Cytoplasmic Structure in Rapid-frozen Axons

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ABSTRACT   Turtle optic nerves were rapid-frozen from the living state, fractured, etched, and rotary shadowed. Stereo views of fractured axons show that axoplasm consists of three types of longitudinally oriented domains. One type consists of neurofilament bundles in which individual filaments are interconnected by a cross-bridging network. Contiguous to neurofilament domains are domains containing microtubules suspended in a loose, granular matrix. A third domain is confined to a zone, 80–100 nm wide, next to the axonal membrane and consists of a dense filamentous network connecting the longitudinal elements of the axonal cytoskeleton to particles on the inner surface of the axolemma.

Three classes of membrane-limited organelles are distinguished: axoplasmic reticulum, mitochondria, and discrete vesicular organelles. The vesicular organelles must include lysosomes, multivesicular bodies, and vesicles which are retrogradely transported in axons, though some vesicular organelles may be components of the axoplasmic reticulum. Organelles in each class have a characteristic relationship to the axonal cytoskeleton. The axoplasmic reticulum enters all three domains of axoplasm, but mitochondria and vesicular organelles are excluded from the neurofilament bundles, a distribution confirmed in thin sections of cryoembedded axons. Vesicular organelles differ from mitochondria in at least three ways with respect to their relationships to adjacent axoplasm: (a) one, or sometimes both, of their ends are associated with a gap in the surrounding granular axoplasm; (b) an appendage is typically associated with one of their ends; and (c) they are not attached or closely apposed to microtubules. Mitochondria, on the other hand, are only rarely associated with gaps in the axoplasm, do not have an appendage, and are virtually always attached to one or more microtubules by an irregular array of side-arms.

We propose that the longitudinally oriented microtubule domains are channels within which organelles are transported. We also propose that the granular material in these channels may constitute the myriad enzymes and other nonfibrous components that slowly move down the axon.

Many membrane-limited organelles in cells exhibit a polarized saltatory motion (37). In neurons this phenomenon is involved in fast axonal transport (9, 15, 23), an energy-requiring process which enables axons to move substances in both the antero-grade and retrograde directions at rates between 0.2 and 5.8 μm/s (15). In addition, there is a slow transport (53) of certain constituents in the axoplasm that ranges in average velocity from 2.3 to 46 nm/s. This slow transport does not involve the translocation of discrete membrane compartments, but does represent a coherent movement of the framework of microtubules and neurofilaments as well as a distinct and coherent movement of soluble cytoplasmic proteins (2, 4, 5).

These examples of cytoplasmic transport in neurons are interesting because they probably represent fundamental processes which occur in all cells, not just nerve cells. In addition to the notion that transport is essential for maintaining the viability of nerve cell processes (53), there is now evidence that cytoplasmic transport mediates an intracellular communication that, in turn, is involved in a poorly understood trophic interaction between neurons, and between neurons and their peripheral target tissues (34, 38).

Despite its obvious fundamental importance, reflected in an immense literature (15), the cellular mechanism and control of axonal transport, indeed of cytoplasmic transport and organelle

motility in general, remain mysterious. This study was spawned by our belief that a deeper understanding of these processes would result from a reexamination of cytoplasmic structure in axons using new techniques of rapid-freezing and cryoprocessing.

Ideas concerning the organization of axonal cytoplasm had not changed significantly from the first electron microscopical studies (33) until a recent investigation based on a variety of new techniques (11). Membrane-limited organelles, microtubules, neurofilaments, and the axolemma were found to be interconnected by a ubiquitous, trabecular network, analogous to the "microtrabecular lattice" visualized by high-voltage electron microscopy of whole, cultured cells (51, 52; see also reference 21). Certain membrane-enclosed organelles have an asymmetric association with the trabecular network which was thought to be relevant to mechanisms underlying organelle motility (11).

The recent improvement of an apparatus for rapidly freezing tissues without prior fixation or cryoprotection (19, 20) provides an opportunity to expose intracellular structure within frozen and fractured cells by sublimating (etching) the frozen intracellular water (17, 18). When the extent of sublimation is minimized and the exposed cytoplasmic surface is rotary replicated and visualized stereographically, cytoplasmic structure is seen from a unique three-dimensional perspective at a resolution near the macromolecular level. Although extraction of nonvolatile, cytoplasmic solutes before freezing has been considered essential to visualize meaningfully the internal structure of some cells with freeze-etching (18), it does not appear to be necessary for axons or fibrous astrocytes in turtle optic nerves.

The present study depends on freezing turtle optic nerves directly from the living state, without any prior extraction in order to be reasonably certain that axoplasmic transport was ongoing at the moment of freezing. Because chemical fixation was avoided, and the frozen axons were only minimally etched, we expect this approach to provide a more realistic look at the organization of cytoplasm not withstanding new artifacts introduced by rapid-freezing or artifacts associated with dissecting living nerves. The resulting conclusions about axonal structure differ in important ways from those of previous studies (11). Furthermore, our images of axonal cytoplasm are relevant to the question of whether the cytoplasmic space in intact cells consists only of discrete components of the cytoskeleton, or whether there is, in addition, an interconnecting matrix of some kind.

MATERIALS AND METHODS

Small adult pond turtles (*Pseudemys scripta elegans* and closely related species) with carapaces measuring 5–10 cm long were decapitated and the eyes, optic nerves, and tecta were dissected out as a single preparation. This was incubated for 5 min to 2 h in a dish of oxygenated Ringer's containing 114 mM NaCl, 3 mM CaCl₂, 2 mM KCl, 2.5 mM glucose, and 5 mM HEPES at pH 7.2. These conditions essentially reproduce those used to keep whole forebrain preparations from the same species viable for up to 4 h (29). The meninges were teased off and in some instances the preparation was first treated with 1 mg/ml protease (Sigma Type X; Sigma Chemical Co., St. Louis, MO) which usually allowed the pia-arachnoid to be peeled off as a single sheet. Enzyme treatment was not required for the smallest animals and did not influence the results in any way that was apparent to us. Optic nerves 4–5 mm long were excised, at their emergence from the eye and at the chiasm, no more than 1 min before freezing them. Excised nerves were mounted on 200-μm thick slabs of gelatin centered on the aluminum freezing disk. Freezing was then accomplished according to methods described in detail elsewhere (19, 20).

Although it was possible to tease away most of the pia-arachnoid before freezing, a layer of damaged pial cells, collagen, and fibrous astrocytic processes inevitably remained on the nerve. Nevertheless, some nerve fibers lay within 5

μm of the pial surface and these were exposed by shallow fractures. It was not uncommon, however, to fracture through vast regions of connective tissue.

Replicas were made in a Balzers 301 modified to fracture specimens at 20 to 30°K, rapidly warm them to 166°K for 10 to 30 min, and then rotary shadow them with metallic alloys designed to give maximum resolution. Briefly, these design goals were achieved by cooling the rotary stage with liquid helium admitted through a side-arm affixed near the nitrogen inlet. The rotary cold stage could then be warmed to 166°K in 6 to 8 min with its built-in heater. The vacuum near the specimen was improved by mounting a metal shroud on the cold microtome arm, and an ambient vacuum of 3–5 × 10⁻⁸ Torr was achieved by the addition of a liquid helium cryopump (CTI Cryogenics, Waltham, MA). The liquid helium-cooled specimens were fractured with a sapphire knife (Adolf Meller Inc., Providence, RI) and rotary shadowed with an alloy of Pt/Ir/Ta in a conventional carbon crucible mounted in a Balzers electron beam gun (Balzers, Hudson, NH). These methods will be described in more detail elsewhere (24).

Frozen nerves were prepared for thin sectioning by freeze-substitution for 3 d at −80°C in 1% osmium tetroxide dissolved in tetrahydrofuran (32). The osmium was then washed out and the nerves were infiltrated in increasing concentrations of Lowicryl HM20 (Polaron, Doylestown, PA) dissolved in tetrahydrofuran (22). The plastic was finally polymerized by exposure to ultraviolet light for 3 d at −60°C.

Specimens were examined in a JEOL electron microscope operating at 100 kV and stereo pairs were obtained with a side entry goniometer stage tilted through 6° to 8°. Micrographs of replicas are printed as negative images in which metal deposits look white and the background dark.

