THE ORGANIZATION OF SYNAPTIC AXOPLASM IN THE LAMPREY (*PETROMYZON MARINUS*) CENTRAL NERVOUS SYSTEM

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ABSTRACT

The fine structure of synapses in the central nervous system of lamprey (*Petromyzon marinus*) ammocoetes has been investigated. Both synapses within the neuropil and synaptic links between giant fibers (including Müller cells) and small postsynaptic units are described. The distribution of neurofilaments and microtubules in nerve profiles over a wide diameter range is described, and the possible role of these structures in intracellular transport is discussed. Electron micrographs indicate that small lucent "synaptic vesicles" occur sparsely throughout the axoplasm and in regular arrays in association with microtubules in the vicinity of synapses. Within a synaptic focus, immediately adjoining the presynaptic membrane, vesicles are randomly arranged and are not associated with microtubules. Neurofilaments are present, generally in large numbers, but these are not associated with vesicles or other particulates. The structural findings are considered in terms of current concepts of fast and slow transport in neurons and the mechanochemical control of intracellular movement of materials.

INTRODUCTION

The cyclostomes, lampreys and hagfish, represent the most primitive living vertebrates, a position that singles them out as of special interest in studies of the evolution and comparative physiology of the nervous system. The preliminary electron microscopic studies of Schultz, Berkowitz and Pease (1956) and the later work of Bertolini (1964) established several fine structural features of the cyclostome nerve cord that reflect the phylogenetic position of these animals. Bertolini points out that these features include the dorsoventrally flattened form of the cord, the horizontal disposition of the gray matter, and the presence in the cord of giant fibers arising from cell bodies within the brain. Furthermore, the lamprey nerve cord shares with that of an insect (Wigglesworth, 1960) the absence of an intramedullary blood supply. It has also been established that the 'white matter' of the cyclostome cord contains no myelin, and Bertolini has described some aspects of neuronal and glial relations in the central nervous system, including some morphological features of central synapses.

A large number of electron microscopic studies on the central and peripheral nervous system of vertebrates and invertebrates have shown that while the neuron cell body contains the nucleus and a varied complement of intracellular components, the axon processes, and dendrites when present, are organized in a characteristic though structurally less complex fashion. The axoplasm of peripheral axons and nonsynaptic regions of the

central nerve cord contain longitudinally oriented microtubules similar in appearance to those described in very diverse situations in animal and plant cells. The microtubules are accompanied, in varying ratios, by similarly oriented but smaller "neurofilaments." Mitochondria accompany these axoplasmic structures, but generally not in impressive numbers. Points of chemically mediated synapse, whether in the central or peripheral regions and irrespective of the chemical nature of the transmitter, are associated with focal concentrations of small vesicles (De Robertis, 1958; Palay, 1958; Katz, 1962; Whittaker, 1968). An attractive hypothesis of synaptic action involves the synthesis and/or storage of transmitter molecules in these vesicles and the subsequent release of transmitter into the synaptic gap upon arrival of excitation at the presynaptic terminal. However, the mechanism by which transmitter molecules are transported to or synthesized at the ending is incompletely understood but represents a crucial aspect of nerve function.

Recent studies have revealed the operation of distinct rapid and slow translocation of materials within the axon, perhaps including materials involved in synaptic function. There is at present much interest in the physical pathways available for intracellular transport of particulate and other components, and the form of the neuron together with the fact that the axoplasm is relatively simple, but includes highly ordered structures, singles this cell out as unusually convenient for such studies. The present work forms part of a general investigation of the central nervous system of the *Petromyzon* ammocoete and provides morphological evidence for an association between vesicles and a linear axoplasmic component that is precisely zoned with respect to the presynaptic surface. The possible functional implications of these findings are discussed.

MATERIALS AND METHODS

Ammocoete larvae of *Petromyzon marinus*, about 12–14 cm in length, were used in this study. The lampreys were obtained from the Ocqueoc River of Lake Huron and kindly provided by Mr. Louis King, Acting Investigation Chief, Bureau of Commercial Fisheries, Hammond Bay Biological Station, Millersburgh, Michigan. Animals were shipped by Air Express to Miami and kept in shallow tanks of aerated water in a 4°C room. Animals were decapitated and a length of the body caudal to the origin of the first dorsal fin was separated with a razor blade. Chilled fixative (2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4, containing 1% sucrose) was gently infused into the perineural space surrounding the spinal cord from a hypodermic syringe. Immediately after, the cord was exposed by cutting into the body wall and stripping off the dorsal meninges; the cord was removed as gently as possible and placed in a vial of chilled fixative for 4 hr. The material was next washed overnight in chilled cacodylate buffer containing 2% sucrose, and at this stage the cord was cut into 2 mm lengths. The segments were placed in chilled 1% OsO_4 in the same buffer for 1 hr, dehydrated in an ethanol series commencing at 50%, and embedded in Araldite (Ciba Ltd., Duxford, Cambridge, England). Sections were cut with glass knives on an LKB Ultrotome III (LKB Instruments, Inc., Rockville, Md.) collected on uncoated grids, stained in 50% ethanolic uranyl acetate (saturated) and lead citrate, and examined in a Philips EM 200. For light microscopy, 1 μ sections of the Araldite-embedded material were stained in hot 1% toluidine blue with 1% borax, and examined in a Zeiss microscope equipped with planapochromatic objectives.

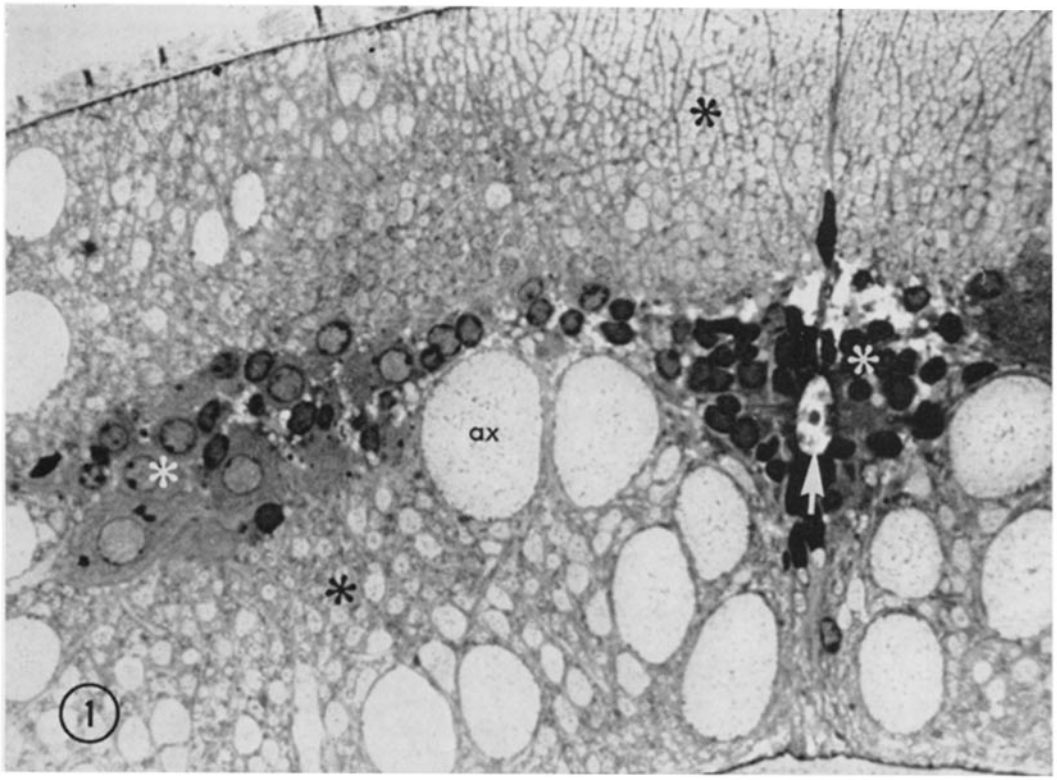
RESULTS

The general organization of the *Petromyzon* ammocoete spinal cord is illustrated in a light micrograph in Fig. 1. It is dorsoventrally compressed, and on either side of the neural canal the gray matter, containing neuron cell bodies, extends as a horizontally disposed flange. The rest of the cord contains glial cells and processes together with nerve axons and dendrites, ranging from extremely small components beyond the resolution of the light microscope to large fibers (of the Mauthner and Müller cells) which, in the larvae used here, reach a diameter of *circa* 30 μ. Fig. 2 includes a group of Müller fibers at higher magnification: the significance of details resolved in such a preparation only become fully apparent when corresponding electron micrographs are examined. The small dots scattered throughout the axoplasm represent filiform mitochondria aligned with the long axis of the cell, while the densely stained patches adjoining the axon surface correspond to the clustered vesicles of the synaptic foci (cf. Fig. 5). The resolution of tight aggregates of synaptic vesicles in the light microscope does not appear to have been reported previously, and may perhaps permit more extensive histochemical analysis of synapses in such specialized neurons as these.

Except for the complete absence of myelin, regions of the cord which may conventionally be

