study of aminergic and cholinergic structures. Unfortunately, formaldehyde vapor destroys cholinesterase activity much more strongly than does formaldehyde solution. Great care is therefore necessary to find an exposure to formaldehyde sufficient to bring about the amine fluorescence but still preserving enough acetylcholinesterase activity to make possible its subsequent demonstration.

Such a technique, a brief report of which has been published (6), has been applied to sympathetic nervous structures of the rat (8–10, 14). It consists of four main steps: 1) freeze-drying; 2) exposure to formaldehyde; 3) fluorescence microscopy; 4) demonstration of cholinesterases. Since the margin of safety is narrow, all of these steps are critical. Although it is not possible to go into all details in the present paper, some essential features of the method will be given.

1. FREEZE-DRYING

Rapid freezing is necessary to avoid ice crystal artifacts and displacement of the amines. Tissue slices less than about 0.5 mm in thickness are placed on copper foils and cooled either with copper disc forceps previously immersed in liquid air (2) or by direct immersion in liquid air or cooled isopentane. The cold tissue is then dried in any good freeze-drying apparatus (see 21). The temperature *of the tissue* must be lower than −40°C. To avoid overly long drying periods the temperature of the tissue holder should be thermostatically controlled, an oil diffusion pump should be used, and a trap cooled with liquid air should be provided close to the tissue holder. With an efficient apparatus the drying time can be reduced to 1 to 2 days.

2. EXPOSURE TO FORMALDEHYDE

After drying, the temperature is increased to +40°C, the vacuum is broken, and the piece of tissue is either directly embedded in paraffin wax under vacuum or before embedding exposed as such to formaldehyde vapor. The former method makes it possible to treat sections individually with formaldehyde, which is helpful in finding out the optimal exposure conditions.

If the relative humidity of the air exceeds 40%, special precautions are necessary to avoid absorption of water vapor by the dried tissue, with resulting displacement of the water-soluble amines. Plastic chambers kept dry with silica gel are essential for storing the tissue both before and after exposure to formaldehyde.

The exposure conditions vary with the nature of the tissue, the size of the piece, the temperature of exposure, the ambient humidity, and the water content of the paraformaldehyde. Accordingly, the optimal conditions must be found by trial and error. We have obtained good results by exposing whole superior cervical ganglia of the rat to formaldehyde gas released from paraformaldehyde at 50°C for 30 min. The paraformaldehyde was first equilibrated with air whose relative humidity was 40%. Paraformaldehyde was then poured onto the bottom of a Petri dish 15 cm in diameter, the warm tissue holder was placed in the center, and the dish was closed and transferred into an incubating oven at 50°C.

After formaldehyde treatment the tissue pieces are embedded in paraffin wax under vacuum and sectioned. Fluorescence microscopy can be done without deparaffinization by melting the paraffin wax; but deparaffinization with, and mounting in, xylene is usually desirable, although some of the fluorescent material, notably epinephrine (E), may be lost.

3. FLUORESCENCE MICROSCOPY

Ultraviolet light has an inactivating effect on cholinesterases. Exposure to it must therefore be kept to a minimum, and this means almost immediate photography. A microscope lamp incorporating a Siemens HBO 200 mercury burner, Schott filters BG 12 and OG 1, a dark field condenser, nonfluorescent glass optics, and any film with an exposure index greater than ASA 200 are sufficient to provide photographic exposures shorter than 30 sec. During exposure, the coordinates of the slide on the microscope stage should be registered for ater relocation.

4. DEMONSTRATION OF CHOLINESTERASES

Immediately after fluorescence microscopy the tissue sections are transferred into the incubation mixture. Acetylthiocholine and butyrylthiocholine are recommended as substrates, the former in combination with 10^{-6} M iso-OMPA (tetra-isopropylpyrophosphoramide) for the selective demonstration of acetylcholinesterase, the latter together with 10^{-5} M B. W. 284 C 51 [1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide] for the selective demonstration of nonspecific cholinesterases (see 21). Successful results can be obtained with Koelle's improved technique (16), with Gomori's simplified version (13), and with the recent modification by Karnovsky and Roots (15). The incubation period should be long enough (often more than 4 hr) to detect the activity weakened after treatment by formaldehyde. The use of the above inhibitors or others more selective than DFP (di-isopropyl fluorophosphonate) is strongly recommended to obtain proper discrimination between acetylcholinesterase and nonspecific cholinesterase (see 21).

