THE DISTRIBUTION OF EXOGENOUS FERRITIN IN TOAD SPINAL GANGLIA AND THE MECHANISM OF ITS UPTAKE BY NEURONS

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ABSTRACT

Toad spinal ganglion cells are individually enclosed in sheaths consisting of one or more attenuated layers of satellite cell cytoplasm surrounded externally by a basement membrane. Narrow (~150 A) extracellular channels separate these layers from one another and from the underlying neuron. In both *in vivo* and *in vitro* experiments it was found that molecules of ferritin, a water-soluble protein, are to some extent able to pass across the basement membrane and through these channels to reach the neuronal plasma membrane. Ferritin particles arriving at the neuronal surface are engulfed by the neuron in 0.1 to 0.2 μ "coated" vesicles. The concentration of ferritin in these vesicles is higher than in the perineuronal space. The ferritin incorporated into the neuron is segregated, apparently intact, in multivesicular bodies. It is inferred that the 150A channels in the satellite cell sheath are patent, aqueous spaces through which molecules with a diameter as large as 95 A are able to pass, and that these neurons are capable of taking up whole protein from their immediate environment by the process of pinocytosis.

INTRODUCTION

One of the characteristic structural features of nervous tissue is the close association of neurons with a class of "supporting cells"—the glia of the central nervous system and the Schwann cells and satellite cells of the peripheral nervous system. In the peripheral nervous system, the supporting cells usually take the form of sheaths which invest the neurons closely and which appear to insulate them from the perivascular connective tissue spaces. In the central nervous system, the formation of such discrete sheaths is less apparent but, as a general rule, glial cells still intervene between the neurons and blood vessels.

Such an architectural arrangement raises questions about the means by which nutrients and metabolites are exchanged between neurons and blood and about the pathways followed by the ions that move during neuronal activity. While it might appear that all such exchanges must take place through the cytoplasm and across the plasma membranes of the supporting cells, electron microscopic examination has consistently demonstrated a thin extracellular space immediately surrounding each neuron. This perineuronal cleft is continuous with similar narrow interstices between the supporting cells themselves. A potential pathway of submicroscopic dimensions thus exists between the neuronal surface and the perivascular space through a system of slender, intercommunicating, extracellular channels. Whether these channels

are, in fact, patent pathways through which ions and nutrients might be expected to flow with relatively little resistance, or, alternatively, whether the channels are occluded by a "cement substance" of low electron opacity cannot be determined from morphological studies alone.

In non-nervous tissues, electron-opaque markers have been employed to follow the passage of materials across cellular barriers and through intracellular compartments. By this means it has been shown that in capillaries, for example, the route taken by a marker from the lumen of a vessel to the perivascular space is predominantly intracellular, by way of pinocytotic vesicles (24, 43), while in the case of the frog cornea the pathway through the endothelium from the aqueous humor into the corneal stroma is almost exclusively extracellular (17). In other instances, a combination of intra- and extracellular pathways is followed (27).

Such is the approach used in the present investigation. The course of ferritin, a water-soluble, electron-opaque protein of known molecular size (95 A; see reference 7) and low toxicity, was followed from the connective tissue spaces in toad spinal ganglia into the neurons in order to determine to what extent this protein is able to penetrate through the satellite cell sheaths, what pathways it follows, whether it is taken up by the neurons, and, if so, by what mechanism. It was shown previously (33) that the relationship between the neurons and satellite cells in this amphibian is typical of that which obtains in other vertebrates.[1]

MATERIALS AND METHODS

Ferritin[2] was introduced into the spinal ganglia of adult toads (*Bufo marinus*) in both *in vivo* and *in vitro* experiments and, after fixation of the specimens, the distribution of the ferritin was studied by means of electron microscopy. In all, eighteen ganglia taken from six animals were used in this study.

In the *in vitro* experiments, toads were pithed and from each animal several lumbar ganglia were removed, transferred to Petri dishes, and barely covered with a balanced salt solution containing 0.1 per cent glucose and either 1 or 7.5 per cent ferritin. Ganglia were allowed to soak for 30 minutes, 1 hour, 2 hours, 3 hours, or 4 hours at room temperature and were then fixed. The composition of the balanced salt solution is as follows:

NaCl	5.10 gm/liter
$NaHCO_3$	1.65 "
KCl	0.30 "
$NaH_2PO_4 \cdot H_2O$	0.13 "
$CaCl_2$	0.18 "
$MgCl_2 \cdot 6H_2O$	0.16 "
Glucose	1.00 "

The pH was adjusted to 7.4 with CO_2 just before use.

It has been shown previously (16) that excised toad ganglia display normal physiological activity for many hours *in vitro* under conditions very similar to those used here. The ganglia are in effect in organ culture and should not be considered moribund.

In the *in vivo* experiments, toads were given 8 ml of a 10 per cent solution of ferritin intraperitoneally. After 3 or 17 hours, the animals were sacrificed and their lumbar ganglia removed and immersed immediately in a fixative solution. One animal received three intraperitoneal injections of 7.5 per cent ferritin over the course of 3 days (12 ml on day No. 1, 13 ml on day No. 2, and 7 ml on day No. 3) and was sacrificed on the 4th day (96 hours).

The most satisfactory fixative consisted of 1 to 2 per cent osmium tetroxide in a balanced salt solution (33) containing acetate-veronal buffer (pH 7.5.) The final concentration of the buffer was approximately 0.028 M for each salt (23). Two ganglia were prefixed for 10 minutes in 1 per cent glutaraldehyde (38) in acetate-veronal buffer (pH 7.5) plus 20 mg per cent $CaCl_2$ before immersion in the osmium tetroxide fixative. The general preservation of detail in these glutaraldehyde-fixed samples was, however, not optimal. Three specimens were fixed in 1.5 to 3.0 per cent potassium permanganate, but in these the ferritin particles could not be visualized. After fixation for approximately 1½ hours, the ganglia were cut in half, rinsed, dehydrated in methanol or ethanol solutions, and embedded in Epon 812 (19). Thin sections were cut with a Porter-Blum microtome, mounted on copper mesh grids coated with carbon and Formvar, stained with a saturated, aqueous uranyl acetate solution (42) for 1 to 3 hours, and examined in an RCA EMU 3G at initial magnifications of 8,000 to 18,000 (accelerating voltage, 50 kv). Lead stains were not used in this study because of the possibility that fine precipitates from the stain might be confused with ferritin particles.

[1] A preliminary report of the present study appears in the abstracts of the third annual meeting of the American Society for Cell Biology held in November, 1963 (35).

[2] The ferritin was obtained from two sources. That from the Pentex Corp., Kankakee, Illinois, was dialyzed against Gey's solution for 1 week at 0° C and then diluted as necessary before use. That from Nutritional Biochemicals, Cleveland, Ohio (2× crystallized, cadmium-free) was used without dialysis, but with the addition of salts as needed.

OBSERVATIONS

Toad spinal ganglia are approximately 1 to 2 mm in diameter and are composed of a cortex of globular nerve cell bodies surrounding a core of nerve fibers. The neurons are unipolar and extend their axons centrally where the latter bifurcate to form peripheral and central branches. A connective tissue investment consisting of innumerable collagen fibrils surrounds the whole ganglion and extends in among the neurons separating them from one another. This connective tissue space constitutes a continuous pathway from the periphery to the core of the ganglion, and distinguishes this ganglion architecturally from central nervous tissue, which has no extensive connective tissue spaces. Capillaries occur in the connective tissue space along with scattered macrophages and fibroblasts.

Each neuron is individually encapsulated by a sheath consisting of one or more attenuated layers of satellite cell cytoplasm, the outermost of which is covered by a basement membrane (Figs. 1 and 2). The layers of the sheath overlap and interdigitate with one another in a complex fashion most striking in the region of the axon hillock. The simple helical patterns seen in compact myelin sheaths (11), and the modified helical patterns in the sheaths of some acoustic ganglion cells (32) are not apparent here.

A narrow (100 to 200 A) extracellular space, the perineuronal cleft, separates each neuron from its surrounding sheath, and the constituent layers of the sheath are, in turn, separated from one another by comparable, channel-like extracellular (or intercellular) spaces (Figs. 1 and 2). Some of these channels extend all the way through the sheath and open into the perineuronal cleft at one end and into the space just under the basement membrane at the other (33).

For the purpose of localizing ferritin in the ganglia, two extracellular compartments may thus be distinguished: (*a*) the collagen-filled connective tissue space, which surrounds the satellite cell sheaths but does not come into direct contact with the neurons, and (*b*) the narrow collagen-free channels between the layers of the sheath and between the sheath and the neuron. These two compartments are separated from each other by the basement membrane which surrounds the sheath without interruption. The second compartment is entirely comparable to the extracellular space of the central nervous system, which also consists of multiple narrow, intercellular channels free of formed collagen.