described as the "white matter" display fine structural features shared with comparable regions of other chordate nervous systems, together with some primitive aspects. Fig. 3 illustrates an electron micrograph of a transversely sectioned group of axons adjoining the dorsal surface of the cord. These are divided into tracts by glial sheets which, as elsewhere in the cord, are replete with cytoplasmic filaments. The general features of axon-glia relations have been described by Schultz et al. (1956) and Bertolini (1964) who confirmed Retzius' (1891) supposition that the cyclostome spinal cord includes only a single type of glial cell. The arrangement of structures within the axoplasm of nonsynaptic areas, on the other hand, presents no obviously unusual features. As illustrated in Fig. 4, both tubular and filamentous structures occur in the axoplasm. Schmitt and Samson (1969) have reviewed the distribution of microtubules and neurofilaments in nerve processes. With the exceptions described by Wuerker and Palay (1969), these components occur in an approximately reciprocal balance—in general, small axons possessing numerous tubules but few filaments, with the reverse holding true in large axons. This general pattern is encountered in the nerve cord of *Petromyzon* where in the material used here the diameter of nerve processes ranges from *circa* 30 to 0.1 μ and (Bertolini, 1964) up to 100 μ in adults. In the smallest processes observed here, three or four microtubules may occur together in the absence of filaments; about 10 of each are present when the diameter of the process is of the order of 0.3 μ. Larger profiles in the range of 1–2 μ have neurofilament:microtubule ratios in the range of 4:1–10:1, while the ratio in axoplasm of the giant fibers is *circa* 20–30:1. As illustrated in Fig. 4, the microtubules occur singly or more rarely in fascicles of up to six or seven in which a minimum intertubule distance of 100 A is maintained and which appear to include linkages between the tubules. As described in some other neurons (Gonatas and Robbins, 1965; Echandia et al., 1968), transverse profiles frequently possess a dense core or "dot," *circa* 50 A in diameter. The latter authors have shown in longitudinal profiles that dense material is discontinuously distributed along the axis of the tubule, apparently accounting for the sporadic occurrence of the core in transverse sections. However, Lane and Treherne (1969), on the other hand, have stained the entire microtubule core with lanthanum nitrate, suggesting that it is uniformly accessible to small molecules. A blank space or halo surrounds each tubule or group, as is usually the case, and the remainder of the axoplasm contains uniformly distributed neurofilaments and occasional mitochondria. Filaments *circa* 100 A in diameter have been described as a special feature of neurons, and within these has been resolved a 30 A electron-lucent core (Palay, 1964; Wuerker and Palay, 1969; Schmitt and Samson, 1969; Wuerker, 1970). In lamprey neurons, the neurofilaments appear to be significantly smaller than elsewhere (Fig. 4); their overall diameter is only 60 A. These resemble in size the glial filaments described by Wuerker (1970), but unlike these and in common with other neurofilaments, wispy cross-bridges are present in the lamprey axoplasm extending between filaments, which have a minimum intervening space of 300 A. In *Petromyzon*, the conspicuous glial filaments are *circa* 60 A in diameter (Fig. 11), and the minimum interfilament space is likewise *circa* 60 A. Fig. 5 illustrates a transversely sectioned field including a small portion of the peripheral axoplasm of a giant fiber (cf. Figs. 1, 2), probably the axon of a Müller interneuron (Rovainen, 1967) linking input from a variety of sensory sources to motor units controlling muscle movements. These fibers engage in profuse and extremely localized synapses with small postsynaptic units, probably dendrites of motor neurons. As Bertolini noted, the synapses are demarcated by the localized grouping of synaptic vesicles at the periphery of the axon. Two synaptic foci are included in Fig. 5, illustrating with almost diagrammatic clarity the structural features recognized (Palay, 1958; De Robertis, 1958, 1967; and others) as associated with sites of chemically mediated synapse. Clusters of small lucent vesicles are grouped around a *circa* 0.5 μ length where the plasma membranes of presynaptic (giant) cell and small postsynaptic member are juxtaposed across a synaptic gap *circa* 150 A in width. This field also includes two groups of synaptic vesicles stopping just short of the plasma membrane and approaching their points of synapse which lie a few tenths of a micron out of the plane of this section. Examination of serial sections (600 A in thickness) suggests that areas of close membrane apposition are approximately circular with a diameter of *circa* 0.5 μ.

In myoneural junctions, synaptic vesicles are grouped into axon endings that are small by comparison with the innervated cell, and the same is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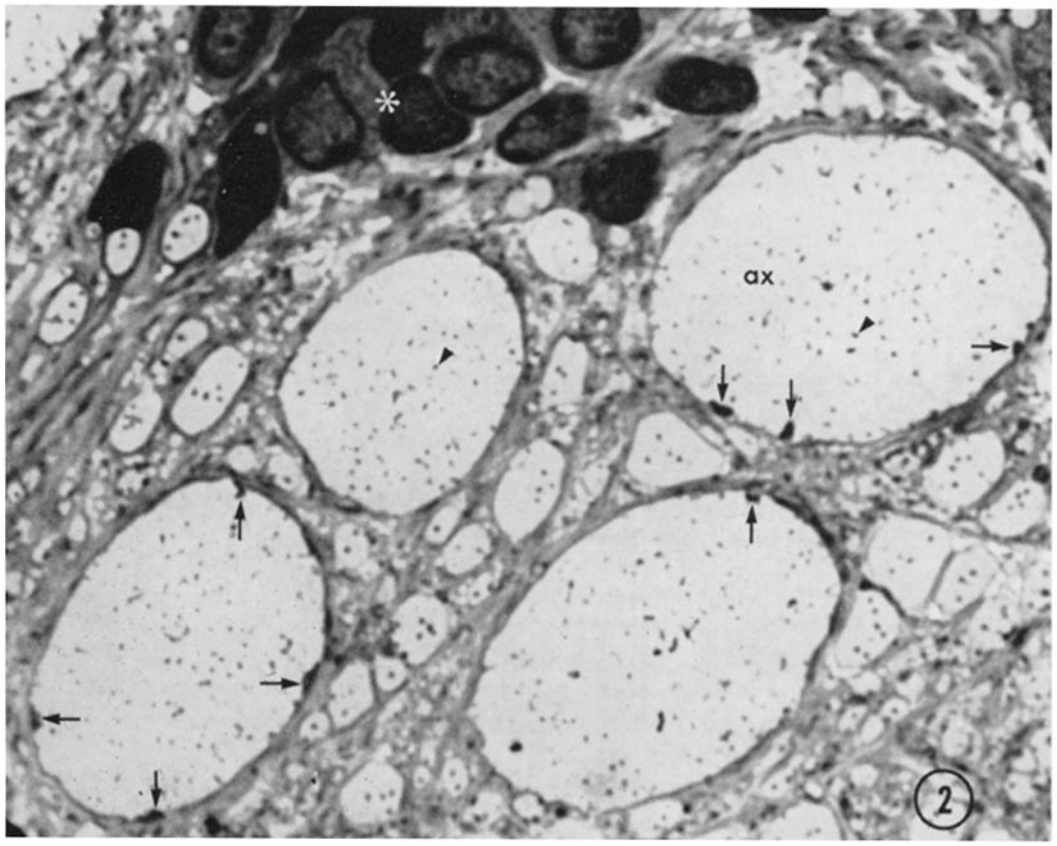
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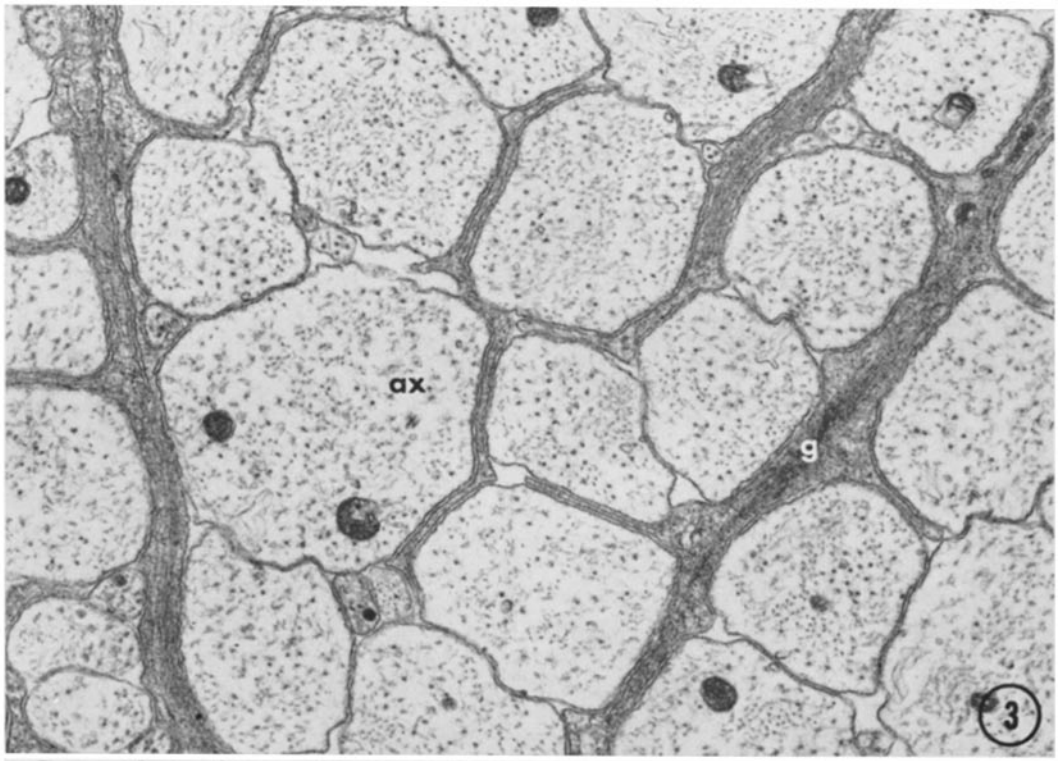
true of synapses onto nerve cell bodies. In *Petromyzon* giant fibers, on the other hand, a very different situation exists. The presynaptic axon surface is a mosaic: a cylinder that is associated with a very large number of discrete punctate synapses. In proportion to the entire cell, these vesicle clusters merely represent small irregularities in the smooth axon cylinder, while the bulk of the axoplasm contains great numbers of neurofilaments (*circa* 250–350/μ^2) and 20 to 30 times fewer microtubules. The disposition of these oriented structures with respect to the synapse is seen at higher magnification: Fig. 6 includes a pair of almost confluent Müller cell synapses, involving spherical synaptic vesicles 450–550 A in diameter, in a concentration of about 3000–4000/μ^3. This field also includes the synaptic cleft and the often noted "thickening" particularly of the postsynaptic membrane, which in this instance results from two quite separate structures: (*a*) wispy dense material lining the cytoplasmic surface of the postsynaptic cell, and (*b*) particles projecting into the synaptic cleft from the postsynaptic cell membrane.

Similar groups of synaptic vesicles occur profusely throughout the neuropil regions of the *Petromyzon* spinal cord, linking innumerable smaller and as yet unidentified nerve processes. In addition, as elsewhere in vertebrate central nervous systems and in sharp distinction to arthropod central ganglia, many processes terminate on cell bodies within the gray matter.

