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Stabilization of tetramethylbenzidine (TMB) reaction product at the electron microscopic level by ammonium molybdate

Carl F. Marfurt [1], Dennis F. Turner [2] and Catherine E. Adams [3]

[1] *Department of Anatomy, Northwest Center for Medical Education, Indiana University School of Medicine, Gary, IN 46408 (U.S.A.),* [2] *Department of Oral Biology, University of Michigan, Ann Arbor, MI 48109 (U.S.A.) and* [3] *Department of Anatomy, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033 (U.S.A.)*

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The ability to use the tetramethylbenzidine (TMB) method for studying neuronal connections at the electron microscopic level is often difficult because the conditions of osmification and dehydration used in processing the tissue may result in significant loss and/or decreased electron density of the reaction product. In the present study, we report that stabilization of TMB reaction product with 5% ammonium molybdate (AM) prior to osmificating the tissue results in the formation of TMB-AM crystals that are many times more electron dense and resistant to ethanol extraction than non-stabilized TMB crystals. The nature of the chemical interaction that underlies the stabilization of TMB by AM is uncertain, but it may involve the formation of an insoluble salt between molybdic ions and the TMB polymer. The use of this simple procedure increases the sensitivity of the TMB procedure at the electron microscopic level and may be used to label neuronal pathways in the peripheral and central nervous systems with equal success.

Introduction

Tetramethylbenzidine (TMB) is presently the most sensitive and widely used chromagen for the light microscopic demonstration of intra-axonally transported horseradish peroxidase (HRP). Unfortunately, the successful extension of the TMB method to the electron microscopic level has been difficult because the conditions that are necessary for preparing TMB-reacted tissue for ultrastructural analysis result in significant loss and variable electron density of the TMB crystals. TMB polymer is increasingly soluble at pH's above 4.0 and is extremely unstable in graded and absolute ethanols (Mesulam, 1978). Several investigators have recently shown that the stability of the TMB reaction product may be improved by osmicating the tissue under conditions of elevated pH and temperature (Sakumoto et al., 1980; Schönitzer and Holländer, 1981; Sturmer et al., 1981; Carson and Mesulam, 1982a; Henry et al., 1985). However, even with these modifications, significant loss of reaction product from the tissue and reduced electron density of the crystals remain acknowledged problems (Carson and Mesulam, 1982b) and techniques that work well for some workers produce inconsistent results for others. Thus, the sensitivity of the TMB technique at the electron microscopic level remains in the hands of most investigators at a level well below the sensitivity of the technique at the light microscopic level.

Recently, two groups of investigators have reported using ammonium molybdate (AM) as an

Correspondence: C.F. Marfurt, Department of Anatomy, Northwest Center for Medical Education, 3400 Broadway, Gary, IN 46408, U.S.A.

effective stabilizing agent for TMB reaction product at the light microscopic level. Fujii and Kusama (1984) have shown that TMB crystals may be rendered resistant to ethanol extraction and time-dependent fading for up to 4 years if the sections are soaked briefly in a 5.0% solution of AM immediately following the TMB procedure. Subsequently, Olucha and coworkers (1985) have reported that substituting AM for sodium nitroferricyanide in the TMB solution produces crystals of reaction product in numbers comparable to those that may be obtained by using Mesulam's original protocol (Mesulam, 1978) but without the non-specific crystalline precipitates and tissue shrinkage that often accompanies the original procedure. Other workers have since shown that, under the appropriate conditions of osmification and dehydration, AM stabilization may also be used successfully to enhance the preservation of TMB reaction product at the electron microscopic level (Marfurt et al., 1986; Joosten et al., 1987). In the current investigation, we have extended our original observations (Marfurt et al., 1986) and report that the AM procedure of Fujii and Kusama (1984), when coupled with the modified osmification procedure of Carson and Mesulam (1982a), is an excellent method for labeling neural elements of both the peripheral and central nervous system at the electron microscopic level with TMB crystals that are extremely electron dense and resistant to ethanol extraction.