Extracellular Distribution of Ferritin

In ganglia which were soaked in ferritin solutions prior to fixation, particles of ferritin are present in large numbers in the connective tissue spaces, the concentration depending on the length of exposure to ferritin, the dilution of the ferritin solution, and the distance of the region examined from the exterior of the ganglion.[3] The highest concentration occurs at the very periphery of the ganglion; hence this is the region studied most intensively. In the *in vivo* studies very little of the injected ferritin reaches the connective tissue spaces of the ganglia after 3 hours. (Most of the injected ferritin is still in the peritoneal cavity at this time.) At 17 hours the concentration in the connective tissue of the ganglia is moderate, and at 96 hours it approaches the concentration in the soaked ganglia.

Ferritin is taken up from the connective tissue space by macrophages, which display large cytoplasmic vacuoles containing multiple ferritin molecules in a concentration higher than that in the surrounding connective tissue matrix (Fig. 1, inset).

In addition, in both *in vivo* and *in vitro* experiments, ferritin particles are, to a limited extent, able to penetrate through the basement membrane of the satellite cell sheaths and can be detected in the light zone between the basement membrane and the plasmalemma of the outermost satellite cell layer (Fig. 2). Some of the particles that traverse the basement membrane also gain access to the narrow channels in the sheath (Figs. 1 and 2) and to the narrow perineuronal space between the neuron and the sheath (Figs. 1, 5, 6, and 17). The concentration of ferritin in these channels is, however, very low even when the concentration in the connective tissue is high, suggesting either that the basement membrane impedes the passage of ferritin here as it does in the normal renal glomerulus (6) or that the channels themselves offer some resistance to the passage of ferritin molecules. The caliber of the channels is not constant; varicosities occur particularly where the channels bifurcate or bend abruptly (Fig. 2). Generally, ferritin particles tend to accumulate

[3] Although the ferritin molecule is about 95 A in diameter, only the dense, iron-containing core is visible in these preparations. This part of the molecule is about 55 A in diameter (7).

in the dilated segments, but are not restricted to these regions and occur singly even where the channels are of their narrowest caliber (Figs. 1 and 2).

Intracellular Localization of Ferritin

Ferritin particles are detectable in the neurons by 1 hour in the soaked specimens and by 17 hours in the *in vivo* studies. In all cases, ferritin molecules occur in membrane-limited structures—multivesicular bodies and vesicles of a special variety, which we have termed "coated" vesicles (35). Ferritin is not found free in the cytoplasm; nor has it been observed in mitochondria or cisternae of the granular endoplasmic reticulum or Golgi apparatus. Because of the high intrinsic density of the lysosome-like inclusion bodies (Fig. 1), it has not been possible to determine whether ferritin occurs in these structures or not. Ferritin is, however, present in bodies intermediate in structure between multivesicular bodies and the dense, lysosome-like inclusion bodies.

Along the neuronal surface, ferritin particles accumulate in distinctive indentations of the plasma membrane (Fig. 3). The indented segments of membrane are approximately 0.1 to 0.2 μ across and are almost perfectly semicircular in section. They usually appear denser than adjacent portions of the plasma membrane and they exhibit unit membrane structure (Fig. 3, inset). These indentations are further specialized in that they are coated on their convex, or cytoplasmic, surface with an ill defined, radially striated material which forms a layer approximately 120 A deep (Figs. 3 to 12). The coating is often indistinct, but in some specimens it appears to consist of hair-like projections. Frequently, only the distal tips of the projections are visualized (Fig. 3, inset, and Fig. 8). The coating bears a striking resemblance to that which occurs on certain virus particles (4) and which has been shown by negative-staining techniques to consist of ridges or spikes.

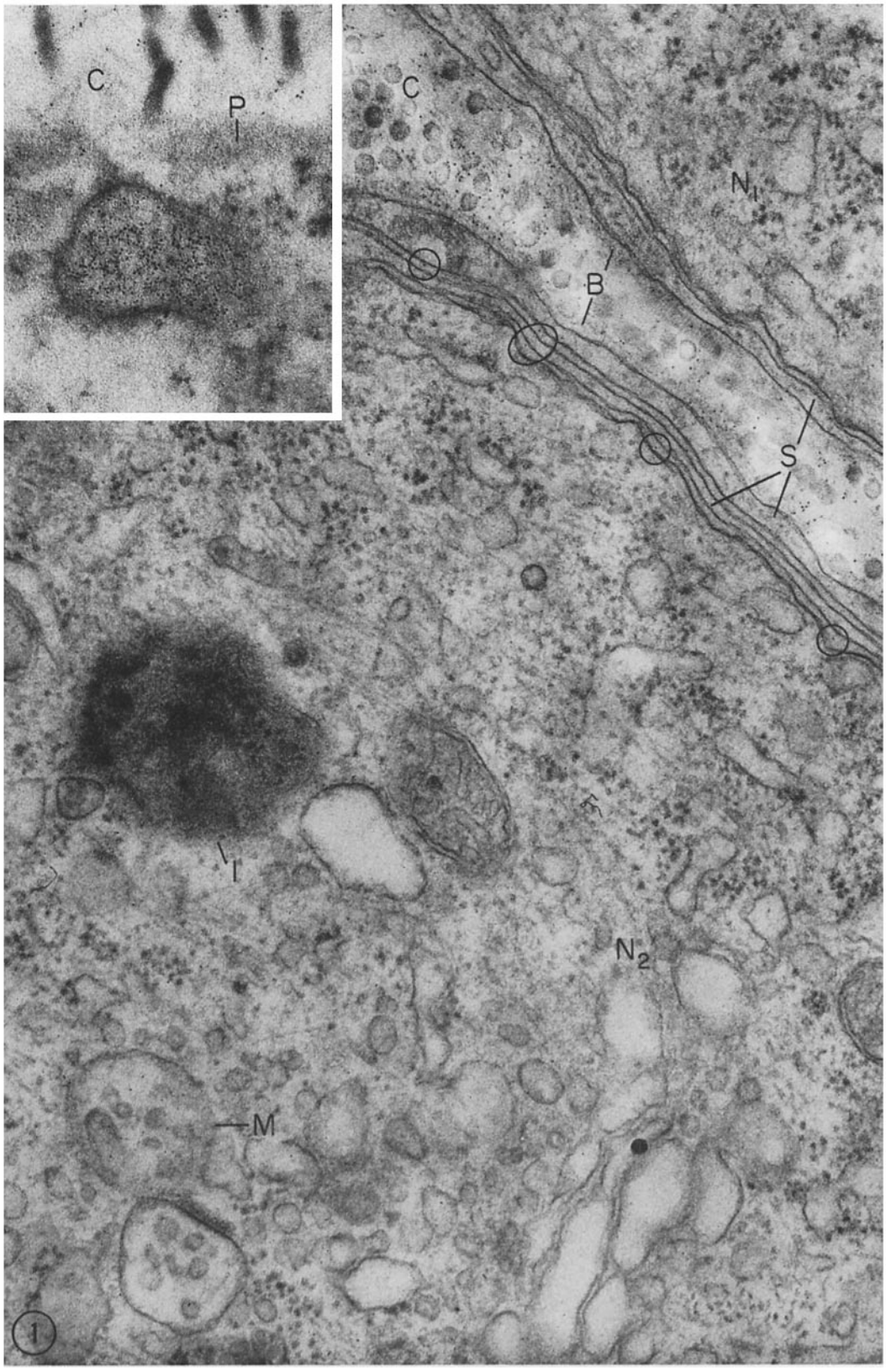
In addition to the coating on the cytoplasmic surface of the indentations there also appears to be a vague, radially striated coating on the concave surface (Figs. 3 to 12). The latter coating is especially difficult to delineate, however, because of the curvature of the indented segment within the thickness of the section. Sometimes the radial elements of the two coatings appear to be continuous across the plasma membrane (Figs. 4 and 9).

Bulbous vesicles coated in the same manner as the indentations and also containing multiple ferritin molecules form at the ends of invaginations of the neuronal plasma membrane. The membrane-limited channel connecting such a vesicle to the neuronal surface may be short (Figs. 6 and 7) or long (Fig. 8). Occasionally two vesicles occur at the end of the same invagination, either as a pair (Fig. 9) or in tandem (Fig. 10).

In addition to ferritin particles, coated vesicles may also contain smaller, uncoated vesicles approximately the same size as those which are present in multivesicular bodies (Figs. 11 and 12). Coated vesicles can be seen both in osmium-fixed specimens and in ganglia prefixed in glutaraldehyde, but not in permanganate-fixed tissues. Presumably, the permanganate does not preserve the coating. It should be emphasized that the concentration of ferritin in coated vesicles is considerably higher than in the perineuronal cleft.

