A STUDY OF POTASSIUM PERMANGANATE FIXATION FOR ELECTRON MICROSCOPIC DETECTION OF SMALL GRANULAR VESICLES IN THE SYMPATHETIC NERVOUS SYSTEM

JUNZO OCHI

*Department of Anatomy (Director: Prof. Y. SANO), Kyoto
Prefectural University of Medicine, Kyoto*

Received for Publication February 1, 1969

A 3% solution of potassium permanganate is effective at acid pH as a fixative for the small noradrenaline-containing granular vesicles of the sympathetic nervous system. The observed high electron density of the central granules in the vesicles is probably due to precipitated MnO_2 which is reduced KMnO_4 in a coupled oxidation of noradrenaline to noradrenochrome. A 3% solution of KMnO_4 in dichromate buffer (pH 6.0) is particularly suitable for the fixation of the vesicles. This may be due to the additional precipitation of a chromic compound reduced from the dichromate.

Routine OsO_4 fixation is not always suitable for electron microscopic detection of the small granular vesicles with a diameter of ca. 500 Å in the sympathetic nervous system. These vesicles have central electron dense granules which are believed to contain noradrenaline (3, 11, 6). Various attempts have been made by several investigators to preserve the central granules of the granular vesicles (12, 2, 10, 5, 8).

The method of Richardson (10), Hökfelt (7) and Ochi *et al.* (9), potassium permanganate fixation (3% KMnO_4 , pH 7.0-7.3), has proved most suitable for the detection of vesicles, although the general fixation of the tissues was somewhat less than excellent. The aim of the present study is to examine the influence of the pH of the KMnO_4 solution on the fixation of the granular vesicles and to determine the origin of the high electron density of the central granules in the vesicles. An attempt has also been made to improve the fixation quality of the tissues by use of a modified KMnO_4 solution.

MATERIALS AND METHODS

Materials used were rabbit iris, normal and reserpinized (5 mg/kg, 4-12 hr before sacrifice), and rat brown adipose tissue. Rabbits were sacrificed under Nembutal anesthesia and rats with an occipital blow.

To determine the influence of the pH of 3% KMnO_4 solutions on the fixation of the granular vesicles, the following fixatives were prepared using different buffer systems covering a pH range from 1.0 to 10.0;

- 1) 3% KMnO_4 in HCl-KCl buffer (pH 1.0)
- 2) 3% KMnO_4 in phosphate buffer (pH 5.8, 7.0 and 7.9)
- 3) 3% KMnO_4 in borax-NaOH buffer (pH 10.0).

In addition, 3% KMnO_4 in 0.1 *M* potassium dichromate-KOH buffer at pH 6.0-7.0 was also employed in the expectation that a reaction product of dichromate with noradrenalin would lead to increased electron density of the granules in the vesicles.

Small pieces of tissue were fixed for 2 hr at 4°C, then dehydrated in a graded series of acetones, and routinely embedded in Epon 812. Ultrathin sections were cut with a Porter-Blum MT-1 ultramicrotome and examined in a JEM-7 electron microscope, either without staining or after light staining with uranyl acetate to enhance background contrast.

Some unstained sections mounted on platinum grids were treated with fuming hydrochloric, nitric and sulfuric acids or conc. sodium hydroxide for 5 min after electron microscopic pre-examination, and immediately after that re-examined, especially with regard to changes of the granular vesicles in the identical areas which had been pre-examined.

RESULTS

1. The validity of KMnO_4 fixation for the granular vesicles.

Two apposed, fine unmyelinated axons, one with a number of small granular vesicles and the other with agranular vesicles, covered by the cytoplasm of a Schwann cell between the dilator layer and the sphincter zone.