Methods

A total of 10 adult Sprague–Dawley rats of either sex, weighing 250–400 g, were anesthetized with i.p. doses of pentobarbital (50 mg/kg b. wt.). The surgical procedures and injection protocols used in the current report were the same as those described in our earlier studies (Marfurt and Turner, 1983; Turner and Marfurt, 1983). Briefly, the left trigeminal ganglion of each animal was exposed on the floor of the middle cranial fossa by removing a small portion of the parietal bone and aspirating the underlying brain tissue. At each of 3 sites along the anteroposterior axis of the ganglion, 0.5 μl of 2% HRP-WGA (Sigma) in saline was injected using a glass micropipette with a tip diameter of 50–70 μm. The animals were sacrificed 24–48 h later via perfusion-fixation through the left ventricle with 200 ml of warm, heparizined, buffered saline. This was followed immediately by 500 ml of 1.25% paraformaldehyde–2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 0.012% $CaCl_2$, pH 7.4, for 15–20 min, and then finally by an additional 15–20 min flush with 500 ml of ice-cold (4° C) 0.1 M cacodylate buffer, in order to remove excess fixative.

Immediately following the perfusion, the brainstem and those portions of the ipsilateral maxilla and mandible containing the 3 molar teeth were removed. The dental tissues were decalcified over a period of 2–3 weeks in daily changes of buffered, ice-cold, 0.2 M sodium (tetra)ethylene diamine tetra-acetate (EDTA) at pH 7.4. The teeth and brainstem were then sectioned at 40 μm on a Lancer vibratome and collected in fresh, ice-cold, 0.1 M phosphate or cacodylate buffer. Equal numbers of sections were processed for HRP histochemistry at the electron microscopic level according to one of the following TMB procedures:

Procedure 1. (TMB procedure of Carson and Mesulam (1982a), with minor modifications.)

(1) Rinse twice for 30 s each in ice-cold 0.1 M acetate buffer, pH 3.7.

(2) Incubate for 20 min at room temperature in a TMB medium prepared according to Mesulam (1978), except at pH 3.7 and 0.1 M, with no preincubation step.

(3) Rinse 3 times for 1 min each in ice-cold 0.1 M acetate buffer, pH 3.7.

(4) Osmicate in 1% OsO_4 in 0.1 cacodylate buffer, pH 6.0, at 45° C, for 45 min.

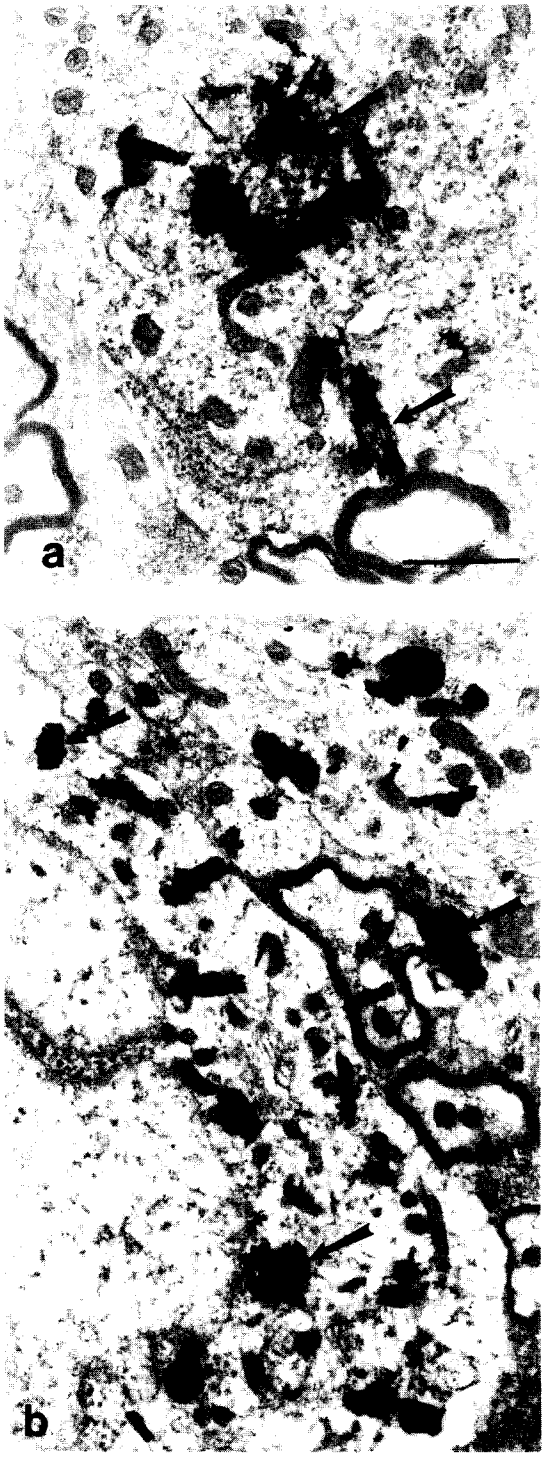
(5) Rinse twice for 5 min each in ice-cold 0.1 M cacodylate buffer, pH 6.0.