The second structure to which ferritin particles have access is the multivesicular body. These are approximately 0.5 μ in diameter and may be either "dark" or "light" (Fig. 13). The former type contains many small vesicles lying in a scant matrix of moderate density; the latter type contains relatively few vesicles in a large matrix of very low density. In occasional sections, no vesicles can be seen in the latter type. In both types of multivesicular body a felt-like coating (28) may cover one or more segments of the limiting membrane (Figs. 12 and 13). This coating is also radially striated, but appears to be

FIGURE 1 Survey picture of ganglion cells. The upper neuron (*N1*) is covered by a single satellite cell layer (*S*), and the lower one (*N2*) by two. Ferritin is visible among collagen fibrils in the connective tissue space (*C*) between the two neurons, in a multivesicular body (*M*), and in the extracellular channels in one of the satellite cell sheaths (circles). None is present in cisternae of the granular endoplasmic reticulum or Golgi apparatus. Dense inclusion bodies (*I*) occur normally in these neurons. *B*, basement membrane. × 60,000. *Inset.* Ferritin-filled vacuole in a macrophage in the connective tissue of a ganglion. The concentration of ferritin in the vacuole is much higher than in the connective tissue itself (*C*). *P*, plasma membrane sectioned obliquely. × 75,000.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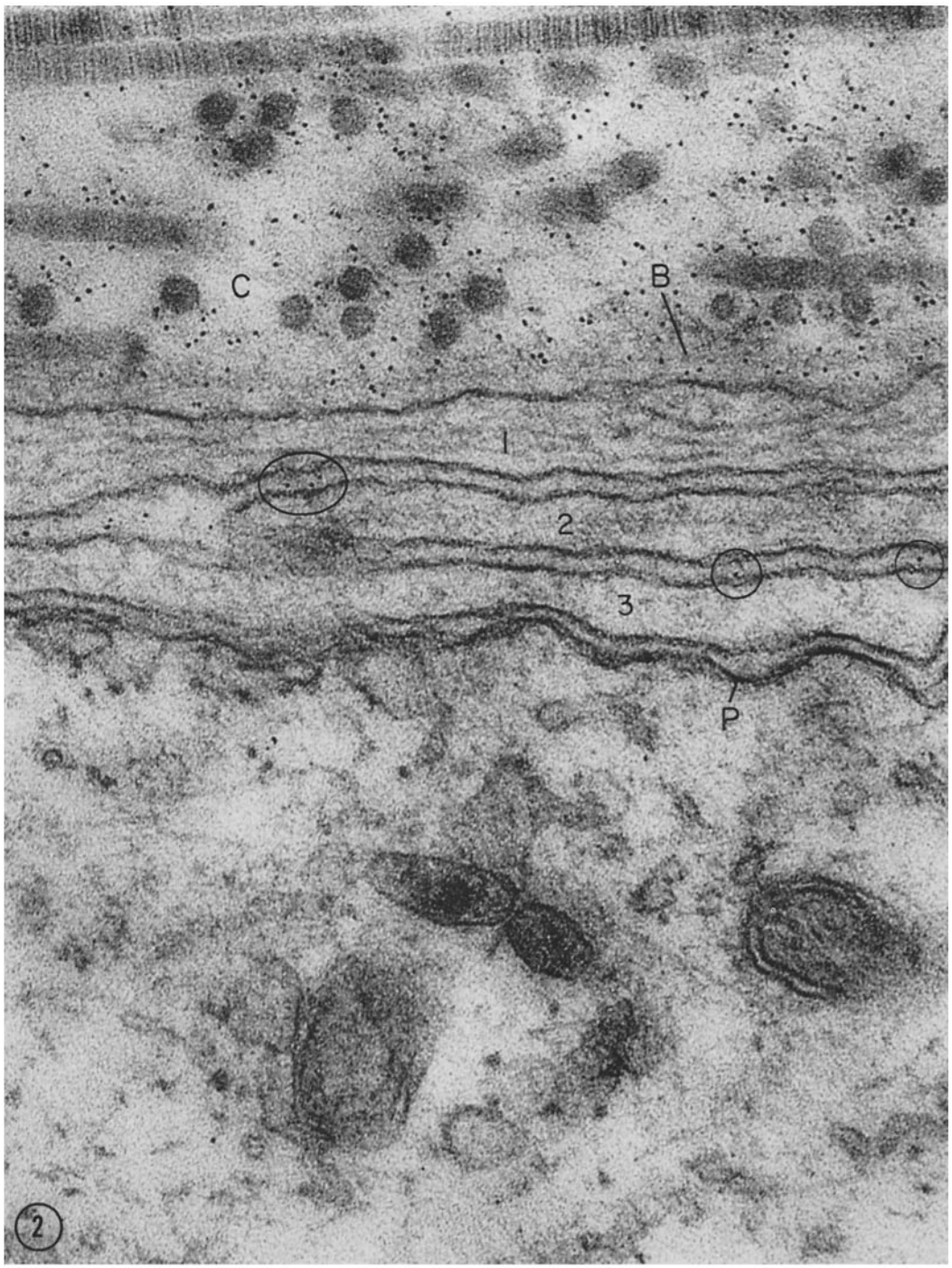


FIGURE 2 Ferritin particles in extracellular channels in a satellite cell sheath. The sheath is composed of three layers (*1*, *2*, and *3*) at the center of the field. At this magnification, individual molecules are clearly visible both in narrow segments of the intercellular channels (circles) and in the dilated portion at the left. Numerous particles have penetrated from the connective tissue space (*C*) through the basement membrane (*B*) covering the sheath. *P*, neuronal plasma membrane. × 115,000.

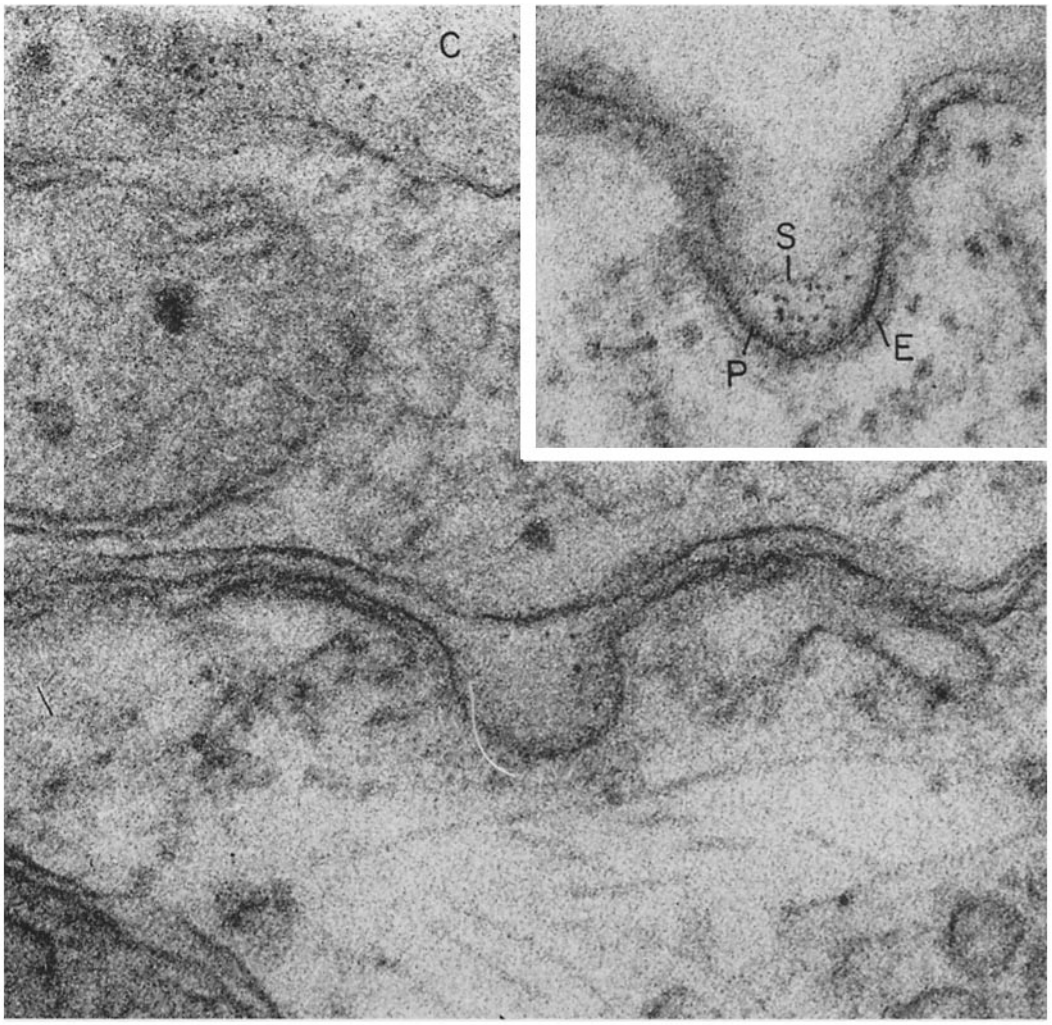


FIGURE 3 Inpocketing of the neuronal plasma membrane. Ferritin particles are present within the inpocketing and in the connective tissue (*C*) at the upper left. × 140,000. *Inset.* The satellite cell plasma membrane (*S*) bulges into an indented segment of the neuronal plasma membrane (*P*). Unit membrane structure is visible in the membrane of the inpocketing, and a felt-like coating (*E*) covers its cytoplasmic surface. Numerous ferritin particles are present in the pocket between the two membranes. × 162,000.

