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Electron Microscopic Demonstration of Neural Connections Using Horseradish Peroxidase:

A Comparison of the Tetramethylbenzidine Procedure with Seven Other Histochemical Methods[1]

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Eight methods for the electron microscopic demonstration of horseradish peroxidase (HRP) labeling have been compared in adjacent series of vibratome sections of mouse lumbar spinal cord. The tracer, a HRP-wheat germ agglutinin (WGA) conjugate, was injected into the gastrocnemius muscle complex. Following retrograde axonal transport to the lumbar motor neurons and transganglionic anterograde transport of the tracer to the dorsal horn, the HRP activity was demonstrated in eight series of adjacent sections of lumbar spinal cord using eight methods. These included procedures using tetramethylbenzidine (TMB), benzidine dihydrochloride (BDHC), *o*-tolidine, paraphenylenediamine-pyrocatechol (PPD-PC), and 4 methods using 3,3′-diaminobenzidine (DAB). All eight methods were able to demonstrate both retrograde labeling of motor neurons and transganglionic anterograde transport into the dorsal horn. However, there were differences in the appearance of the various reaction products under the electron microscope. In addition, differences in the distribution of the reaction products were observed by both light and electron microscopy. The largest distribution of reaction product was observed with TMB. BDHC and *o*-tolidine were next, followed by the DAB procedures and PPD-PC. The TMB, BDHC, and *o*-tolidine reaction products were all found to be suitable for electron microscopy. The TMB reaction product was electron dense and had a very distinctive crystalloid appearance that made identification of HRP-labeled neuronal profiles easy and unequivocal.

KEY WORDS: Horseradish peroxidase ultracytochemistry; Neuronal tracing; Axonal transport; Tetramethylbenzidine; Benzidine dihydrochloride; *o*-Tolidine; Paraphenylenediamine-pyrocatechol; 3,3′-Diaminobenzidine.

Introduction

Quantitative light microscopic comparisons among nine procedures have shown that the choice of chromogen and incubation parameters influences the completeness with which neural connections are demonstrated with horseradish peroxidase (HRP) neurohistochemistry (Mesulam and Rosene, 1979). For example, a method based on tetramethylbenzidine (TMB) as the chromogen was shown to have a lower threshold than the others for detecting tissue-bound HRP activity. This increase in histochemical sensitivity resulted in the demonstration of connections that eluded detection by other less sensitive methods. Similar comparative studies based on different combinations of chromogens and incubation parameters have not been done at the level of electron microscopy.

The electron microscopic demonstration of HRP is most commonly accomplished by using 3,3′-diaminobenzidine (DAB) as the chromogen (Graham and Karnovsky, 1966; LaVail and LaVail, 1974). This chromogen affords a reliable ultrastructural method that has enabled the subcellular compartments of HRP transport to be investigated in detail. However, at least at the light microscopic level, the methods of Graham and Karnovsky (1966) and of LaVail and LaVail (1974) have a comparatively low level of sensitivity in comparison to methods based on other chromogens, such as benzidine dihydrochloride (BDHC) and TMB. Recently it has been shown that both TMB (Sakumoto et al., 1980; Stürmer et al., 1981) and BDHC (Hanker et al., 1981) can be used for

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electron microscopy. The results reported below show that individual histochemical methods yield markedly different levels of sensitivity at the electron microscopic level of analysis and that a TMB procedure adapted for ultrastructural analysis offers advantages that parallel its desirable properties for light microscopic analysis of neural connections.

Materials and Methods

Eight histochemical methods were used for detecting HRP activity in adjacent series of sections of the lumbosacral spinal cord in mice that had received HRP-wheat germ agglutinin (WGA) injections in the gastrocnemius muscle group. This system was chosen because it provided the opportunity to compare, in the same section, the distribution of retrograde transport to the ventral motor neurons and transganglionic transport across the dorsal root ganglia to the dorsal horn. The HRP-WGA conjugate was used as the tracer in order to maximize uptake and transport (Gonatas et al., 1979; Brushart and Mesulam, 1980). The conjugation of the HRP and WGA was done by the methods of Avrameas and Ternynck (1971) as described by Gonatas et al. (1979).

Twelve adult female mice (C57 BL/6J, Jackson Laboratories, Bar Harbor, ME) were used in this study. Prior to the injection of the tracer the mice were anesthetized by intraperitoneal injection of Chloropent (0.08 ml per 25 g body weight). Ten microliters of 10% (W/V) conjugate solution were injected into the gastrocnemius muscle complex on one side using a 50 μl syringe with a 27 gauge needle.

Forty-eight hours after the injection the mice were anesthetized with Chloropent (0.1 ml per 25 g of body weight) and gravity perfused through the left ventricle with saline until the organs and effluent were cleared of blood. This was followed by perfusion with about 300 ml of 1.25—4% glutaraldehyde (TAAB Laboratories Equipment, Reading Berkshire, U.K.) with 1% depolymerized paraformaldehyde in 0.1 M phosphate buffer at pH 7.3. A 2 cm segment of caudal spinal cord including the lumbar enlargement was excised and immersed in the same fixative for 1 to 4 hr at 4°C. At this point the cord segment was cut with a vibratome (Ted Pella, Inc., Tustin, CA), either immediately or after overnight storage at 4°C in phosphate buffer. Although this postfixation and the absence of a terminal perfusion with buffer may cause additional depression of HRP enzyme activity (Rosene and Mesulam, 1978), this procedure was followed in order to increase preservation of ultrastructural detail.

The spinal cord segment was embedded on end in 7% agar that had been melted and cooled to 40°C. The agar block containing the tissue was further cooled in a refrigerator for 10 min. The block of agar was trimmed and glued to a vibratome chuck using a quick-drying cyanoacrylate adhesive. The spinal cord segment was cut coronally on a vibratome at a thickness of 75 microns and divided into 8 series of approximately 20 sections each. The sections were rinsed in buffer from 4 to 24 hr.