(6) Dehydrate for 7 min each in graded ethanols (50, 70, 95, 100 and 100%).

(7) Infiltrate with spurrs epoxy resin and embed in fresh spurrs resin between Teflon-coated coverslips.

Procedure 2. (Ammonium molybdate stabilization procedure of Fujii and Kusama (1984) adapted for electron microscopy.)

(1) Steps 1–3 (Procedure 1).



(2) Incubate for 15 min at room temperature on a rocker table in a solution of 5% ammonium molybdate (Molybdic acid, Sigma Chemical Co., St. Louis, MO, cat. #M-0878) in 0.1 M acetate buffer, pH 3.7. The ammonium molybdate solution should be prepared fresh 1–3 h before use. (Prolonged storage of the solution results in a slight discoloration which we interpret as a possible time-dependent chemical degradation.) The solution appears slightly cloudy when first mixed, and should be filtered before use.

(3) Rinse 3 times for 1 min each in ice-cold 0.1 M acetate buffer, pH 3.7.

(4) Osmicate for 45 min in 1% OsO_4 in 0.1 M cacodylate buffer according to *one* of the following procedures:

(a) pH 6.0, 45 °C (referred to henceforth as Procedure 2a);

(b) pH 6.0, 45 °C, with 1.5% potassium ferricyanide (Langford and Coggeshall, 1980) added to the solution (Procedure 2b);

(c) pH 7.3, 4 °C (Procedure 2c);

(d) pH 3.7, 4 °C (Procedure 2d).

(5) Steps 5–7 (Procedure 1).

Procedure 3. (Same as Procedure 2a, but with the TMB and AM reactions carried out at pH 6.0 in 0.1 M phosphate buffer.)

(1) Steps 1–3 (Procedure 2), but with all incubation and rinse solutions prepared in 0.1 M phosphate buffer, pH 6.0.

(2) Step 4a (Procedure 2).

(3) Step 5 (Procedure 2).

Following polymerization, the 80–100 μm thick resin wafers were trimmed and glued with quick-setting epoxy to the end of an epoxy resin block. Semi-thin (1–2 μm) plastic sections were

Fig. 1. Electron micrographs illustrating the difference in appearance between non-posttreated TMB crystals (Procedure 1) (a, arrows) and TMB crystals that had been posttreated with ammonium molybdate (Procedure 2) (b, arrows). Figs. a and b were photographed and printed under similar conditions of illumination and contrast. Note the altered internal structure and superior electron density of the crystals posttreated with AM (b). The micrographs reveal HRP-labeled neurons and surrounding neuronal elements in pars caudalis of the trigeminal brainstem nuclear complex. The bar in Fig. 1a is 1 μm and is applicable to both figures.

cut with glass knives on a Sorvall MT-6000 ultramicrotome, air-dried on slides, coverslipped, and examined with a light microscope. Areas of interest were identified, the sections retrimmed, and thin (80–100 nm) sections cut with a diamond knife. The sections were then collected on 150 mesh copper grids and viewed, unstained, in a Jeol JEM 100CXII electron miroscope.

