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EDITED BY

G. H. BOURNE

J. F. DANIELLI

ASSISTANT EDITOR

K. W. JEON

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Electron Microscopic Study of Retrograde Axonal Transport of Horseradish Peroxidase

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I. Introduction

One of the earliest reports on retrograde transport of materials within axons, i.e., toward the cell body, was the *in vitro* observation of Matsumoto (1920), who followed the rapid movement of vesicles stained with neutral red within outgrowing sympathetic fibers. Experiments carried out by Kerkut *et al.* (1967) and by Watson (1968) indicated that radioactive labeled materials injected into muscles appeared in the perikarya of nerve cells innervating them.

The retrograde axonal transport of horseradish peroxidase (HRP), which was originally demonstrated in peripheral motorneurons by Kristensson and Olsson (1971) and by Kristensson *et al.* (1971), has arroused great interest among neuroanatomists. It was shown that HRP is transported intraaxonally from the terminal region of an axon retrogradely to the parent cell body (Kristensson *et al.*, 1971; LaVail and LaVail, 1972; Hanson, 1973; Jones and Leavitt, 1973; Kristensson and Olsson, 1973a,b; Ralston and Sharp, 1973; Warr, 1973; Graybiel and Devor, 1974; Kuypers *et al.*, 1974; Nauta *et al.*, 1974; Ito *et al.*, 1981; Carlson and Mesulam, 1982a).

This technique has now become widely used as an experimental tool for demonstrating neuronal connections both in the central and in the peripheral nervous systems (i.e., Kristensson, 1975; LaVail, 1975; Cullheim and Kellerth, 1976; Kitai *et al.*, 1976; Adams, 1977; Hedreen and McGrath, 1977; Hunt *et al.*, 1977; Luiten and van der Pers, 1977; Keefer, 1978; Malmgren and Olsson, 1978; Kalia and Davies, 1978; Vanegas *et al.*, 1978; Satomi *et al.*, 1979; Contreras *et*

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al., 1980; Panneton and Loewy, 1980; Arvidsson and Gobel, 1981; Kuo *et al.*, 1981; Nicholson and Severin, 1981; Nomura and Mizuno, 1981, 1982; Ross *et al.*, 1981; Stuesse, 1982).

A major advantage of the use of the enzyme HRP is elucidating the connections of the nervous system, that neuronal cell somata are labeled in a way which enables the determination of the cells inducing a particular fiber pathway (LaVail *et al.*, 1973; Ralston and Sharp, 1973; Sherlock *et al.*, 1975; Price and Fisher, 1978). For example, LaVail and LaVail (1974) observed retrograde transport of HRP from the region of retinal ganglion cell bodies after injection of HRP into the chick optic tectum.

The enzyme HRP has been shown in electron microscopic studies to flow in an orthograde as well as in a retrograde direction (Hanson, 1973; Lynch *et al.*, 1973, 1974; Repérant, 1975; Winfield *et al.*, 1975; Jones and Hartman, 1978). Some studies have demonstrated that orthograde transport of HRP can be used to reveal the central distribution of afferent fibers of peripheral nerves both at the light microscopic (Repérant, 1975; Scalia and Colman, 1974; Luiten, 1975; Gwyn *et al.*, 1979; Mesulam and Brushart, 1979; Kalia and Mesulam, 1980) and the electron microscopic level (Muller and McMahan, 1976; Proshansky and Egger, 1977; Rastad *et al.*, 1977; Bettie *et al.*, 1978; Gobel and Falls, 1979; Ohara and Lieberman, 1981).

The cells projecting to regions injected with HRP are identifiable with histochemical procedures (Graham and Karnovsky, 1966) due to the accumulation of vesicular packets of reaction product, which presumably represent the pinocytotic incorporation of the protein at axon terminals (LaVail and LaVail, 1972; LaVail, 1975; Hedreen and McGrath, 1977).