much denser than that on the coated vesicles. It is about 300 A deep and it is usually separated from the limiting membrane of the multivesicular body by a thin light zone (Figs. 12 and 14). Sometimes the coating appears to consist of two layers with a narrow light zone intervening (*cf.* 25). The area of each coated segment on a multivesicular body is approximately the same as the surface area of one coated vesicle. Multivesicular bodies may exhibit one or more evaginations of their limiting membrane (Fig. 13) and, in some cases, the lumen of the body continues into a slender elongated "neck" which extends out into the cytoplasm (Fig. 16). The diameter of the neck is comparable to the width of the invaginations of the neuronal plasma membrane to which coated vesicles may be attached (Figs. 8 to 10). Multiple ferritin molecules occur in both types of multivesicular body. The particles are contained not within the included vesicles, but rather in the matrix surrounding these vesicles (Fig. 13).

On occasion, forms intermediate between the

coated vesicles and multivesicular bodies occur near the neuronal surface. These are small membrane-limited structures which may be either circular or elongated in profile and which contain only a few vesicles (Fig. 15).

The satellite cells resemble the neurons rather than the macrophages of the connective tissue space with regard to their behavior towards ferritin. Although it is occasionally possible to find a large vacuole containing multiple ferritin molecules forming at the surface of a satellite cell, more typically the satellite cells take up ferritin in coated vesicles quite like those which occur at the neuronal surface. These vesicles may form at any portion of the satellite cell plasmalemma, but most commonly they arise from the surface facing the neuron (Fig. 17, inset). Multivesicular bodies which, like those in the neurons, have radially striated coatings on segments of their limiting membrane also occur (Fig. 4), and these, too, contain ferritin particles (Fig. 17). An occasional satellite cell displays many ferritin particles free in its cytoplasm, but such cells show evidence of damage, including cytoplasmic swelling and interruptions in the limiting membrane. The passage of ferritin into the cytoplasmic matrix of these cells is, therefore, interpreted as an artifact.

After fixation with osmium tetroxide, both neurons and satellite cells may display numerous serried vesicles, some of which also contain ferritin particles. It was pointed out previously (33) that vesicles of this type are not present after permanganate fixation, and in the present study they were not visible after combined glutaraldehyde-osmium tetroxide fixation either. Thus, there is reason to suspect that such chains of vesicles are artifacts of osmium tetroxide fixation arising by fragmentation of continuous membrane-limited channels, and that the ferritin particles they contain are, in reality, extracellular. Should future experiments bear out this supposition, then the coated vesicles alone would remain as the pinocytotic apparatus involved in the incorporation of ferritin into these neurons and satellite cells.

DISCUSSION

Intercellular Clefts as Aqueous Pathways

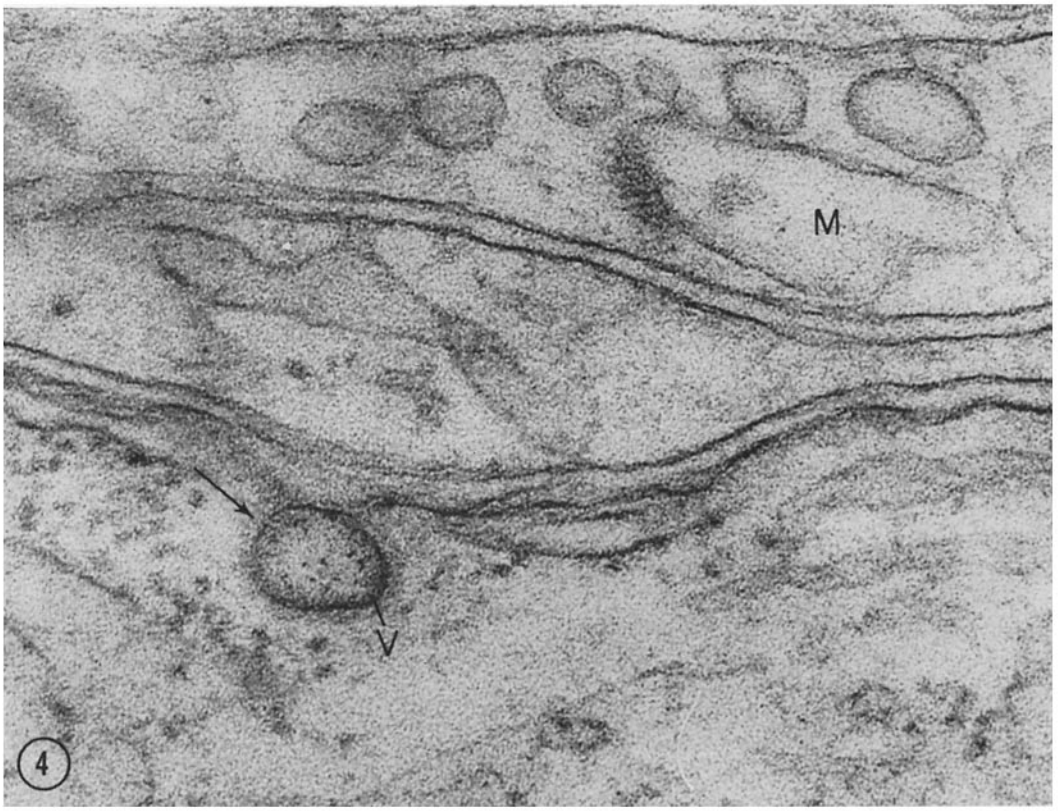
From the standpoint of their dimensions, the clefts separating satellite cell layers from one another seem to be patent. The total width of each cleft and the plasma membranes bordering it is about 300 A. The membranes themselves have been shown in permanganate-fixed specimens to be typical trilaminate unit membranes (33), each of which is about 75 A thick (31). (After osmium tetroxide fixation only the innermost, or cytoplasmic, lamina of the unit membrane is visualized.) The gap between the membranes should therefore be approximately 150 A wide. This figure is intermediate between the value of 200 A used by Horstmann and Meves (15) in their calculations of extracellular space in the central nervous system and the figure of 60 A reported by Villegas and Villegas (41) for the width of intercellular clefts in the sheaths of squid giant axons.

These estimates must all be considered only as approximations, however. The dimensions of the spaces may be altered before or during fixation by small deviations of the bathing solution or of the fixative from isotonicity (*cf.* 30), or by abnormalities in the divalent ion content of these solutions (*cf.* 12). After fixation, there may be dimensional changes during dehydration and embedding (*cf.* 9). Moreover, if, as Pethica suggests (29), long range chemical and electrical forces act to maintain adjacent cell membranes at a fixed distance from one another, it would not be surprising to find that the changes taking place in membranes during fixation and embedding alter the balance of these forces and consequently distort the intervening space. We are also not justified in

FIGURES 4 and 5 Coated vesicles forming at the neuronal surface.

FIGURE 4 A coating is present on both the concave and convex surfaces of the vesicle (*V*). In one region (arrow) radial elements of the coating appear to extend across the limiting membrane of the vesicle. A multivesicular body (*M*) whose limiting membrane has a radially striated coated segment is present in the outer satellite cell layer. × 139,000.