With care, satisfactory results can be obtained. Figure 1a shows the distribution of NE in the superior cervical ganglion of the rat. Figure 1b is the same field after demonstration of acetylcholinesterase activity. Identification of the same individual cells in both photomicrographs is readily possible, and it can be seen that intense acetylcholinesterase activity can be associated with a weak, moderate, or strong fluorescence, and *vice versa*. Cells with an intense fluorescence and an intense enzyme activity are of special interest in view of the hypothesis of Burn and Rand postulating both adrenergic and cholinergic transmission mechanisms in the same neurone (see 18).

Figure 2a shows formaldehyde-induced fluorescence in the rat iris. Acetylcholinesterase activity of the same field is shown in figure 2b. Correlation of the fluorescence and the enzyme reaction in the individual fibers is difficult when the pictures are side by side, because the fluorescent fibers exhibit a pattern essentially different from that of the cholinesterase positive fibers. Spatial correlation is facilitated by examination of superimposed prints over a strong light. It can thus be seen that many of the fine, strongly fluorescent fibers with synaptic enlargements are exactly in the same site as some of the cholinesterase positive fibers. While other interpretations are possible, it is attractive to speculate that such fibers, which are far fewer than fibers which are *either* fluorescent *or* cho-

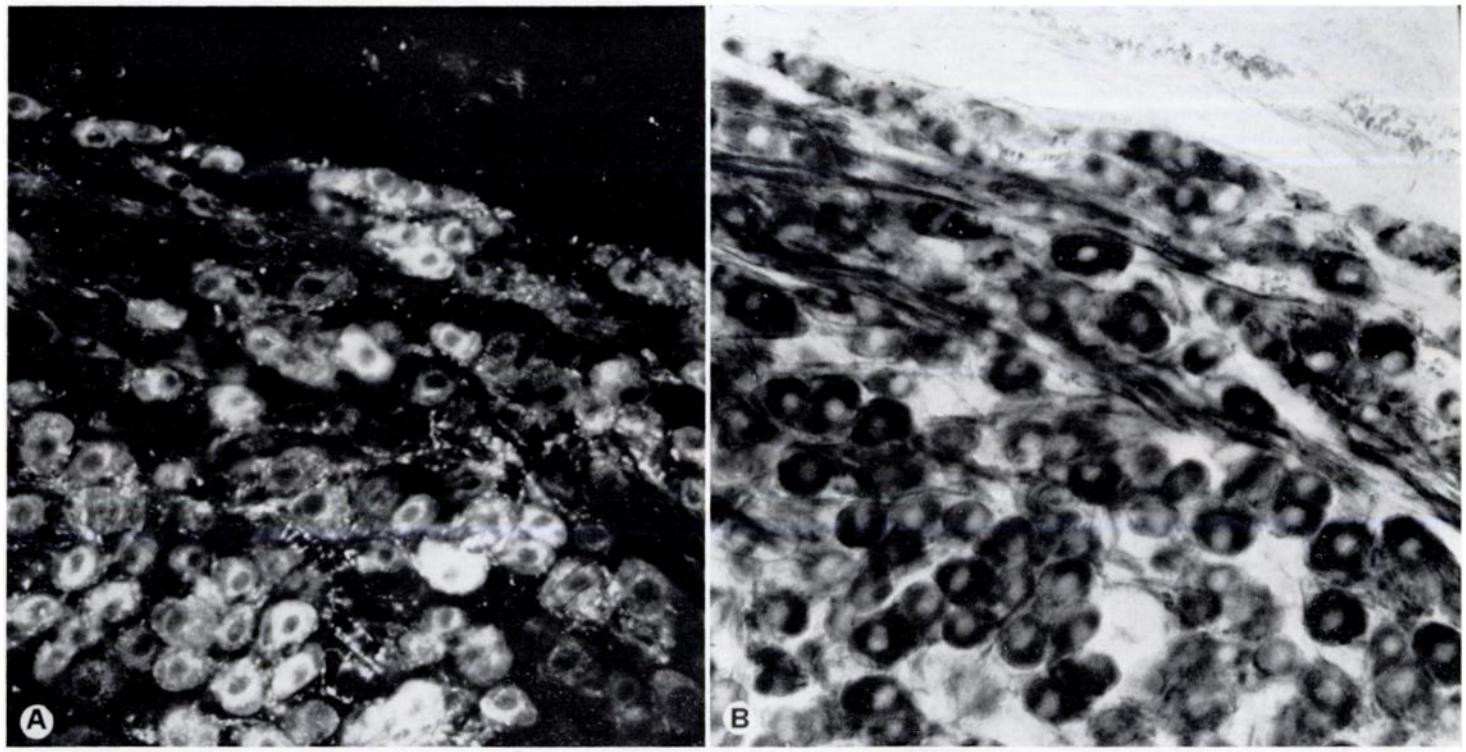

FIG. 1a. Superior cervical ganglion of the rat. Fluorescence photomicrograph of tissue exposed to formaldehyde vapor at 50°C for 30 min.
FIG. 1b. The same field as in figure 1a after demonstration of acetylcholinesterase activity.