RESULTS

Freeze-substituted and Cryoembedded Axons

In transverse sections of freeze-substituted and cryoembedded axons from the turtle optic nerve, the axoplasm appears to consist of two types of domains: bundles of tightly packed neurofilaments; and regions containing microtubules in a loose, granular matrix. Microtubules are less regularly packed than neurofilaments and, on the average, are much further apart (Fig. 1). The main difference between these images and those from conventionally fixed or freeze-substituted preparations embedded in epoxy resin is that the axoplasmic matrix around the discrete cytoskeletal filaments and tubules now appears evenly distributed though it is somewhat different in the two domains. By contrast, even the best conventional micrographs of axons show clumps of dense material adhering to the discrete intracellular structures, and a network of "empty" regions (33). Thus the preservation of the cytoplasmic matrix around discrete components of the cytoskeleton appears to be more realistic after low-temperature embedding.

The border between microtubule- and neurofilament-associated domains is typically quite sharp but in some areas there is a more gradual transition from one domain to the other. Membrane-limited organelles are specifically associated with the microtubule-containing regions. Although some may lie at borders of neurofilament domains, they are never enveloped in neurofilaments. The exception is smooth axoplasmic reticulum which is found in both domains (Fig. 1).

Freeze-fractured Axons

The axoplasm of frozen axons which have been fractured longitudinally and etched to expose intracellular structure (Figs. 2 and 5) appears to consist of longitudinally oriented filaments interconnected by a network of transverse crossbridging material. The regularity of spacing as well as the amount and distribution of material between these longitudinal filaments varied most markedly between nerves and less so between axons within a single frozen specimen. These variations are related to the rate and extent of etching and to the size of ice crystals, which in turn are related to the depth of the fracture. In fact, differences in etching temperature of 1°–2°C noticeably affected the depth of etching.
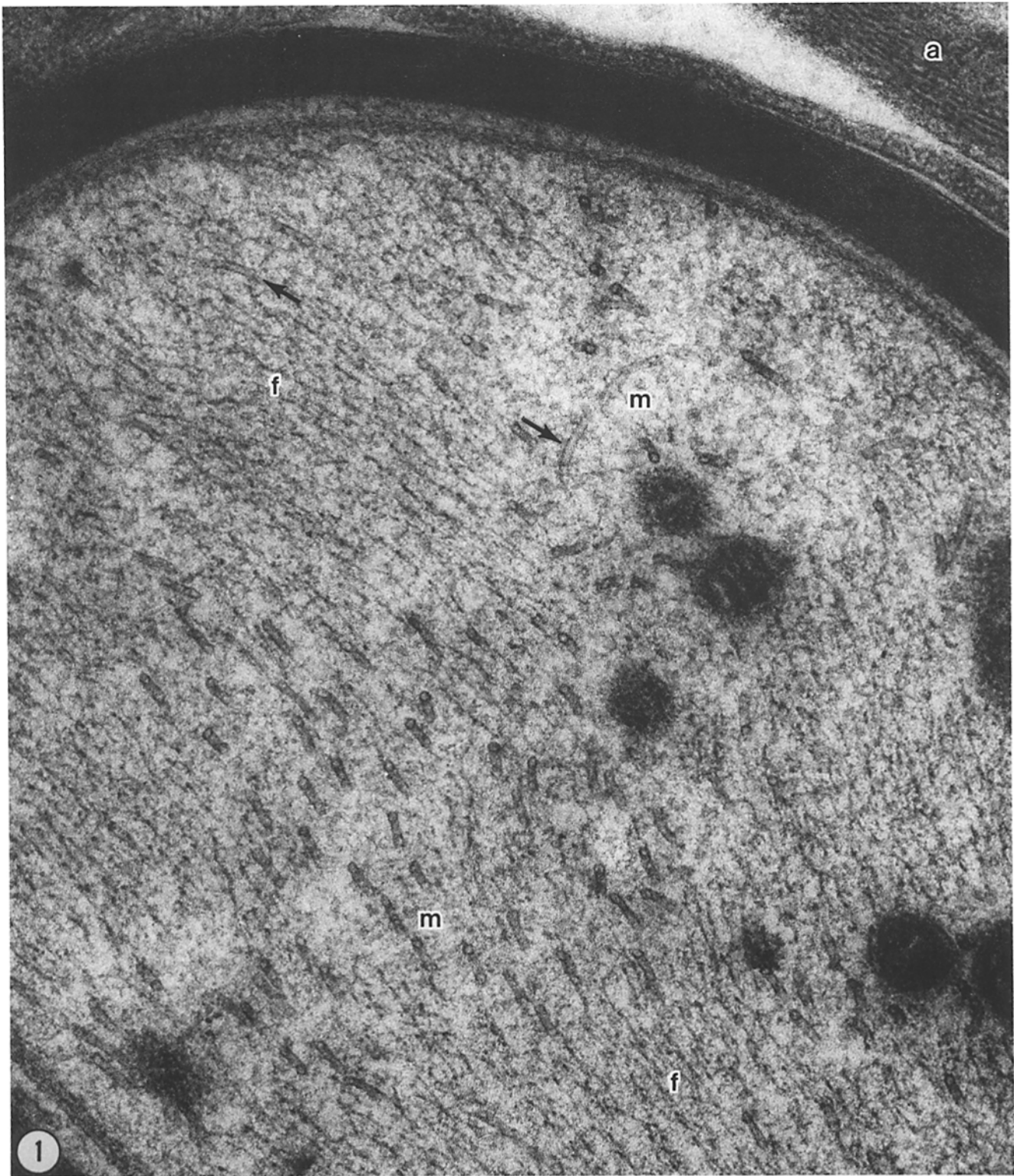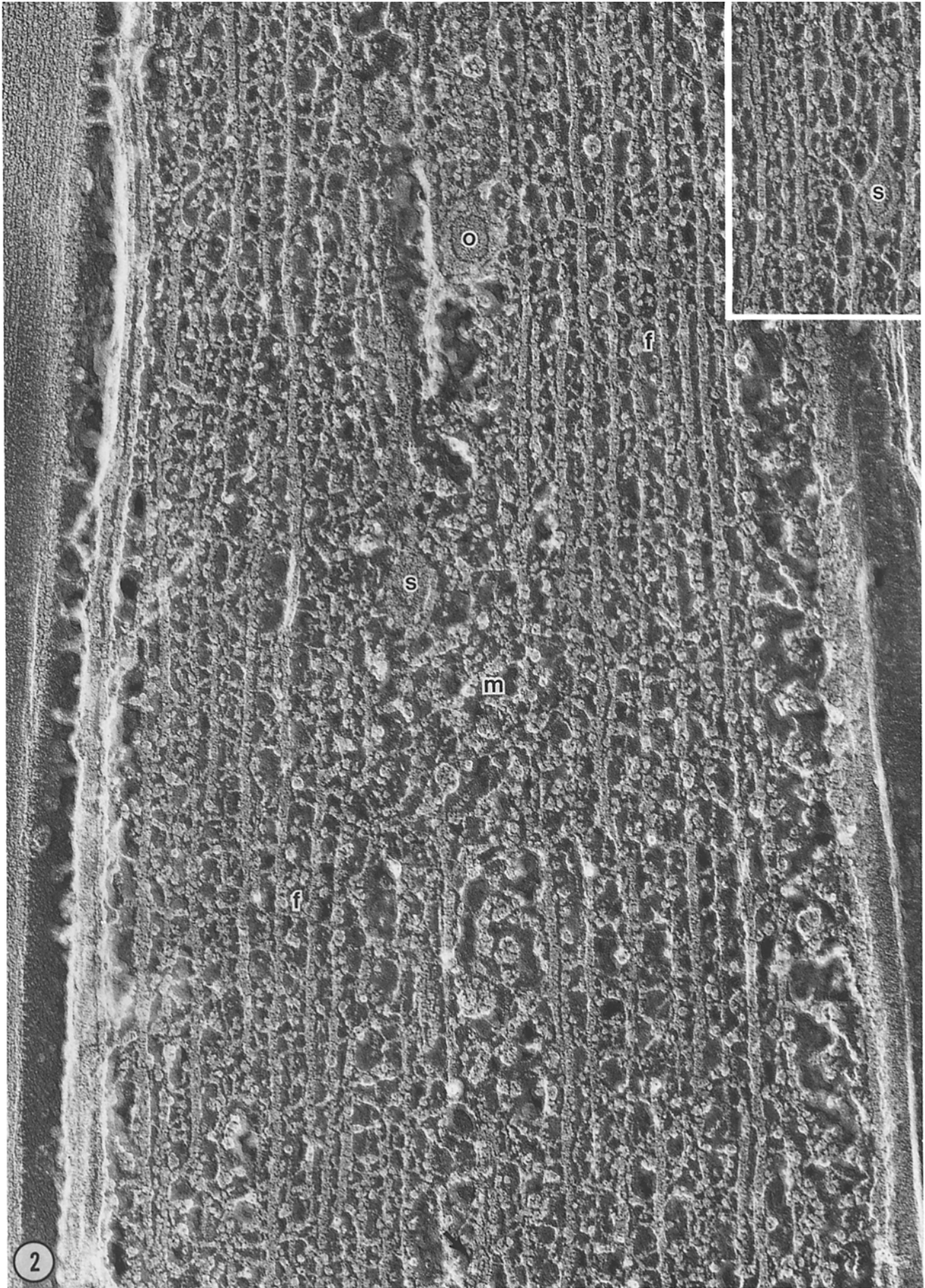
FIGURE 1   Cross section of a myelinated axon from a rapidly frozen and freeze-substituted turtle optic nerve. Surface of nerve, which contacted the copper cold block, is at upper right. Two domains or types of axoplasm can be distinguished: (*i*) bundles of neurofilaments (*f*) and (*i*) microtubule-containing regions (*M*) where the tubules appear suspended in a granular matrix. Both cut ends of the microtubules are seen as they pass out of the plane of section because this embedding resin scatters fewer electrons than conventional resins and the tubules are stained entirely through the section. Most organelles are seen in the microtubule domains, often near the interface with the neurofilament bundles, but axoplasmic reticulum is found in both domains (arrows). Mitochondria are intimately apposed to microtubules (lower right). Astrocyte processes (*a* at upper right) are characterized by densely packed intermediate filaments. × 92,000.