Several sections from each group were isolated prior to the osmification step and processed for light microscopic analyses. Immediately following the TMB or ammonium molybdate stabilization step, the sections were mounted on gelatin-coated slides, air-dried, dehydrated rapidly through a graded series of ethanols, coverslipped, and examined critically with an Olympus BH-2 light microscope.

Results

The results of the current investigation showed that HRP-WGA injected into the trigeminal ganglion was taken up by the neuronal cell bodies and transported intra-axonally in large quantities into both the peripheral and central processes of these cells (see Marfurt and Turner, 1983). Twenty-four to 48 h postinjection, numerous HRP-filled sensory fibers were visible in the ipsilateral tooth pulps (Marfurt and Turner, 1983; Turner and Marfurt, 1983) and HRP-labeled terminal fields and transneuronally-labeled neurons were observed within the ipsilateral trigeminal brainstem nuclear complex (Marfurt and Adams, 1984).

Light microscopic observations

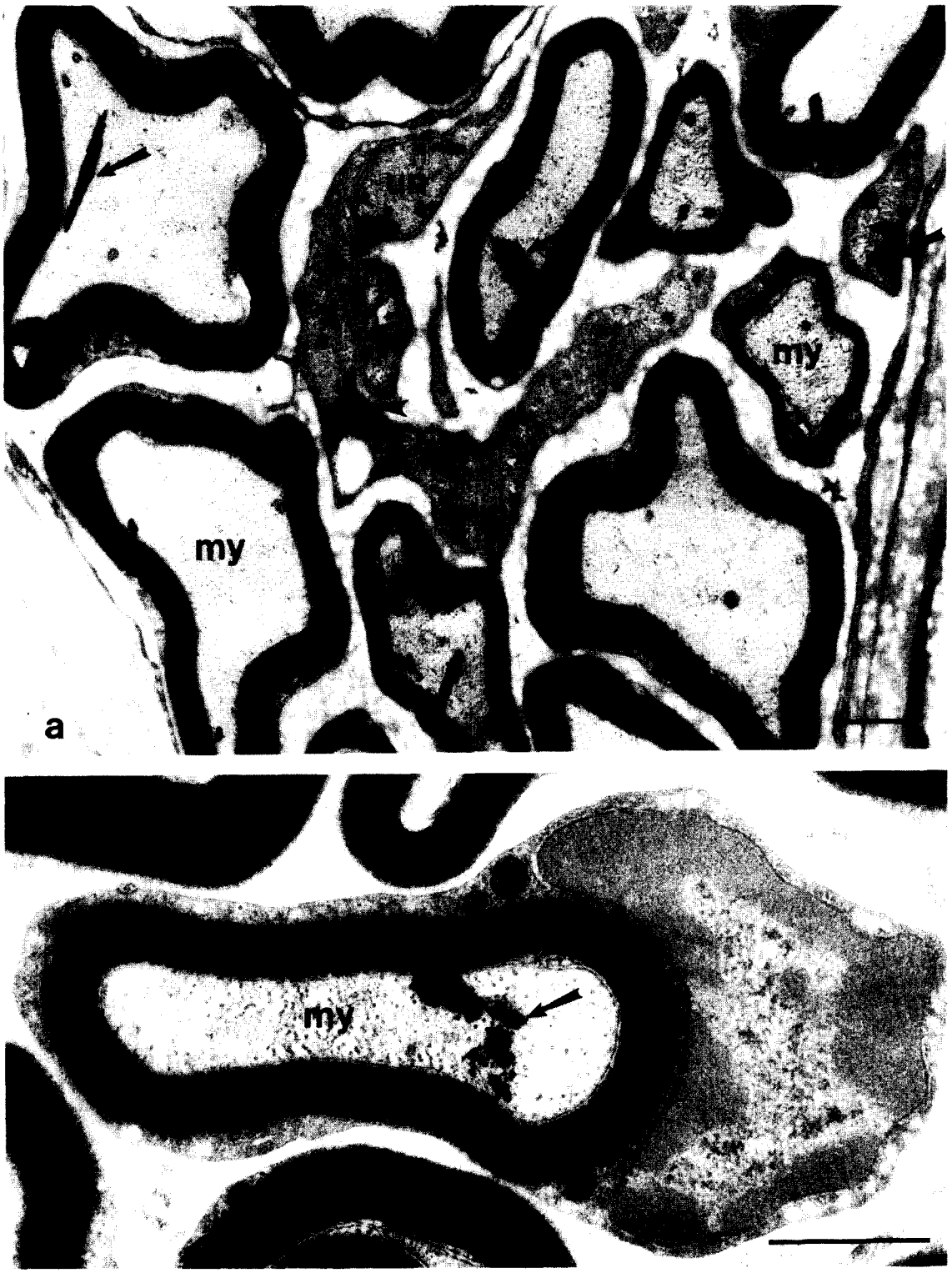
Bright field, light microscopic examination of sections processed according to Procedures 1 and 2 (without and with AM stabilization, respectively) revealed no significant differences in quantity, size, shape, or color of the reaction product crystals. However, when viewed under darkfield optics, untreated TMB-crystals appeared bright orange-yellow, whereas TMB crystals stabilized with ammonium molybdate (referred to throughout the remainder of the paper as TMB-AM crystals) were pale lavender in color. Sections processed according to Procedure 3 (incubation in TMB and AM solutions prepared in 0.1 M phosphate buffer at pH 6.0) contained very little visible reaction product at the light microscopic level.