In view of the possibility that axoplasmic structures may be involved in transport of materials from the perikaryon, perhaps including materials destined from synaptic areas, it is of interest to establish whether neurofilaments and/or neuronal microtubules show any special features such as preferential location in the vicinity of synapses. Counts of these structures in giant fibers and smaller profiles lead to the following conclusions. (*a*) Neurofilaments are uniformly distributed virtually throughout the neuron profile except in the smallest profiles where they are not numerous and, except within the clusters of synaptic vesicles, where they appear to be absent. (*b*) Microtubules are not strikingly concentrated within synaptic foci, occurring at a concentration of *circa* 10–20/μ^2 both in these regions and elsewhere in profiles of giant and medium-sized axons in *Petromyzon*. However, despite this apparent lack of "recognition" of synaptic foci by linear axoplasmic structures, the synaptic vesicles display an extremely precise and specific affinity for microtubules in a restricted zone of the synaptic focus, described in a preliminary account (Järlfors and Smith, 1969) and here illustrated further in Fig. 7. This micrograph includes numerous synaptic vesicles within an axon *circa* 1 μ in diameter, but illustrates a configuration that occurs equally in the largest fibers of the ammocoete nerve cord (cf. Fig. 6). Randomly scattered vesicles are accompanied by petal-like clusters of vesicles, but incomplete groups frequently occur. The crispness of the vesicle profiles within a rosette is often variable, suggesting that the vesicles are not simply arranged in a plane transverse to the long axis. Moreover, the rosette pattern is slightly asymmetric; adjacent vesicles are either separated by a narrow gap or show slight overlap. The center-to-center distance between the vesicles and the microtubule core is *circa* 400 A, and vesicles are separated from the tubule surface by a space of 80–120 A, in which no structure has

FIGURE 1 Light micrograph of a transversely sectioned *Petromyzon* ammocoete nerve cord embedded in Araldite after processing for electron microscopy, cut at a thickness of 1 μ, and stained with toluidine blue. The medial neural canal is indicated by an arrow. A narrow flange of gray matter (white asterisks) occupies the central region of the ribbon-like cord on either side of the neural canal (arrow); this contains neurone cell bodies, and the bulk of the cord contains small nerve processes (black asterisks) corresponding to the white matter of higher chordate nerve cords, though as noted by Schultz et al. (1965) these lack myelin. Large axons (*ax*) include the giant Müller fibers. × 750.

FIGURE 2 A section similar to Fig. 1 at higher magnification. Cell bodies of small neurons in the gray matter are included (*) beneath which lie axon profiles ranging from the limits of the light microscope to large cells (*ax*) *circa* 25 μ in diameter, representing the 'giant' Müller fibers. The details of axoplasmic organization in such preparations become clear only after examination of electron micrographs, as in Fig. 5. The small dots in the axoplasm (arrowheads) evidently represent the filiform mitochondria. More interestingly, the discrete heavily stained patches, each about 1 μ in width (arrows) adjoining the axon surfaces, correspond to the compact foci of synaptic vesicles observed in electron micrographs (cf. Figs. 5 and 6). × 2000.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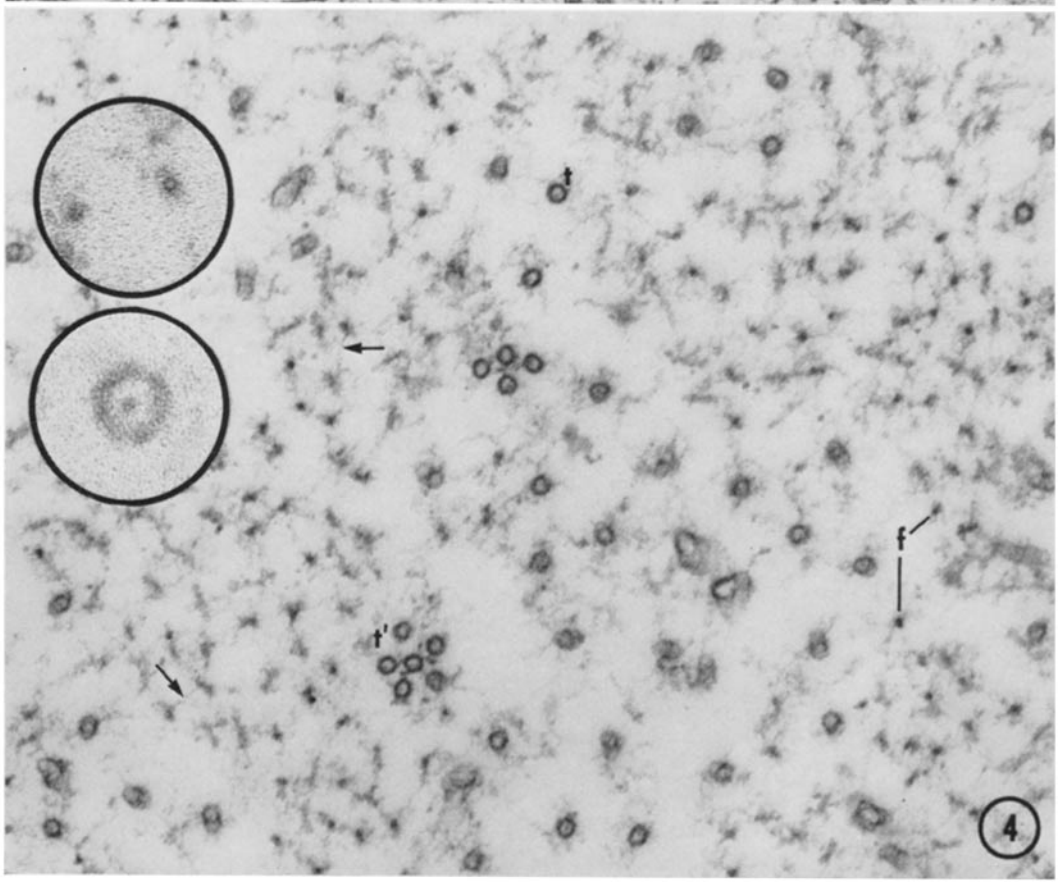
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yet been resolved. No evidence has been found of lateral bridges or other linkage devices (such as the neurofilament side arms) between the vesicles and tubules.

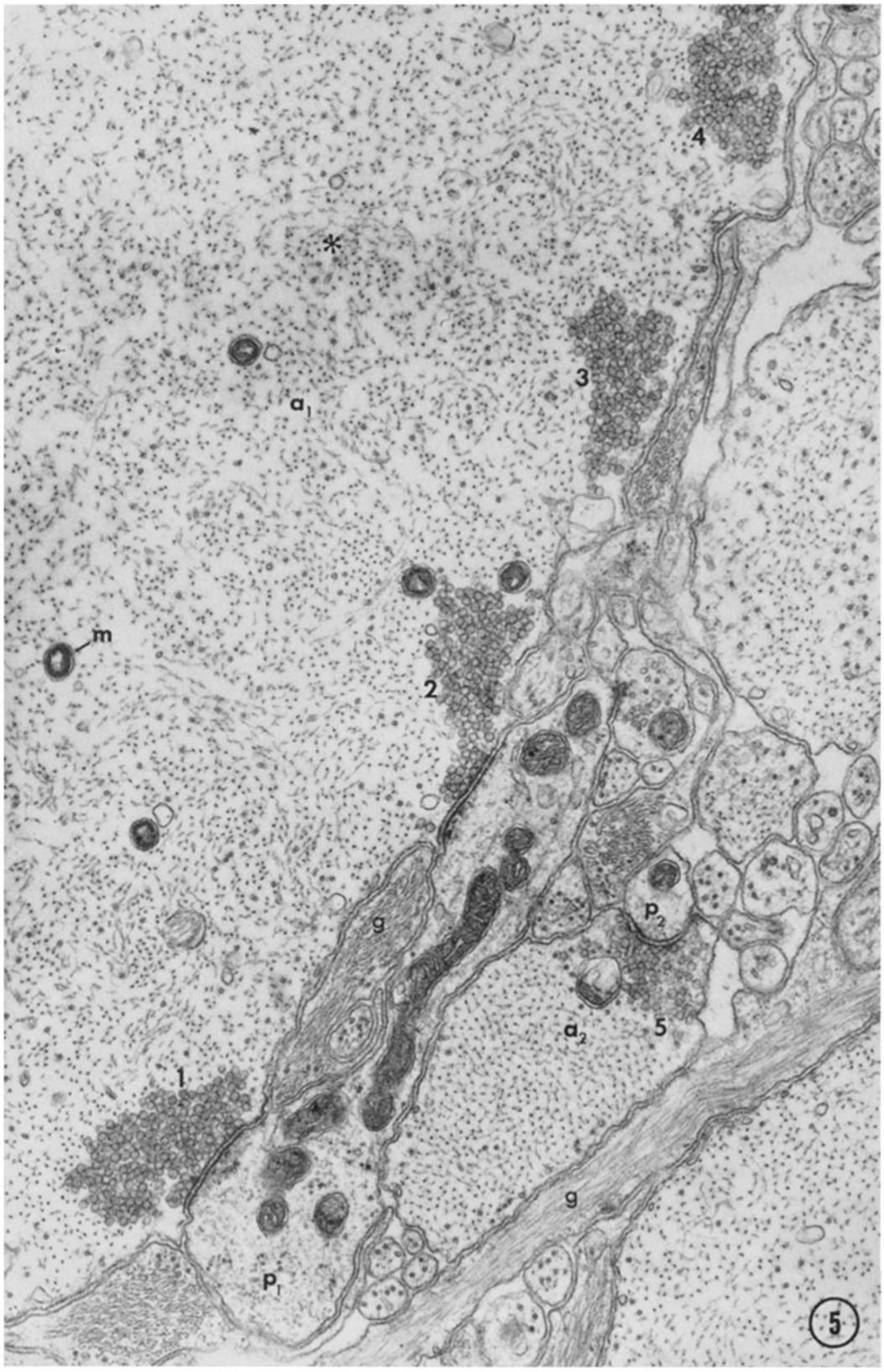
In longitudinal aspect, this association is still more striking. The low-power survey field shown in Fig. 8 illustrates an unusually extensive alignment of synaptic vesicles on a framework of microtubules, over a distance of 5 μ. A similar configuration is shown at higher magnification in Fig. 9, in which a tight column of electron-lucent vesicles is arranged on a linear tubular scaffolding adjoining, at a short distance, a virtually unaccompanied tubule. Transverse sections (Figs. 6 and 7) indicated that the vesicle-to-tubule association is a generally regular one, and longitudinal profiles reveal that the petal-like grouping results from the close packing of vesicles around microtubule cores.

The vesicle-clad tubules in Figs. 8 and 9 may well adjoin synaptic areas, but if so the apposed pre- and postsynaptic membranes lie out of the plane of section. The restriction of microtubules to the general axoplasm and the outer zone of the synaptic focus and their absence among the vesicles immediately adjoining the presynaptic membrane (Fig. 6) are general features of transverse profiles, and this disposition is also evident in suitable longitudinal sections. In Fig. 10 a vesicle-clad tubule passes within $\frac{1}{2}$ μ of a point of synapse: in this $\frac{1}{2}$ μ zone microtubules are absent, and randomly arranged and unassociated vesicles cluster around the presynaptic membrane (cf. Fig. 6). Similarly, in Fig. 11 a large axon adjoins a small postsynaptic unit; vesicles are tightly grouped around microtubules (as in Fig. 9) some distance from the apposed synaptic membranes, but closer to the latter tubules are associated with vesicles only to the level of the synapse. In Fig. 11, unattached vesicles extend to the localized patch of the axon surface thought to be site of transmitter release, while below this level in the micrograph extend naked microtubules.