Eight different procedures were used for the histochemical demonstration of HRP activity. Each spinal cord series was reacted according to one of these eight histochemical methods. Seven of these methods have been described in detail by the respective authors and include the following: DAB after LaVail and LaVail (1974), DAB after Malmgren and Olsson (1977), DAB after Streit and Reubi (1977), DAB-glucose oxidase after Itoh et al. (1979), paraphenylenediamine-pyrocatechol (PPD-PC) after Hanker et al. (1977) and Carson et al. (1980), *o*-tolidine after Somogyi et al. (1979), and TMB after Mesulam (1978). The BDHC procedure used in these cases was identical to the TMB procedure, except that 50 mg of BDHC was substituted for the 5 mg of TMB. No stabilization bath was used for the TMB or BDHC procedures (Mesulam et al., 1980). In general, the steps in each protocol were similar and included the following: 1) a brief rinse of the sections in the same buffer as that used in the incubation medium, 2) preincubation of the sections for 20 min at room temperature in the respective histochemical medium without hydrogen peroxide, or, in the case of the DAB-glucose oxidase method, without glucose, 3) incubation in the full medium for the appropriate time, and 4) rinsing of the sections in buffer. During the final rinsing period the sections were placed in petri dishes and viewed under a low power stereomicroscope to select sections with maximum staining in the dorsal and ventral horns. Selected sections could then be cover slipped wet and photographed with a light microscope. Some sections from each series were mounted on subbed slides, air dried, dehydrated in ethanol and xylene, and mounted in Permount for detailed examination with a light microscope. The remaining sections were processed further for electron microscopy.

In 7 of the 8 series of sections, osmication was done in a routine manner by immersing the sections in a 1% osmium tetroxide solution in 0.1 M phosphate buffer, pH 7.3, for 60 min at room temperature. Osmication conditions for the TMB series were based on the results of Sakumoto et al. (1980) and Stürmer et al. (1981) and were subsequently modified according to our results. Our original efforts were to osmicate the TMB-containing sections at a pH less than 4, since the TMB reaction product is increasingly soluble above pH 4 (Mesulam, 1978). While these conditions did not seem to interfere with reduction of osmium by tissue components, the TMB reaction product was lost from the sections. Indeed, Sakumoto et al. (1980) and Stürmer et al. (1981) have shown that osmication at neutral pH and at room temperature or above is necessary for preservation of the TMB reaction product through dehydration and embedding in epoxy resins. Sakumoto et al. (1980) reported that optimal conversion of the blue TMB reaction product into the dark, osmicated insoluble product occurred at 45°C and pH 7.2. In order to detect the optimal compromise of osmication and preservation of reaction product, we varied the temperature (37 or 45°C) and pH (5.0, 5.5, 6.0, 6.5, and 7.0) of the osmium tetroxide solution and examined by light microscopy the quantity of reaction product in 2 micron epoxy sections of tissue osmicated under each of the ten sets of conditions.

Except for the TMB series, all of the sections were dehydrated through a series of ethanols (50%, 70%, 95%, 100%, 100% acetone, acetone) in 10 min steps. The TMB series was dehydrated in a different series of ethanols (80%, 90%, 95%, 100%, 100%, acetone, acetone) in 7 min steps, as recommended by Stürmer (personal communication). All sections were infiltrated with a mixture of acetone and epoxy resin (1:1, v/v) for 2 hr and then put in 100% resin for 12–18 hr. Epoxy resins used included Spurr-Epon (Coleman et al., 1976), Epon 812, and Epon-Araldite. The results were equivalent with each. The sections were flat-embedded in the form of wafers between Teflon-coated cover slips (Romanovicz and Hanker, 1977) and polymerized at 65°C for 36 to 72 hr. The cover slips were separated from the wafers that were viewed with a light microscope. Optimal areas were selected, cut out with a scalpel, and glued with quick setting epoxy to the ends of epoxy resin blocks. Two micron sections of the wafer-embedded tissues were cut with glass knives, floated onto water drops on glass slides and dried on a hot plate at 80°C. The sections were cover slipped in Permount without counterstaining. These sections were examined in detail with a light microscope to select areas for thin sectioning and photography. The blocks were retrimmed and silver thin sections were cut with a diamond knife ad picked up on 200 mesh uncoated copper grids. Sections were viewed with a Philips 200 or Philips 301 electron microscope without poststaining.

Results

Osmication of the Tetramethylbenzidine Reaction Product

To determine the conditions of temperature and pH of osmication for the TMB reaction product for maximal conversion to the insoluble electron-dense product, ten series of adjacent sections of lumbar spinal cord from injected mice were osmicated at pH 5.0, 5.5, 6.0, 6.5, and 7.0, each at 37°C or 45°C. In all ten cases a reaction product was formed that proved resistant to loss during dehydration, infiltration, and embedding for electron microscopy. This reaction product was readily visible by light microscopy in 2 micron epoxy sections. Osmication at pH 6.0 and 45°C for 45 min offered an optimal combination for preservation of osmicated reaction product in our test system. All TMB reacted sections used for subsequent comparisons were osmicated under the optimal condition of 45°C and pH 6.0 for 45 min. In distinct contrast, our previous efforts to osmicate the TMB-containing sections at pH 3.7 and 4°C had failed, even though these conditions are optimal for preserving the reaction product for light microscopic studies (Mesulam et al., 1980). The use of osmium tetroxide with 1.5% potassium ferricyanide (Langford and Coggeshall, 1980) resulted in complete loss of the reaction product.