Electron microscopic observations

At the electron microscopic level, dramatic differences in stability and electron density of the reaction product were observed. Tissue that had been processed according to Procedure 2a (with AM stabilization) contained many times more reaction product than sections processed in identical fashion but without previous AM stabilization (Procedure 1). TMB-AM crystals were of similar size and shape as non-posttreated TMB crystals, but the two forms of reaction product differed significantly in internal structure and electron density. Non-posttreated TMB crystals resembled densely-packed, parallel stacks of fine, needle-like profiles of moderate electron density (Fig. 1a). In contrast, TMB-AM crystals demonstrated an internal structure that was more blocky and smooth and were extremely electron dense, even in the absence of poststaining with lead citrate and uranyl acetate (Fig. 1b). As a result, the contrast between TMB-AM reaction product and other tissue components of both the central and peripheral nervous system was consistently excellent (Fig. 2).

Strict adherence to the recommended osmification procedure of Carson and Mesulam (1982a;

Fig. 2. Electron micrographs illustrating the extremely high electron density of TMB crystals (arrows) that have been posttreated with ammonium molybdate. The tissue is unstained and has not been treated with lead citrate or uranyl acetate. TMB-AM crystals constitute with rare exceptions the most electron dense structures in the tissue and are even more dense than myelin. At high magnification (b), note the characteristic blocky, amorphous internal structure of the reaction product. Occasionally, lysosome-like dense bodies (arrowhead in a) are observed that possess an electron density similar to that of the TMB-AM crystals, but the characteristic shape and internal morphology of the reaction product crystals make their identification clear and unambiguous. The micrographs illustrate HRP-labeled myelinated (my) and unmyelinated (un) sensory fibers in the pulp canal of a first maxillary molar tooth. The bar in Fig. 2a is 1 μm and in Fig. 2b 0.5 μm.