FIGURE 5 Ferritin particles are present within the coated vesicle (*V*), in the cleft between the neuronal and satellite cell plasma membranes (arrows), and in the connective tissue (*C*) at the upper left. The segment of plasma membrane from which the vesicle arises is cut obliquely and appears to overlap the vesicle itself. × 139,000.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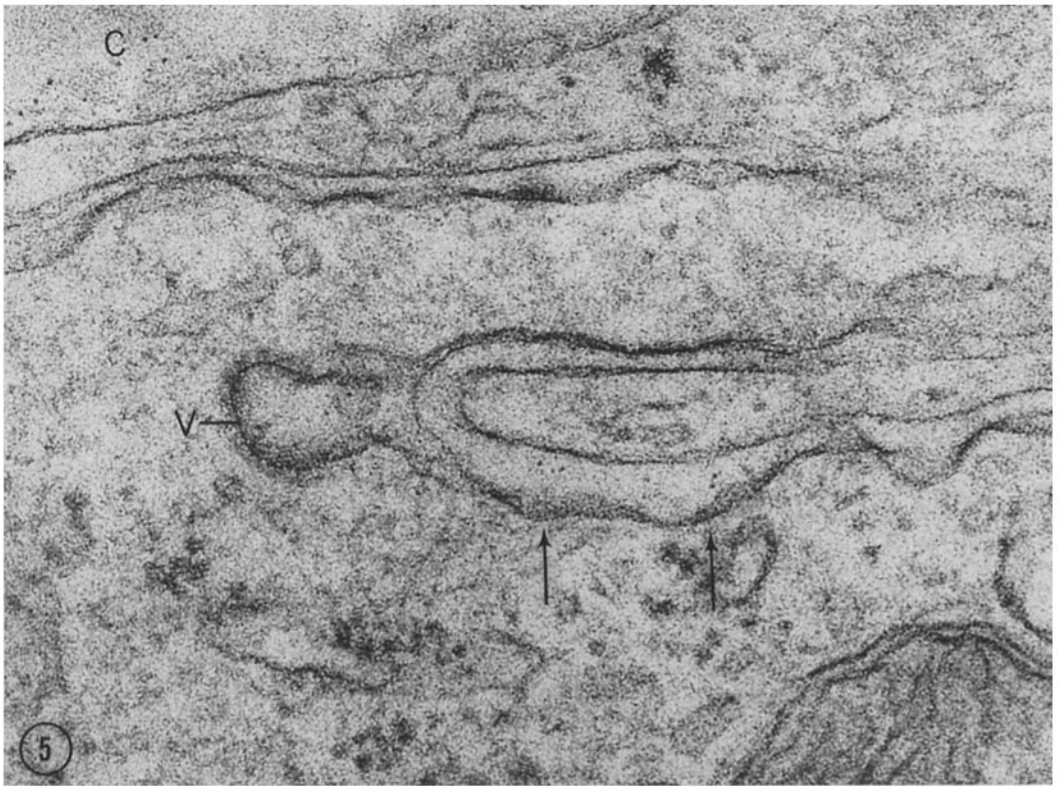
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making any assumptions about the *content* of intercellular clefts from their appearance alone or about the physiological properties of the material filling the clefts.

Indirect evidence from physiologic experiments is consistent with the contention that the clefts are patent at least as far as the penetration of water and small ions is concerned. Frankenhaeuser and Hodgkin (10) found that potassium ions escaping from squid giant axons follow a low resistance pathway through the investing sheaths to the surrounding medium. They suggested that the tortuous channels between Schwann cells described by Geren and Schmitt (12) could serve as such a pathway. More recently Villegas and Villegas (41) have arrived at the same conclusion after studying the diffusion of water from the interior of giant axons to the surrounding medium.

Our experimental findings unequivocally support the hypothesis that the narrow clefts between satellite cell layers are patent passages for water and its solutes by demonstrating that a large, water-soluble protein can pass through them to reach the perineuronal space. Scattered evidence indicates that intercellular clefts are probably patent in nervous tissues generally. Lasansky and Wald (18) reached this conclusion on the basis of their study of the distribution of ferrocyanide in the toad retina; the same inference was made from the presence of a spicular material of endogenous origin in the clefts between glial cells in an invertebrate ganglion (34); exogenous ferritin has also been noted in some intercellular spaces in the vertebrate central nervous system (3).

Patency of intercellular clefts in nervous tissue does not, however, imply that all exchanges between neurons and blood or connective tissue spaces need follow this route or that passage through these channels is unrestricted. Lipid-soluble materials undoubtedly can pass directly across the plasma membranes of supporting cells. In the present study, it is clear that the total number of ferritin molecules reaching the neuronal surface is small despite exposure of some ganglia for protracted periods to a rich concentration of this protein. Obviously, the sheath or its basement membrane constitutes a partial barrier to ferritin. It is conceivable that supporting cells exercise some control over traffic through the clefts, obstructing some materials, and permitting others to pass, perhaps even assisting them by active movements of the plasma membranes lining the clefts (*cf.* 39).

The Uptake of Protein by Neurons

Although the turnover of protein in neurons is high, one does not ordinarily think of neurons as cells which engulf whole proteins from their environment. Yet it is clear that, in toad spinal ganglia, ferritin not only penetrates through the satellite cell barrier but is taken up by the neurons in much the same manner as it is incorporated into ascites tumor cells (37), protozoa (40), or erythroblasts (1).

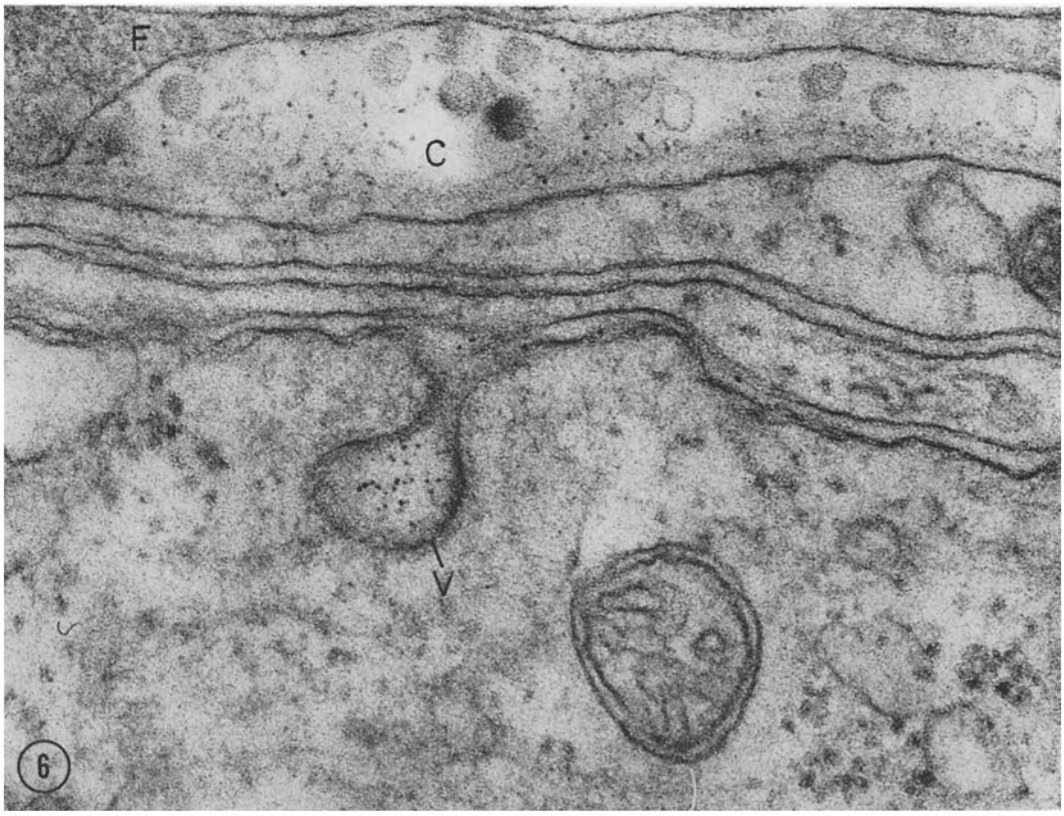
This finding is not so surprising when one considers that neurons, in addition to being highly specialized functional units concerned with impulse propagation and transmission, are at the same time very large cells, which have their own metabolic needs to take care of. As such, they can be expected to display many of the characteristics of undifferentiated or even free-living cells. These comments apply equally well to neurons of the central nervous system as to the spinal ganglion cells described here. We may, therefore, speculate that neurons in general, including those of the central nervous system, take up whole protein from their immediate environment as a part of their normal nutritive and metabolic activity.

The extent to which this behavior is quantitatively significant is difficult to assess. It has been shown that pinocytosis in amoebae can be stimulated by increasing the protein content of the surrounding medium (2, 14). The possibility therefore exists that, to some extent, it is the

FIGURES. 6 and 7 Coated vesicles arising from short invaginations of the neuronal plasma membrane.

FIGURE 6 The concentration of ferritin in the vesicle (*V*) is somewhat higher than that in the connective tissue (*C*). Part of a fibroblast (*F*) passes across the top of the field. × 113,000.