The distribution of reaction product helps to clarify under the light microscope the type of neuron, on the basis of its shape, size (Wilson and Groves, 1981), and dendritic and axonal arborization pattern. In a subsequent step the synaptic connections of this identified neuron can be examined by electron microscopes (Jankowska *et al.*, 1976; Muller and McMahan, 1976; Cullheim *et al.*, 1977; Ralston *et al.*, 1978, 1980; Réthelyi *et al.*, 1979; Robson and Mason, 1979; Langerbäck *et al.*, 1981). The reaction product of the retrograde transport of HRP is usually filling the soma and proximal dendrites of the neuron; this means, first, that the type of neuron—that is labeled—can be determined, and second, that boutons undergoing anterograde degeneration following lesion of an afferent neuron can be traced to the soma and proximal dendrite. HRP is particularly useful for demonstrating the arbors of axons and should be applicable to the study of the intrinsic organization of any region with well defined afferent connections. The intracellularly applied HRP is an invaluable marker for tracing neuronal projections, to resolve the detailed morphology of individual neurons, and for marking cells in synaptological analysis with the electron microscope

(Cowan and Cuenod, 1975; Winer, 1977; Kristensson, 1975; LaVail, 1978; Eckert and Boshek, 1980; Elekes and Szabó, 1982).

II. Visualization of Horseradish Peroxidase

The method for visualizing the activity of HRP at the electron microscopic level was introduced by Graham and Karnovsky (1966) who have used it to study membrane recycling and to follow the path of the retrograde transport from the synaptic region.

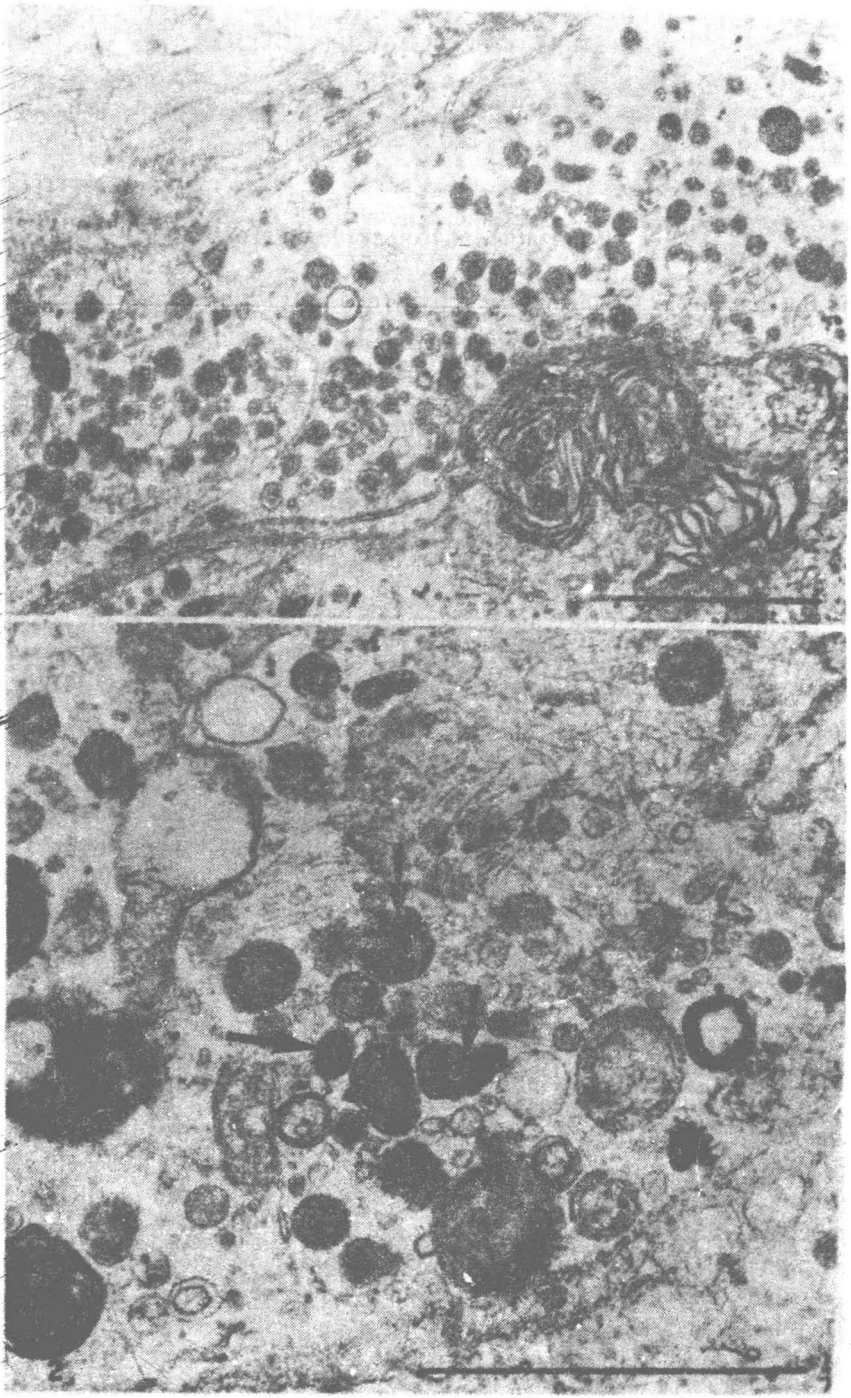
HRP being a protein of molecular weight of about 40,000 will not pass across cell membranes unless an invagination of the membranes does not occur. Visualization of the reaction product of the enzyme inside the terminal should therefore imply a membrane event of this kind. Membrane infoldings supposedly resulting from the release of transmitters have been described by several groups of workers (Holtzman *et al.*, 1971; Ceccarelly *et al.*, 1973).