my
my
a
my

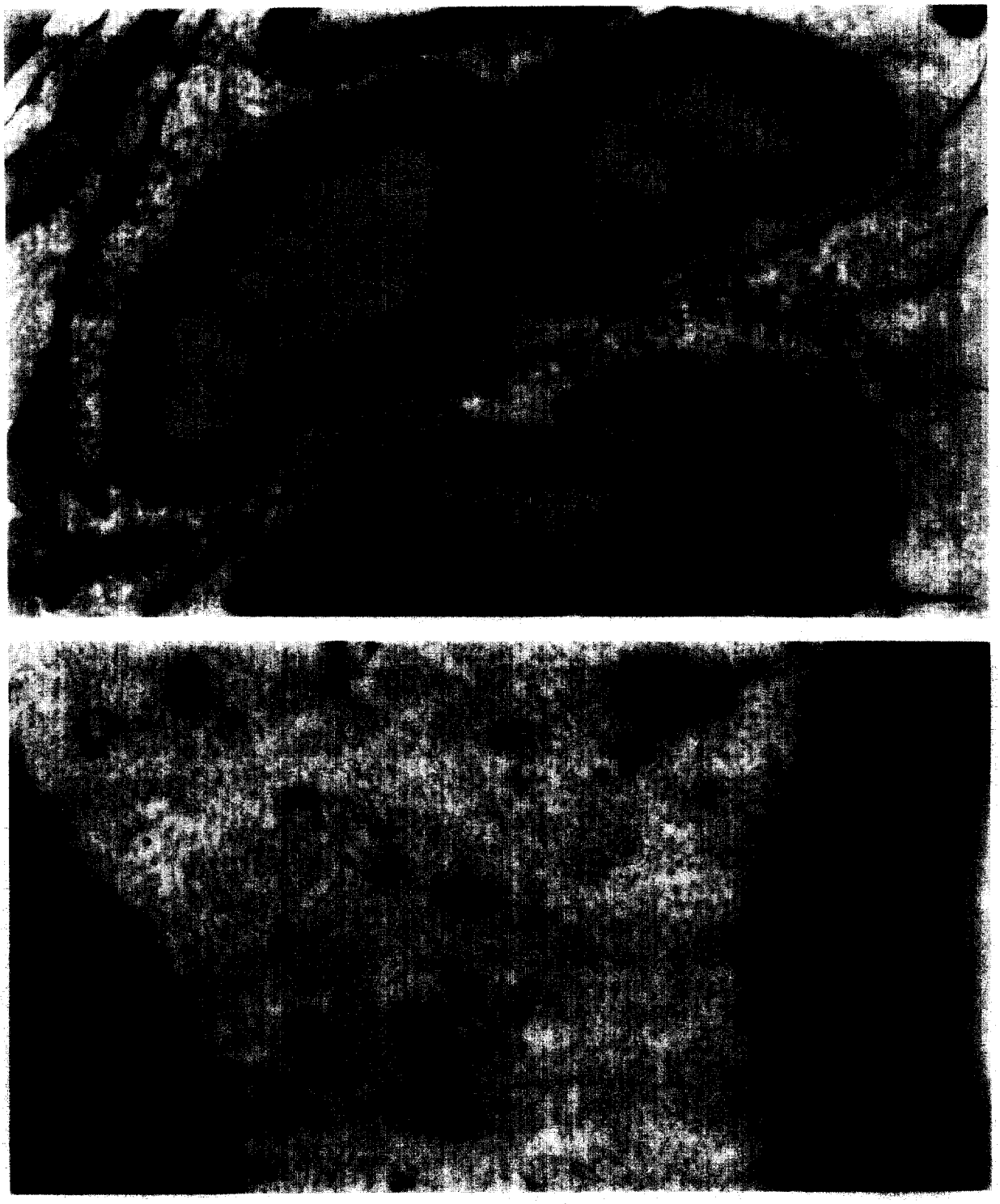


Fig. 3. Electron micrographs illustrating the typical appearance of TMB-AM crystals (arrows) that had been osmicated at pH 6.0 at 45 °C in the presence of 1.5% potassium ferricyanide (Procedure 2b). While the basic internal structure of the TMB-AM crystals remains unchanged from that shown in Figs. 1b and 2, note the marked reduction in electron density of the crystals osmicated in the presence of potassium ferricyanide. The micrographs illustrate HRP-labeled axons in a first maxillary molar tooth pulp. my, myelinated nerve fiber; un, unmyelinated nerve fiber; S, Schwann cell nucleus. The bar in Fig. 3a is 1 μm and in Fig. 3b 0.5 μm.