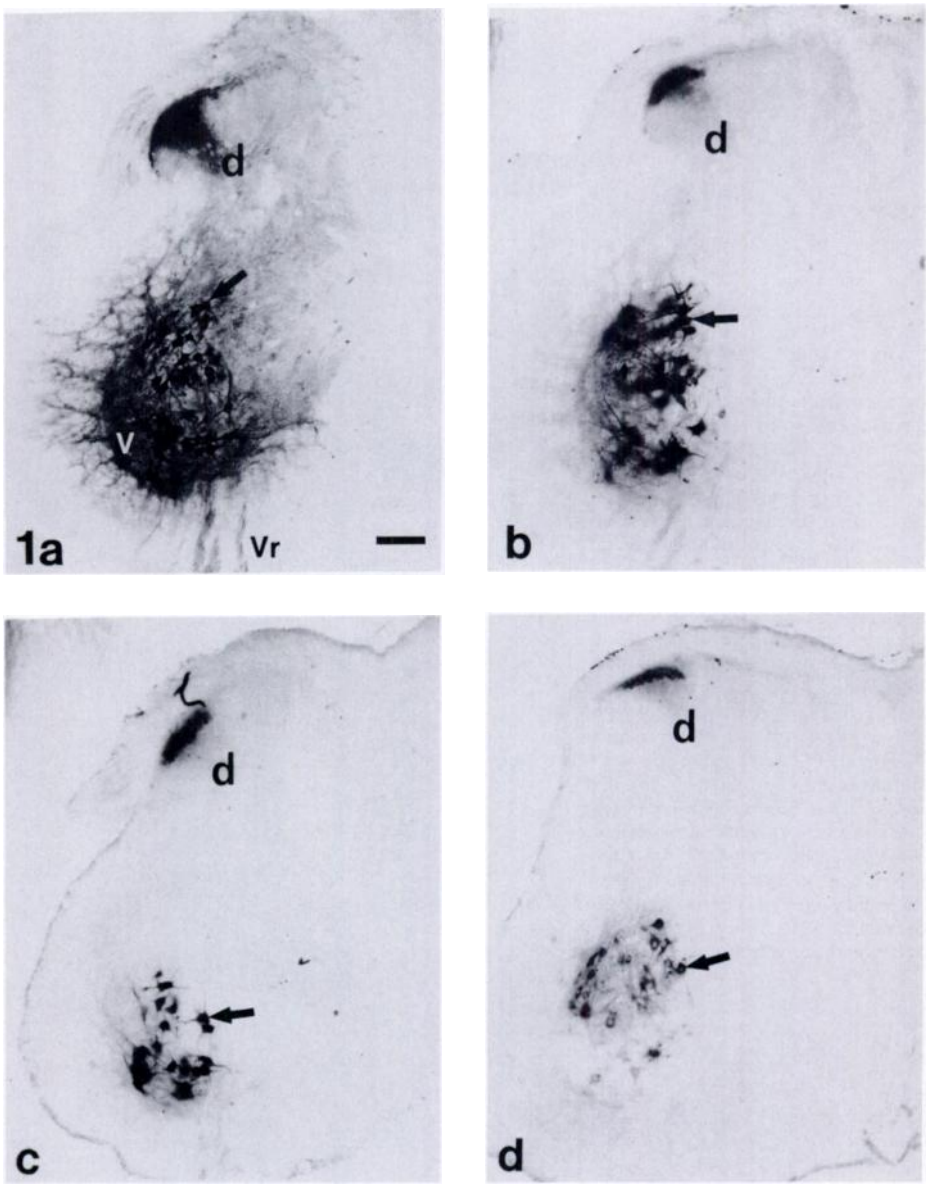


Figure 1. Light photomicrographs of 75 micron vibratome sections of the hemichord ipsilateral to the injected hind limb. Bar = 100 microns. (a) Tetramethylbenzidine method: HRP-WGA-labeled ventral horn motor neurons are densely stained (arrow). Reaction product is present in the ventral horn neuropil (V), and in several ventral root axons (Vr). In the dorsal horn, the substantia gelatinosa is heavily stained and many stained axons extend into deeper layers of the dorsal horn (d). Several dorsal root and dorsal column axons are also stained. (b) Benzidine dihydrochloride method: Labeled motor neurons in the ventral horn are darkly stained (arrow). The substantia gelatinosa contains heavy deposits of reaction product, but the number of stained axons penetrating into deeper layers is reduced compared to the TMB sections (d). (c) *o*-Tolidine method: Ventral horn motor neuron perikarya and proximal dendrites are filled with reaction product (arrow). The substantia gelatinosa is heavily stained, but very little reaction product is present in deeper layers of the dorsal horn (d). (d) Streit and Reubi DAB method: Ventral horn motor neuron perikarya are moderately stained (arrow). In the dorsal horn the substantia gelatinosa is also moderately stained. There is very little reaction product in deeper layers of the dorsal horn (d). (From Carson and Mesulam, 1982; with permission of Wiley and Sons, Inc.)

Light Microscopic Comparisons

Vibratome sections with maximal staining were selected for detailed light microscopic examination from each of the eight series prior to osmication. The criteria for comparison of each method were 1) the density of staining in the ventral horn neurons and the degree of dendritic labeling, and 2) the intensity and distribution of axonal labeling in the dorsal horn. At this level of examination, there were readily visible differences between the eight methods. In all eight series many ventral horn motor neurons were heavily stained, but the degree of staining varied between methods. The TMB, *o*-toluidine, and BDHC sections had dense brownish-blue reaction product that filled the perikaryal cytoplasm and demonstrated significantly more of the motor neuron dendrites than the other procedures (Figure 1). The DAB and PPD-PC methods yielded a brown granular reaction product that was predominantly in the perinuclear cytoplasm. The Streit and Reubi (1977), Malmgren and Olsson (1977), and DAB-glucose oxidase (Itoh et al., 1979) methods demonstrated a moderate number of dendrites, while the LaVail and LaVail (1974) and PPD-PC (Hanker et al., 1977) exhibited less dendritic reaction product. In the dorsal horn, the differences in the distribution of reaction product were more distinct. In the TMB series the substantia gelatinosa was heavily stained and a wedge of reaction product extended more ventrally into the dorsal horn (Figure 1a). The *o*-tolidine and BDHC methods also stained the substantia gelatinosa heavily, but the ventrally extending wedge in the dorsal horn was less obvious (Figures 1b,c). The Streit and Reubi, Malmgren and Olsson, and DAB-glucose