The synaptic vesicles that have been illustrated in the accompanying electron micrographs are uniformly spherical and electron lucent. Before this section is concluded, two further axoplasmic components that have been noted in the ammocoete spinal cord must be mentioned. (*a*) A larger vesicle varying widely in diameter and containing a denser pellet or core is present consistently, but infrequently, in populations of the smaller lucent vesicles. In Figs. 12 and 13, the size range of this component is 700–1200 A, though slightly smaller and larger examples have been noted. These vesicles do not seem to be preferentially located near the presynaptic surface, nor have they been noted in the columns of vesicles surrounding the microtubules. (*b*) Each of the major structural features of ammocoete synapses described so far is duplicated in central axon profiles smaller than the giant fibers, and distinguishable by the shape of its synaptic vesicles. Several authors (Atwood and Jones, 1967; Uchizono, 1965, 1967; Nakajima, 1970; and others) have reported ellipsoidal or discoidal synaptic vesicles in preparations that also include neurons containing the spherical type. It has been suggested by these authors that by a fortunate chance, from the point of view of interpretation of electron micrographs, the flattened vesicles may be associated with inhibitory rather than excitatory endings. Similar flattened vesicles occur frequently in thin sections of the *Petromyzon* spinal cord. Their distribution relative to the spherical vesicles in this material will be described in detail elsewhere (Smith and Beránek, manuscript in preparation) but it may be mentioned in summary that an axon appears never to contain a mixture of lucent spherical and nonspherical vesi-

FIGURE 3 A group of axons (*ax*) within the central nerve of a *Petromyzon marinus* ammocoete. The transverse nerve profiles are separated into tracts by sheets of fibrous glial cytoplasm (*g*). As in other nonsynaptic regions the axoplasm contains microtubules, neurofilaments (seen at higher magnification in the next figure), and occasional mitochondria. × 21,000.

FIGURE 4 Illustrating typical nonsynaptic axoplasm of a *Petromyzon* central axon in more detail than in Fig. 1. The principal structural components are microtubules, *circa* 250 A in diameter, and neurofilaments, *circa* 60 A in diameter. The former occur single (*t*) and in compact groups (*t'*) with a center-to-center spacing of *circa* 400 A. Microtubules frequently exhibit an opaque core (lower *insert*). Neurofilaments (*f*) are situated in the surrounding axoplasm; they appear to be linked by side arms (arrows), and as illustrated in the upper *insert*, the filament includes an electron-lucent core. × 100,000; *inserts*, × 370,000.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cles, and that the arrangement of vesicles with respect to axoplasmic microtubules and synaptic foci described here is duplicated in axons exhibiting flattened vesicles.

The foregoing account has been primarily concerned with the organization of synaptic axoplasm. Certain features of the cytoplasm of axons more or less remote from synaptic junctions must be noted. Irregular agranular cisternae, of variable width and orientation, occur in all *Petromyzon* central axons, but Bertolini's proposal (1964) that these are highly developed and connected with the plasma membrane has not been confirmed. Furthermore, no evidence has been obtained suggesting that synaptic vesicles are formed by fragmentation of cisternae, microtubules or other components of the axoplasm. However, as illustrated in Fig. 14 *a–e*, objects structurally identical with the spherical vesicles concentrated at synapses occur with low frequency throughout the axoplasm, typically in association with microtubules. The axoplasm adjoining the synaptic surface thus appears to be quantitatively rather than qualitatively distinct from other regions of the axon.

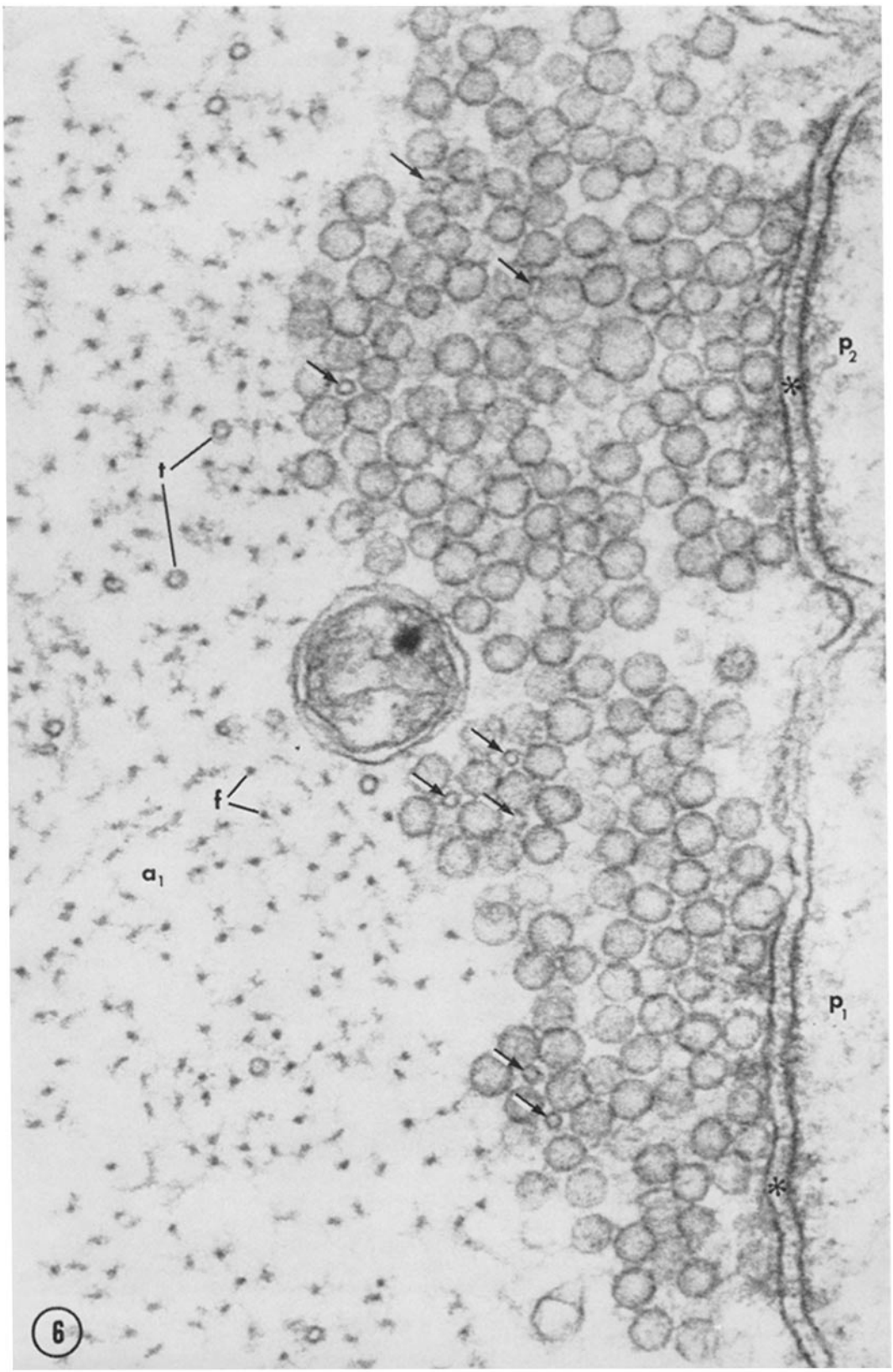
DISCUSSION

One of the central problems in neurobiology concerns the functional relation between the perikaryon and the often distant terminals of the nerve cell. The complex cytoplasmic region surrounding the nucleus maintains the cell, and net passage of materials down the axon has been documented in earlier studies on the cellular behavior of neurons, as discussed by Weiss (1969). However, it has become clear that some synthetic processes may occur at points outside the perikaryon, including the nerve terminals (see Barondes, 1969, for references). Recent studies have not only cast some light upon the factors involved in directional movement of materials and structures within the cytoplasm of a variety of cells but have also drawn attention to oriented systems occurring in axons, other elongated cell processes, and elsewhere that have been implicated as a possible framework for directional intracellular translocation.

While a wide variety of materials are undoubtedly subject to oriented movement in cells, the process of translocation is most striking when the material moved is resolvable by light or electron microscopy. The behavior of chromosomes upon the spindle is a long-established example of precisely directed intracellular translocation. Rebhun (1967) has described discontinuous or saltatory non-Brownian movements of a variety of granules in invertebrate eggs and in melanocytes of *Fundulus* embryos, generally occurring at a rate of 0.5–2 μ/sec. Bikle et al. (1966) further investigated the bidirectional movements of pigment granules in the melanocyte. Porter and Tilney (1965) have studied the translocation of particles along the slender axopodia of *Actinosphaerium*. Similar displacements of a variety of particles, including fat spheres and pinocytic droplets, have been described by Freed (1965) in cultured mammalian cells, and Holmes and Choppin (1968) have found that in virus-induced syncytia of kidney cells newly contributed nuclei migrate directly to the center of the fused mass. The distal flow of axoplasm in nerve cells has been recognized for some time (Weiss et al., 1962; Barondes, 1969; Schmitt, 1968), and it now appears that in addition to the massive but relatively slow bulk flow, specific materials may be transported far more rapidly. The list of these materials already reflects the importance of the cell body in maintaining and regulating the function of distant reaches of the cell, and includes rapidly transported glutamate (Kerkut et al., 1967), proteins (McEwen and Grafstein, 1968) and also neurosecretory granules synthesized in the vicinity of the nucleus (Jasinski et al., 1966).

The identification of a single structure under-

FIGURE 5 Micrograph illustrating the organization of axoplasm of a Müller cell (a_1) in the *Petromyzon* ammocoete. The bulk of the axoplasm contains microtubules and neurofilaments (*) as in the small axons illustrated in Fig. 1, together with small mitochondria (*m*). Peripherally, precisely demarcated regions of the axoplasm are differentiated as synaptic foci, opposite small postsynaptic elements. These regions contain dense concentrations of synaptic vesicles. At *1* and *2*, vesicle clusters approach the axon plasma membrane very closely in regions *circa* 0.5 μ in width. These two synaptic foci are associated with a single postsynaptic (probably dendritic) nerve process (p_1) while the clusters of vesicles at *3* and *4* lie a short distance from the presynaptic surface and are evidently approaching points of synapse. At *5*, a comparable group of vesicles is associated with a synapse between a small axon (a_2) and a small postsynaptic process (p_2). Glial processes are indicated (*g*). × 28,000.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lying intracellular translocation is an attractive, but perhaps illusory goal. Electron microscopic studies have shown that over a wide range of cell types the most striking subcellular structures seen in thin sections in the region of transported cytoplasm or particles are filaments and microtubules, respectively *circa* 50–100 and 200–250 A in diameter. While the latter have most frequently been implicated in transport mechanisms, there is evidence that filaments may sometimes be involved, and the picture is further complicated by the fact that each structure is composed of resolvable subunits which have on occasion been considered as building blocks common to both types of linear structure.