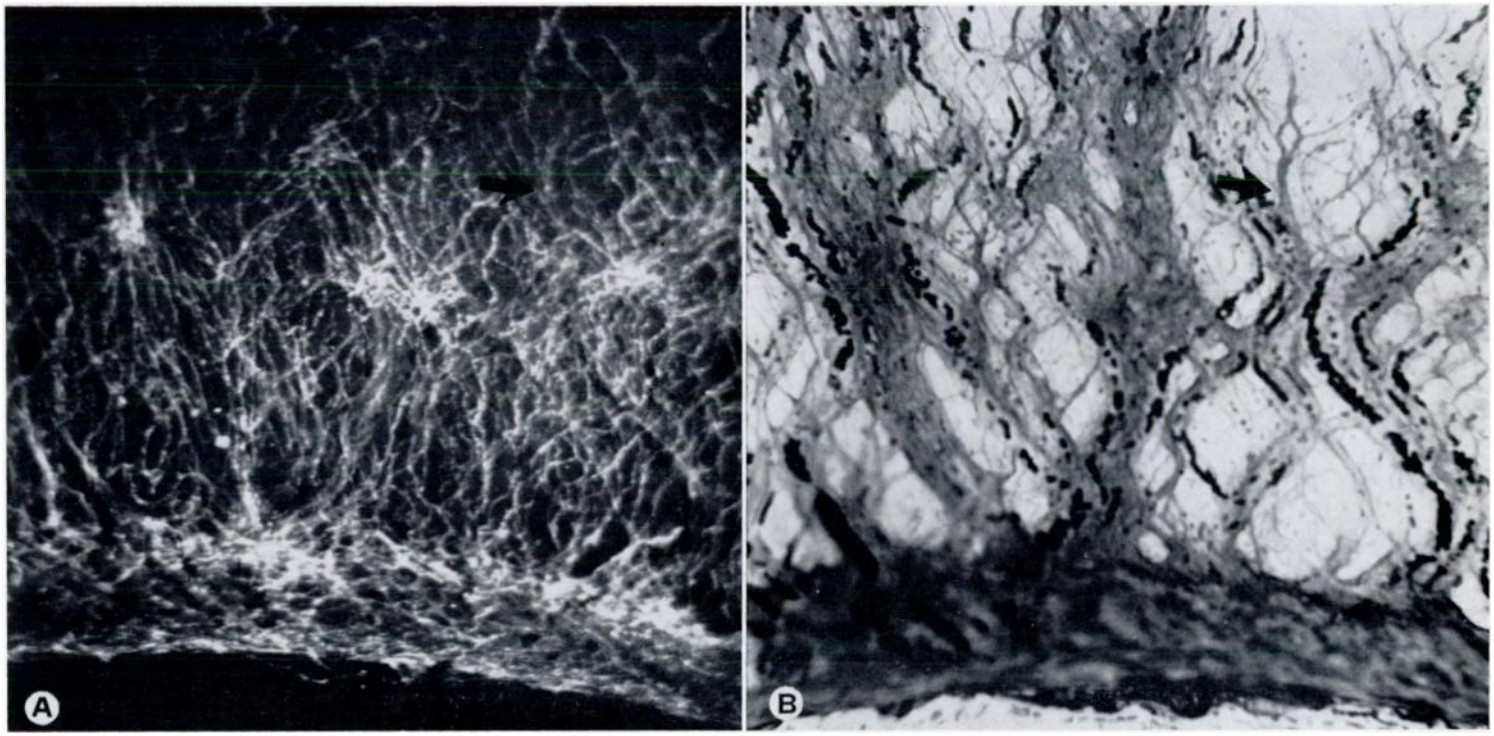

FIG. 2a. Whole mount of the rat iris. Fluorescence photomicrograph obtained after the tissue was exposed to formaldehyde vapor for 20 min at 40°C.

FIG. 2b. Same field as in figure 2a after demonstration of acetylcholinesterase activity. The pattern is different but fine fibers with the same course as that of the fluorescent fibers can be found (arrow). The black rows of dots are red blood cells.

linesterase positive ones, originate from those cells of the superior cervical ganglion which were shown to contain both an intense fluorescence and an intense acetylcholinesterase activity.

These examples may suffice to indicate the potential value of the combination technique. Its application to the study of individual synaptic boutons appears especially interesting, and work is in progress to study this problem.

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