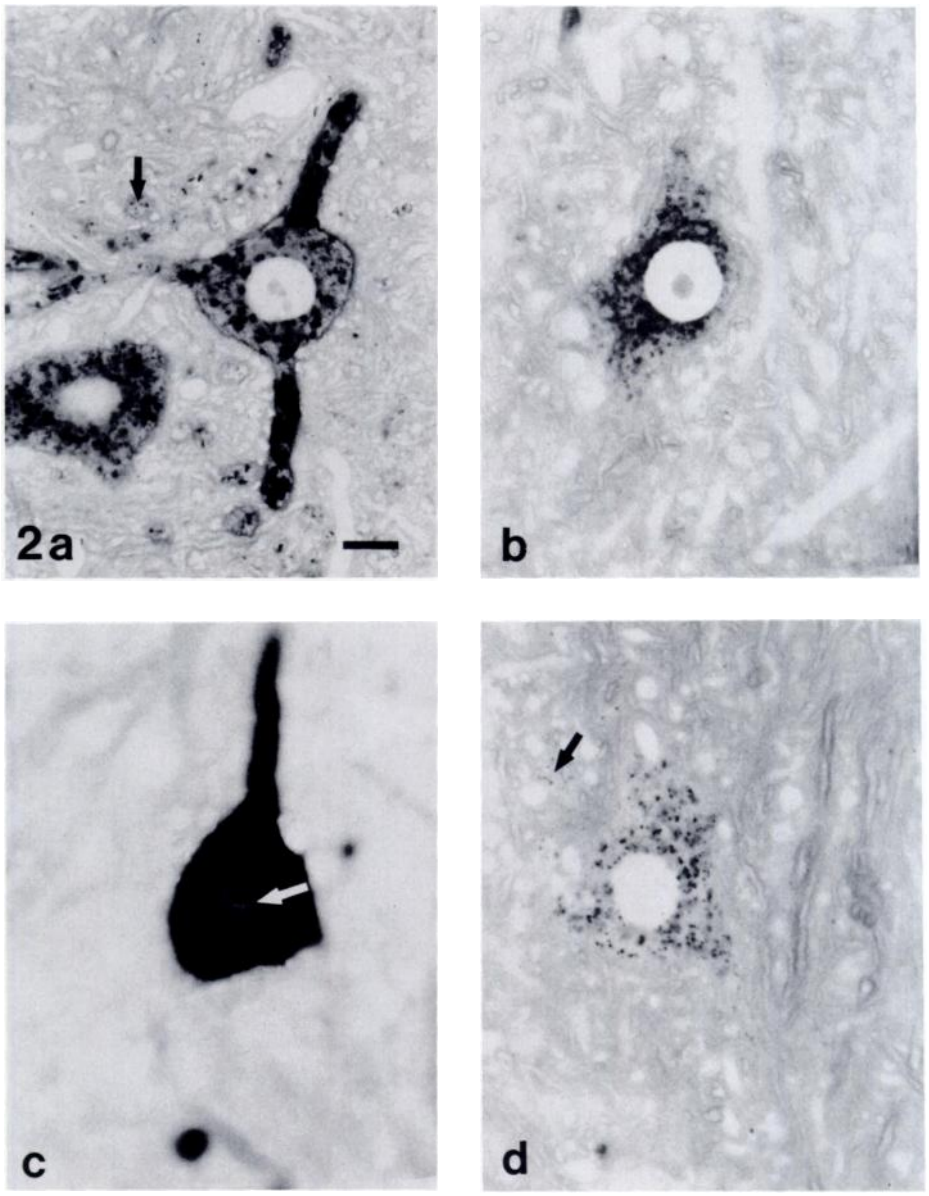


Figure 2. Light photomicrographs of two micron epoxy sections of labeled ventral horn motor neurons, no counterstain. Bar = 10 microns. (a) Tetramethylbenzidine method: Dense aggregates of reaction product fill the perikaryal cytoplasm and dendrites of labeled motor neurons. Several profiles of dendrites in the neuropil also contain reaction product (arrow). (b) Benzidine dihydrochloride method: Reaction product is present in the perinuclear cytoplasm of this labeled motor neuron. (c) *o*-Tolidine method: Very dense reaction product completely fills the perikaryon and proximal dendrite of this motor neuron. The nucleus (arrow) also appears to contain reaction product. (d) Streit and Reubi DAB method: Discrete granules of reaction product are present in the perinuclear cytoplasm of a labeled motor neuron and in adjacent dendritic profiles (arrow). (From Carson and Mesulam, 1982; with permission of Wiley and Sons, Inc.)

oxidase methods produced moderately heavy staining of the substantia gelatinosa, but the wedge was very faint if present at all (Figure 1d). The LaVail and LaVail and PPD-PC methods exhibited less dorsal horn staining with no sign of the ventral extension of reaction product. These light microscopic differences observed in the vibratome sections were also present after osmication, dehydration, and embedding in the 2 micron epoxy sections (Figures 2, 3).

Electron Microscopic Comparisons

The fine structural features and distributions of the reaction products of the eight histochemical methods were compared on the basis of several criteria: 1) the contrast between reaction product and tissue, 2) the morphology of the reaction products and the ease with which differentiation from endogenous tissue components could be made, 3) the intracellular compartments that contained or were associated with the reaction product, and 4) estimation of the frequency that reaction product containing neuronal structures was encountered. The sections were not poststained so that endogenous electron densities would not be erroneously identified as reaction product.

In general, during low magnification scanning of grids with the electron microscope, recognition of the TMB and BDHC reaction product aggregates was easiest. This was due, at least in part, to their unique and irregular appearance. The angular crystalline shapes could be distinguished from any endogenous electron-dense structures. Sections not incubated for the relevant histochemical reaction did not contain any similar crystalloid deposits. The high frequency of profiles containing