Axons that are etched deeper than a single layer of longitudinal filaments (10–20 nm) tend to show the most variability in filament spacing. With further etching, adjacent filaments collapse together creating crevices and the transverse or cross-bridging material assumes a progressively simpler appearance. With the most extreme over-etching (40 min at 166°K) axoplasmic structure appears completely and obviously disrupted. The images of deeply or moderately etched axoplasm (lowering the water table by ~50 nm, e.g., Fig. 6) give the impression of "clean" filaments and cross-bridges and, at first glance, are easier to examine compared to minimally etched specimens (10–20 nm) which look "cluttered" by comparison (Fig. 2). However, views created by a minimum of etching should more accurately represent cytoplasmic structure in its intact, living state. In fact, the two cytoplasmic domains evident in cryoembedded nerves can only be identified in freeze-fractured axons that have been minimally etched. Except for Fig. 6, all freeze-etch micrographs in this paper are from specimens etched to a depth which exposes slightly more than one layer of neurofilaments, on the order of 20 nm.

Freezing that was poor enough to alter contours of membranes (20) also created holes or spaces within the axoplasm that were even larger in over-etched nerves; this artifact is probably also present in Fig. 6. In fact, subtle manifestations of this artifact appeared before any indications of ice crystal damage to membranes.

The axoplasm was often obscured by another prominent artifact which was never brought entirely under control. This appears as smearing of the fracture surface, with distortion or even obliteration of the underlying filamentous structures. Many regions in all our replicas were affected in this way. We suspect that the most useful fractures occur when the knife edge initiates a natural cleavage plane between or along filaments. After rejecting the various artifacts listed above, we were left with ~200 stereo views from optic nerves. These views, which showed a uniformity of structure free of these gross artifacts, are described below.

Axoplasm

The most conspicuous longitudinally oriented fibrils in fractured and etched axons are unambiguously identified as neurofilaments by their diameter (11 nm), abundance and close-packing (Figs. 2 and 3, and see Fig. 8). In replicas with the highest resolution, a regular transverse substructure is sometimes seen. In our best specimens, characterized by small ice crystals, untwisted nerve fibers, very shallow etching, and a fracture plane parallel with the neurofilaments, these filaments are regularly spaced ~20 nm apart. Although fractured ends of the neurofilaments are obvious, we could not identify any natural ends or branch points.

The neurofilaments are interconnected by a cross-bridging network (Figs. 2 and 3, and see Fig. 8) which has no apparent regularity in its organization and is, therefore, difficult to characterize in a precise way. Moreover, the appearance of this network is critically dependent on the depth of etching. In moderately or deeply etched specimens, the cross-bridges tend to appear as discrete structures (Fig. 6). In minimally etched specimens there appears to be a cross-bridging network consisting of an anastomosing, filamentous material, 3–6 nm in diameter, that is coated with granules (Figs. 2 and 3). In some regions the fracture plane passed between neurofilaments and exposed en face views of the cross-bridging network; even from this perspective there is no apparent regularity other than the spacing between filaments. We could find no indication of a 40-nm periodicity in the spacing of cross-bridges (11, 21), but the shallowness of our etching would make a three-dimensional lattice difficult to see. Otherwise, our images are consistent with the idea of a "trabecular lattice" connecting neurofilaments (11).

Microtubules are identified in replicas of axoplasm by their size and substructure (18). They average 23 nm in diameter and on their outer surface they have longitudinal arrays of subunits that represent the 13 protofilaments of tubulin (Figs. 2, 4, 5, and 7–9). Views of the insides of split microtubules show regular oblique striations, 4 nm apart (Fig. 7), corresponding to the proposed staggered relationship between individual protofilaments (1, 12).

In perfect correspondence to their appearance in thin sections through cryoembedded axons, the microtubules appear in groups (Figs. 4 and 5), are less frequently encountered than neurofilaments, are far less regularly spaced, and, on the average, are much further apart. Unlike neurofilaments, microtubules are not integrated within a cross-bridging network. Instead, they are situated within a matrix consisting of granules varying in diameter up to 40 nm. The specific association of this "granular axoplasm" with microtubules is best demonstrated in oblique fractures, where only short fractured pieces of microtubules are evident (Fig. 4). In precisely longitudinal fractures, granular zones of axoplasm look "disorganized" and stand out against the regularly organized bundles of neurofilaments (Fig. 2), but the shallow etching and large spacing between microtubules means that they are only rarely encountered in these regions. Few cross-bridges comparable to those between neurofilaments connect elements of this granular matrix to microtubules, or microtubules to each other, though cross-bridges, if infrequent, might be obscured by the granular material.

The microtubule domains were less frequently encountered in freeze-etched specimens than would be expected from their relative abundance in thin sections. We suspect that planes of fracture tend to follow neurofilament bundles, thus avoiding