FIGURE 7 The width of the "neck" of the coated vesicle (*V*) is approximately the same as that of the channel between the neuron and satellite cell. The coating on the vesicle does not extend onto the neck. *C*, connective tissue. × 113,000.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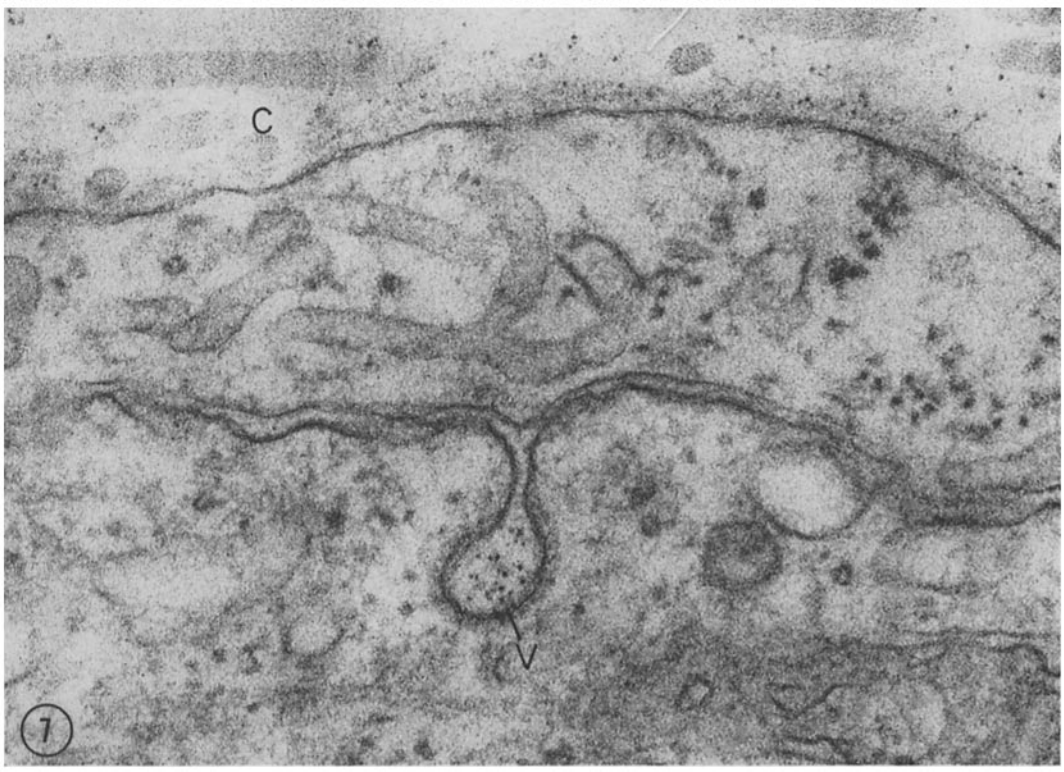
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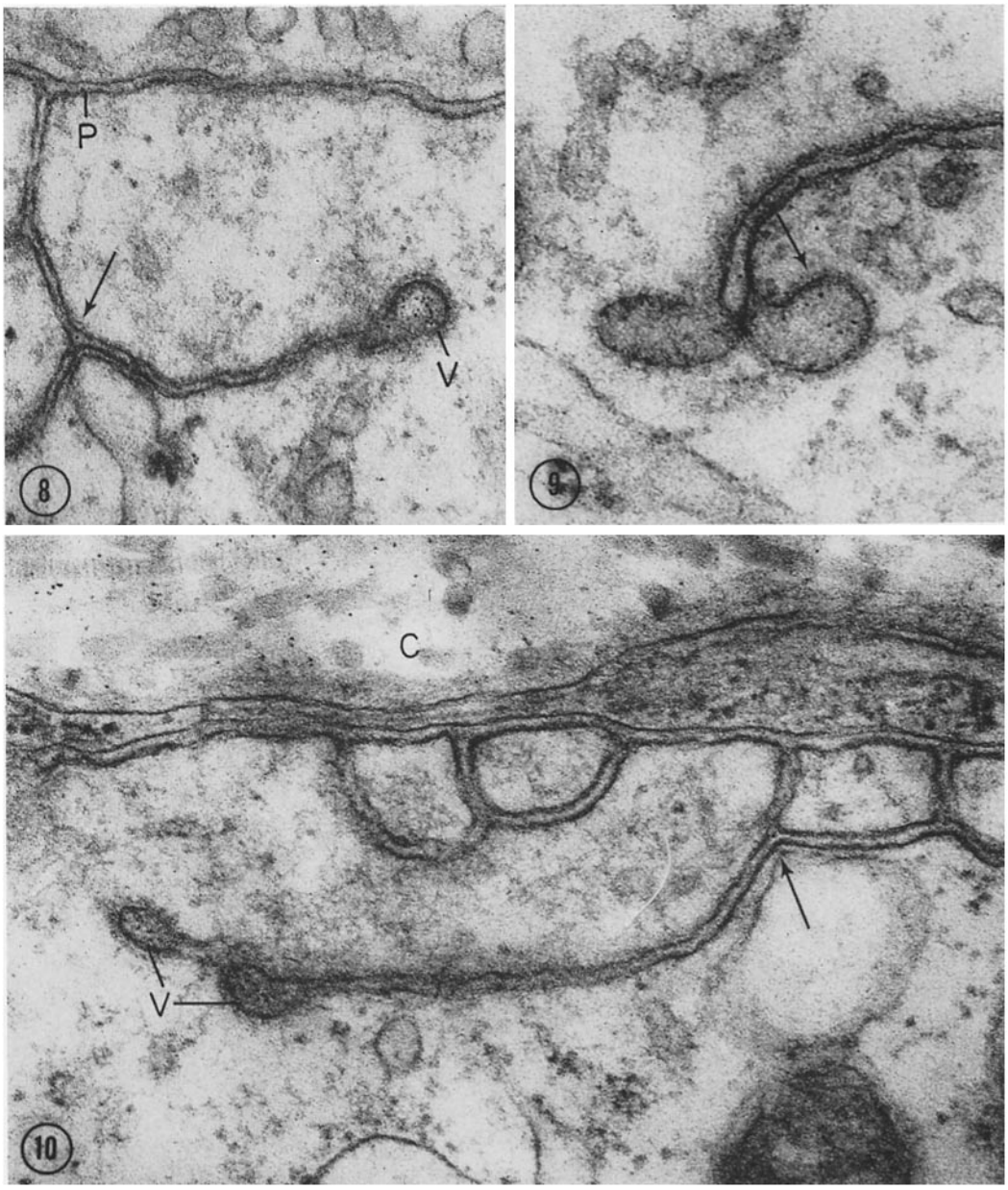
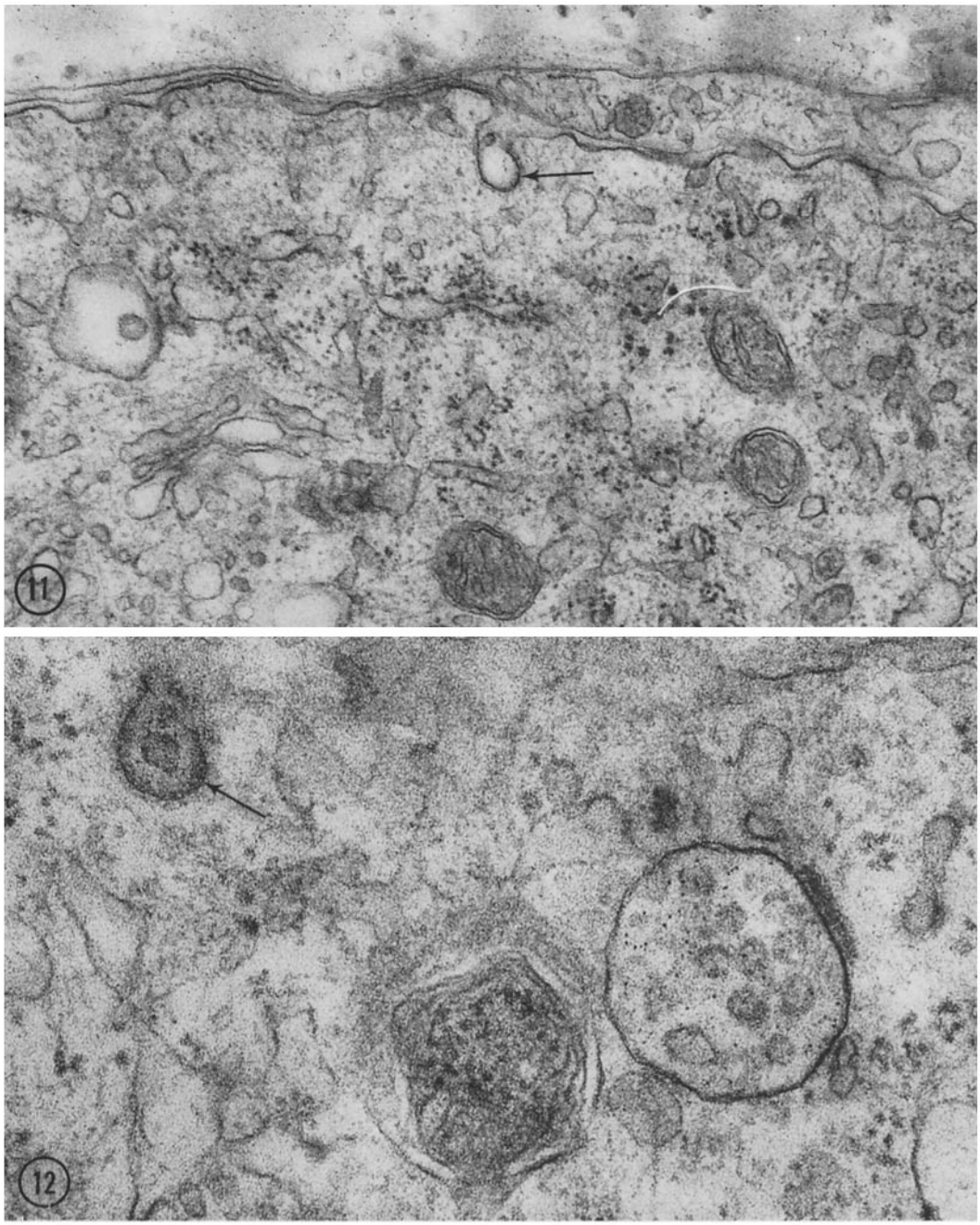


FIGURES 8 to 10 Coated vesicles at the ends of long invaginations of the plasma membrane.