In cytoplasmic droplets of the alga *Nitella*, Rebhun (1967) associated both saltatory and streaming movements with bundles of 50 A microfilaments. Bikle et al. (1966), on the other hand, observed microtubules extending along the arms of melanocytes; they suggested that these do not act like the strings of a marionette, pushing and pulling the pigment granules, and moreover may not merely act as a supporting framework for the asymmetric cell, but may act as guiding tracks for moving particles, and conceivably provide the means of propelling these structures. They consider the microtubules as stationary structures upon which the granules are moved and discuss the intriguing question of the operation of a single (central) or dual motive source in a two-directional system. Porter and Tilney (1965) likewise implicated an elaborate arrangement of microtubules in the demarcation of regions of cytoplasm in the axopodia of *Actinosphaerium* within which cytoplasmic movements occur. The nuclear migration into a parainfluenza virus-induced syncytium observed by Holmes and Choppin (1968) has been correlated with microtubules and filaments, and isolated rows of nuclei have been obtained, apparently strung together by microtubules. Inoué and Sato (1967) have suggested that chromosomes move against a superstructure provided by microtubules of the spindle, and Rudzinska (1965, 1967) has proposed an elaborate microtubular array as a probable pathway for translocation of digestive enzymes and particles in the suctorian ciliate *Tokophrya*.

If it could be established that microtubules and filaments are self-sufficient, stable, and mutually exclusive structures, the question of their possible involvement in intracellular transport would be greatly clarified. The structural uniformity of microtubules is not paralleled by uniform stability. Behnke and Forer (1967) recognized four microtubule populations in insect spermatids, distinguished by their response to conditions of fixation. Schultz and Case (1968) found that neuronal microtubules disintegrate in the presence of bicarbonate ions and acrolein. The axopodia of *Actinosphaerium* are reversibly retracted by virtue of dissociation of oriented microtubules by low temperature and high pressure (Tilney et al., 1966; Tilney and Porter, 1967). The disappearance of the 220 A microtubules in the cold is accompanied by the formation of short segments of a larger tubule 340 A in diameter, and at high pressure, by the appearance of fibrillar material, supposedly derived respectively by transformation and disintegration of the normal structure. Holmes and Choppin (1968) described an increase in the count of 80 A filaments accompanying the colchicine-mediated disappearance of 250 A microtubules in virus-induced syncytia, and a similar effect occurs in interphase HeLa cells (Robbins and Gonatas, 1964). Microtubules and neurofilaments appear to exist together, though in varying proportions, in almost all axons throughout the phyla. There is a general reciprocity between populations of these components; when one predominates the other is relatively less conspicuous. Furthermore, as has already been mentioned, axon size seems to play a part in determining the ratio of these two structures; in the vertebrate, microtubules are generally most abundant in small unmyelinated axons, while in large myelinated axons neurofilaments predominate. Schmitt and Samson (1969) consider the evidence suggesting that micro-

FIGURE 6 A synaptic region of Müller cell axoplasm (a_1) similar to that shown in Fig. 3, at higher magnification. This field includes a pair of synaptic foci involving two small postsynaptic elements (p_1, p_2). The axon surfaces at the synapse are opposed across a cleft of 150 A (*). Scattered microtubules (t) and neurofilaments (f) occur throughout the axon (cf. Fig. 3) except for a striking modification within synaptic vesicle clusters. Neurofilaments are sparse or absent within the synaptic foci, and microtubules are restricted to the outer zone of vesicles, where they form the central element of eight rosettes (arrows), further illustrated in Fig. 7. × 130,000.

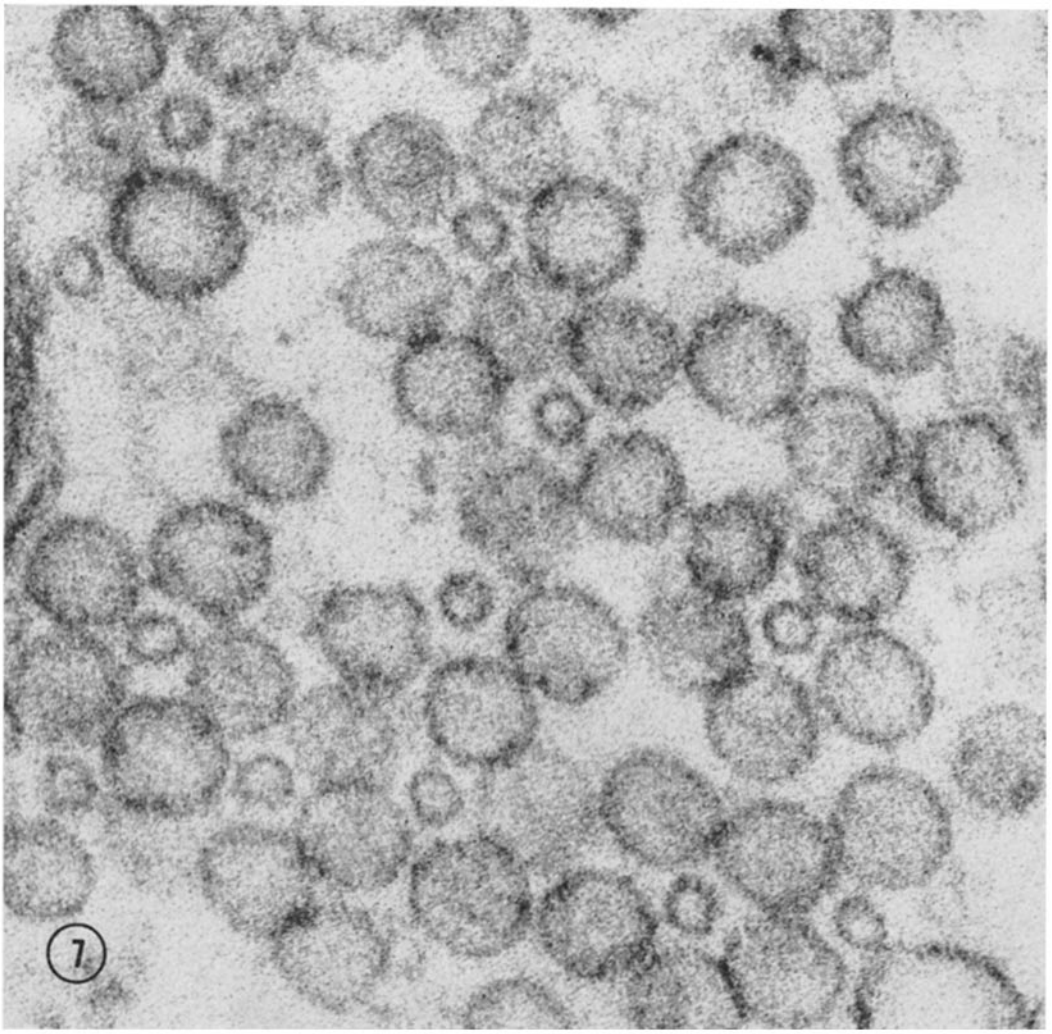


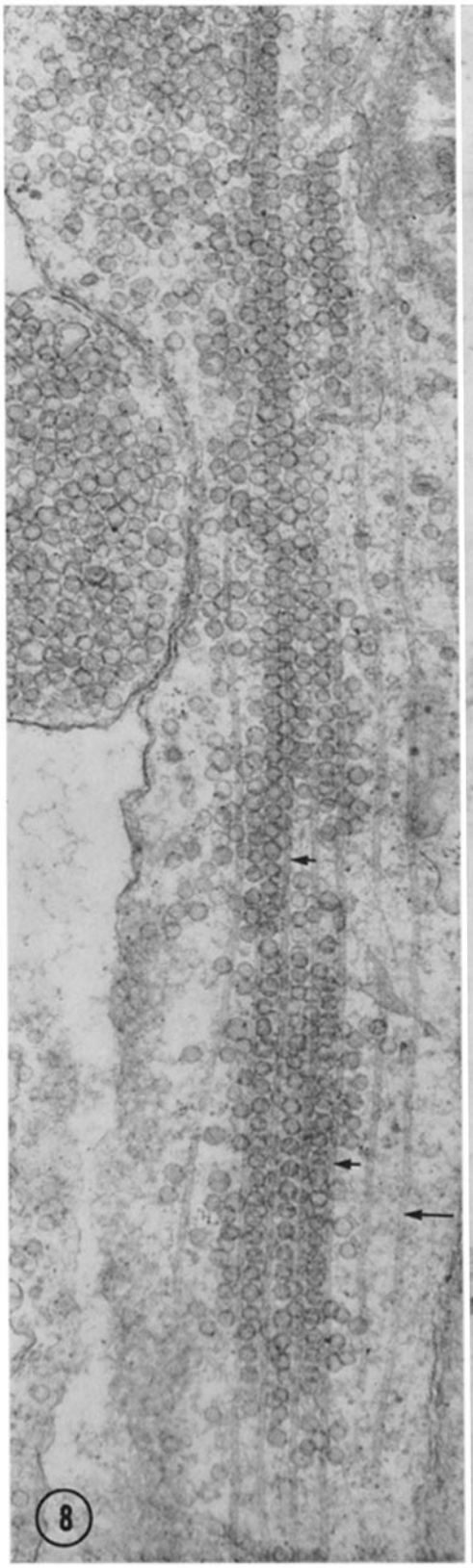
FIGURE 7 A micrograph further illustrating the association between synaptic vesicles and microtubules in an axon of the *Petromyzon* central nerve cord. 'Complete' rosettes comprise five synaptic vesicles and, in their immediate vicinity, lie randomly scattered vesicles and isolated microtubules. × 300,000.

tubules and filaments may be interconvertible, comprising a common protein subunit. Peters and Vaughn (1967) have supposed, from studies on developing neurons, that microtubules may progressively give rise to filaments—a view also put forward by Wisniewski et al. (1968).