FIGURE 2 Axon prepared by direct freezing, freeze-fracturing, minimal etching, and rotary shadowing. Parallel, longitudinally oriented neurofilaments (f) are interconnected by a cross-bridging network of filamentous and granular material. The true cytoplasmic surface around a small vesicular organelle (o) has been exposed by sublimation of ~10 nm of the surrounding ice, which also exposes a single layer of neurofilaments. This organelle lies in a narrow, longitudinal domain of granular axoplasm (m), which is distinct from the neurofilaments and their cross-bridges. This domain extends to the bottom of the figure, where it includes a fractured piece of microtubule (arrow, just above lower border of micrograph). A gap in the granular axoplasm lies below and to the left of the vesicular organelle. Smooth axoplasmic reticulum (a large cistern is shown at s, and a more typical one at s in the inset), recognized by its varicose shape and is associated with both the granular, microtubule-domains, and the cross-bridged neurofilament-domains of the axoplasm (inset). Particularly evident near the axonal membrane at the right is an 80–100 nm wide zone from which neurofilaments are excluded. This subaxolemmal domain consists of transversely oriented filamentous structures interconnecting the sides of the neurofilaments and the inner surface of the axonal membrane. X 140,000. Inset, X 140,000.
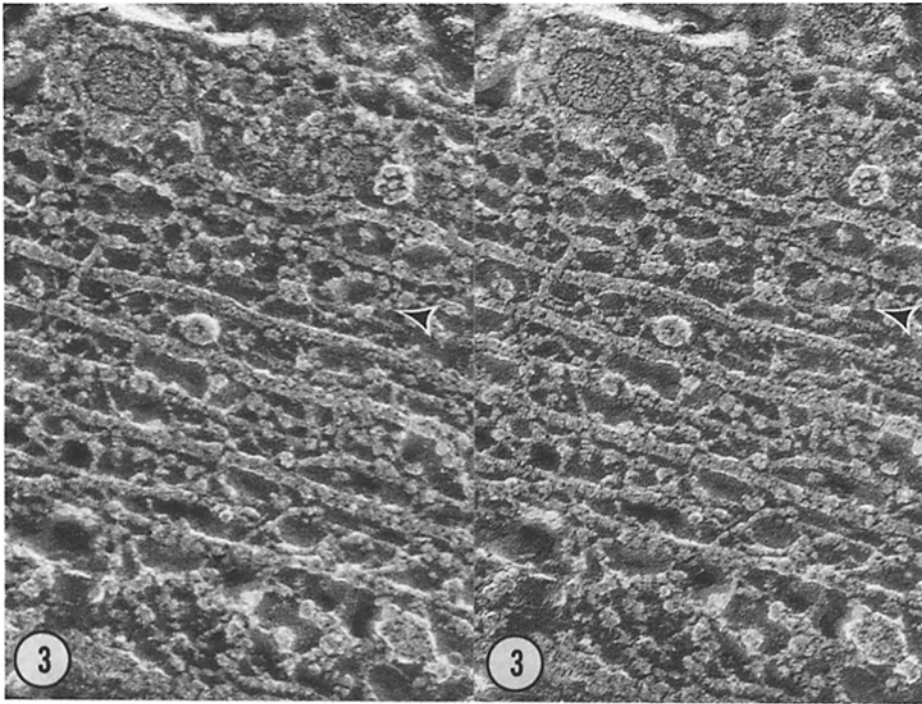
FIGURE 3 Stereo view of a neurofilament-associated domain (from Fig. 2). The cross-bridging network is composed of fine, irregularly shaped filaments coated with globular elements. An *en face* view of the network is exposed where the plane of fracture goes between adjacent filaments (arrowhead). × 170,000.
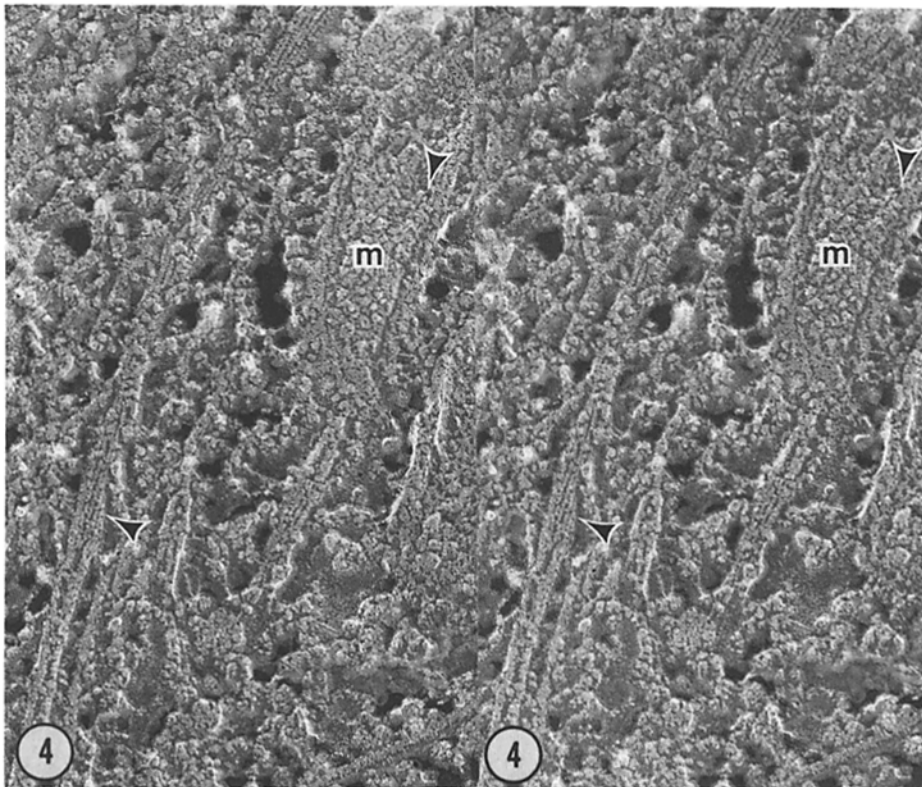
FIGURE 4 Stereo view of microtubule-associated domain. The fractured pieces of six microtubules can be identified on the basis of their size and substructure (e.g., at lower arrowhead). Two organelles identified as mitochondria (m) by their shape and membrane structure lie in a granular matrix. This matrix is different from the cross-bridging network associated with neurofilament domains. Upper arrow head indicates a close apposition between a mitochondrion and a microtubule which is partly underneath it. × 140,000.

regions containing microtubules. Occasionally microtubules adjacent to neurofilaments form cross-bridges to them (Fig. 9). We assume that these images result from fracturing at borders between a microtubule region and a bundle of neurofilaments because cross sections of freeze-substituted axons show that some microtubules are associated with the neurofilament cross-bridging network at borders between the two domains.

A third domain of axoplasm, shown in Fig. 5, occupies a zone up to 100 nm wide next to the axolemma. Here, the

longitudinal array of neurofilaments gives way to an array of filamentous material distinct from both the cross-bridging network between neurofilaments and the granular material around microtubules. The neurofilaments do not penetrate the subaxolemmal zone, but filamentous material extends between them and the inner surface of the axolemma. Some granular material, similar to but less concentrated than that in microtubule domains, is also found in the subaxolemmal domain. Where the axolemma is obliquely fractured, exposing its external leaflet,
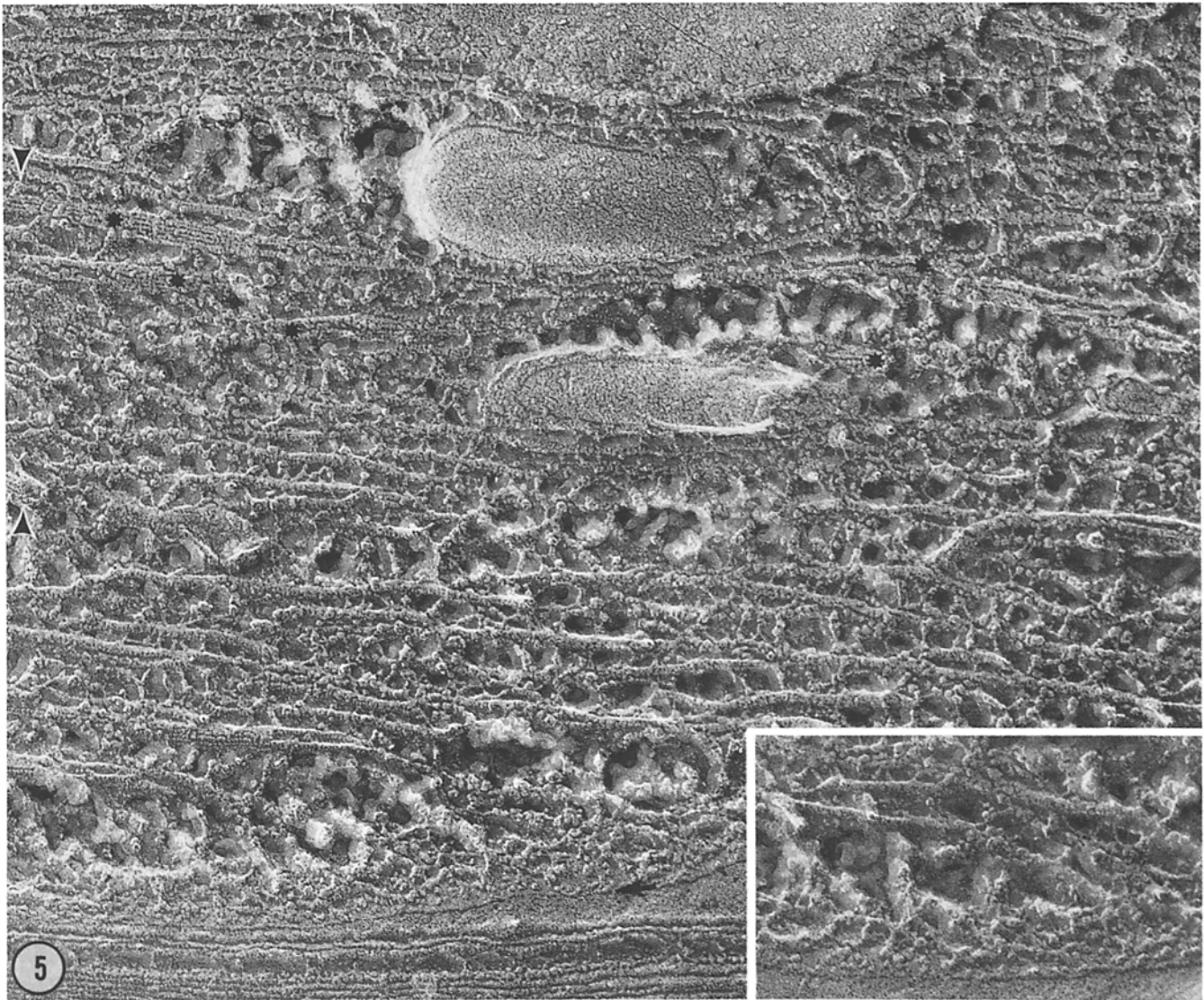
FIGURE 5   Zone near the axolemma where filamentous material contacts particles on the inner axolemmal surface (arrow, near the bottom of figure, indicates where plane of fracture steps up from the E-fracture face, to the true inner membrane surface exposed by etching). At the top of figure are two sausage-shaped organelles which probably correspond to components of the retrograde transport system. The left end of the larger one is associated with a gap in the axoplasm. These organelles are in a microtubule domain of the axoplasm (bracketed by arrow heads at left) which passes obliquely through the plane of fracture and consequently has a very irregular outline. Pieces of at least five microtubules (stars) are evident in the vicinity of the organelles. × 105,000. *Inset*: The true inner axolemmal surface shown at a higher magnification. Filamentous material unlike that in other domains projects transversely from the sides of the neurofilaments to branch on or near the inner surface of the axolemma. These branches appear to contact particles on this surface. *Inset*, × 130,000.