Procedure 2a) was critical to the preservation and enhanced electron density of the TMB-AM stabilized crystals. The addition of 1.5% potassium ferricyanide to the osmium solution (Procedure 2b) resulted in a significant loss of reaction product from the tissue, as well as a marked decrease in electron density of the individual crystals (Fig. 3a,b). However, addition of this compound to the osmium solution appeared to have a slight beneficial effect on the overall level of tissue preservation. Attempts to osmicate the TMB-AM crystals under conditions of pH and temperature different from those recommended by Carson and Mesulam (1982a; e.g. Procedures 2c, d) resulted in a complete loss of reaction product from the tissue.

Sections incubated in TMB and AM solutions prepared in 0.1 M phosphate buffer at pH 6.0 (Procedure 3) contained only modest amounts of reaction product within the tissue. TMB-AM crystals produced under these conditions (Fig. 4) were somewhat more flocculent in appearance and of diminished electron density compared to TMB-AM crystals produced and stabilized at pH 3.7. However, on the positive side, the quality of ultrastructural preservation was clearly superior in tissue reacted at pH 6.0; this was particularly evident with regards to the structure of the neuropil of the trigeminal brainstem nuclear complex (Fig. 4).

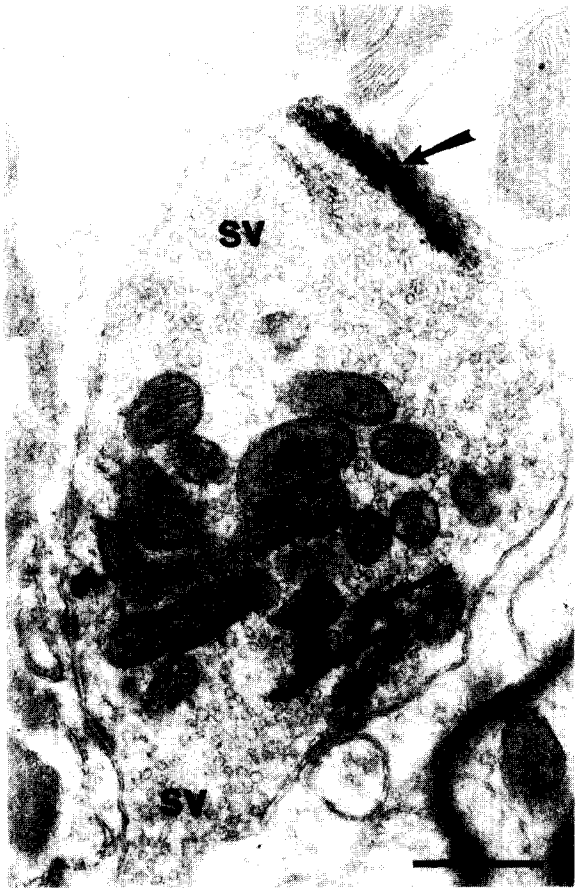


Fig. 4. Electron micrograph illustrating an HRP-labeled axon terminal in pars caudalis of the trigeminal brainstem nuclear complex. The tissue was reacted with TMB at pH 6.0, followed immediately by ammonium molybdate stabilization (i.e. Procedure 3). This procedure results in a decrease in electron density of the TMB-AM reaction product (arrows; compare with the electron density of the TMB-AM reaction product illustrated in Figs. 1b and 2), but an increase in the quality of ultrastructural preservation. m, mitochondria; sv, synaptic vesicles. Bar = 0.5 μm.