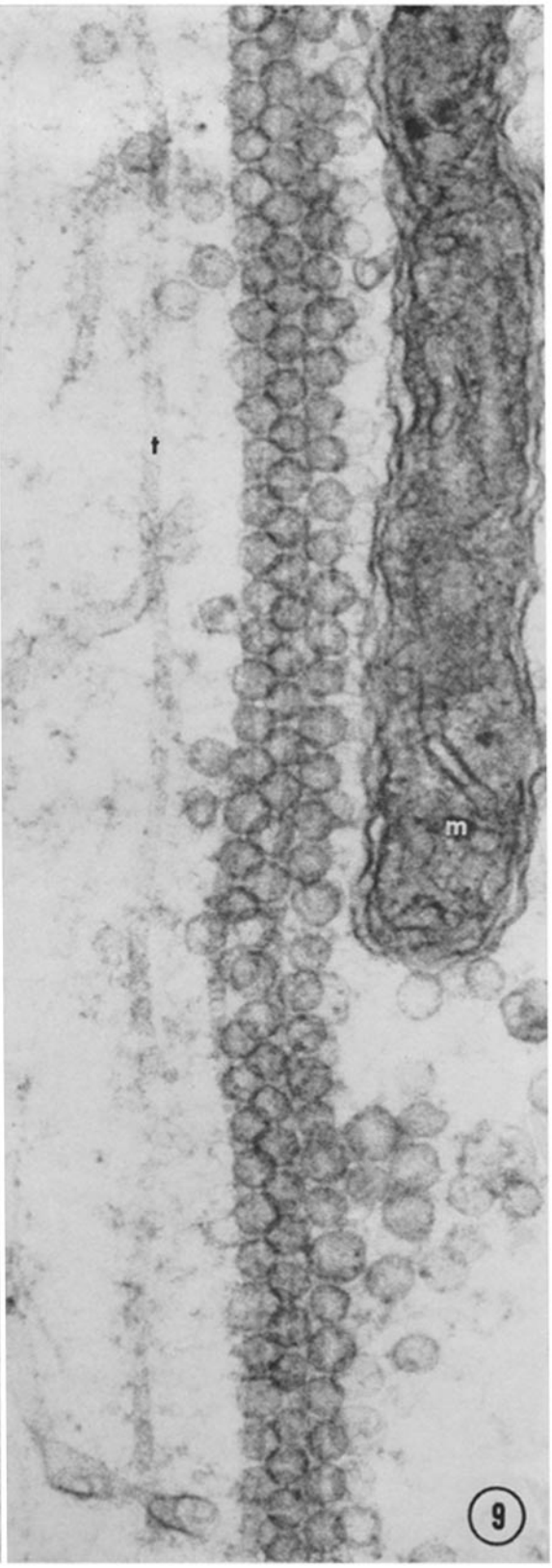
In assessing the possible role of longitudinally oriented structures in transporting materials along

FIGURE 8 A field within a longitudinally sectioned central nerve cord of a *Petromyzon* ammocoete, illustrating the striking alignment of synaptic vesicles within the axoplasm over a distance of about 5 μ. Microtubules are evident within (short arrows) and alongside (long arrows) the vesicle groups. × 45,000.

FIGURE 9 Further illustrating, in longitudinal aspect, the grouping of synaptic vesicles around microtubules in a *Petromyzon* axon. In the center of the field, vesicles are distributed in a close-packed hexagonal pattern around microtubules, two or three of which lie within the plane of section. This specific association is revealed as the rosette pattern in transverse profiles (cf. Figs. 4, 5). On the left lies an unaccompanied microtubule (*t*) and on the right, an elongated mitochondrion (*m*). × 110,000.



8



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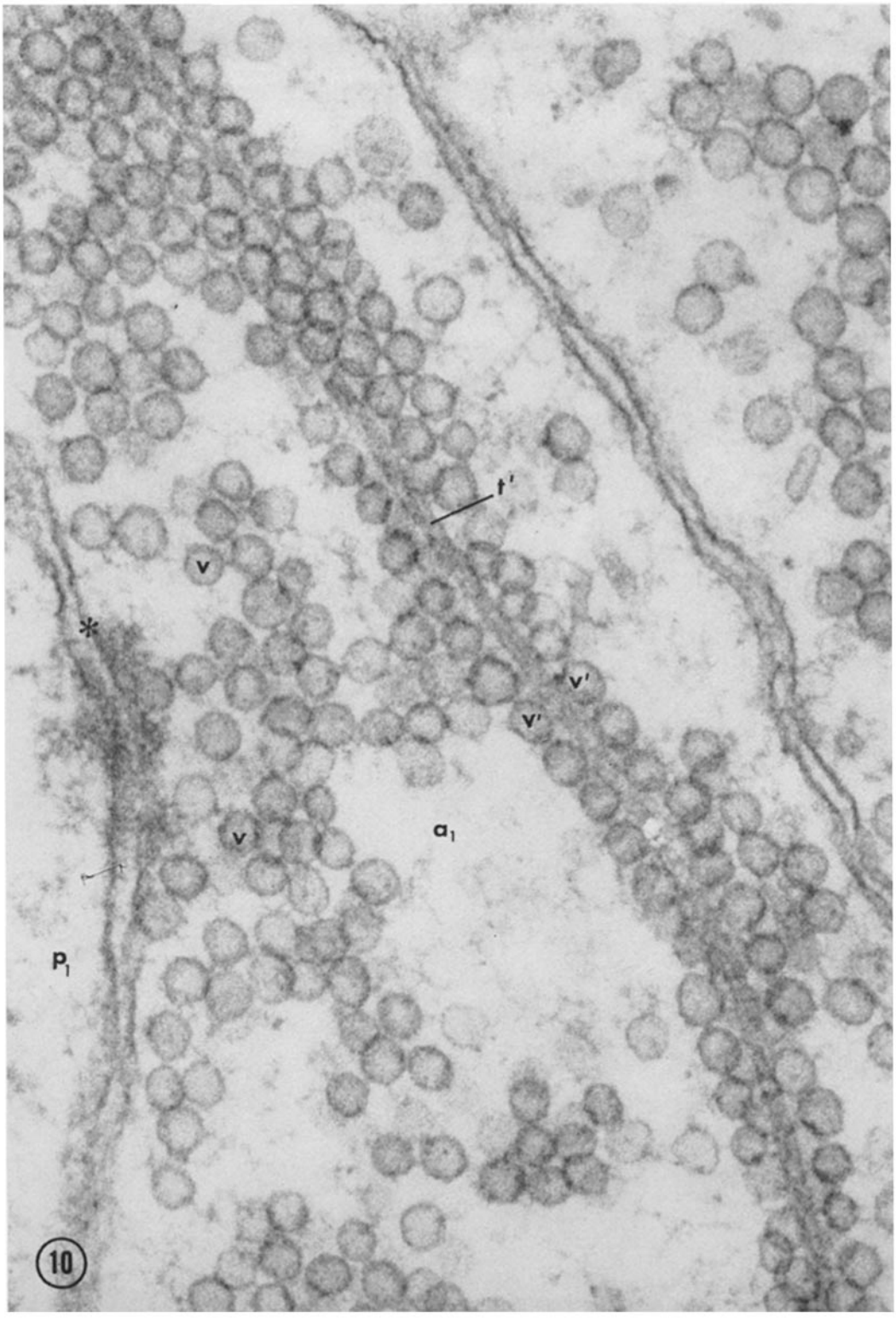


FIGURE 10 Longitudinal section of an axon (a_1) of *Petromyzon* including a point of synapse with a post-synaptic nerve process (p_1). Synaptic vesicles (v) converge on the presynaptic membrane (*), but lie freely and apparently randomly in the axoplasm. Nearby, a single microtubule (t') is closely flanked by a double row of synaptic vesicles (v')—an optical section of the close-packed configuration shown in Fig. 7. × 130,000.