etching provides a view of its true inner or cytoplasmic surface. This surface is covered with 8- to 9-nm particles, some of which contact the terminations of the subaxolemmal filaments. Microtubules, unlike neurofilaments, occasionally approach the axon membrane. Not enough views of the inner surface of the axon were obtained in order to be certain that its entiere inner surface, e.g., at nodes, fits this pattern.

We were unable to find any clear examples of microfilaments anywhere in the axoplasm. Within the subaxolemmal network of filaments were two examples of short lengths of filaments with an appropriate diameter (8–9 nm after shadowing (14), but the transverse spacing in the filaments was not definite enough to equate it with the 5.5 nm spacing characteristic of actin (19).

Membrane-enclosed Organelles

Our classification of organelles in turtle optic nerve optic nerve axons is limited by those distinctions we are able to make in replicas of fractured and etched axons. All the membrane-limited organelles typical of axons in general are encountered in thin sections of freeze-substituted turtle optic nerves: mitochondria, axoplasmic reticulum, multivesicular bodies, lysosomes, lipoidal granules, and vesicles of various sizes (33).

In replicas we can distinguish three classes of organelles. Mitochondria (Figs. 4 and 9) are recognized by their size and elongated shape, by their double membranes, and, when cross-fractured, by their cristae. Their fractured membranes have a characteristic rough, particle-laden surface which aids in iden-
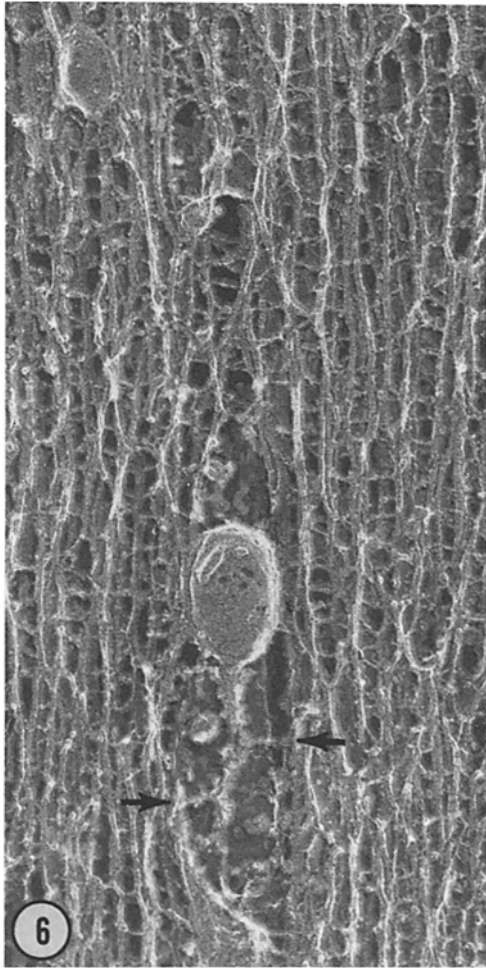
FIGURE 6 Extensively etched axon showing collapsed neurofilaments and large spaces within the cross-bridging network. Under these conditions, discrete cross-bridges are evident between neurofilaments. Because the globular structures around the vesicular organelle have been pushed aside or collapsed, a larger than usual piece of its longitudinally oriented appendage is exposed. Arrows indicate side-arms arising from the appendage. There are also a few structures bridging the space between the sides of the organelle and the neurofilaments. A microtubule lies above the spherical organelle but boundaries of domains are not certain in such collapsed axoplasm. This area of the nerve is also likely to have been poorly frozen judging from the tendency for the spaces between the neurofilaments to be circular. × 78,000.

tifying smaller mitochondria where shape and size alone do not suffice to make their identification certain (e.g., Fig. 4). Fractured pieces of the axoplasmic reticulum (Figs. 2, 7, and 8) are easily recognized as narrow, tubular membrane compartments with varicosities ~70 nm in diameter (69 ± 20 nm; n = 21) at regular intervals. It is thought that the axoplasmic reticulum is an anastomosing network that extends for long distances through the axon, (36, 48) and is possibly one continuous system extending from cell body to nerve terminal. Our views only exposed short segments of this network and were not useful for determining continuity over long distances.

The third and only other type of organelle we can distinguish are round or tubular vesicles ranging from 80 to 260 nm in diameter (138 ± 48 nm; n = 11; Figs. 2 and 5–8). These had unremarkable fracture faces containing particles that cleave with the P-face. At least some of these organelles must corre-

spond to the class of discrete, membrane-limited compartments involved in the retrograde transport of horseradish peroxidase (27) or the more specific, receptor-mediated transport of lectins (39), tetanus toxin (40–42), and nerve growth factor (38, 41). These include multivesicular bodies and lysosomes which, in turn, have been identified with particles that undergo polarized, saltatory movements as visualized in living axons (3).

Vesicular organelles and mitochondria have a consistent and unique association with the adjacent axoplasm. Solitary vesicular organelles are associated with narrow, longitudinal zones of granular axoplasm at one or both of their ends (Figs. 2, 5, and 8). This axoplasm is the type which is associated with microtubules (Fig. 4) and it differs clearly from the axoplasm containing well organized bundles of neurofilaments and their cross-bridging network. However, the 10–20 nm depth of view in minimally etched replicas does not allow any direct inference regarding the full longitudinal extent of these zones. Thus, we cannot tell from freeze-fracture data alone whether the zones of granular axoplasm are in isolated pockets, or in continuous columns that pass in and out of the plane of the replica. To decide whether these organelle-associated zones are residua from saltatory movement of organelles or are preexisting channels to which the organelles are confined requires a synthesis of freeze-etch and thin-section views. This issue will be considered further in the Discussion.

Vesicular organelles have other distinctive structural features in addition to their consistent association with granular, microtubule-associated domains of axoplasm. A gap in the surrounding granular axoplasm occurs at one or both ends of the organelle (Figs. 2 and 5–8) and sometimes is shaped as if the organelle had just been pulled away (Fig. 5; see also reference 11). A single appendage originates from the end associated with the gap and passes longitudinally through the surrounding space either to disappear in the unetched axoplasm below or to be severed at the plane of fracture, so that usually only very small pieces of the appendage are exposed. Even in the clearest examples, appendages are decorated with an amorphous substance that obscures their surface detail, making them appear non-uniform in size and preventing their characterization (Figs. 2 and 6). It is impossible to determine whether these structures are polymeric filaments or narrow, tubular membrane compartments. Finally, material on the sides of vesicular organelles may make lateral contacts with neurofilaments (7) that border the microtubule-associated zones.

Although vesicular organelles and mitochondria are both specifically associated with the microtubule-containing domains of axoplasm, the vesicular organelles rarely, if ever, contact microtubules. Mitochondria on the other hand, almost always lie close to several microtubules and either make very close contacts with them or, more typically, are connected by irregular side-arms (Figs. 1 and 9). Compared to vesicular organelles, mitochondria are less frequently associated with gaps in the axoplasm and do not have a longitudinally oriented appendage at one of their ends.