The Sigma Type VI HRP used contains mainly the basic isoenzyme. According to Giorgi and Zahnd (1978) it is only this isoenzyme that is taken up and transported retrogradely at detectable levels by undamaged nerve cells (Bunt *et al.*, 1976; Bunt and Haschke, 1978; Malmgren *et al.*, 1978).

The sections of materials used were processed to demonstrate the presence of HRP using tetramethylbenzidine (TMB) or 3,3′-diaminobenzidine (DAB) according to the method of Mesulam (1978) and to Graham and Karnovsky (1966), respectively. The distribution of reaction product was much greater in the TMB incubated tissue than in the DAB incubated tissue under the light microscope. This is consistent with the previous observations on the greater sensitivity of the TMB method (Mesulam and Rosene, 1979; Dietrichs *et al.*, 1981; Carlson and Mesulam, 1982a). In the dorsal horn according to Carlson and Mesulam (1982b) DAB reaction product was localized within membrane-bound bodies located in synaptic terminals. These labeled bodies were generally larger than synaptic vesicles and some were elongated rather than circular in profile (Figs. 1 and 2).

In contrast to the DAB reaction product, the crystalloid TMB reaction product was not confined to membrane-bound organelles and frequently filled significant portions of the entire synaptic terminal. It has been reported by several authors (Beattie *et al.*, 1978; Gobel and Falls, 1979) that application of HRP to the proximal ends of dorsal roots and subsequent ultrastructural examination of DAB reaction product in the spinal cord showed labeling on the cytoplasmic side of the axolemma and on the external surface of synaptic vesicles and mitochondria. They concluded that this suggests such labeling occurs mostly by diffusion within the cytoplasm rather than by membrane-bound transport.

According to Somogyi *et al.* (1979) a highly electron-dense reaction product



was formed when 3,3′-diaminobenzidine was used as substrate at pH 7.4. Only slightly electron dense, but of a characteristic appearance reaction product formed when 0.02% *o*-tolidine is used at the same pH. The reaction from *o*-tolidine at pH 7.4 is found in membrane-limited particles, including multivesicular bodies.

The cobalt–glucose oxidase method is also used for HRP reaction by Itoh *et al.* (1979) and by Nakamura *et al.* (1981).

