# UPTAKE OF PROTEIN BY MAMMALIAN NEURONS

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## INTRODUCTION

Although the uptake of macromolecules by neurons often figures in theories of function in the nervous system (see reference 18), such uptake has actually been demonstrated only in a few instances, usually under special circumstances. In general, exogenous tracer proteins have been shown either to be stopped by "barriers" between the circulation and the nervous system (see references 3, 29) or to enter nonneuronal cells of nervous tissue, such as Schwann cells and capsule (satellite) cells (see references 21, 34). However, Rosenbluth and Wissig (30) using organ-cultured ganglia or massive intraperitoneal injection, have demonstrated uptake of ferritin by perikarya of toad ganglia. Also, Brightman (6, 7), using ferritin and peroxidase, and Becker et al. (2), using peroxidase, have reported protein uptake by rat and mouse central nervous system neurons after injection of the protein directly into cerebrospinal fluid or into extracellular spaces in the brain. In these several studies coated vesicles, multivesicular bodies, and dense bodies were found to be the chief intraneuronal sites of exogenous protein. In addition, several authors (1, 21, 35) have interpreted morphological observations as indicating pinocytic vesicle formation at neuronal surfaces.

The present report deals with the uptake of horseradish peroxidase demonstrable in the perikarya and axons of the autonomic system which are scattered through the rat adrenal gland (the perikarya are of the ganglion cells; see references 14, 33 for additional details and references) and in neurons of mouse dorsal root ganglia grown in culture.

## MATERIAL AND METHODS

### *Adrenal Gland Neurons*

Rats were injected, through a tail vein, with 1.5 cc of a 2% solution of horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.; Type II) in normal saline. From 15 min to 2 hr after injection, fixation was initiated by perfusion, through the heart, of 2–3% glutaraldehyde in cacodylate or phosphate buffer. Glutaraldehyde fixation lasted for approximately 1 hr and was terminated by rinsing in cacodylate or phosphate buffer; details of fixation methods are described elsewhere (15). For cytochemical studies, frozen sections were cut at 10$\mu$ for light microscopy and at 40 $\mu$ for electron microscopy. Additional tissue was fixed for electron microscopy without freezing or incubation.

### *Cultured Neurons*

Mouse dorsal root ganglia were explanted from fetal mice and grown in culture for a minimum of 3 wk by methods previously published (8, 27). Cultures were exposed for 4–19 hr to 0.2–0.3% peroxidase added to their usual growth medium. They were fixed by immersion in 2% glutaraldehyde in phosphate or cacodylate buffer (15). Fixation lasted 15–30 min for cytochemistry, or up to 2 hr for electron microscopy of unincubated preparations. After fixation, the cover slips carrying cultures to be incubated were frozen on the head of a freezing microtome, and the neurons, with the collagen on which they are grown, were dissected off and incubated as free-floating preparations.

For demonstration of peroxidase activity, tissue was incubated, at room temperature, in the $H_2O_2$ and 3-3′-diaminobenzidine medium of Graham and Karnovsky (12). Preparations incubated in substrate ($H_2O_2$)-free medium showed no reaction product.[1] Tissues that had not been exposed to peroxidase also were negative when incubated in full medium (or in substrate-free medium), except for occasional axon endings that showed some dense granular deposits associated with packed synaptic vesicles.

All material was postfixed in 1% osmium tetroxide in phosphate or Veronal-acetate buffer, embedded in Epon, and stained with lead citrate by usual procedures (15). Photographs were taken with an RCA EMU-3F microscope at magnifications of 2–16,000.