Discussion

The results of the present investigation have demonstrated that posttreatment of TMB reaction product with ammonium molybdate forms a TMB-AM complex that is many times more stable and electron dense than standard TMB polymer. TMB-AM crystals survive the modified osmification and dehydration steps used in TMB histochemistry at the ultrastructural level in numbers far greater than standard TMB crystals. Thus, the frequency with which reaction product is encountered and, therefore, the sensitivity of the TMB technique at the electron microscopic level, is greatly increased through the use of AM stabilization.

The nature of the chemical interaction that underlies the ammonium molybdate 'stabilization' of TMB crystals demonstrated in the present study is not clear, in part because the precise chemical composition of TMB polymer is still unknown. However, the alteration of internal structure that occurs when TMB crystals are stabilized with AM suggests that the heavy metal, molybdenum, may be bound to, or deposited on, the TMB polymer to form an insoluble salt. According to Olucha and coworkers (1985), molybdic ions are capable

of forming ion-paired complexes with benzidine derivatives, but only when the nitrogen of the amino-benzidine groups has a positive charge, as is the case when the benzidine derivatives are partially oxidized by HRP-H_2O_2 complexes. Molybdic ions are also strong precipitating agents for a variety of other positively charged molecules, such as proteins and quaternary ammonium compounds (Tsuji et al., 1983) and will form insoluble salts with these cations to 'fix' them in situ. Indeed, molybdenum compounds such as phosphomolybdic acid and ammonium molybdate are widely used in a variety of histologic and histochemical techniques as staining and contrasting agents for both light and electron microscopy (Ferrer et al., 1984).

An alternative explanation for the AM 'stabilization' of TMB crystals seen in the current investigation may be that molybdic ions interact with the TMB polymer to unmask active groups on the TMB molecule that will accelerate or enhance reduction of osmium tetroxide. This, in turn, could result in the formation of increased quantities of insoluble, and electron dense, osmium black.

The uncertain chemical nature of AM 'stabilization' of TMB polymer is further complicated by the fact that TMB-AM crystals survive the osmification, dehydration and embedding steps only when the osmification procedure is carried out at 45°C and pH 6.0. If the temperature of the osmium solution is lowered to 4°C, or if the pH is changed to either 3.7 or 7.3 (Procedures 2c, d), there is a near total loss of reaction product from the tissue. Thus, AM 'stabilization' does not appear to alter the conditions under which TMB polymer and osmium tetroxide will interact, but rather acts somehow to promote osmium black formation under these optimal conditions.

Another advantage of posttreating TMB crystals with AM besides the increase in reaction product preservation and electron density is that it enables one to add potassium ferricyanide (Langford and Coggeshall, 1980) to the osmium solution, if desired (Fig. 3a, b). This procedure may be useful in situations where the investigator is willing to sacrifice some of the sensitivity of the TMB-AM technique in favor of increased membrane definition. In marked contrast, addition of 1.5% potassium ferricyanide to the osmium solution during processing of tissue that has not been posttreated with AM causes a total loss of all TMB reaction product from the tissue (Carson and Mesulam, 1982b).

Finally, the results of the present study have shown that incubating tissue sections in TMB and AM solutions prepared with 0.1 M phosphate buffer at pH 6.0 (Procedure 3) markedly decreases the sensitivity of the technique, although it does result in improved ultrastructural preservation of some tissue types, e.g. the central nervous system. Recent work by one of the authors (Marfurt, unpublished data; see also Crutcher and Marfurt, 1988) has shown that most of the loss in sensitivity that accompanies the use of Procedure 3 may be prevented simply by substituting acetate buffer (also at pH 6.0) for phosphate buffer in all incubation and rinse procedures (Schonitzer and Hollander, 1981; Westman et al., 1986). Thus, with additional experimentation it may be possible to bring about even further improvements in the AM stabilization procedure beyond those reported here.

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