In our observations to obtain information on the ultrastructural localization of HRP, materials were processed by the method of Somogyi *et al.* (1979). In each cat 0.3–0.04 μl at 20% solution of HRP (Sigma Type VI) in 0.05 *M* phosphate buffer was injected into the mesenteric nerves between the layers of the mesothelium under semisterile conditions over a period of 13–20 minutes. After 2 or 3 days survival the animals were perfused through the left ventricle with 1% glutaraldehyde and 1% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) according to Benedeczky and Somogyi (1975). Small pieces of the intestine (the middle part of the intestine innervated by the injected nerves) were excised and then cut by a Vibratome in 30-μm sections. The slices were washed for several hours in phosphate buffer and placed for 30 minutes in a medium containing 0.05% 3,3′-diaminobenzidine and 0.03% hydrogen peroxide in 0.1 *M* phosphate buffer for 1 hour. The slices were then postfixed in osmium acid, dehydrated, and embedded in Araldite. Ultrathin sections were mounted on single-hole grids, contrasted with uranylacetate and lead citrate.

At the control examination—processed in a similar way—of these sections no labeled cells and processes were found either on light or on electron microscopy.

III. Morphology and Histology of the Reaction Product

A. Localization of Horseradish Peroxidase in the Nerve Cell Bodies

The usefulness of HRP as a neuronal marker at the electron microscopic level has already been demonstrated via the use of intracellularly applied HRP by several authors (Cullheim and Kellerth, 1976; Jankowska *et al.*, 1976; Snow *et al.*, 1976; Rastad, 1978; Rastad *et al.*, 1977; Réthelyi *et al.*, 1982). In the labeled neurons large (300–700 nm in diameter), highly electron-dense profiles, identified earlier as residual bodies or secondary lysosomes (Broadwell *et al.*,

FIG. 1. Labeled nerve processes in the myenteric plexus. Bar scale= 1 μm. ×42,000.

FIG. 2. Arrows show the labeled membrane-bound bodies in the nerve terminal. Bar scale= 1 μm. ×72,000.

1980) were found. Multivesicular bodies were also common and had a variable morphology; a portion of their limiting membrane was often coated on its cytoplasmic surface. Electron-lucent vesicles (40–80 nm in diameter), HRP-labeled vesicles with or without an external coat, and vacuoles (100–300 nm in diameter) of various shape were apparent in all the preparations, usually accumulated closely to the Golgi zones but also at other cytoplasmic sites (Fig. 3).

The peroxidase reaction product eventually filled many of the lysosomal residual bodies in the perikarya (Colman *et al.*, 1976; Takeuchi *et al.*, 1982).

In vesicles, smooth endoplasmic reticulum and membrane-limited granules, the end product fills the space right up to the limiting membrane; in contrast, dense-core vesicles which are not labeled and occur in all neurons have a granular matrix, usually of higher electron density than the HRP reaction product, and there is a translucent zone between the matrix and the limiting membrane (Figs. 4 and 5).

Lipofuscin pigment is normally found in ganglion cells and appears to increase significantly with age. However, an accumulation of pigment that may be misinterpreted as HRP vesicles could be ruled out since these animals were young and the control materials showed the absence of these pigments.

The distribution and cytological features of the labeled neurons were carefully examined and compared with those of the unlabeled neurons. The labeled neurons were seen in both the myenteric and the submucosal plexuses. They were medium-sized (30–50 μm) and spindle-shaped, multipolar, triangular, or oval (Fig. 6). These data are similar to those obtained by light microscopy (Fehér and Vajda, 1982b). The shape and distribution of the labeled neurons resembled the medium-size cells stained by silver impregnation (Fehér and Vajda, 1972).

According to Dogiel (1895) and Type II nerve cells in the wall of the intestine once were believed to be sensory in nature. Later, Kadanoff and Spassowa (1959) described the sensory function of the bipolar and unipolar neurons in the gut. Kuntz (1922) traced nerve fibers from the submucosal plexus into the mucous membrane and suggested that some of the fibers were likely to originate from afferent neurons in the submucous ganglia.