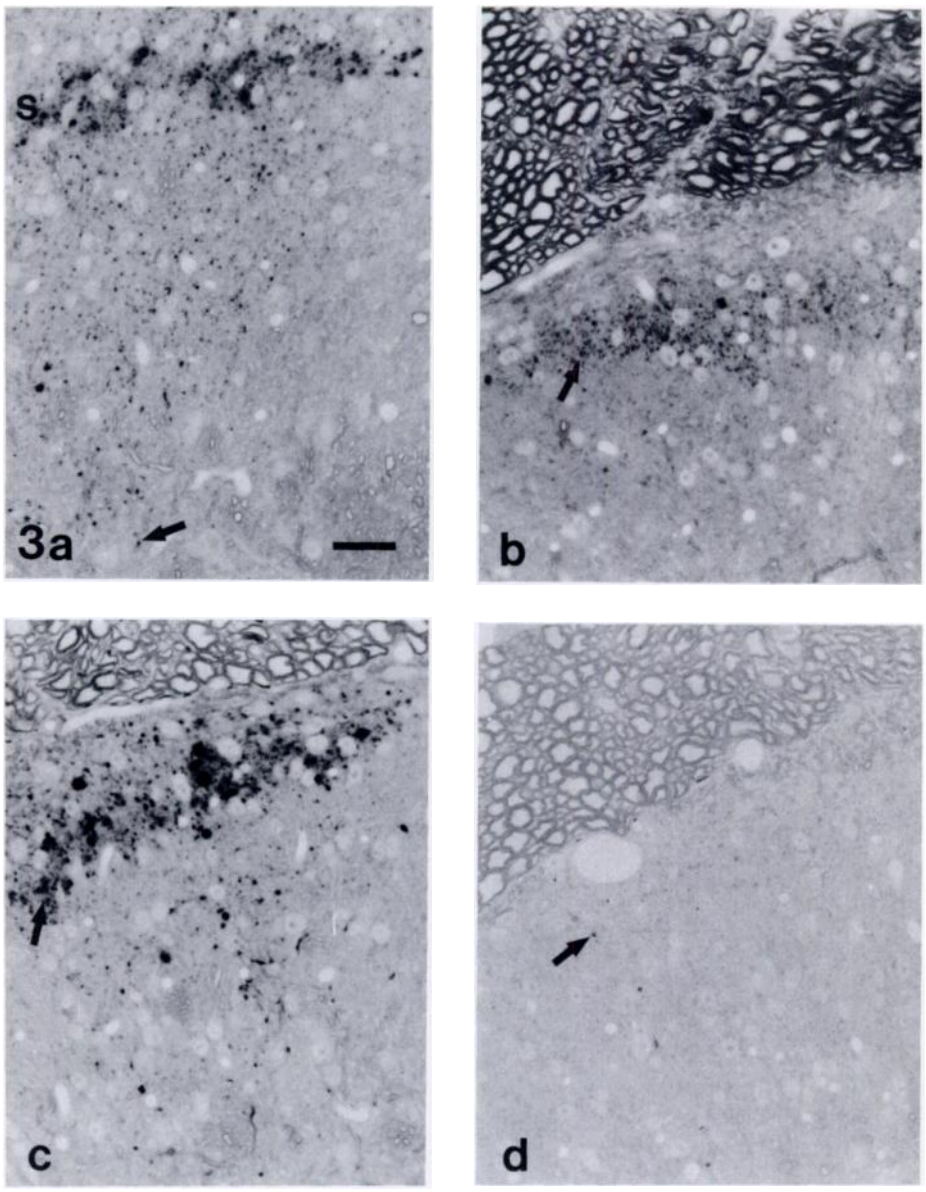


Figure 3. Light photomicrographs of two micron epoxy sections of labeled axons and terminals in the dorsal horn. Bar = 20 microns. (a) Tetramethylbenzidine method: Many labeled axon and synaptic terminal profiles contain reaction product. The substantia gelatinosa(s) contains the largest number of labeled profiles. Many stained axons extend into deeper layers of the dorsal horn (arrow). (b) Benzidine dihydrochloride method: Many labeled axons are present in the substantia gelatinosa (arrow). Relatively few stained axons are present in deeper layers of the dorsal horn. (c) *o*-Tolidine method: A large number of labeled axons in the substantia gelatinosa are densely stained (arrow). Several labeled profiles are present in more ventral layers of the dorsal horn. (d) Streit and Reubi DAB method: The substantia gelatinosa (arrow) contains relatively few stained axons. (From Carson and Mesulam, 1982; with permission of Wiley and Sons, Inc.)

TMB, BDHC, and *o*-tolidine reaction products observed with the electron microscope indicated that the apparent quantitative differences observed by light microscopy in the vibratome sections and in the 2 micron epoxy sections were also manifested at the ultrastructural level. The cellular compartments containing the DAB and PPD-PC reaction products were similar to those reported in previous studies (LaVail and LaVail, 1974; Nauta et al., 1975; Carson et al., 1980). In the DAB and PPD-PC material it was also possible to confirm a previous report that the HRP-WGA conjugate labels the GERL region of the Golgi apparatus (Figure 9a) following retrograde axonal transport (Harper et al., 1980). No labeled neurons were observed in the dorsal horn with any of the histochemical methods.

Tetramethylbenzidine

Ventral horn motor neurons labeled by retrograde axonal transport of the tracer were readily visible at low magnification under the electron microscope due to the presence of many aggregates of electron-dense reaction product in the cytoplasm (Figure 4a). This reaction product invariably extended into dendrites branching from labeled perikarya. Many dendritic profiles, both large and small, in the ventral horn neuropil also contained aggregates of reaction product. At higher magnification (Figures 4b, 5), the electron density and contrast of the reaction product aggregates could be more fully appreciated. Their unusual angular, crystalline shapes were very distinct from the cellular organelles (Figures 4b, 5). The spacing of lamellae in some selected aggregates of reaction product varied from 300–700 Å. Frequently, these dense aggregates were observed closely associated with lysosomes and Golgi saccules in the neuronal cytoplasm. However, the reaction product was not confined within these organelles. Although it is conceivable that these organelles contained HRP, the reaction product clearly exceeded their boundaries. In other instances, crystalline aggregates of reaction product were observed in the neuronal cytoplasm and were not clearly associated with a particular organelle. No reaction product was observed in the nucleus. In rare instances, it appeared that a segment of the reaction product actually penetrated the plasma membrane of the labeled neuron. In addition to perikaryal and dendritic staining, reaction product was also detected in synaptic boutons making contact with motor neuron dendrites. In some cases, motor neurons receiving these labeled boutons also contained reaction product.

In the dorsal horn many axonal profiles and boutons contained the reaction product (Figure 5). The frequency of these profiles was greatest in the substantia gelationsa, but there were also many in more ventral areas of the dorsal horn. In axons of the dorsal root and dorsal column (Figure 6) there were no large crystalline aggregates. Instead, smaller electron-dense clumps of reaction product were observed in the axoplasm, perhaps because of the lower HRP concentrations at these sites. The reaction product was not observed within neuronal perikarya located in the dorsal horn. This was the case even for neurons surrounded by reaction product-containing boutons. The aggregates of reaction product observed in the TMB-treated sections had variable morphology. In some instances elongated crystalloid bodies appeared to be uniform, whereas in other instances there was a lamellar appearance.

Benzidine Dihydrochloride

The BDHC reaction product, like that of TMB, was easily visible and unique in appearance. It was clearly electron dense, but in contrast to the crystalline structure of the TMB reaction product, the BDHC product had a filamentous morphology (Figure 7). The distribution of the BDHC reaction product in the ventral horn was similar to that of the TMB product, but the frequency of stained profiles was lower. In the dorsal horn, labeled profiles were numerous in the substantia gelatinosa. Reaction product-containing profiles were present in deeper layers of the dorsal horn also, but were less numerous than in the TMB sections.