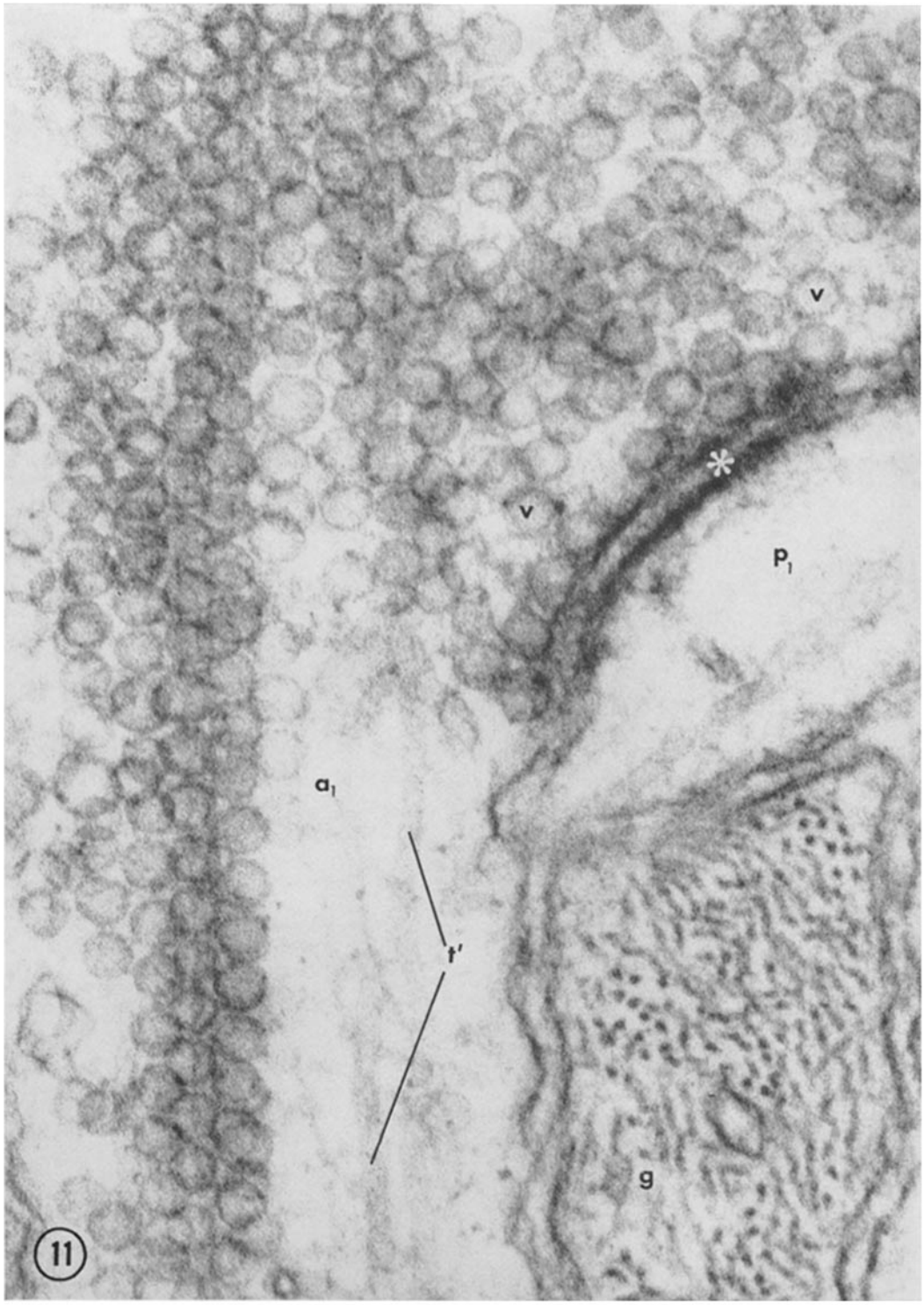
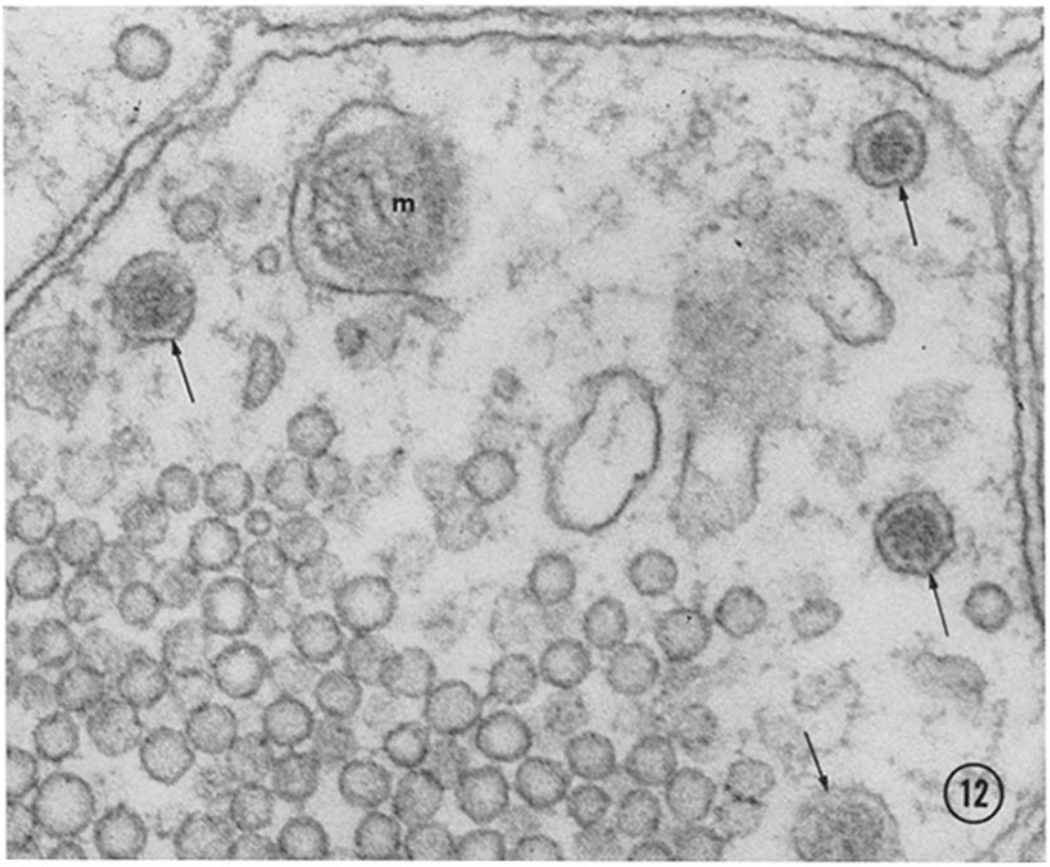
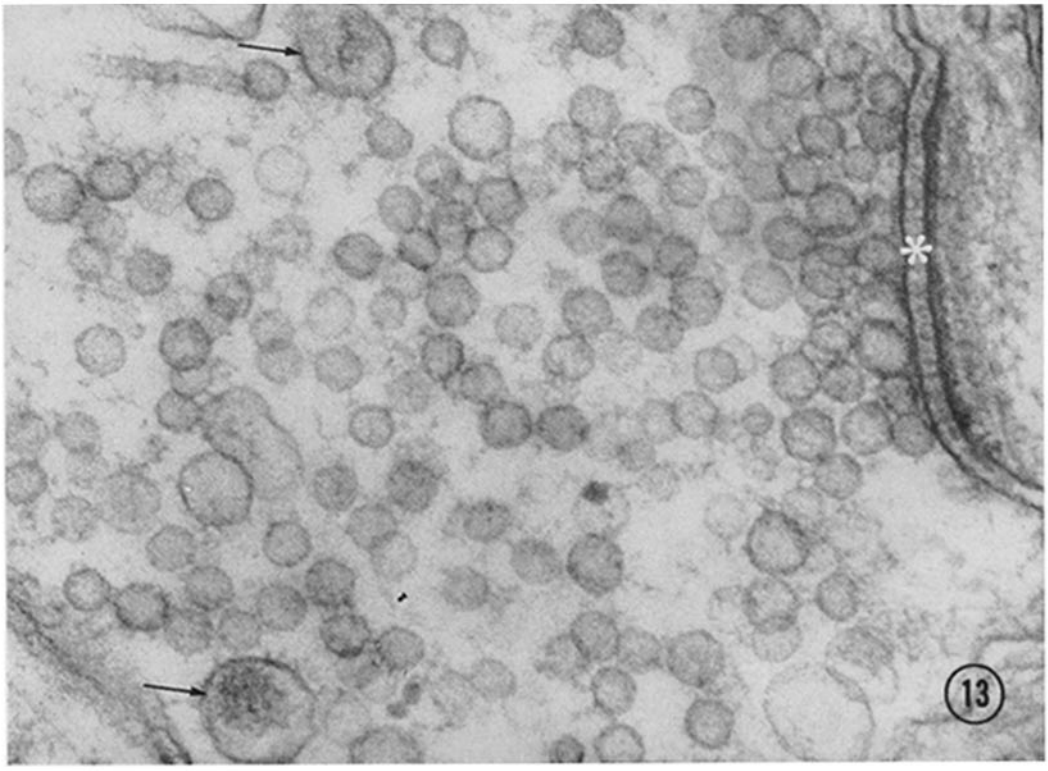


FIGURE 11 A micrograph further illustrating the distribution of synaptic vesicles in the vicinity of a central synapse. The field includes an *en passant* synapse between a large axon (a_1) and small postsynaptic component (p_1), across a synaptic gap indicated by an asterisk. Glial cytoplasm, containing numerous filaments, is included at *g*. Abundant but unoriented synaptic vesicles (*v*) are clustered around the presynaptic focus at upper right. The left of the field is traversed by longitudinally aligned microtubules tightly ensheathed by synaptic vesicles (cf. Figs. 8–10). In the center of the field, similar grouping occurs above the level of the synapse, but beneath, isolated microtubules (*t'*) emerge. × 170,000.


m
12


13

the axon, it is important to establish whether or not the obvious candidates, the microtubules and the filaments, are chemically similar. Schmitt and Samson point out that neuronal microtubules are more stable than structurally similar components of some other cells, being unaffected by exposure to low temperature (4°C)—a finding confirmed in the present *Petromyzon* material. On the other hand, neuronal microtubules are dissociated by colchicine or other mitotic inhibitors (Wisniewski et al., 1968) with the concomitant appearance of filamentous structures, supposedly abnormal polymers of the same protein aggregates normally organized into the microtubule. This does not, however, indicate that tubular and filamentous components of normal axoplasm are interconvertible, since no rigorous method was employed for distinguishing between a possible mixed population of normal and induced filaments.

There is, however, good reason to suppose that neuronal microtubules (formerly termed "neurotubules") are homologous with microtubules of other cells, including those implicated in intracellular movement, mentioned above. Borisy and Taylor (1967) found that colchicine-^{3}H complexes specifically with a protein in nervous (brain) tissue, cilia, and dividing cells, shown in the last instance (Shelanski and Taylor, 1967) to be the subunit of microtubules. Weisenberg et al. (1968) have isolated from mammalian brain a protein similar in a variety of biochemical properties to cilia protein, believed to be the building unit of the neuronal microtubule.

Schmitt and Samson (1969) stress that neurofilament protein, investigated by Davison (1970) differs from that of microtubules (Borisy and Taylor, 1967) in lacking GTP-binding sites and colchicine-binding properties characteristic of microtubule subunits. Furthermore, Davison (1970) has recently provided the telling evidence that squid axon neurofilament protein and microtubule protein differ in their respective amino acid composition, indicating that one component could only be derived from the other by synthetic reassembly of the constituent amino acids, and not by direct interconversion. On fine structural grounds, Wuerker (1970) strengthens the conclusion that the tubules and filaments of the axon are not interconvertible structures, but rather two distinct populations, each existing independently and presumably subserving separate functions.

Wuerker and Palay investigated in detail the distribution of neurofilaments and microtubules in rat anterior horn cells, with special reference to dendrites. They confirmed that the microtubule wall contains 11–14 possibly inhomogeneous 60 A subunits, leaving a central core of 150 A, sometimes including a medial 50 A dot judged to be a filamentous structure traversing the microtubule (cf. Echandia et al., 1968). The neurofilaments, on the other hand, are reported to be *circa* 100 A in diameter, likewise including a translucent core, but comprising a wall of three to six subunits, each only 30 A in diameter. Wuerker and Palay point out that direct transformation of one component into the other (as by unravelling of the single tubule to provide neurofilaments) cannot be achieved by any obvious sequence of events. They also point out, in support of the nonequivalence of microtubules and filaments, that depolymerization of tubules by low temperature (Echandia and Piezzi, 1968) produces a fibrous material distinct from the neurofilaments, which persist unaffected by the experimental treatment. Wuerker and Palay are especially concerned with the possible role of neurofilaments as transporting organelles. They have correlated the observations of Pomerat et al. (1967) on bidirectional streaming within the cell body with channels occupied by neurofilaments. In certain neurons, Wuerker and Palay further report a close association between bundles of neurofilaments and Nissl bodies and suggest that

FIGURE 12 An axon within the spinal cord of *Petromyzon* which contains, in addition to the spherical electron-lucent synaptic vesicles illustrated in Figs. 3–11, a second axoplasmic component, indicated by arrows. This takes the form of a larger membrane-limited structure, *circa* 1000 A in diameter, including a dense core of variable size, partially filling the vesicle. A mitochondrion is included at *m*. × 130,000.