## RESULTS

At the earliest times studied (15 min for the adrenal gland, 4 hr for the cultures), peroxidase is demonstrable, in both systems (Figs. 2, 6, 8), in the spaces between perikarya and the capsule cells that sur-

[1] Initial efforts to extend our observations to mice have been much hampered by apparent extensive reaction, in peroxide-free medium, of many cells in he adrenal glands of peroxidase-injected animals.

round them, in the spaces between axons and the Schwann cells that surround them, and between gland cells and axon endings. At 30–35 min, peroxidase is seen within the neurons of the adrenal gland (Fig. 3); although the perikarya are relatively rare, they can be distinguished from gland cells both on morphological grounds and on the basis of the distribution of "marker" enzyme activities such as acid phosphatase (activity in lysosomes and Golgi-associated membrane systems) or thiamine pyrophosphatase (Golgi apparatus). At 4 hr, peroxidase is present within the cultured mouse neurons (Fig. 2). By 17–19 hr, the cultured neurons show the distribution of peroxidase-containing bodies indicated in Fig. 1; this is reminiscent of the distribution of lysosomes (multivesicular bodies, dense bodies) in these neurons as visualized by cytochemical incubation for phosphatase and sulfatase activity (14).

Within the neurons, much of the exogenous protein is found in perikarya. However, peroxidase-containing structures are also regularly encountered in axons in both systems (Figs. 2, 8, 9); such structures are seen in the unmyelinated axons and axon endings of the adrenal medulla at 35 min and in the unmyelinated axons of the cultures at 4 hr. Myelinated axons were not seen to contain peroxidase.

Both in vivo and in culture, Schwann cells (Fig. 6) and capsule cells take up considerable amounts of exogenous protein.

In the neurons and in the Schwann and capsule cells, peroxidase-containing structures include small vesicles that appear to form from the cell

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All figures, except Fig. 1, are electron micrographs. With the exception of Fig. 5, all thin sections were stained only with lead citrate. For peroxidase-incubated preparations, incubation times are indicated in parentheses. The terminology employed in referring to regions of neurons is that of Bodian (4). Bar length equals 0.25 $\mu$ in all electron micrographs.