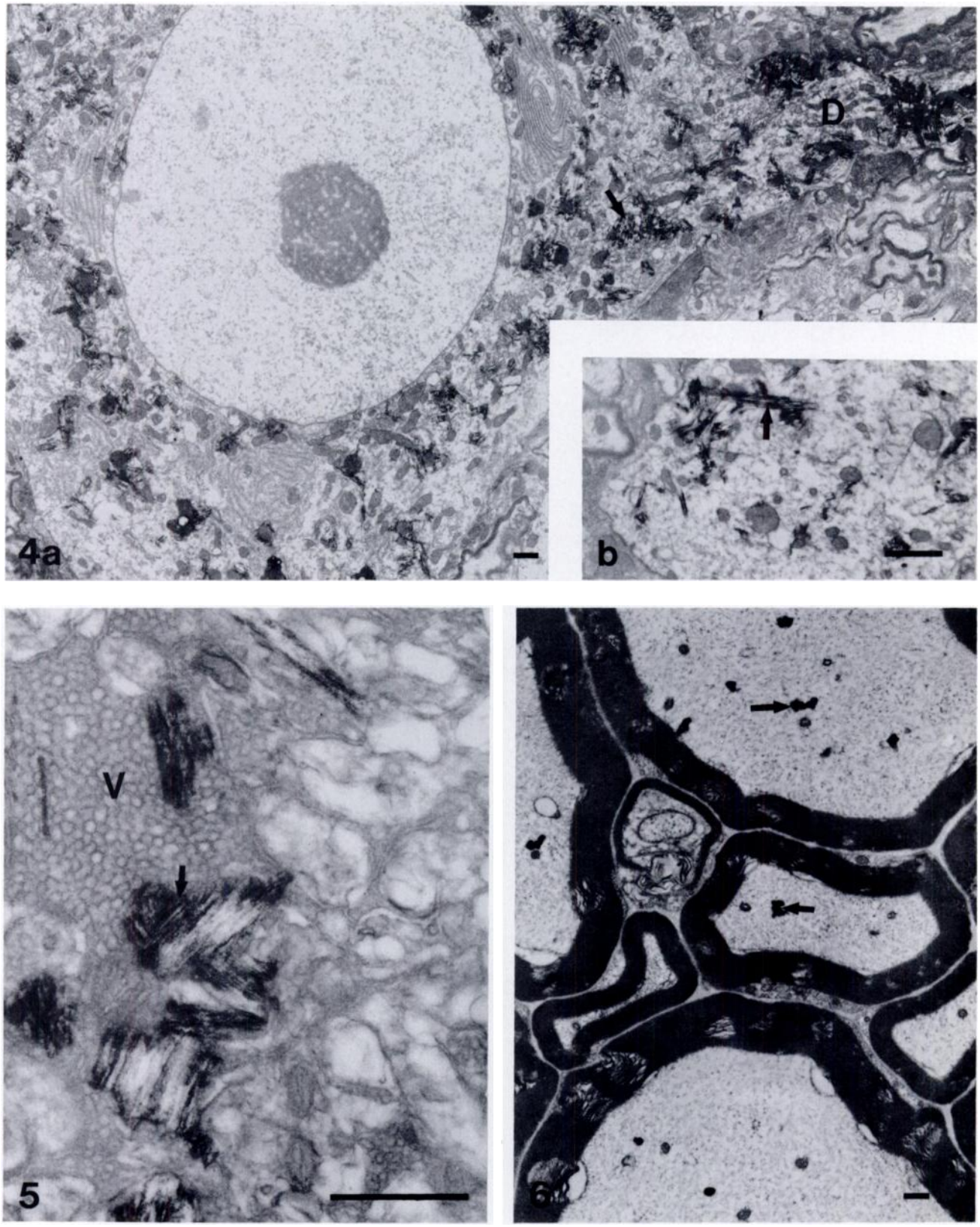
o-Tolidine

The *o*-tolidine reaction product was distinctly different from both TMB and BDHC. By light microscopy, labeled neurons appeared to be filled with a nongranular blue-brown reaction product. By electron microscopy, the *o*-tolidine reaction product appeared as a very fine moderately electron-dense deposit throughout the cytoplasm of labeled ventral horn motor neurons (Figure 8a). At low magnification, labeled motor neurons could be identified by their amorphous dense cytoplasm. Dendrites branching from the perikarya also contained this reaction product. Other labeled dendrites could be distinguished in the ventral horn neuropil adjacent to pale unlabeled dendrites. In the dorsal horn, labeled boutons had dark cytoplasm that caused the synaptic vesicles to stand out as pale circles (Figure 8b). The frequency of labeled structures in the dorsal and ventral horn was lower than that observed in the TMB series. Labeled neuronal processes were somewhat more difficult to distinguish with this method, particularly in cases where the amount of reaction product was low.

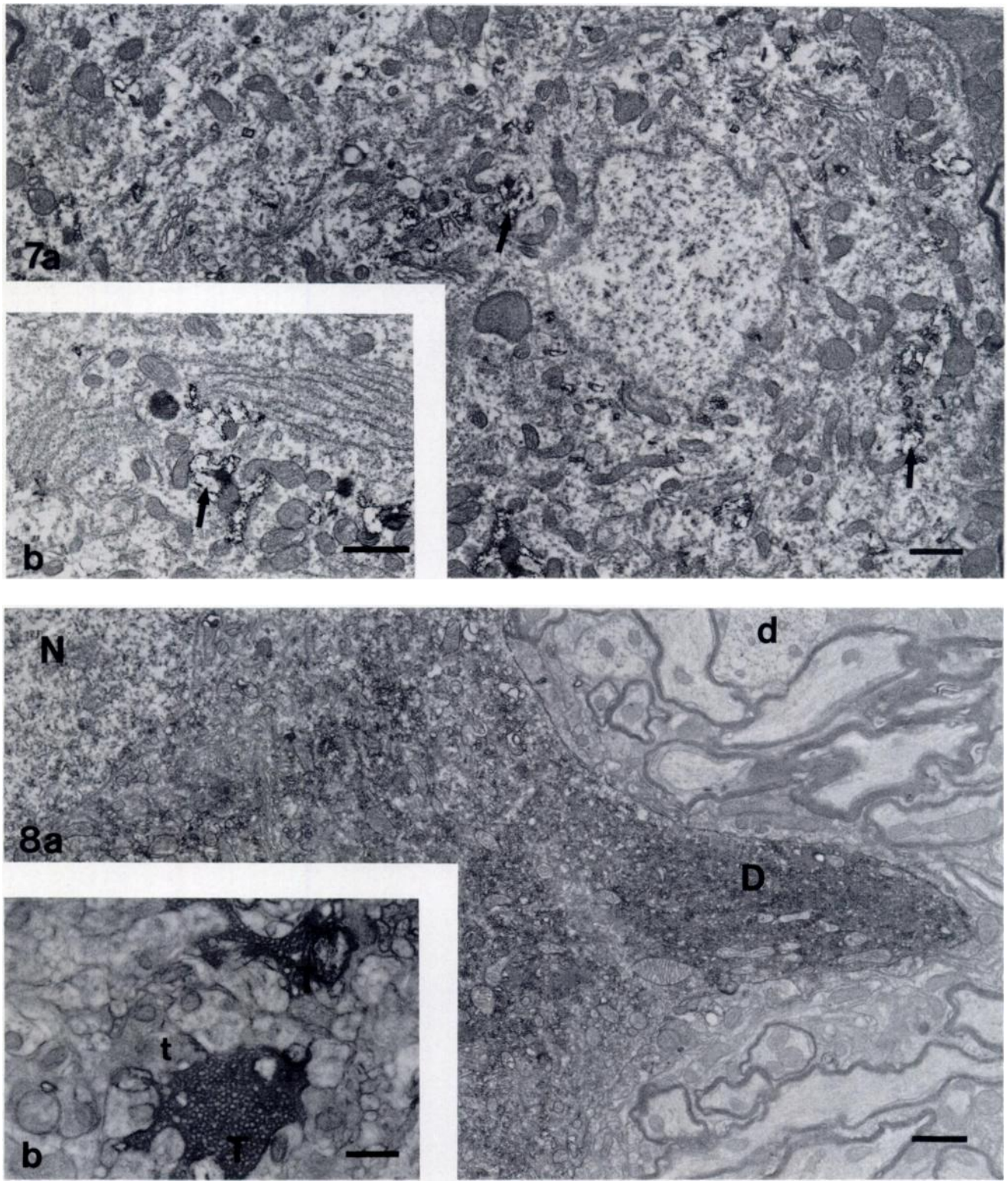
Figure 4. Electron-dense tetramethylbenzidine reaction product (arrow) is scattered throughout the cytoplasm of this motor neuron and extends into a proximal dendrite (D). The unique appearance of the reaction product facilitates identification of cells and processes of labeled neurons. (b) A large dendritic profile in the ventral horn contains several aggregates of TMB reaction product (arrows). The reaction product frequently has a distinct lamellar substructure. No poststain. Bar = 1 micron.