FIGURE 13 A field similar to the last, but including a point of pre- and postsynaptic membrane apposition (*). Spherical lucent vesicles are primarily involved in the synaptic focus, but occasional larger structures (arrow) (cf. Fig. 11) containing an irregular core of dense material are included, but do not seem to be preferentially located near the synaptic axon surface. × 120,000.

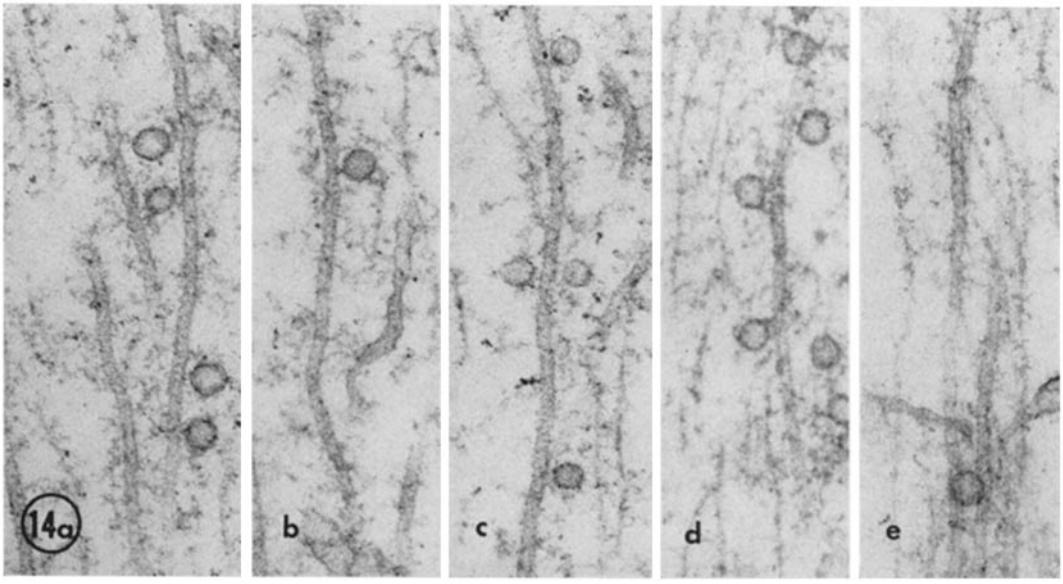


FIGURE 14 *a–e* A group of electron micrographs representing fields within large axons in the *Petromyzon* spinal cord. These areas do not adjoin synapses. Close examination of the nonsynaptic axoplasm reveals electron-lucent vesicles, singly or in small groups, preferentially located beside microtubules. As illustrated here, these are structurally similar to the spherical synaptic vesicles included in Figs. 5–11. In axons possessing ellipsoidal or discoidal vesicles in the synaptic foci (see text), similar vesicles are sparsely associated with microtubules outside the synaptic areas. In both of these neuron types, larger vesicles with an electron-opaque core (Figs. 12, 13) have occasionally been noted beside microtubules in areas some distance from synaptic terminals. × 80,000.

the production of the bundles may be controlled by the Nissl cisternae, and that neurofilaments may be actively concerned in the centrifugal transport of materials. They have described the microtubules, in mammalian material, as arranged at intervals of 100 μ and suspended in a web of lateral threads. In the lamprey material, lateral threads link the neurofilaments, but neither this suspensory web nor comparable regularity marks the microtubules.

It is entirely possible that the neurofilaments perform a transporting function in all nerve cells, following the model proposed by Wuerker and Palay. The morphological evidence they present is impressive and the neurofilament is certainly a conspicuous and virtually universal component of neurons. For several reasons, these authors conclude that microtubules and neurofilaments are distinct, noninterconvertible entities. Schmitt and Samson (1969) have considered biochemical evidence that leads to the same conclusion. From observations on the ammocoete nerve cord material, we feel that while the generalization concerning proportionality of filaments and microtubules vis-à-vis size of nerve profile holds good; the ratios, reaching a limiting count of 20–30:1 do not show any evidence of direct or simple interconversion such as dissociation of a microtubule into filamentous subunits.

We are conditioned to movement along restraining pathways—turnpikes, cycle tracks, escalators, footpaths, and so on, each providing for an appropriate speed range or type of traffic. It may well be that intracellular traffic in the neuron is analogously varied. This cell type contains two prime candidates for providing intracellular tracks, the microtubules and neurofilaments, and while the evidence suggests that these are self-sufficient and distinct structures, it should also be remembered that both fast and slow transport occurs concurrently and selectively in the axon. It is tempting to suppose that not only do the axoplasmic fibrous proteins of the axoplasm play a part in transport, but that the filaments and tubules may represent dual transport pathways. Schmitt (1968) has suggested that the slow, nonspecific bulk flow of axo-

plasm away from the cell body may be effected by the neurofilaments, and that rapid and specific translocation, in neurons and other cells, may be controlled by microtubules. Schmitt (1968) and Schmitt and Samson (1969) further proposed that microtubules extend as stationary tracks from cell body to extremities of the neuron, and that they may incorporate part of a mechanochemical transduction mechanism responsible for rapid transport. According to their hypothesis, a microtubule-particulate association functionally and perhaps biochemically comparable with myosin-actin interaction of myofilaments may be responsible for moving certain components along the axon at rates up to several hundred times that of the slowly moving axoplasm, and they discuss the possibility that directionality is imposed by the sense of the helical disposition of subunits of the axonal fibrous systems.

The results described here in no way conflict with the above suggestions, and they document, for the first time, an association between microtubules and another axoplasmic structure; these results are remarkably close to diagrammatic representations outlined by Schmitt and Samson. To interpret the accompanying electron micrographs, some assumption must be made and recognized. (*a*) Preliminary counts indicate that microtubule distribution is similar in synaptic foci to that in the general axoplasm, even in the "giant" neurons of *Petromyzon*. We have no reason to suppose that microtubules are divided into two populations, one destined to reach synapses and another not, so we assume that each synapse receives its due complement of microtubules, presumably stemming from the perikaryon. Thus a microtubule in the center of, for example, a giant Müller fiber in the ammocoete is envisaged as being en route for one or probably more synapses further along the spinal cord. (*b*) Current observations on the *Petromyzon* cord indicate that microtubules do not terminate at synapses; electron micrographs repeatedly show these tubules bypassing the presynaptic membrane at the periphery of the synaptic focus. This is most obvious in the giant fibers, but occurs also in smaller units. A giant fiber 20 μ in diameter (such as that illustrated in part of Fig. 5) contains approximately 1500–3000 microtubules counted in transverse profile (together with some 75,000–100,000 neurofilaments). Individual synaptic foci commonly include about five microtubules. If the tubules ended at the foci, such a giant cell might contain of the order of 300–600 synaptic junctions with small nerve processes (primarily dendrites), but the arrangement of microtubules appears to ensure that each is associated with a sequence of foci, and the count of synaptic junctions within a cell is unknown and probably very much greater than the above figure. (*c*) Electron-lucent vesicles, whether spherical or discoidal, occur sparsely throughout the axoplasm, but become regularly disposed around the microtubules in the vicinity of synapses. This observation is consistent with the hypothesis that the vesicles are passed along the tubules to accumulate opposite locally differentiated regions where release of chemical transmitters takes place. Since the tubules do not appear to be confined to a single synaptic region, it is possible that each region may be supplied with vesicles only to a hypothetical saturation point, permitting overspill and sequential distribution of vesicles to more than one synapse. (*d*) If we assume that the association of vesicles with microtubules reflects a mechanism for vesicle transport, then the focal clustering of "free" vesicles immediately adjoining the presynaptic surface may indicate that vesicles are released from the adjacent microtubules, perhaps destined for nearby receptive sites on the axon membrane (postulated by Katz, 1962; Hubbard et al., 1968) at which transmitter release takes place. (*e*) The speculative nature of these interpretations is clear, and it should be pointed out that the chemical nature of the transmitters present in the lamprey spinal cord are not yet known. We are as yet unable to assess, in this material, the relative importance of the perikaryon and extensions of the neurons, including synaptic regions, in transmitter synthesis.

The presence of 700–1200 A vesicles with a dense core in lamprey neurons deserves comment. Smaller dense-cored vesicles are established as a conspicuous component of adrenergic nerves, but distinct larger-cored structures have been reported in a variety of neurons not generally recognized as hormone secreting. De Iraldi and De Robertis (1968) propose that these are probably present in small numbers in all types of neuron but found them to be particularly abundant in nerves regenerating after damage, a finding confirmed by Van Arsdall and Lentz (1968). Richardson (1964) also noted that the distribution of these cored vesicles may include cholinergic neurons. Their significance in the lamprey spinal cord and elsewhere, and in particular their possi-

ble relationship to the smaller lucent vesicles and to the synapse, has yet to be established.

Links between cellular structure and function generally pay little regard to phyletic boundaries. Certainly, it is expected that so fundamental a function as rapid and specific transport in nerve cells of diverse animals would share a common structural basis. It will be important to ascertain, in the future, whether the relationship between microtubules, vesicular components of the axoplasm, and presynaptic areas, described in *Petromyzon*, occurs in the nervous system of other animals or indeed throughout the larval and adult life of the lamprey. Meanwhile, the ammocoete promises to afford convenient material for continued experimental studies on the organization and function of microtubules and other intracellular components of nerve cells.

RADAN BERÁNEK. *1923–1969.* Dr. Beránek was head of the Laboratory of Cellular and Comparative Neurophysiology, the Czechoslovak Academy of Science, Prague. He came to the University of Miami School of Medicine in November, 1968, as visiting Professor of Physiology, at which time he introduced us to ammocoetes and we started a fine structural study of the central nervous system of *Petromyzon*. We were able to discuss several of the features described in this paper and had begun to consider longer term studies at the time of Radan Beránek's death in March 1969. He, and his collaboration, is greatly missed.

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Note Added in Proof: Electron microscopic evidence has recently been obtained of structural links between synaptic vesicles and microtubules in the ammocoete nerve cord. These preliminary findings, made after the present paper went to press, will be reported at a Discussion Meeting of the Royal Society of London on "Subcellular and macromolecular aspects of synaptic transmission" held in London, May, 1970. (D. S. Smith, *Proc. Roy. Soc. (London) Ser. B.*, manuscript in preparation).

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