Modified Technique of Mesulam's Tetramethylbenzidine Method
to Prevent Fading of the Color of the Reaction Product

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Summary: Trials to prevent fading of the color of tetramethylbenzidine reaction product were performed after injection of horseradish peroxidase (HRP) into the vitreous body of albino rats. Sections mounted on slides were dried immediately after the end of the HRP histochemical reaction and soaked directly in chilled xylene. These procedures were performed in a dark room and the preparations were stored in a refrigerator. By means of this technique, almost all of the HRP positive fibers were observable even after 14 months of storage.

Horseradish peroxidase (HRP) histochemistry was first introduced by Straus (1957) and applied to the peripheral nervous system by Kristensson and Olsson (1957). LaVail and LaVail (1972) further applied this method to the central nervous system. HRP was utilized as a retrograde transport method to examine the afferent fibers to the injected site, but efferent connections of the injection site were also demonstrable by this method (Lynch et al., 1974; Repérant, 1975). Nevertheless, it was found difficult to obtain an effective and consistent visualization of the anterograde movement of HRP. The difficulty in visualizing such movement appeared to be due to the chromogen used. Diaminobenzidine which was employed as a chromogen appears to show a low level of sensitivity for demonstrating the anterogradely transported HRP. The subsequent introduction of a more sensitive histochemical method using tetramethylbenzidine (TMB) as the chromogen to demonstrate HRP activity, permitted a more effective and reproducible demonstration of the efferent connections (Mesulam, 1978). Further, Mesulam and Rosene (1979) reported that the TMB procedure was distinctly superior to the other currently available methods of HRP neurohistochemistry. The TMB method is at present one of the most frequently used techniques in tracing the neural connectivity within the central nervous system. Recently, it has been demonstrated that the anterograde migration of HRP is subserved by fast axonal transport (Mesulam and Mufson, 1980). Nevertheless, there is a shortcoming with this method; namely, the product in the terminal region has a tendency to disappear rapidly. The present modified technique was devised to overcome this shortcoming and to make long-term storage of the preparation possible.

Materials and Methods

Adult albino rats of Wistar strain were...
lightly anesthetized with ethyl ether, and a solution of about 3 μl of 20% HRP (Sigma Type VI) dissolved in saline was injected into the unilateral vitreous body. Forty-eight hours after this injection, the animal was sacrificed by intracardial perfusion of saline followed by a mixture of 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and then by 10% sucrose solution in the same buffer at 4°C. The brain was removed, placed immediately into the sucrose solution for 12 hours at 4°C, and then cut sagittally or horizontally into 40 μm thick frozen sections. These were kept at 4°C in the phosphate buffer for several hours, mounted on glass slides which had been coated with a chrome-gelatin solution (Zwemer, 1933), and stored in a refrigerator until being dried. The slides were briefly washed in the phosphate buffer at 4°C and histochemical procedures were performed according to Mesulam's TMB method (1978). These procedures were carried out in a dark room. As soon as the reaction had finished, the sections on the slides were desiccated with a drier and cleared directly in chilled xylene. After application of cover slips, the sections were observed under a dark field microscope. These procedures were also carried out in the dark room. The preparations were stored in a refrigerator to preserve the reaction product for a longer period.

Results and Discussion

In the preparations treated by Mesulam's TMB method (1978) or the present technique, all the retinal fibers showed an HRP positive reaction at the time of reaction. Although Mesulam (1978) reported that the dark-blue color was maintained at least for as long as 10 months, the color of the very fine optic nerve fibers in the terminal regions stained by his method faded away with time. To prevent such fading of the blue TMB reaction product, we attempted many modifications. Eventually, the method described here, although a simple treatment, was found to be the most effective one tested to prevent the fading of the blue color in the preparation. By this method, it was demonstrated that even after 14 months of storage, the fading was so limited as to permit demonstration of nearly all the retinal fibers observed at the time of reaction. The improved points in the present technique compared to the original method are: 1) the whole procedure is carried out in a dark room; 2) the sections are dried immediately after the reaction without using an ascending series of ethanol and are cleared by chilled xylene; and 3) all sections are mounted serially on several slides which have been coated with a chrome-gelatin solution (Zwemer, 1933).

The present incubation medium contains hydrogen peroxide (H₂O₂) and TMB which assumes a densely colored form only in the oxidized state. At sites containing HRP activity, the general reaction

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\text{HRP} \quad \text{TMB} + 2\text{H}_2\text{O} \quad \rightarrow \quad \text{H}_2\text{O}_2 + \text{TMB}
\]

occurs and the color of the oxidized TMB becomes visible. The subsequent fading should be considered as due to deoxidization of the oxidized TMB by certain factors. The first process described above was introduced to prevent fading of the color caused by exposure to light, and the second process aimed to prevent loss of the reaction product from the tissue after the reaction. It should be pointed out that the TMB reaction product is known to be vulnerable to ethanol (Warr et al., 1981). The third process served to make the handling of the sections easier and faster in the dark room. Similar good results were also obtained with preparations treated by Mesulam and Mufson's method (1980) in which the
stabilization step was eliminated from the original TMB procedure (Mesulam, 1978).
By utilizing the above method, the authors were able to confirm the existence of terminals of the retinal fibers in the inferior colliculus (Yamauchi and Yamadori, 1982) and also the courses of the accessory optic tract in the rat (Yamadori and Yamauchi, 1983). This technique is thought to be useful for repeated observation using semi-permanent or permanent preparations which is indispensable for investigating the exact courses and delicate terminals of specific nerve fibers in the central nervous system.

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References

Explanation of Figures

Plate I

Dark-field photomicrographs of preparations preserved for 6 to 14 months after the HRP reaction by means of the present modified method. × 160.

Fig. 1. Horizontal section through the optic tract. Very fine optic nerve fibers in the anterior fasciculus (AF) of the accessory optic tract (arrows) can be observed. Six months after the reaction. OT: optic tract.

Fig. 2. Sagittal section of the medial terminal nucleus of the accessory optic system (MN). Fourteen months after the reaction.

Fig. 3. Sagittal section through the superior colliculus. Optic nerve fibers and their terminals are clearly observed. Fourteen months after the reaction. OS: optic stratum of the superior colliculus, SG: superficial gray stratum of the superior colliculus.