Fig. 1. Normal rabbit iris, fixed with 3% KMnO_4 in phosphate buffer (pH 7.0). Two apposed unmyelinated axons covered by a Schwann cell between the dilator layer and the sphincter zone. Note that the axon above (A_1) has only agranular vesicles, whereas the axon below (A_2) a number of the granular vesicles. $\times 53,000$
 Fig. 2. Reserpinized rabbit iris, fixed with the same fixative as in Fig. 1. Two unmyelinated axons, one with agranular vesicles (A_1) and the other with almost no vesicles (A_2), in the stroma close to the dilator. $\times 48,000$

Fig. 3. Normal rabbit iris. Influence of the pH of 3% KMnO₄ solutions on the granular vesicles. a, pH 1.0; b, pH 5.8; c, pH 7.9; d, pH 10.0. Observations were made with unstained sections. Note the more conspicuous appearance of the granules in the granular vesicles at the range of acid pH. $\times 50,000$, $\times 57,000$, $\times 54,000$, and $\times 53,000$, respectively

cell, were clearly demonstrated between the iris dilator and sphincter muscles by the use of a KMnO₄ fixation solution (Fig. 1). This finding offers us evidence to confirm that not all synaptic vesicles appear granular after KMnO₄ fixation. It seems highly probable that these granular vesicles are the storage site for noradrenalin since the granules in the vesicles were almost completely depleted by prior administration of reserpine, a catecholamine-depleting drug (Fig. 2).

2. The influence of the pH of the fixation solutions of the granular vesicles.

The pH range tested was from 1.0 to 10.0. As is shown in Fig. 3, the lower the pH of the fixation solution, the more conspicuous the granules of the vesicles appear. However, the fixation quality of other tissue components was considerably poorer in cases in which the pH of the fixation fluid was extremely far from neutrality.

Fig. 4. Normal rabbit iris, fixed with 3% KMnO_4 in phosphate buffer (pH 7.0). Treatment of an unstained section with fuming HCl for 5 min. a, before the treatment; b, after the treatment. An arrow in Fig. 4 a indicates a typical granular vesicle, and an arrow in Fig. 4 b the identical vesicle with that indicated in Fig. 4a. Note the loss of high electron density of the granular vesicles after the treatment and the resulting reverse contrast similar to that obtained by negative staining. $\times 54,000$

Considering these facts, a 3% KMnO_4 solution at a pH of ca. 6.0 is recommended for the fixation of granular vesicles.

3. The nature of the high electron density of the granules in the granular vesicles.

After treatment of sections with fuming hydrochloric acid, the granules in the vesicles were completely freed of highly electron dense material (Fig. 4). Treatment with other mineral acids or sodium hydroxide produced no remarkable change. This result suggests that the high electron density of the granules was caused by the precipitation of MnO_2 which is soluble in fuming hydrochloric acid.

4. Improvement of the KMnO_4 fixation method for granular vesicles.

The best fixation is obtained in materials fixed in 3% KMnO_4 in dichromate-KOH buffer at pH 6.0. The granular vesicles appear more constantly with central granules, the granularity of the fixed tissue is relatively fine and membrane systems are quite satisfactorily preserved (Fig. 5). We also find that this fixation solution is superior to the other KMnO_4 fixatives tested, especially for the detection of granular vesicles in tissues rich in lipids, like brown adipose tissue (Ochi *et al.*, to be published).

Fig. 5. Normal rabbit iris, fixed with 3% KMnO₄ in dichromate buffer (pH 6.0). A nerve terminal containing the granular vesicles (Tg) is closely contacted with an iris dilator muscle (Md). Note the relatively good preservation of the granular vesicles and other cell components. $\times 45,000$

DISCUSSION

The mechanism of fixation with KMnO₄ for the granular vesicles in the sympathetic nervous system can be considered as follows: KMnO₄, a strong oxidizing agent, presumably oxidizes noradrenaline to noradrenochrome. Noradrenochrome may escape from the vesicles (4). KMnO₄ itself is reduced to MnO₂ which combines with or precipitates on carrier lipo- or glycoproteins. The deposited MnO₂ gives sufficient electron density to the vesicles for electron microscopy.