Axoplasmic reticulum, recognized by its varicose shape and small diameter, differs from both vesicular ogranelles and mitochondria in that it seems to have no special relationship to other structures within the axon. It can be incorporated within bundles of neurofilaments, where it is integrated within the network of cross-bridging material (Figs. 2 and 8); it can lie in the granular microtubule domains near other organelles (Figs. 2, 7, and 8); or it can be near the axolemma. It is not associated with gaps in the axoplasm.
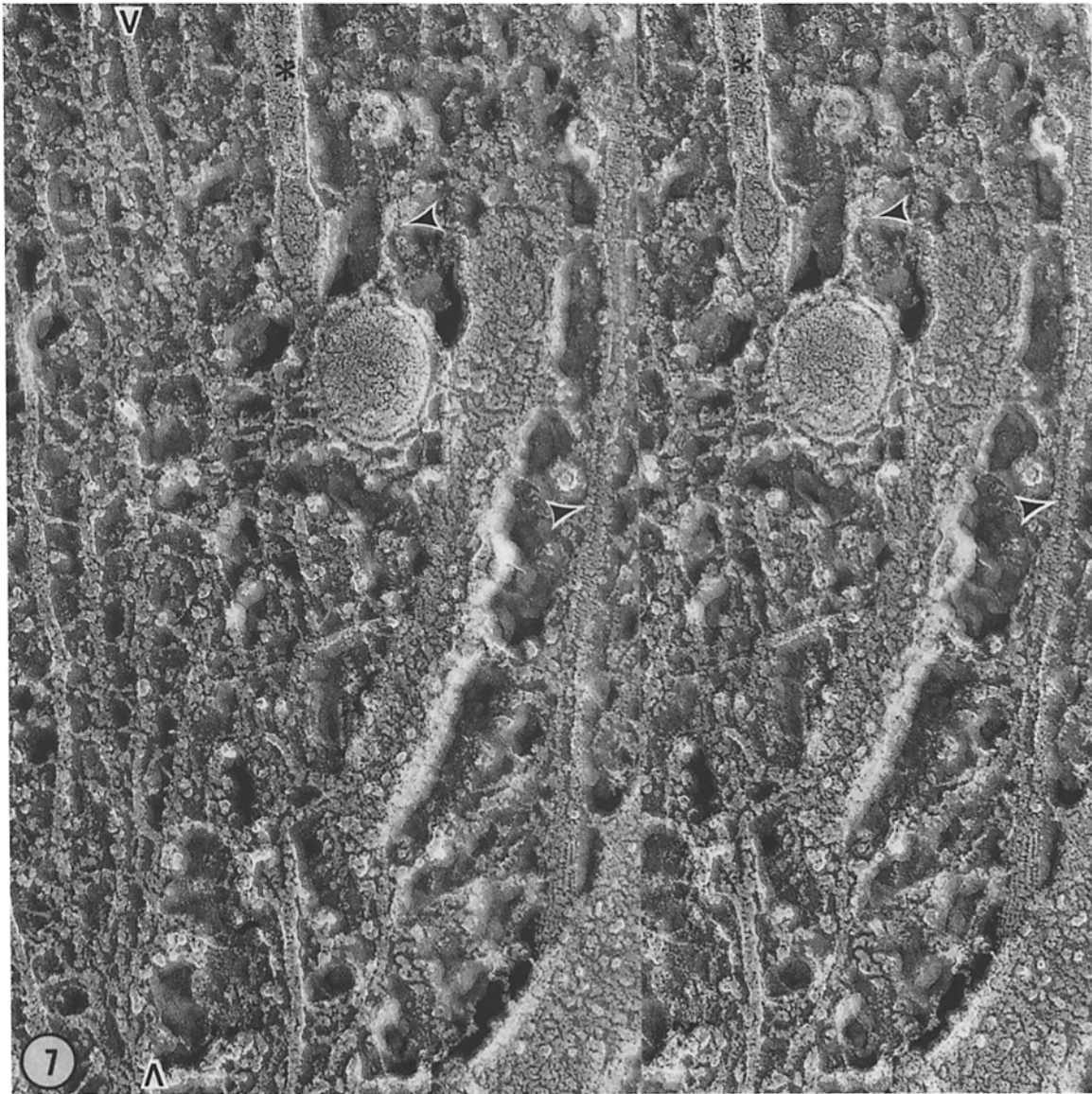
FIGURE 7 Stereo view of microtubule-associated domain of axoplasm including a vesicular organelle with a smooth E-fracture face, and a cistern of axoplasmic reticulum (asterisk near top). The vesicular organelle has an appendage (upper arrow heads) arising from one end as well as several cross-bridges to adjacent structures. The concentration of granular material is lower at both poles of the vesicular organelle. A microtubule extends from top to bottom at the extreme right. Oblique striations (arrowheads on right) are evident where this tubule has been fractured open, exposing its inner surface. To the left, outside the area of stereo overlap, is the interface with neurofilament-associated axoplasm (between pointers at top and bottom of picture). × 145,000.

Interfascicular Fibrous Astrocytes

Profiles of astrocytes found in thin sections are filled with closely packed glial filaments and little else (Fig. 1). This view is consistent with the fractured and etched preparations where tightly packed glial filaments lie in bundles filling glial processes (Fig. 10). Like neurofilaments, glial filaments are 11 nm in diameter but they have no indication of a transverse substructure and therefore can be distinguished from neurofilaments by the pattern of rotary shadowed metal coating them. They are also more tightly packed than many filaments and they lack cross-bridging material which therefore appears to be specific for neurofilaments. Microtubules are seen occasionally near the cell membrane, and are not surrounded by a granular matrix. Organelles such as mitochondria may be enveloped within the glial filaments, with no indication of associated cytoplasmic gaps like those found in axoplasm. A

narrow zone next to the cell membrane is evident and, like axonal cytoplasm, consists of a network of transverse, filamentous material.

DISCUSSION

Axoplasm

The views of axoplasm presented here do not depend on cytoplasmic extraction or chemical fixation. However, nerves were maintained in vitro, with eyes and brains attached, for at least 10 min before excising and immediately freezing them. Niether the time in vitro nor partial dissection of the pia-arachnoidal sheath affected our results, but if any of these manipulations had rapid and enduring effects, they could not have been recognized. We selected the turtle optic nerve for this study because of the ability of the turtle central nervous system to function in vitro (29), the large size of its optic nerve,
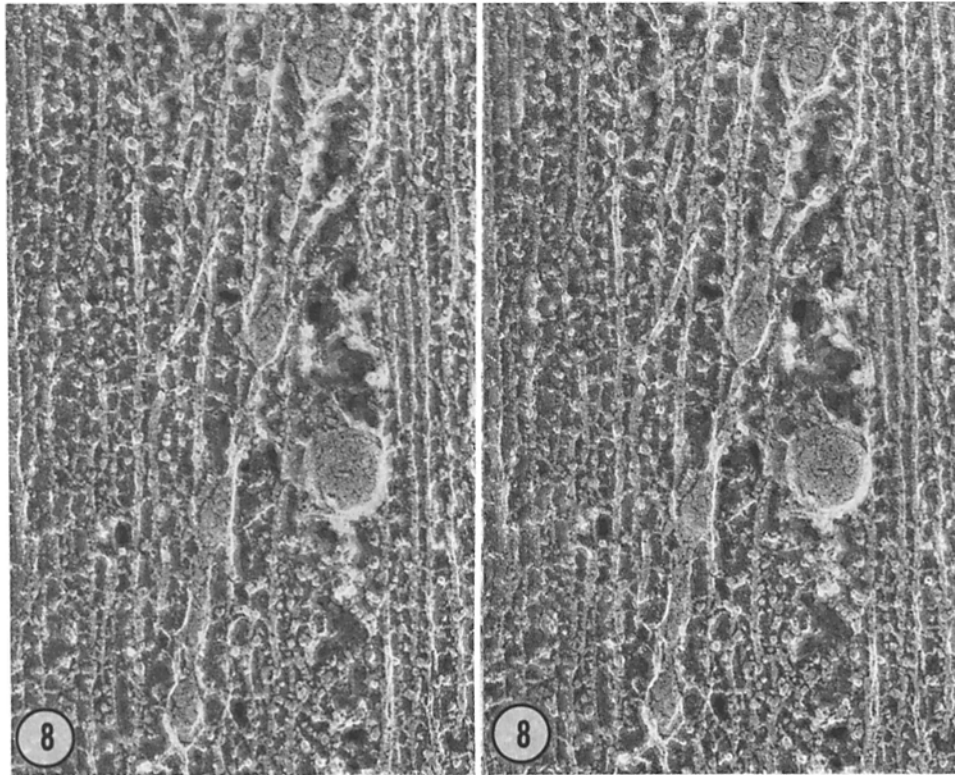
FIGURE 8   Stereo view of a vesicular organelle lying within a longitudinal zone of granular axoplasm. This domain of cytoplasm extends in both directions well beyond the field of view shown here, and is associated with pieces of fractured microtubules (not included here). An elongated cistern of axoplasmic reticulum lies next to the vesicular organelle. While the varicosities of axoplasmic reticulum are also roughly spherical, they are typically smaller than the discrete vesicular organelles. Gaps in the granular axoplasm occur at both ends of this vesicular organelle, but the one at the top is more pronounced. An appendage is associated with the lower end of the organelle. × 85,000.

and the relatively thin myelin sheaths around the axons, a factor which contributes to good freezing and, in addition, provides a greater frequency of fractures through axoplasm. We are not certain, however, to what extent our findings can be generalized to other nerves.