It has also been proved with degeneration methods (Schofield, 1960, 1968; Fehér and Vajda, 1974) that some of the enteric neurons project centripetally along mesenteric neurovascular bundles. The combined anatomical and physiological studies by Bülbring *et al.* (1958) proved the presence of afferent neurons that innervate the mucous membrane. It has also been shown that with regard to ultrastructural features the small intestine contains different types of nerve cells (Fehér and Csányi, 1974; Cook and Burnstock, 1976). Physiological studies have also demonstrated that the intrinsic nerve plexus of the small intestine is composed of at least three types of neurons (Milton and Smith, 1956; Wood, 1975; Furness and Costa, 1980).

The labeled neurons have oval nuclei, contain the usual cytoplasmic orga-

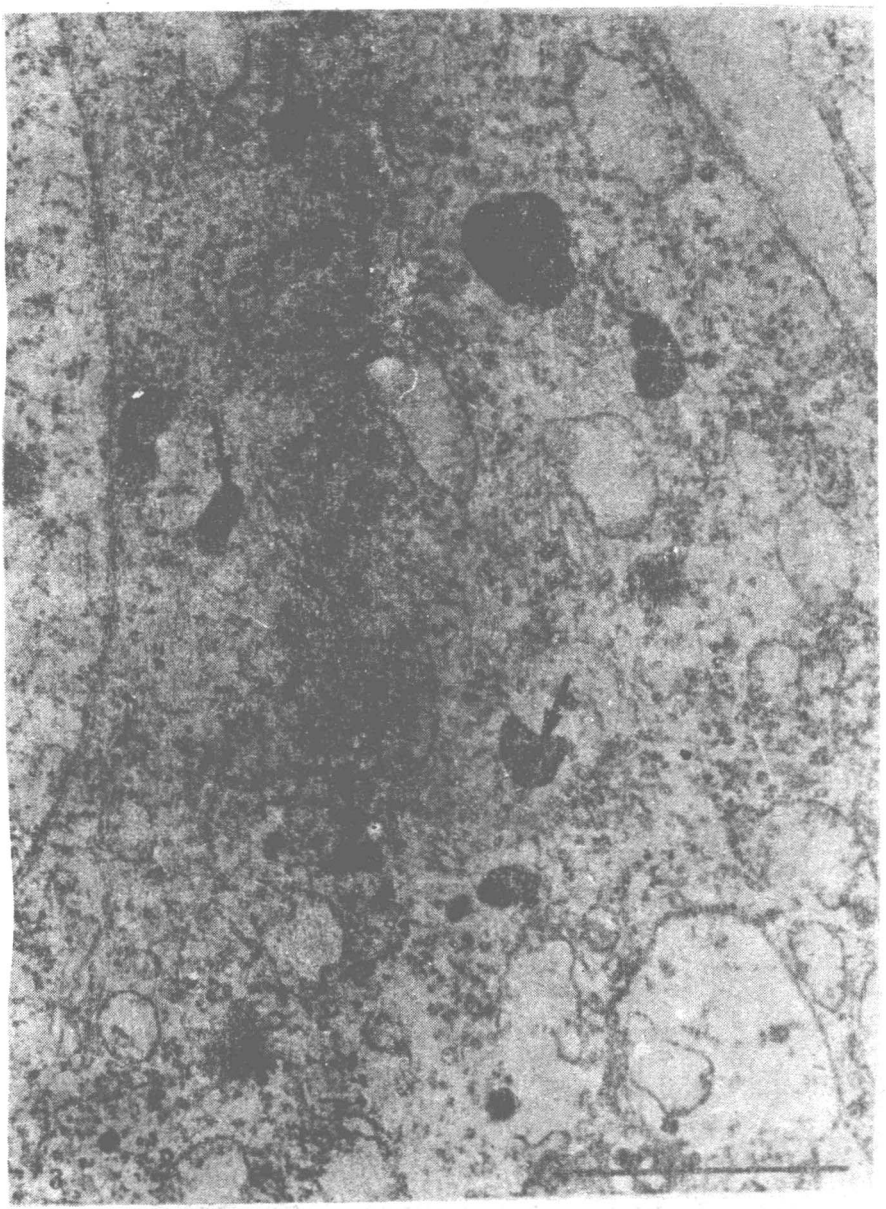
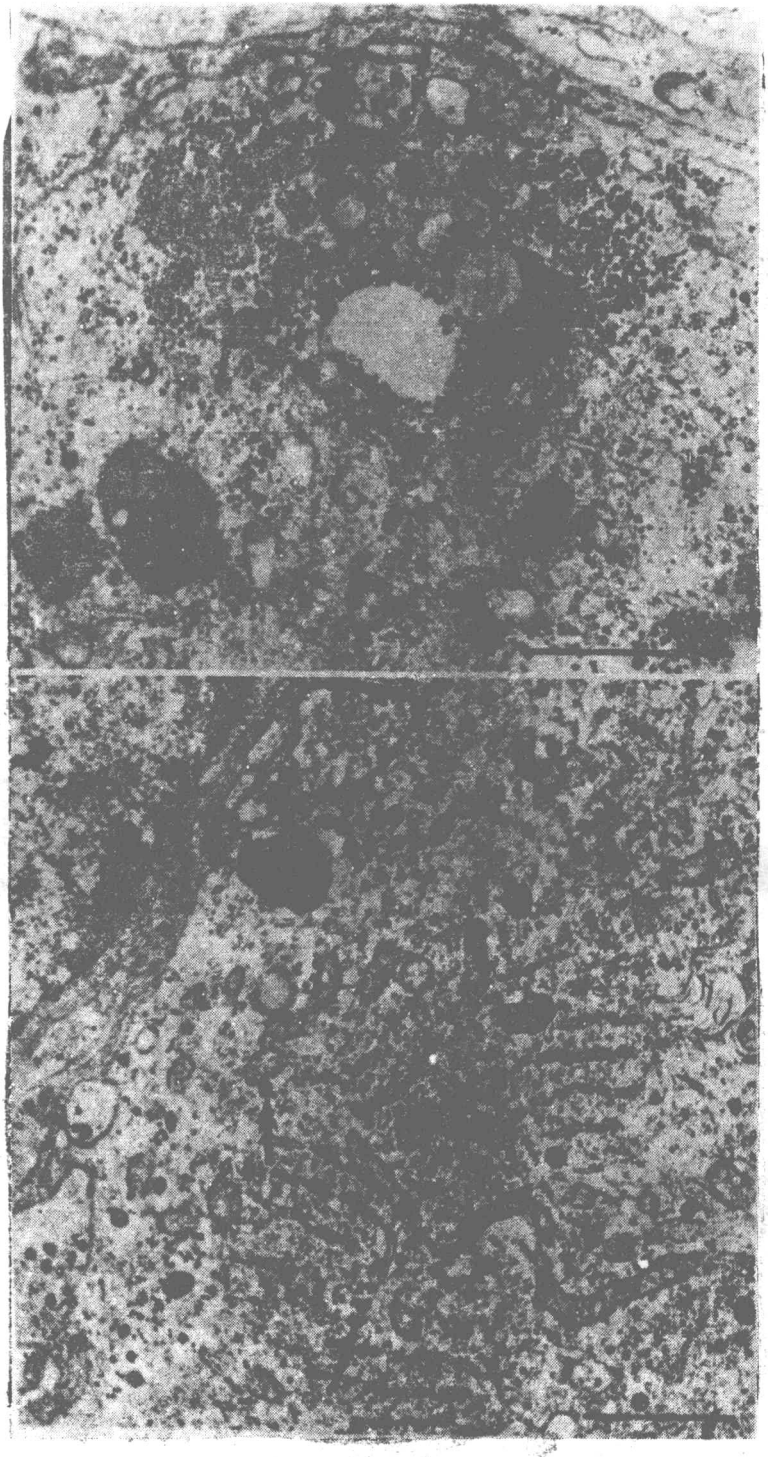


FIG. 3. Labeled nerve cell in the submucous plexus. Arrows point to the HRP-labeled vesicles close to the Golgi zones. Bar scale= 1 μm. ×54,000.



