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A SIMPLIFIED METHOD FOR SELECTIVE SILVER IMPREGNATION OF DEGENERATING AXONS AND THEIR TERMINALS IN THE CENTRAL NERVOUS SYSTEM

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Takashi YAMADORI. *A Simplified Method for Selective Silver Impregnation of Degenerating Axons and Their Terminals in the Central Nervous System.* Kobe J. Med. Sci. **23**, 1-5, December 1977----A simplified method for selective silver impregnation of degenerating axons and their terminals was developed, modifying the Nauta-Gygax method and was used in experiments on the fiber connection in the central nervous system of mice, rats and dogs with satisfactory results. An electron microscopic observation was also performed to confirm the result. The main merits of this method are 1) the stability of the result, always producing dark, and dot-like degenerating axons and their terminals against a clean, light-golden background, and 2) the very much shortened time of the whole procedure which enables the staining of a large number of sections in 30 to 40 minutes.

INTRODUCTION

Since Nauta and Gygax (1954) reported a method of silver impregnation for degenerating axons, many investigators utilized it in studying fiber connections in the central nervous system. At the same time many modified methods including Fink and Heimer's two methods (1967) which have been used frequently in recent years, were reported. Recently the author also developed two more effective and simplified methods of silver impregnation for degenerating axons and their terminals (1975). Due to stability of the result as well as the short time of the whole staining process, which takes only 30 to 40 minutes, these methods proved very beneficial for impregnating many sections at one time rapidly and evenly in experiments on the brain of mice, rats and dogs. However, the first method, using only 0.05% potassium permanganate, 1:1 solution of 1% oxalic acid and 1% hydroquinone, 0.5% phosphomolybdic acid, an ammoniacal silver nitrate solution and Nauta reducer, offers cleaner and more beautiful results than the second method in which uranyl nitrate is used instead of

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phosphomolybdic acid. Therefore, a slight modification of the first method here described is presently used routinely in the author's laboratory instead of the Nauta-Gygax (1954, 1957) and Fink-Heimer (1967) methods which had been used previously. In this paper the author wishes to present the procedure of this impregnation method as well as findings of electron microscopy which was performed to confirm the result of the impregnation.

IMPREGNATION METHOD

Usually adult mice, rats and dogs of either sex are used in the laboratory. On the 6th (mice and rats) or 8th (dogs) day after injuring the right retina or producing a lesion in the brain with an electrode, the animals are sacrificed by vascular perfusion of a 10% formalin from the left ventricle. Soon after fixation the whole brain is taken out and immersed in the same percent of formalin for the next 2 weeks. Then, the brain is embedded in 25% aqueous gelatin and immersed again in the same percent of formalin for 2 days. The whole block is frozen, serially sectioned at 25 or 30 μm thickness and preserved in the same fixative at about 5° C for up to 4 months. At the time of impregnation 15 to 35 sections are put into a shallow plastic basket of either 10 or 13 cm diameter which has a fine meshwork and can be immersed in a Petri dish of either 12 or 15 cm diameter. If the basket is separated into 2 parts by a septum, 2 series of sections can be impregnated at one time. Therefore, to transfer the basket from one dish to the next and to agitate is enough for each step and there is no need to handle each section individually with a curved glass rod except at the final step of mounting.

The procedure of impregnation is as follows:

1. Wash sections briefly in distilled water.
2. Transfer sections to 0.05% potassium permanganate and agitate intermittently for 5 to 15 minutes.
3. Wash sections briefly in distilled water.
4. Transfer sections to 1:1 solution of 1% oxalic acid and 1% hydroquinone and agitate until completely decolorized.
5. Wash sections in 3 dishes of distilled water.
6. Soak sections in 0.3 or 0.5% phosphomolybdic acid for 5 to 10 minutes.
7. Wash sections thoroughly in at least 3 dishes of distilled water.
8. Transfer sections to freshly made ammoniacal silver nitrate solution (100 ml 1.5% silver nitrate, 50 ml absolute ethanol, 10 ml ammonium hydroxide, 8 ml 2.5% sodium hydroxide) and agitate for 2 to 10 minutes.
9. Transfer sections to Nauta-Gygax's reducing fluid and agitate vigorously for about 1 minute. A large amount of the fluid can be prepared in advance (27 ml 10% formalin, 27 ml 1% citric acid, 90 ml absolute ethanol, 800 ml distilled water), and used according to need.
10. Wash sections in distilled water and soak them briefly in 1% sodium thiosulfate.

Then the sections are washed and put into gelatin alcohol according to Albrecht's mounting method (1954). They are mounted onto slides, dehydrated in the air, put into xylol for 3 to 6 hours and covered for observation.

For electron microscopy, only a well impregnated small portion was selected and cut out from the section which had been impregnated by this method. In this portion, many fragments of degenerating nerve fibers and fine granules which were thought to be degenerating terminals were seen. This small portion of the section was fixed again with phosphate buffered 1% osmic acid, dehydrated in the ethanol series and finally embedded in Maraglas 655. Ultrathin sections were prepared by Porter-Blum II type ultramicrotome and stained with Reynolds' lead citrate solution. A HU-12AS type electron microscope was used for the observation.