The turtle optic nerves maintained in vitro were excised in their living state only seconds before rapid-freezing and the components of axoplasm exposed by simply fracturing the axons and sublimating the intracellular water just enough to expose a thin layer of cytoplasm. We believe that minimizing the etching accounts for many of the differences between our views of cytoplasmic structure in axons and those previously reported (11). Indeed, we are able to distinguish microtubule-associated axoplasmic domains from neurofilament-associated domains only in minimally etched axons.

Soluble proteins and salts should remain with the tissue during the etching process, but they do not seem to obscure the discrete components of the cytoskeleton to the extent reported in fibroblasts, where soluble components must be extracted before freezing in order to visualize clearly cytoskeletal structures (17, 18). We chose not to pursue this approach because of the inevitable interference with the native cytoplasmic organization and the processes of fast and slow transport. While we acknowledge that the precipitation of soluble, nonvolatile material could contribute to the images of axoplasm in our preparations, the nature of this contribution seems quite unpredictable. For the time being we regard major structures such as the cross-bridges between neurofilaments and even the granular material around neurotubules as components of native, hydrated axoplasm, since such structures are not evident in

astrocytic cytoplasm prepared in the same way.

The replicas reveal that round and tubular organelles, of a type likely to be transported (3, 27), lie within a granular axoplasm which differs from other regions of axoplasm characterized by neurofilaments and associated cross-bridging material. One possibility is that these granular regions represent a "wake" that the organelle makes as it moves through the axon. If this were so, one would expect them to be polarized with respect to the organelle and limited in their longitudinal extent, perhaps to the length of a single saltatory movement. The fact that granular regions often emanate from both poles of organelles seems to contradict this idea. Moreover, granular regions can be considerably wider (Fig. 7, for example) than would be predicted from the linear movements organelles appear to make in living axons (3, 9, 13, 23).

A second explanation, the one we favor, is that longitudinal channels of axoplasm containing organelles such as lysosomes, multivesicular bodies, and mitochondria lie between the bundles of cross-bridged neurofilaments. Although fractured pieces of microtubules are often associated with these organelle-containing zones, the essentially planar views of longitudinally fractured axons provided by the minimal etching makes this image difficult to interpret. Fortunately, cross sections of freeze-substituted, cryoembedded nerves yield complementary information which confirms that organelles are preferentially associated with the microtubule domains of the axoplasm. We therefore suggest that axoplasm consists of domains of neurofilaments, tightly bound together by their associated cross-bridging network, and domains of microtubules suspended in a granular matrix. The microtubule-associated domains com-
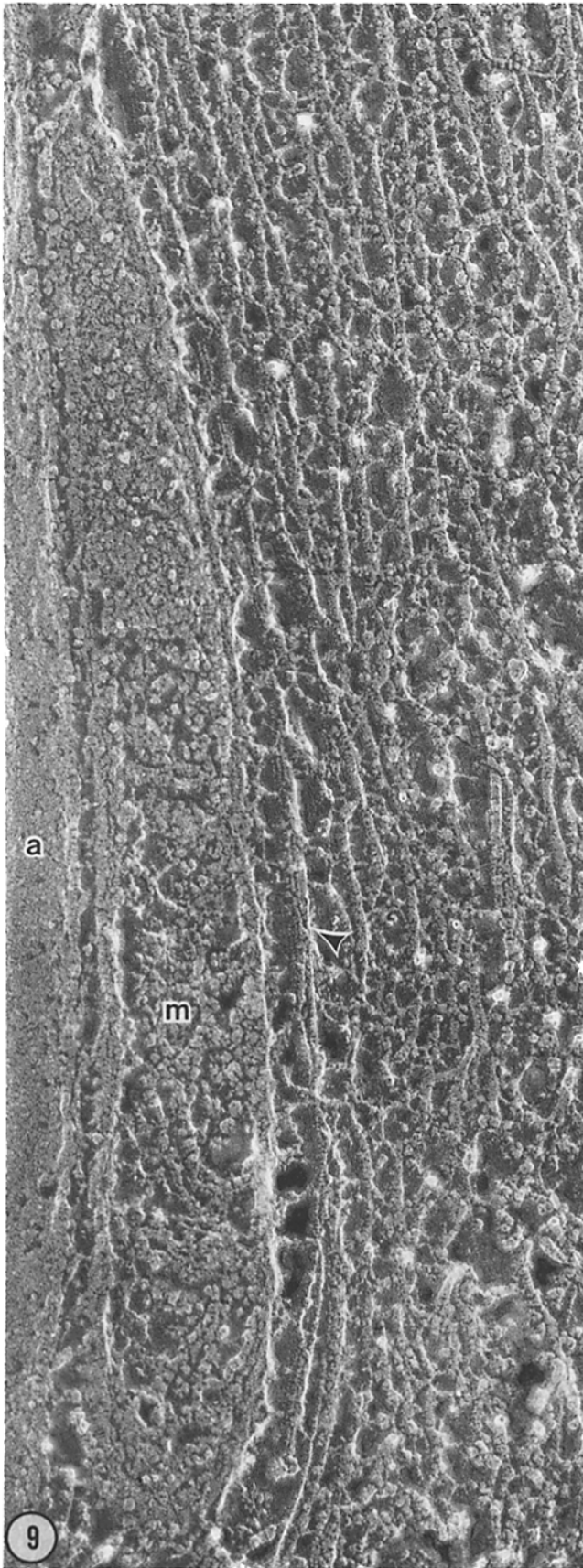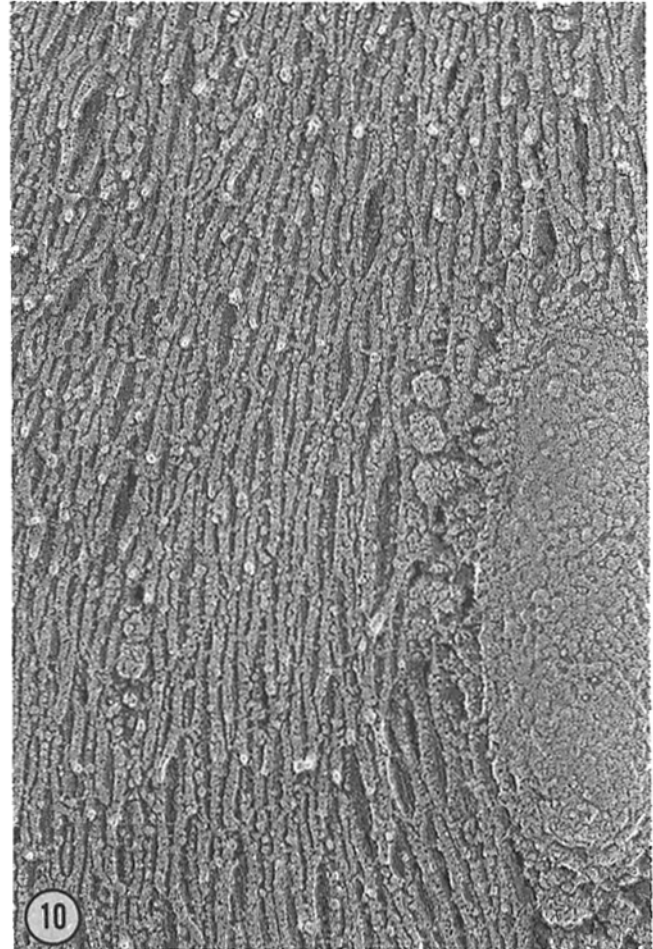
FIGURE 10 Cytoplasm of an astrocyte process. The glial intermediate filaments are not associated with cross-bridging or extensive granular material, and the organelle to tbe right of the figure, a mitochondrion, is not associated with microtubules or a microtubule-associated domain of cytoplasm. Glial intermediate filaments are also more densely packed than neuronal filaments. × 100,000.

prise the longitudinal channels through which organelles are transported.