Figure 5. This labeled synaptic terminal in the dorsal horn contains many vesicles (V) and large aggregates of electron-dense TMB reaction product (arrow). No poststain. Bar = 0.5 micron.

Figure 6. In dorsal column and dorsal root axons the TMB reaction product was in the form of small irregular electron-dense bodies (arrows) scattered in the axoplasm. No poststain. Bar = 1 micron. (From Carson and Mesulam; 1982; with permission of Wiley and Sons, Inc.)



Figures 4–6



Figures 7 and 8

Figure 7. (a) The BDHC reaction product (arrows) in this labeled motor neuron has a distinctive morphology, a filamentous appearance, and is less dense than TMB. (b) At higher magnification the appearance of the BDHC reaction product (arrow) is more easily observed. No poststain. Bar = 1 micron.

Figure 8. (a) Moderately electron-dense *o*-tolidine reaction product fills the perikaryal and dendritic (D) cytoplasm of this labeled neuron. Compare this to the appearance of the unlabeled dendrite (d). The nucleus (N) also appears to contain reaction product. Bar = 1 micron. (b) In the dorsal horn, labeled synaptic terminals (T) containing *o*-tolidine reaction product had electron-dense axoplasm that contrasted with the pale synaptic vesicles. Compare this with the unstained bouton (b). No poststain. Bar = 0.5 micron.

Diaminobenzidine and Paraphenylenediamine-Pyrocatechol Methods

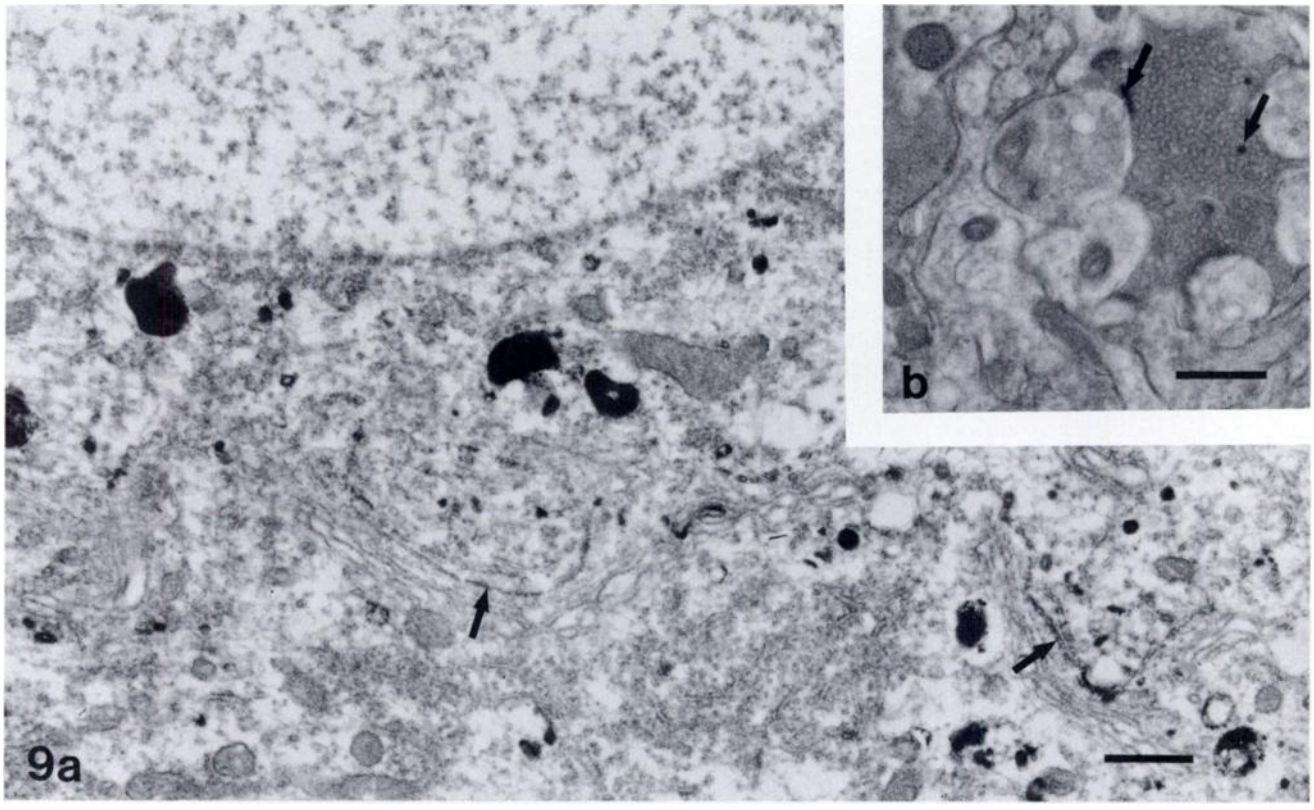
In general, the appearance and distribution of these reaction products were similar. However, there appeared to be differences between them in the frequency of labeled profiles in the ventral and dorsal horns. Among these methods, the Streit and Reubi (1977) and DAB-glucose oxidase (Itoh et al., 1979) procedures produced the largest numbers of labeled profiles. The frequency observed with the Malmgren and Olsson (1977) procedure was next and the PPD-PC (Hanker et al., 1977) and LaVail and LaVail (1974) methods followed. Labeled ventral horn motor neurons exhibited the typical reaction product distribution described by many investigators. Electron-dense reaction product was present in circular and irregular membrane-bound organelles as well as in tubular profiles (Figure 9a). Labeled dendrites sectioned along their long axis contained tubular profiles filled with reaction product. In the dorsal horn, labeled boutons contained circular and irregular membrane-bound bodies filled with reaction product (Figure 9b).