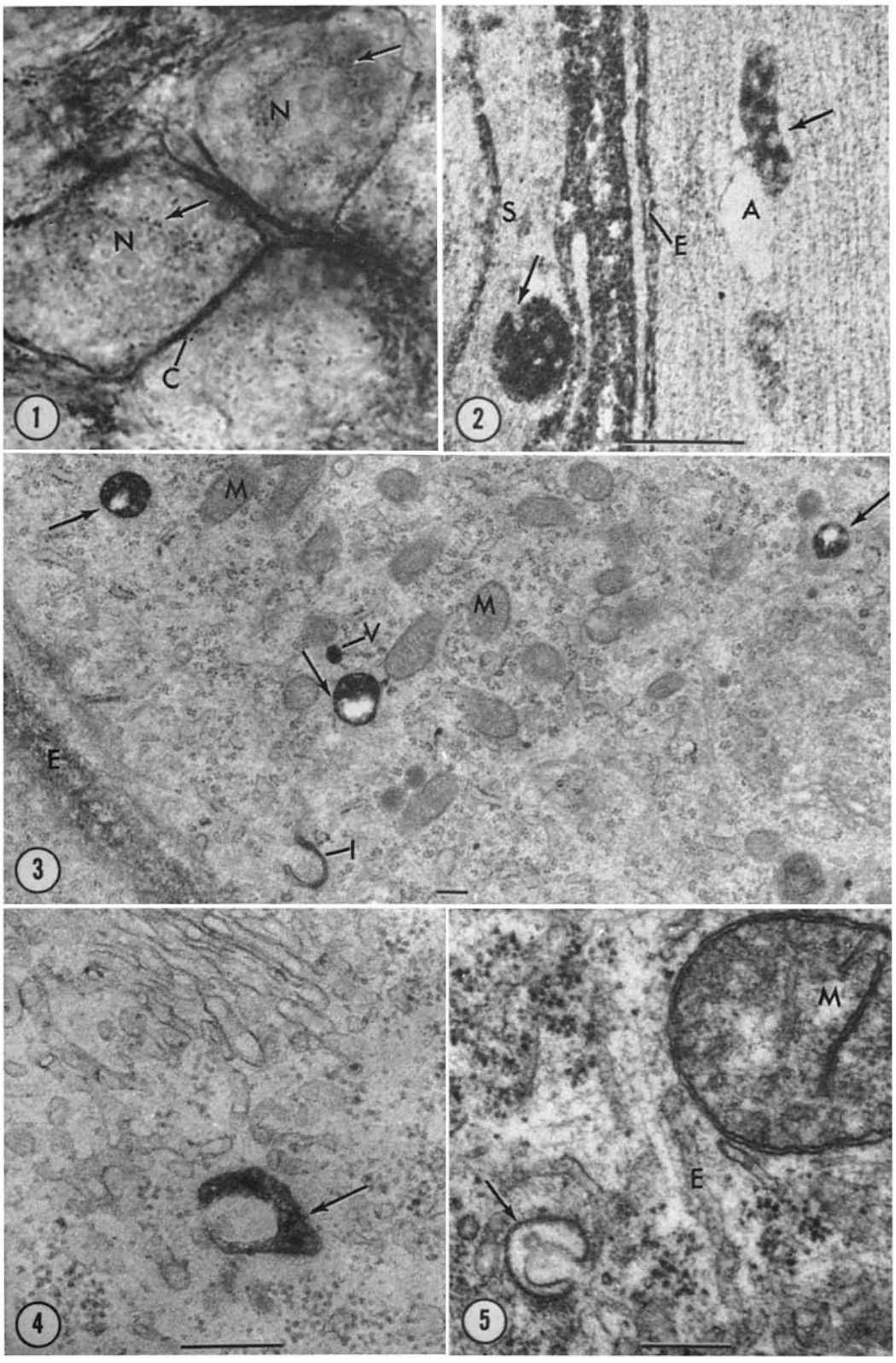
FIGURE 1 Light micrograph of culture neurons fixed after 17 hr exposure to peroxidase and incubated (35 min) for peroxidase activity. This is a whole mount showing unsectioned neurons. Reaction product is seen at the borders (*C*) between perikarya and capsule cells. In the perikarya, peroxidase activity is demonstrated in small bodies (arrows) that show some tendency to group near the nuclei (*N*). × 1,250.

FIGURE 2 Portion of a cultured preparation fixed after 4 hr exposure to peroxidase and incubated (40 min) for peroxidase activity. The arrows indicate two bodies containing reaction product; probably these are multivesicular bodies. One body is present in an unmyelinated axon (*A*); *E* indicates the extracellular space that separates this axon from the Schwann cell surrounding it. The other body is in a Schwann cell (*S*) associated with another unmyelinated axon. × 75,000.

FIGURE 3 Portion of a perikaryon from an adrenal gland fixed 35 min after peroxidase injection and incubated (38 min) for peroxidase activity. Reaction product in the extracellular space is seen at *E*. Activity in the perikaryon is demonstrated in several large bodies (arrows) and in a small vesicle (*V*). The structure at *I* is probably a deep infolding of the cell surface. *M* indicates mitochondria. × 20,000.

FIGURE 4 Portion of a perikaryon from the same peroxidase-incubated culture used for Fig. 1. Reaction product is present in a cuplike body (arrow) near the Golgi apparatus. × 65,000.