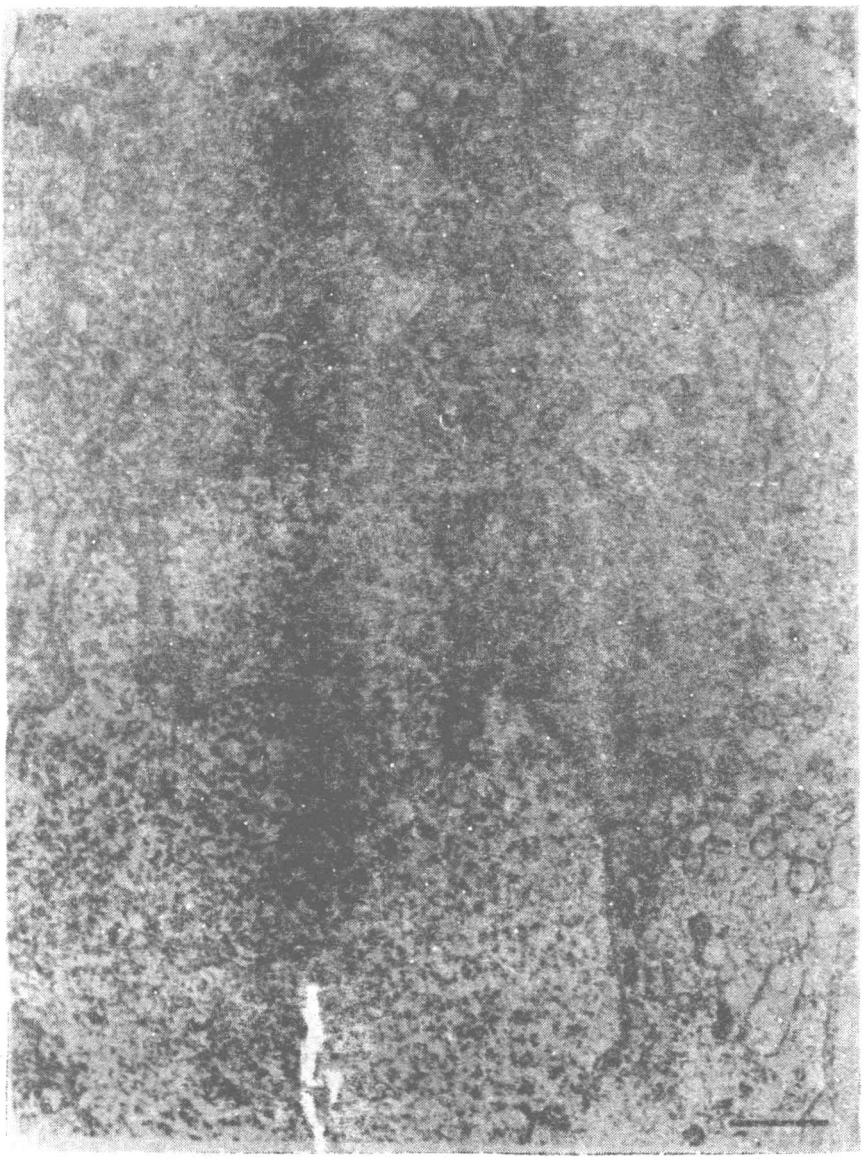


FIG. 6. A medium-size oval-shaped neuron. Peroxidase is evident in a variety of sizes of vesicles and tubules of the neuron soma. Bar scale= 1 μm. ×18,000.

FIG. 4. Cytoplasm of the labeled nerve cell. Arrows show the dense-core vesicles. Bar scale= 1 μm. ×42,000.

FIG. 5. Cytoplasm of the labeled nerve cell in the myenteric plexus. Note the abundant dense-core vesicles (arrows) occurring in the cytoplasm. Bar scale= 1 μm. ×30,000.