The idea that organelles are transported within channels is not new (16). Organelles in pathologically swollen axons accumulate in discrete, longitudinal islands (28, 46), suggesting that they are normally transported within "streets" (28). A similar suggestion has been made to account for organelle movements that occur in living axons (23).

Membrane-limited Organelles and Fast Axonal Transport

Retrograde axonal transport involves multivesicular bodies, lysosomes, and vesicles of various shapes and sizes (3, 27, 42), but does not involve the axoplasmic reticulum (27). The compartments mediating anterograde transport are not known with certainty, although autoradiographic experiments (36) indicate that the axoplasmic reticulum is involved. The vesicular organ-

FIGURE 9 Large mitochondrion (m) lying between the axolemma (a), and a microtubule (arrowhead). The microtubule bends around the mitochondrion and shares side-arms with its outer membrane surface and with adjacent neurofilaments. The fractured inner mi-

tochondrial membrane (above) has a characteristic rough appearance and particle distribution which aids in identifying smaller mitochondria (e.g., Fig. 4). × 100,000.

elles seen in freeze-etched axons have a greater diameter than the varicosities of axoplasmic reticulum, but they are similar in size and shape to retrogradely transported organelles (3, 27, 38–42). Therefore, the special associations which these large vesicular organelles have with the cytoskeleton are probably most relevant to mechanisms of retrograde transport. It is uncertain, however, whether some large, discrete vesicles associate with the axoplasmic reticulum, although examining it with high-voltage electron microscopy after selective impregnation indicates that this is generally not true (36, 48).

Electron microscopical studies have repeatedly shown a close morphological association between microtubules and various organelles in axons (8, 35, 44, 45), and the role of microtubules in organelle movement has been explored in a variety of experimental preparations. This is typically done by determining whether drugs (e.g., colchicine) or physical treatment (e.g., low temperature) which depolymerize microtubules also block organelle movement. Usually there is a positive correlation (reviewed in reference 1), but contradictory results have been reported (7). Contradictory results may occur because the procedures for disrupting microtubules are not specific (43), and the morphological assay for microtubules, requiring fixation, may itself affect their frequency. Even a positive result would not distinguish between a direct and indirect role of microtubules in organelle movement. In other cells, where it is easier to visualize organelle movements directly, the orientation and distribution of microtubules suggest that they somehow direct these movements, and disrupting the microtubules usually interferes with this movement (14, 30, 37, 47). However, it is still not clear to what extent microtubules are directly involved in mediating the motive force via a contractile type of mechanism (37), as opposed, for instance, to providing the backbone of a channel having the necessary conditions for generating saltatory movements (10).

The picture of axonal cytoplasm presented here is most consistent with the idea that microtubules delineate channels for organelle movement. Moreover, our results are inconsistent with a transport mechanism that requires the formation of direct connections between microtubules and organelles because only mitochondria, which in living axons are noted for their relative immobility (9, 13), are typically attached to microtubules. Indeed, it has been suggested (13), and we agree, that mitochondria with close contacts and cross-bridges to microtubules are in an arrested state.

There is not enough information, at this point, to decide whether the microtubule-containing channels directly mediate transport by a streaming mechanism (15, 16) or indirectly support some other mechanism that directly drives organelle movements. However, there are two structural features associated with the vesicular organelles that could be indicative of a contractile mechanism: the cross-connections to surrounding neurofilaments, (noted previously, reference 11), which border the microtubule-associated channels; and the appendages associated with one of their ends. Single filamentous appendages of this kind would probably be missed in thin sections, although Nagai and Hayama (31) noticed polar attachments of filaments to organelles that undergo movement in *Chara* internodal cells, and confirmed this association by isolating the organelles and visualizing them with negative stain. We have not been able yet to determine where these appendages end in the cytoplasm, nor even to define any substructure typical of known filaments. Indeed, it is possible that some of these, particularly those connected to the smaller spherical organelles, are minute,

tubular membrane compartments of the axoplasmic reticulum (6).

Slow Axoplasmic Transport

Proteins travel down the axon in at least two coherent waves distinguished by their rate of movement away from the cell body (26, 49, 50). The slower wave, slow component *a*, contains little but microtubule and neurofilament proteins, and probably corresponds to formed neurofilaments and microtubules as well as the cross-bridges between the neurofilaments (5, 50). The cross-bridges we and others (11) have found between microtubules and neurofilaments may explain how they move down the axon at the same rate. A somewhat faster slow wave has a far more complex composition, containing a range of metabolic enzymes and other soluable components such as calmodulin (4) and actin (2, 5).

The present study shows that granular material is concentrated in the microtubule-associated domains of the axon. Many of these granules, which may be lobulated and larger than 10 nm, are unlikely to be single cytoplasmic proteins; instead they could represent several proteins associated in a particle. If these granular structures correspond to associations of proteins which constitute the slow component *b*, this association could provide part of the answer to the question how the diverse soluble components in slow component *b* can travel down the axon as a coherent wave (5). However, our findings provide no explanation for the mechanism of this movement.

The Organization of Cytoplasm

The long standing assumption that the "ground substance" of cytoplasm is random and amorphous has recently been questioned (51, 52). The use of high voltage electron microscopy to visualize whole, cultured cells that were fixed, stained, and critical-point-dried without an embedding matrix has provided images that argued for a "microtrabecular lattice" consisting of slender strands 3 to 6 nm in diameter. These strands are organized into a three-dimensional network that contacts the cell surface, membrane-limited organelles, and discrete cytoskeletal components such as microtubules and the filaments in the stress fibers. This concept has recently been extended to include axoplasm (11).

A microtrabecular lattice is missing, however, in the cytoskeletons of cultured fibroblasts which are extracted with detergent and then fixed, rapid-frozen, freeze-dried, and rotary shadowed (18). In these cytoskeleton preparations the extraction removes the cell membrane and all membrane-limited organelles, leaving discrete filaments which are clearly shown to be microtubules, intermediate filaments, or actin fibers, organized in a way which is consistent with their disposition in intact cells. Because no cross-bridging structures of any kind which could be related to the microtrabecular lattice were found, it must be concluded that either the microtrabecular meshwork represents some component lost during the extraction (which, in the intact cell, is superimposed on the cytoskeleton of actin, intermediate filaments, and microtubules), or that the microtrabecular meshwork is a representation of discrete filaments clumped during fixation, staining, or critical-point-drying (18). The latter explanation has been favored for two reasons: the most compelling is that freeze-dried cytoskeletons show only individual actin filaments in lamellipodia, where high voltage electron microscopy shows only microtrabeculae, which suggests that some aspect of the preparative

method imposes a trabecular appearance on the actin; furthermore, first fixing and then extracting cells before rapid-freezing and etching them partially agglutinates and coarsens the appearance of filaments so that their discrete nature is obscured (18).

One can still ask, however, whether the discrete nature of the filaments in freeze-dried cytoskeletons is itself a result of detergent extraction and fixation. Since the views of directly frozen, fractured, and etched fibroblasts have been difficult to interpret (18), it has remained unclear whether intact cells contain interconnecting cytoplasmic elements in addition to the discrete components of the cytoskeleton.

We suggest that one important reason why our images of axonal cytoplasm prepared by direct freezing, fracturing, and etching are comprehensible, while corresponding views from fibroblasts are not, is because microtubules and intermediate filaments are specifically oriented in axons. A second reason is that minimizing the etching avoids the collapse of cytoplasmic components. We are thus able to offer a third view of the organization of native cytoplasm, based on direct freezing and relatively shallow etching. Our results show that the cytoplasmic domains around neurofilaments, around microtubules, and adjacent to the axonal membrane can each be distinguished by their structural organization in freeze-etched and freeze-substituted axons. We therefore regard the notion of a morphologically homogeneous microtrabecular lattice (11) as inadequate for axonal cytoplasm. On the other hand, to consider only the discrete axoplasmic filaments is to disregard the fact that the matrix between them is heterogeneous and can be structured more (in neurofilament-associated axoplasm) or less (in microtubule-associated axoplasm). It is now important to identify the macromolecules present in each of the three types of cytoplasmic zones and to define the factors controlling their assembly and interaction.

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