FIGURE 5 Portion of a perikaryon from a culture that had not been exposed to peroxidase. The arrow indicates a cuplike body similar to the one seen containing peroxidase in Fig. 4. Similar bodies in other tissues (15, 16) have been tentatively identified as precursors of multivesicular bodies with internal vesicles presumably derived in part from the infolded membrane of the cup. Perhaps one such vesicle is in the process of formation in the body shown in this figure. *M* indicates a mitochondrion, and *E* identifies endoplasmic reticulum. The thin section was stained with uranyl acetate and lead citrate. × 58,000.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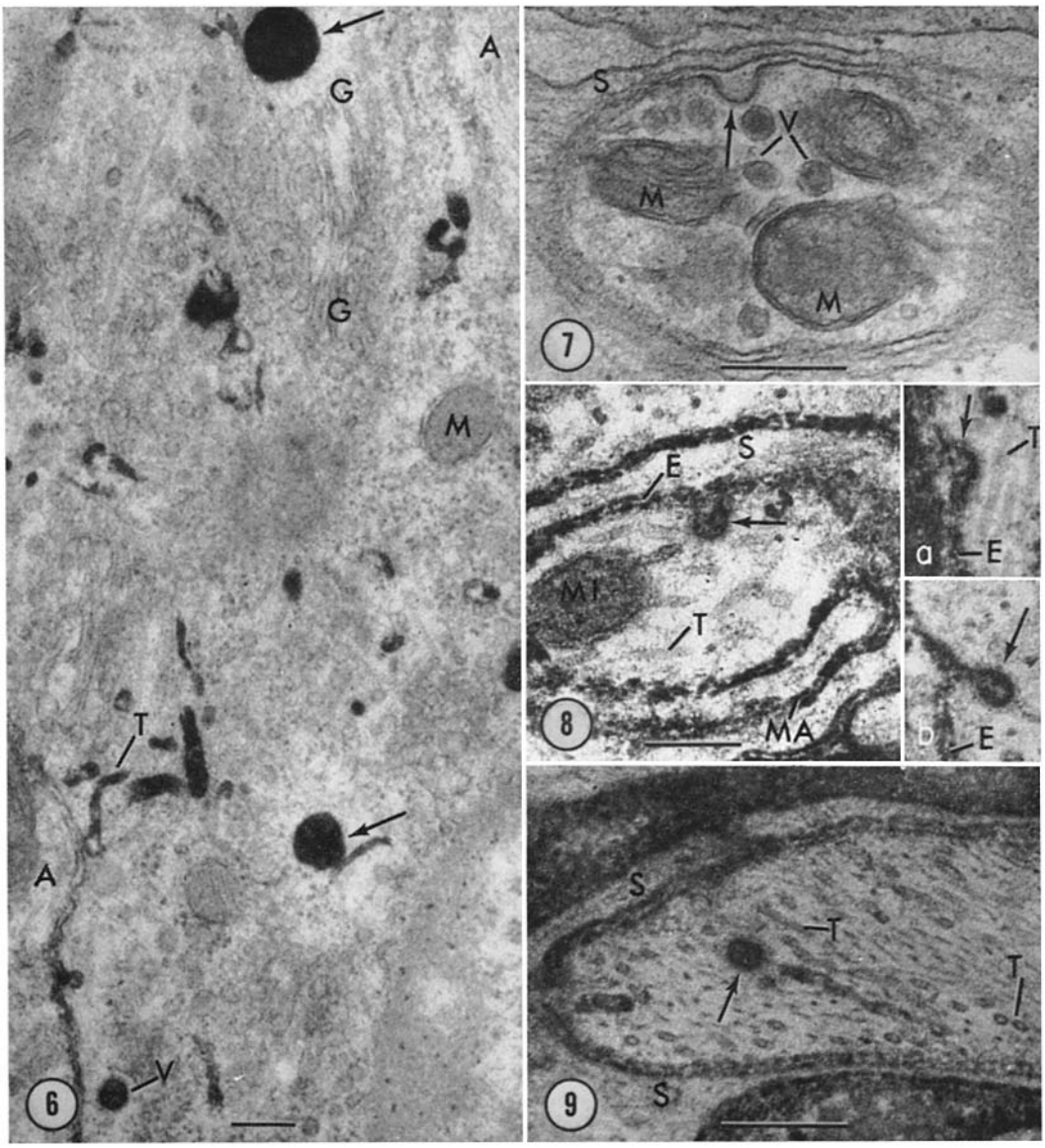


Figs. 6, 8, and 9 are all from the same adrenal gland fixed 35 min after peroxidase injection and incubated (38 min) for peroxidase activity.