FIGURE 8 A single coated vesicle (*V*) containing multiple ferritin particles forms the blind end of an invagination of the neuronal plasma membrane (*P*). A single particle is present in the invagination itself (arrow). × 73,000.

FIGURE 9 Two coated vesicles apparently forming simultaneously at the end of an invagination of the neuronal plasma membrane. At the arrow, elements of the coating appear to extend across the limiting membrane of the vesicle. × 103,000.

FIGURE 10 Two coated vesicles (*V*), forming in series from an invagination of the neuronal plasma membrane. A single ferritin particle is present in the invagination at its origin (arrow). *C*, connective tissue. × 92,000.



FIGURES 11 and 12 Vesicles within coated vesicles.

FIGURE 11 An inpocketing (arrow) of the neuronal plasma membrane containing several ferritin particles and one smaller vesicle. × 44,000.

FIGURE 12 A coated vesicle (arrow) containing one or two smaller vesicles. The latter are approximately the same size as those within the multivesicular body at the right. A radially striated coating covers the cytoplasmic surface of one segment of the multivesicular body. × 92,000.

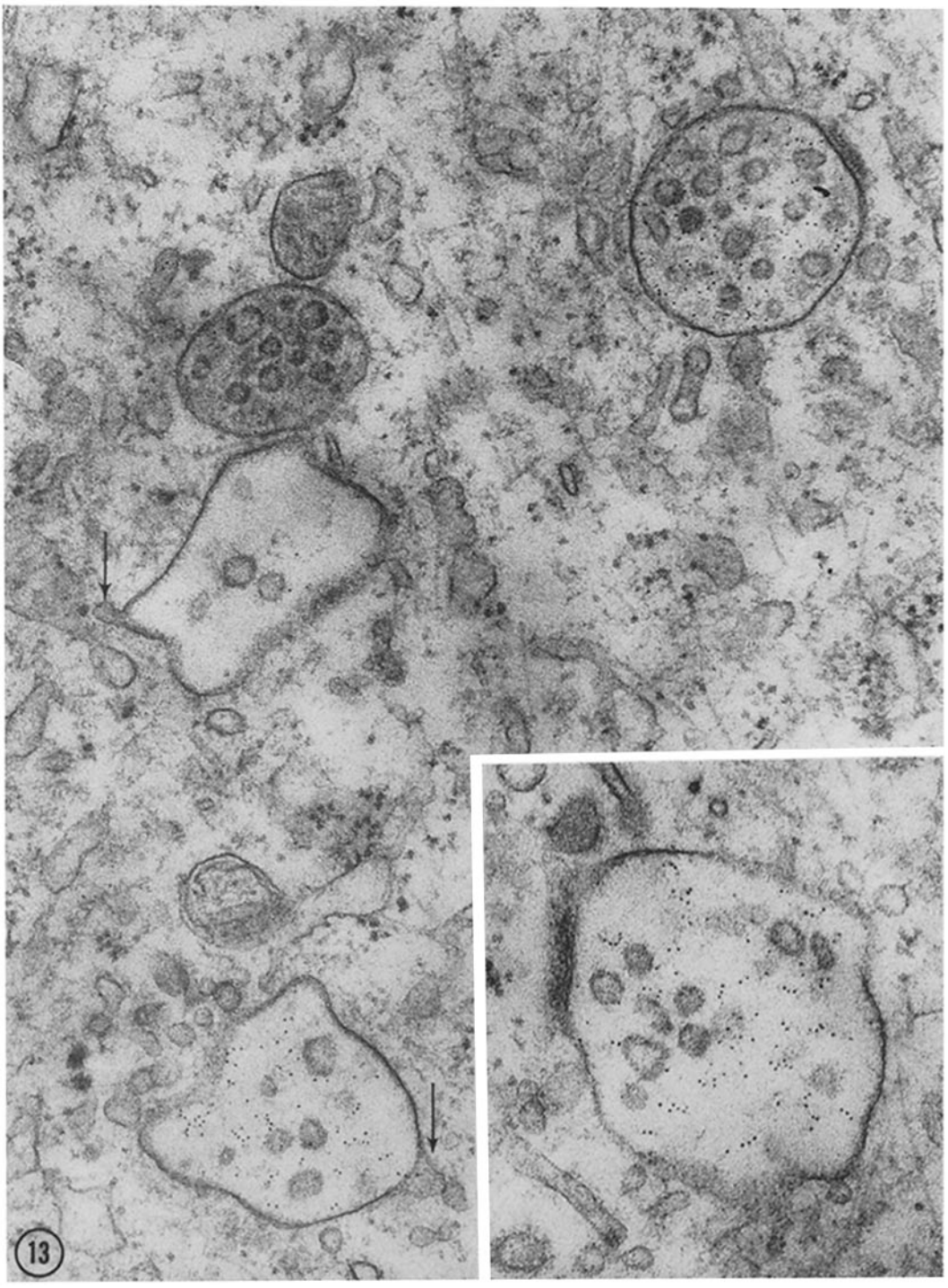
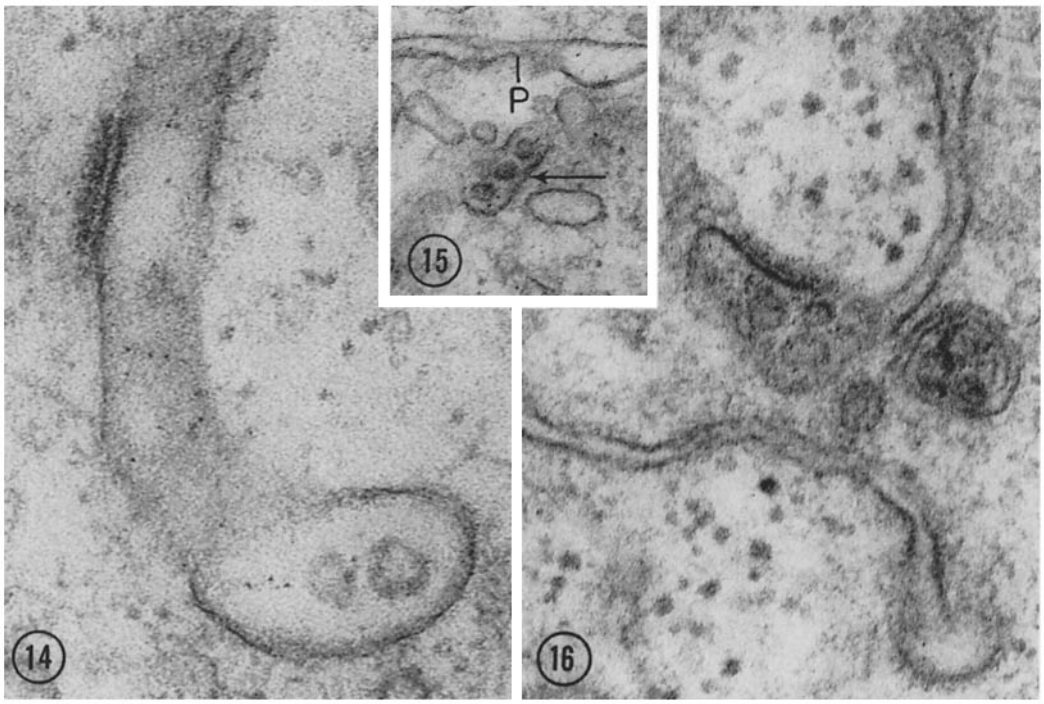


FIGURE 13 Dark and light multivesicular bodies in a neuron. Ferritin particles occur in both types, but not free in the cytoplasm. The concentration of ferritin varies markedly from one multivesicular body to the next, perhaps reflecting the formation of these bodies at different times. Evaginations of the limiting membranes of the multivesicular bodies are shown at arrows. × 68,000. *Inset*, × 84,000.