The experiment on sections with conc. mineral acids or alkali supports this hypothesis because MnO₂ is slowly dissolved only in cold fuming hydrochloric acid, but not in nitric or sulfuric acids, nor in conc. sodium hydroxide. This was confirmed by an *in vitro* test. Considering the more vigorous oxidizing action of KMnO₄ in acid solution than in alkali, it is easily understood that KMnO₄ fixation for the granular vesicles is more effective at acid pH.

As described in the results, the best quality of fixation for the vesicles can be obtained from a 3% KMnO₄ solution using potassium dichromate as buffer. In this case potassium dichromate probably acts not only as a buffer, but also as a fixative, especially for lipids in the tissue, as is observed in light microscopy. An increase in electron density of granules in the vesicles might be due to a reduced chromic compound of K₂Cr₂O₇ precipitated on the granules, in addition to MnO₂. This consideration is based on the fact that chrome or a chromic compound resulting from the chromaffin reaction gives a high electron density to catecholamine-

containing granules in electron microscopy (1).

ACKNOWLEDGMENT

The author's thanks are due to Prof. Y. Sano for his interest in this work and for his kind criticism of this manuscript.

Note added in proof: Since this paper was received for publication, a paper by Hökfelt and Jonsson (*Histochemie* 16; 45, 1968) has come to the author's attention. Independently of this study, they also consider that during the fixation with KMnO_4 noradrenaline reduces probably KMnO_4 to MnO_2 , which is the main constituent of the precipitate on the cores in the small granular vesicles.

REFERENCES

1. Arnold, M. und Hagar, G.: Zum Kontrast von Catecholamingranula im Elektronenmikroskop. *Histochemie* 14; 297, 1968.
2. Bloom, F. E. and Barrnett, R. J.: Fine structural localization of noradrenaline in vesicles of autonomic nerve endings. *Nature* (Lond.) 210; 599, 1966.
3. De Robertis, E. and Pellegrino de Iraldi, A.: A plurivesicular component in adrenergic nerve endings. *Anat. Rec.* 139; 299, 1961.
4. Devine, C.E. and Laverty, R.: Fixation for electron microscopy and the retention of ^3H -noradrenaline by tissues. *Experientia* (Basel) 24; 1156, 1968.
5. Devine, C. E. and Simpson, F. O.: The fine structure of vascular sympathetic neuromuscular contact in the rabbit. *Anat. Rec.* 121; 153, 1967.
6. Grillo, M. and Palay, S. L.: Granule-containing vesicles in the autonomic nervous system. In *Electron Microscopy*, vol. 2, U-1, edited by S. S. Breese jr., Academic Press, Inc., New York, 1963.
7. Hökfelt, T.: On the ultrastructural localization of noradrenaline in the central nervous system of the rat. *Z. Zellforsch.* 79; 110, 1967.
8. Machado, A. B. M.: Straight OsO_4 versus glutaraldehyde- OsO_4 in sequence as fixatives for the granular vesicles of sympathetic axons of the rat pineal body. *Stain Technol.* 42; 293, 1967.
9. Ochi, J., Konishi, M., Yoshikawa, H. and Sano, Y.: Fluorescence and electron microscopic evidence for the dual innervation of the iris sphincter muscle of the rabbit. *Z. Zellforsch.* 91; 90, 1968.
10. Richardson, K. C.: Electron microscopic identification of autonomic nerve endings. *Nature* (Lond.) 210; 756, 1966.
11. Wolfe, D. E., Potter, L. T., Richardson, K. C. and Axelrod, K.: Localizing tritiated norepinephrine in sympathetic axons by electron microscopy and autoradiography. *Science* 138; 440, 1962.
12. Wood, J. D. and Barrnett, R. J.: Histochemical demonstration of norepinephrine at a fine structural level. *J. Histochem. Cytochem.* 12; 197, 1964.