FIGURE 6 Most of the field is occupied by a Schwann cell containing peroxidase reaction product in large bodies (arrows), tubules (*T*), and a vesicle (*V*). *A* indicates unmyelinated axons associated with this Schwann cell. *G*, the Schwann cell Golgi apparatus; *M*, mitochondria. × 36,000.

FIGURE 7 Unincubated preparation from an adrenal gland that had not been exposed to peroxidase. The arrow indicates an invagination of the plasma membrane of an unmyelinated axon; a faint coating is seen at its surface. Mitochondria are present at *M*, and small vesicles of the type that accumulate in large numbers at the endings of axons are seen at *V*. *S* indicates a portion of the Schwann cell that surrounds this axon. × 67,000.

FIGURE 8 Portion of an axon showing peroxidase reaction product in an invagination of the membrane (arrow) similar to the invagination of membrane seen in Fig. 7. Reaction product is also present in the space (*E*) between axon and Schwann cell (*S*), including the mesaxon space seen at *MA*. *T* indicates a segment of a microtubule, and *MI* indicates a mitochondrion in the axon. × 52,000. Inserts *a* and *b* show other stages in the formation of vesicles from the membranes of two other axons in the same preparation as Fig. 8. The coated invagination at the arrow in *a* is probably an early stage, while the coated structure at the arrow in *b* presumably is about to pinch off as a free vesicle; *b* is from an axon near its ending, so *E* in this case indicates the axon-gland cell space. *a*, × 69,000; *b*, × 61,000.

FIGURE 9 Portion of an unmyelinated axon. At the arrow, a peroxidase-containing coated vesicle is seen in the axoplasm. Microtubules are indicated by *T*. Part of the Schwann cell that surrounds the axon is seen at *S*. × 67,000.

surface (Figs. 3, 6–9). Neuronal vesicle formation is not confined to perikaryal surfaces and axon endings but is also observed along the axons (Figs. (6–9). Exogenous protein is also demonstrable intracellularly in tubules (Fig. 6) and in larger bodies (Figs. 1–6), including some bodies resembling multivesicular bodies and other bodies like the cuplike bodies (Figs. 4, 5) that have been seen to accumulate peroxidase in other tissues (15). None of these structures is unique to peroxidase-treated neurons; all of these structures are seen (without peroxidase activity) in material that has not been exposed to the protein (Figs. 5, 7).

## DISCUSSION

Both in the cultures and in the adrenal gland the results indicate that exogenous protein can penetrate into the spaces between neurons and associated cells and can, by pinocytosis, enter perikarya and axons. Of particular interest is the observation of uptake of peroxidase by unmyelinated axons. To our knowledge, the only previous demonstration that exogenous protein can enter axons is that of Brightman, who has noted some ferritin and peroxidase in occasional naked axons in the central nervous system after protein injection into cerebrospinal fluid (6, 7). The axons used for the present study, like most axons outside the central neuropil, are covered by closely associated nonneuronal cells (in the cultures and adrenal gland, the axons are covered by Schwann cells). Much of the peroxidase that enters the axons almost certainly is taken up directly from the Schwann cell-axon space rather than being transported from the perikaryon. This is indicated both by observations suggesting vesicle formation by the axon membrane and by the fact that exogenous protein is present in the adrenal gland axons and presynaptic axon endings by 35 min, which seems too fast to be accounted for by known rates of flow from perikaryon to axon (see reference 22 for review).