FIGURES 14 to 16 Possible intermediate forms between multivesicular bodies and pedunculated coated vesicles.

FIGURE 14 Boot-shaped multivesicular body with a radially striated extraneous coating on the "ankle." A narrow light zone intervenes between the coating and the limiting membrane of the multivesicular body. × 123,000.

FIGURE 15 A small, slender, elongated multivesicular body (arrow) near the neuronal plasma membrane (*P*). × 68,000.

FIGURE 16 Multivesicular body whose lumen continues into a long neck extending towards the top of the figure. The width of the neck is approximately that of a plasma membrane invagination. Note that ferritin particles occur in the expanded portion of the multivesicular body and in the uppermost portion of the neck. A pedunculated coated vesicle occurs at the bottom of the field. × 86,000.

ferritin itself which has stimulated pinocytosis in these neurons. The pinocytotic activity cannot be wholly dependent on the presence of exogenous ferritin, however, for the structures involved in ferritin uptake—coated vesicles and multivesicular bodies—also occur in these cells in the absence of exogenous ferritin (33).

Coated vesicles are widely distributed in the animal kingdom. They occur not only in various tissues of vertebrates (1, 6, 13, 26, 33, 36, 44) but also in protozoa (8) and in other invertebrates (36). They evidently subserve some general metabolic function which, from our data, would appear to be the sequestration and uptake of macromolecular material intact from the immediate environment. Roth and Porter (36) have reported the occurrence of such vesicles in cells known to accumulate protein and have inferred that vesicles of this type are engaged in the uptake of protein specifically. Their hypothesis is consistent with the results reported here as well as with the earlier observations of Farquhar, Wissig, and Palade (6) and the recent observations of Maunsbach (21).

The structural pattern of the coated vesicles is extremely stable phylogenetically, apparently having persisted unchanged from protozoa to the highest mammals. Unfortunately, we have no specific information about the chemical compo-

sition of the coating or even about its precise morphology and we can, therefore, only guess what its function might be. The radial components of the cytoplasmic coating may represent adsorbed molecules which act as a kind of detergent to permit deformation of the plasma membrane into the shape of a pocket. Or they may constitute elements of new plasma membrane being added to the cell surface. A more provocative possibility is suggested by the observation that ferritin is much more concentrated in the coated vesicles and indentations than it is in the perineuronal space. If, as our observations suggest, the radial elements of the coating protrude through the membrane of the vesicle into its lumen, they may be regarded as molecules which are capable of sequestering and thereby concentrating large molecules, perhaps of specific types, from the immediate vicinity of a coated indentation. There is the further possibility that the coating represents molecules of specific hydrolytic or digestive enzymes. This does not seem likely, however, for the ferritin within the vesicles, and indeed within the multivesicular bodies, shows no sign of gross structural alteration. The characteristic distribution of iron micelles in the molecules is markedly disturbed once the protein itself is attacked by protease (20).

Our findings indicate that the engulfed ferritin does not pile up in coated vesicles, but rather in multivesicular bodies—the only other structure in these neurons to which ferritin has access. The apparent transfer of ferritin from the one structure to the other can be explained by assuming that the vesicles which form at the cell surface become detached from the plasma membrane and then coalesce either with one another or with existing multivesicular bodies, adding their membrane to the limiting membrane of the multivesicular body and at the same time discharging their content into its lumen.

An alternative interpretation is suggested by the fact that coated vesicles and multivesicular bodies seem to be closely related structurally as

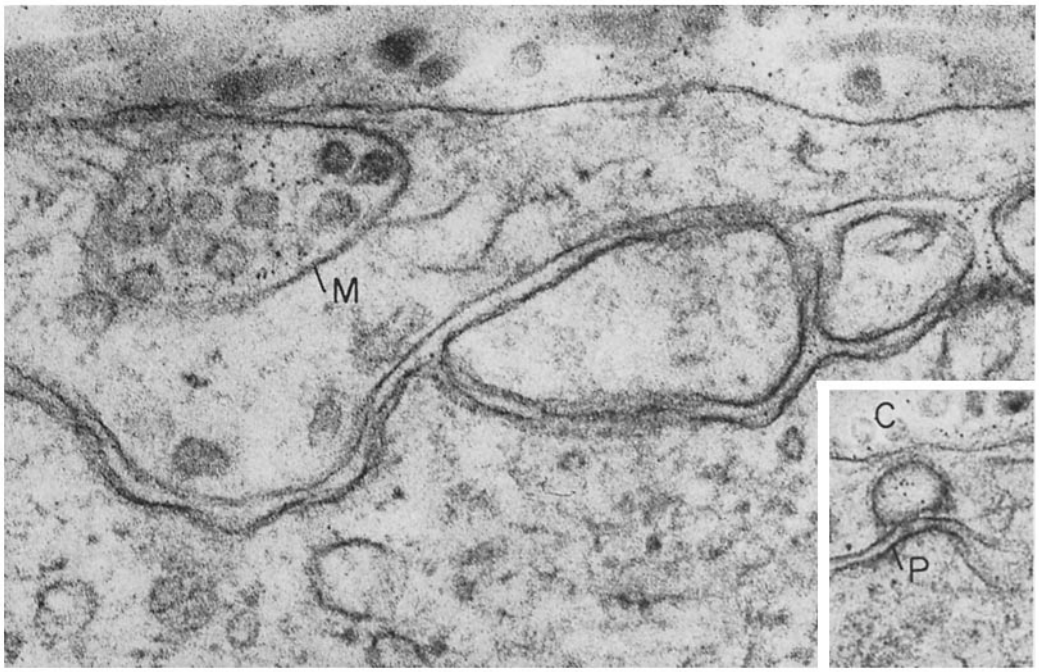


FIGURE 17 Ferritin-containing multivesicular body (*M*) in a satellite cell. Note particles also in the channel between the satellite cell and neuron especially near the right of the figure. × 97,000. *Inset.* Ferritin-containing coated vesicle forming within a satellite cell. *C*, connective tissue; *P*, neuronal plasma membrane. × 70,000.

well as functionally. On the one hand, coated vesicles sometimes contain subsidiary vesicles resembling those in multivesicular bodies, and on the other hand, multivesicular bodies sometimes possess long "necks" which resemble the plasma membrane invaginations to which coated vesicles are often attached. Moreover, although the coating that occurs on multivesicular bodies is thicker and denser than that on the coated vesicles, these are the only two cytoplasmic structures that possess coatings of any kind on their limiting membrane.

These findings suggest as a second possibility that these two apparently separate structures are, in fact, different stages of the same one, *i.e.*, that coated vesicles are multivesicular bodies in the process of formation. According to this view, the limiting membrane of the multivesicular bodies is formed directly from the cell surface by invagination of the plasma membrane. This contention is supported by the observation that the thickness of their limiting membrane matches that of the plasma membrane itself (45). Continual formation of multivesicular bodies from the cell surface in this way could account for the presence of a great deal of ferritin in some of these bodies and its absence in others nearby. Presumably, the latter were formed before ferritin was administered.

Whatever the mechanism of formation of the multivesicular bodies, it is, at least, apparent that these structures, which occur so commonly in neurons, have as one of their functions the uptake and sequestration of macromolecular material from the environment of the neurons. Their general function here appears to be the same as in the renal corpuscle (5). However, it is not clear whether in neurons this type of uptake is quantitatively significant in terms of the cell's over-all protein metabolism, or whether this process is important only as a device for sampling the environment or taking in trace amounts of specialized materials such as hormones. We are equally in the dark about such matters as the origin and significance of the vesicles within the multivesicular bodies, the possibility of conversion of multivesicular bodies into lysosomes (*cf.* 22) or digestive vacuoles, and the ultimate fate of the engulfed ferritin within multivesicular bodies.

At the present time it can, at least, be stated that the first stages of ferritin uptake by neurons entail concentration and incorporation of this protein by coated pinocytotic vesicles followed by segregation of the ferritin into multivesicular bodies. The same mechanism appears to operate in satellite cells, but not in the macrophages that occur in the connective tissue spaces of the ganglia. This apparent difference in uptake mechanism may be a basic one or it may merely reflect local differences in the concentration of ferritin.

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Addendum. Evidence that intercellular clefts in the central nervous system of the leech serve as pathways for the flow of ions and of molecules as large as sucrose has been presented recently by Kuffler, S. W., and Potter, D. D. (*J. Neurophysiol.*, 1964, **27**, 290), and Nicholls, J. G., and Kuffler, S. W. (*ibid.*, 1964, **27**, 645). Further details of yolk protein uptake by mosquito oocytes (36) have been published by Roth, T. F., and Porter, K. R. (*J. Cell Biol.*, 1964, **20**, 313).

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