RESULTS AND DISCUSSION

By this method, degenerating axons and their terminals are clearly demonstrable as dark dots against the golden or yellowish background (Figs. 1 and 2). As

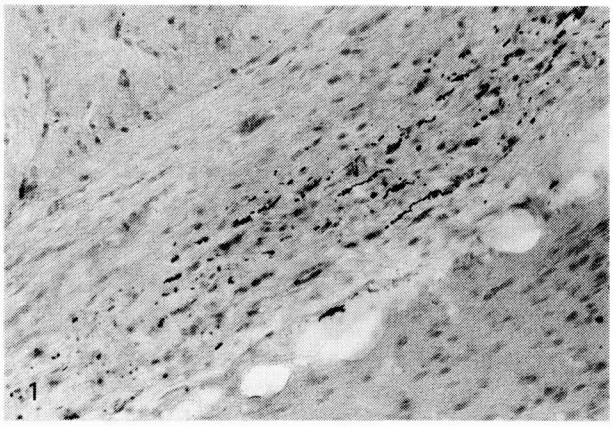

Fig. 1 Degenerating axons in the contralateral optic tract after partial injury of the right retina in the rat. Frontal section. ×170.

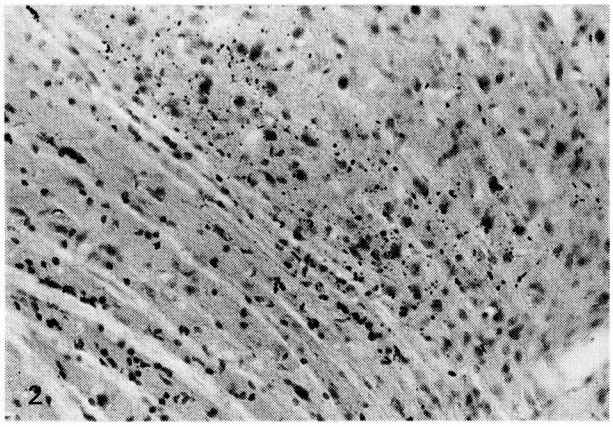

Fig. 2 Degenerating axons and their terminals in the contralateral dorsal nucleus of the lateral geniculate body after partial injury of the right retina in the rat. Frontal section. ×170.

the contrast of degenerating axons and their terminals to the background is so sharp, the general distribution of these axons and terminals can be observed easily with a microscope. By electron microscopy of impregnated sections, the silver that is deposited by the staining procedure can always be observed in degenerating axons and their terminals (Figs. 3 and 4).

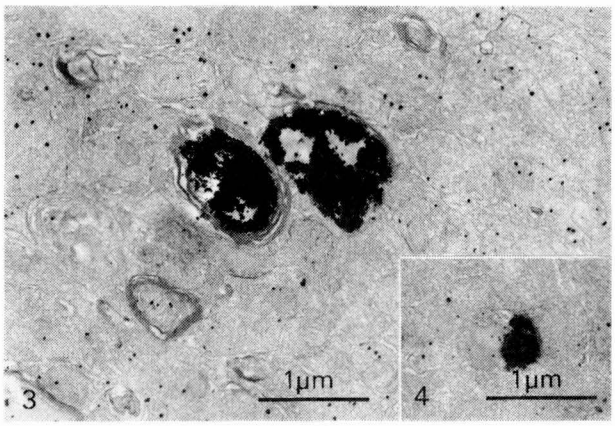

Fig. 3 Electron micrograph of silver granules in degenerating axons of the superior colliculus in the rat. The myelin sheaths are quite free from silver deposit.

Fig. 4 Electron micrograph of silver granules in a degenerating terminal of the superior colliculus in the rat.

In the entire procedure of the present method, the treatment in potassium permanganate is important. As Nauta and Gygax (1954) pointed out in their report, longer treatment will not only inhibit silver impregnation of normal fibers but also limit the impregnation of degenerating axons and their terminals, while shorter treatment will cause the impregnation of some normal fibers in addition to the impregnation of degenerating axons and their terminals. The optimum duration in this solution must be decided by preliminary test staining for each group of sections. The treatment in the phosphomolybdic acid is also important to get a good and stable result. However, 5 to 10 minutes seem to be adequate. If the sections have been preserved in the fixative for rather a long period, a weak solution of phosphomolybdic acid (0.3%) will be preferable. The optimal immersion time in the ammoniacal silver nitrate is usually 2 to 10 minutes. When the immersed sections are colored in light yellow, it is time to transfer.

The result of this impregnation method seems more like that of the Fink-Heimer method (1967) than that of the Nauta-Gygax method (1954). However, the background appears much lighter by this method.

SUMMARY

A simplified method for selective silver impregnation of degenerating axons and their terminals, which is presently used routinely in the author's laboratory

instead of the Nauta-Gygax and Fink-Heimer methods, was reported. To confirm the result of the impregnation, an electron microscopic examination was also performed. The main procedure of this method is as follows:

1. Wash sections briefly in distilled water.
2. Transfer sections to 0.05% potassium permanganate and agitate intermittently for 5 to 15 minutes.
3. Wash sections briefly in distilled water.
4. Transfer sections to 1:1 solution of 1% oxalic acid and 1% hydroquinone and agitate until completely decolorized.
5. Wash sections in 3 dishes of distilled water.
6. Soak sections in 0.3 or 0.5% phosphomolybdic acid for 5 to 10 minutes.
7. Wash sections thoroughly in 3 dishes of distilled water.
8. Transfer sections to freshly made ammoniacal silver nitrate solution (100 ml 1.5% silver nitrate, 50 ml absolute ethanol, 10 ml ammonium hydroxide, 8 ml 2.5% sodium hydroxide) and agitate for 2 to 10 minutes.
9. Transfer sections to Nauta-Gygax's reducing fluid and agitate vigorously for about 1 minute.
10. Wash sections in distilled water and soak them briefly in 1% sodium thiosulfate.

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