The distribution of labeled boutons, dendrites, and motor neuron perikarya was consistent with the known anatomical connections of these spinal cord systems and, therefore, it is very unlikely that the reaction products observed were diffuse artifactual precipitates.

Discussion

Histochemical techniques using TMB, *o*-tolidine, and BDHC are applicable for electron microscopic studies. Together with the DAB and PPD-PC methods they offer several alternatives

Figure 9. (a) The Streit and Reubi DAB reaction product fills many HRP-WGA-containing structures in the cytoplasm of this labeled motor neuron. Note the reaction product in the Golgi saccules (arrows)—a characteristic of the WGA-HRP conjugate. Other HRP-WGA-labeled structures include large irregular bodies and small vesicles. (b) The Streit and Reubi DAB reaction product in synaptic terminals in the dorsal horn was typically present in just a small number of membrane-bound structures in each terminal (arrows). No poststain. Bar = 0.5 micron.



for the investigator who chooses to approach questions of neural connectivity at the fine structural level. All eight of the methods tested demonstrated the HRP-WGA tracer in the spinal cord following its retrograde and transganglionic anterograde transport from the injection site in the hindlimb musculature. However, there was considerable variation in the distribution of tracer shown by these different methods. The TMB, *o*-tolidine, and BDHC methods exhibited the largest distribution of reaction product in the dorsal and ventral horns of the spinal cord. The TMB procedure showed the widest distribution of labeling, especially in dendrites of motor neurons and in the axonal terminals within the dorsal horn. The effect of sensitivity was most evident in the demonstration of labeled dendrites and terminals because these regions probably contained much lower quantities of transported HRP. The results of this comparison of eight methods in the vibratome sections of spinal cord are similar to those of a previous quantitative light microscopic study of nine HRP histochemical techniques (Mesulam and Rosene, 1979). However, this electron microscopic study did not employ quantitative statistical comparisons on matching sections such as those used in the light microscopic investigation. Instead, eight series of at least 20 sections were cut and reacted and those with the most staining were selected for detailed light microscopic examination and processing for electron microscopy. In this way, the best results with each method were used as the basis for comparison.

The appearance of the TMB reaction product under the electron microscope proved to be very distinctive. The electron-dense angular crystalloid aggregates were very easy to identify and facilitated recognition of HRP-labeled neurons and synaptic terminals under the electron microscope, even at very low magnification. Measurements of the spacing of lamellae in these aggregates of reaction product yielded values from 300–700 Å. This varied between different units of reaction product in an apparently random manner. The TMB reaction product appeared to be associated with cytoplasmic organelles in many instances, but it clearly exceeded the membranes of these organelles. Thus it appears that the TMB reaction product exceeds, in size, the distribution of the HRP. This phenomenon of histochemical magnification may be responsible for the high degree of sensitivity of the TMB method. This sensitivity of the TMB method under the electron microscope has important consequences for ultrastructural tracing of neuronal connections where it is desirable to demonstrate all the processes of the labeled neurons. Distal dendrites and synaptic terminals may have the lowest amounts of transported HRP in the labeled neuron, so a sensitive method may be desirable to more fully demonstrate these processes where most of the synaptic interaction occurs.

The possibility that a unit of reaction product may not only penetrate the membrane of the organelle containing the transported HRP, but that it may also extend into the intercellular space or penetrate an unlabeled neuron should be considered. We have seen very little evidence for this possibility. However, further study of serial sections will be necessary to assess the actual potential for problems of this type. In considering this aspect of the TMB reaction product it is important to note that the TMB method is inherently flexible in terms of the capability to control the size of the reaction product aggregates. Incubation of the section at 4°C rather than room temperature and using one-half to one-fourth the typical amount of sodium nitroferricyanide will affect the reaction product formation. However, these manipulations will also alter the sensitivity of the TMB method, so it is likely that a compromise between sensitivity and reaction product aggregate size may have to be made for certain applications of the TMB ultrastructural method.

The results of the TMB, BDHC, and *o*-tolidine methods show that the localization of the reaction products of some histochemical techniques may not accurately reflect the localization of the enzyme itself. This is particularly true in the case of *o*-tolidine, where the cytoplasm of labeled neurons was full of a fine-grained reaction product. And yet adjacent sections from the same animal showed strictly intravesicular localization of reaction product when treated with a DAB method. *o*-Tolidine reaction product also appeared within the nucleus of labeled motor neurons. It appears that the TMB, *o*-tolidine, and BDHC methods are not suitable for studying the subcellular compartments involved in the axonal transport of HRP. The DAB and PPD-PC techniques remain the methods of choice for this type of study. Our results indicate that the Streit and Reubi (1977), DAB-glucose oxidase (Itoh et al., 1979), and the Malmgren and Olsson (1977) methods apparently provided increased sensitivity over the PPD-PC method (Hanker et al., 1977) and the LaVail and LaVail (1974) method.

The application of TMB to the electron microscopic localization of HRP became possible as a result of the initial studies of Sakumoto et al. (1980) and Stürmer et al. (1981). Their work indicated that conversion of the blue TMB reaction product to an electron-dense insoluble product proceeded best at temperatures above 22°C and at a pH of approximately 7.0. The chemical composition of the TMB reaction product is not known so it is difficult to attempt to define the nature of the chemical interactions occurring between the reaction product and osmium tetroxide and why pH and temperature are so crucial to this process. Osmium tetroxide is known to react with many tissue components, including proteins and the double bonds of unsaturated lipids (Hayat, 1981). The conditions of temperature (37–45°C) and pH (6.0–7.0) that favor formation of the electron-dense reaction product by osmium tetroxide also favor dissolution of the TMB reaction product. It is possible that as the TMB product dissolves, active groups are unmasked that reduce osmium tetroxide to form the insoluble electron-dense product. At pH 3.7 and 4°C the TMB reaction product may remain in a nonpolar form that favors stability but also prevents reaction with osmium tetroxide. Therefore, conditions that are not appropriate for exclusively light microscopic investigations become necessary in preparing the tissue for electron microscopy. The chemistry of these interactions remains to be worked out, as does the chemical nature of the TMB reaction product itself. However, it is clear that the TMB ultrastructural method has excellent potential for application to ultrastructural studies of neuronal connectivity.

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