Morphological indications of pinocytosis are observed whether or not peroxidase is present in the animal or culture. Thus it is unlikely that the protein is inducing an abnormal response by the neurons. The axons, endings, and perikarya in the adrenal gland take up protein almost as rapidly as do the secretory cells (15) of the cortex and medulla. This suggests that the Schwann and capsule cells are not particularly effective as "mechanical" barriers and that once material has left the blood stream it has relatively ready access to the neuronal surface. This contrasts, for example, with the situation in the central nervous system (see references 3, 6, 7). Nevertheless, the fact that Schwann and capsule cells do take up much protein (see Fig. 6) indicates that these cells probably can restrict the amounts of exogenous macromolecules that enter the neurons. Recently (15*a*) we have observed apparent peroxidase uptake in Schwann cells of giant crustacean neurons; such Schwann cells also possess elaborate systems of anastomosing tubules which permit passage of peroxidase to the axon surface through a network that provides a large surface area for possible exchanges between Schwann cell and extracellular medium (15*a*).

Study of neuronal protein uptake has depended chiefly on use of culture techniques (30) or direct injection (2, 6, 7) to circumvent the circulation, although Rosenbluth and Wissig (30) observed some ferritin uptake by perikarya of toad ganglia 17 hr after intraperitoneal injection of 0.8 g of protein, and Brightman (6, 7) has suggested that limited amounts of blood-borne peroxidase might pass the choroid plexus by transport in vesicles.

In the present study, intravenously administered protein readily gained access to adrenal gland neurons. However, the permeability of blood vessels is known to alter in peroxidase-injected animals because of histamine-mediated effects (19, 20), and changes in blood vessel permeability to protein have been observed in response to a number of other conditions (see reference 25 for pathological changes). Thus it is too early to evaluate the normalcy of the release of protein from adrenal blood vessels. It may eventually be of interest that, in studies of other rat nervous tissue, intravenously administered peroxidase has not been detected; this is true of central nervous tissue (3, 32), neurons of the nodosal ganglion (Holtzman, E., unpublished data), and peripheral nerve (Straus, reference 32, attributed the small amounts of peroxidase he found in peripheral nerve to protein still in the circulation).

The significance of macromolecule uptake by neurons is open to speculation. Passage of intact nucleic acids into neurons has been postulated as important for neuronal functions or for the interrelations of neurons and glia (18). As have others, Pomerat, by microcinematographic methods, has observed morphological signs of intensive pinocytosis at the tips of growing axons (28). Neurons are

known to accumulate some exogenous pharmacologically active components (see reference 10) at rapid rates. Rosenbluth and Wissig (30) have suggested that pinocytosis may be a mechanism whereby the neuron "samples" its environment. Also, Brandt and Freeman (5) have reported an interesting association between changes in electrical behavior of cell membranes and increased rates of pinocytosis. In several pathological conditions (see reference 25 for further references), increases in protein release from the blood stream into nerves have been reported.

It is clearly not yet possible to evaluate adequately the involvement of the routes of protein uptake, demonstrated in the present study, in any of these phenomena or to decide on the importance of the uptake for nerve function. As in many other tissues (see references 2, 9, 11, 12, 15, 23, 26, 30), most of the exogenous protein is seen in structures that appear to be lysosomes (14, 24) or in vesicles and other bodies that probably contribute material to lysosomes. If this is so, then lysosomal hydrolysis presumably would limit the possibility of direct effects of exogenous material on neuronal metabolism.

Recent studies have shown that the flow of macromolecules within neurons involves several components which operate at different rates and in both directions along the axon (see reference 22 for discussion). Other work suggests, for example, that axons can accumulate catecholamines directly from extracellular spaces (10) and that labeled amino acids can enter axons after passing across Schwann cell cytoplasm and myelin (31). From the present study and from other work referred to above, it seems likely that pinocytosis may be responsible for part of the complexity of the observed patterns of flow and accumulation within neurons and patterns of the exchanges between neurons and the extracellular environment. At least, direct pinocytic uptake represents an important potential route for entry of macromolecules into all portions of neurons, which is usually overlooked but may turn out to have appreciable physiological or experimental significance.

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