Cytoskeletal Architecture and Immunocytochemical Localization of Microtubule-associated Proteins in Regions of Axons Associated with Rapid Axonal Transport: The β,β'-iminodipropionitrile-Intoxicated Axon As a Model System

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ABSTRACT  
Axons from rats treated with the neurotoxic agent β,β'-iminodipropionitrile (IDPN) were examined by quick-freeze, deep-etch electron microscopy. Microtubules formed bundles in the central region of the axons, whereas neurofilaments were segregated to the periphery. Most membrane-bounded organelles, presumably including those involved in rapid axonal transport, were associated with the microtubule domain. The high resolution provided by quick-freeze, deep-etch electron microscopy revealed that the microtubules were coated with an extensive network of fine strands that served both to cross-link the microtubules and to interconnect them with the membrane-bounded organelles. The strands were decorated with granular materials and were irregular in dimension. They appeared either singly or as an extensive anastomosing network in fresh axons. The microtubule-associated strands were observed in fresh, saponin-extracted, or aldehyde-fixed tissue.

To explore further the identity of the microtubule-associated strands, microtubules purified from brain tissue and containing the high molecular weight microtubule-associated proteins MAP 1 and MAP 2 were examined by quick-freeze, deep-etch electron microscopy. The purified microtubules were connected by a network of strands quite similar in appearance to those observed in the IDPN axons. Control microtubule preparations consisting only of tubulin and lacking the MAPs were devoid of associated strands. To learn which of the MAPs were present in the microtubule bundles in the axon, sections of axons from IDPN-treated rats were examined by immunofluorescence microscopy using antibodies to MAP 1A, MAP 1B, MAP 2, and tubulin. Anti–MAP 2 staining was only marginally detectable in the IDPN-treated axons, consistent with earlier observations. Anti–MAP 1A and anti–MAP 1B brightly stained the IDPN-treated axons, with the staining exclusively limited to the microtubule domains. Furthermore, thin section–immunoelectron microscopy using colloidal gold–labeled second antibodies revealed that both anti–MAP 1A and anti–MAP 1B stained fuzzy filamentous structures between microtubules. In view of earlier work indicating that rapid transport is associated with the microtubule domain in the IDPN-treated axon, it now appears that MAP 1A and MAP 1B may play a role in this process. We believe that MAP 1A and MAP 1B are major components of the microtubule-associated fibrillar matrix in the axon.
tubules and neurofilaments in transport is as yet not understood.

Numerous reports of filamentous elements connecting membrane-bounded organelles with microtubules or neurofilaments have appeared (8, 9, 17, 29, 31). Presumably, within this system of cross-links resides the machinery responsible for organelle transport. Ellisman and Porter (8) described a uniform microtubecular network of fine, anastomosing fibers throughout the axoplasm, interconnecting microtubules, neurofilaments, and membrane-bound organelles, and suggested a role for this network in transport. Using the quick-freeze, deep-etch method of electron microscopy, Hirokawa (17) also described a system of fine strands that form a variety of cross-links among microtubules, membranous organelles, and neurofilaments. In addition, a subaxolemmal network including actin-like filaments was found (17, 29). The prominence of cross-bridges between microtubules and membranous organelles observed in this study led to the hypothesis that these particular cross-links are in some way involved in fast transport. In a similar study, however, Schnapp and Reese (29) concluded that the microtubules are embedded in a granular, rather than a fibrillar matrix, and argued the appendages associated with one end of membrane-bounded organelles may, instead, be responsible for the forces that transport organelles. Early evidence based on the use of microtubule-disrupting agents led to conflicting conclusions regarding the role of microtubules in axonal transport (see, for example, reference 1 vs 2). However, more recent studies involving the use of /3,2'-iminodipropionitrile (IDPN) have strongly implicated microtubules rather than neurofilaments in rapid transport. Whereas in normal axons microtubules and neurofilaments are interspersed, the two types of filament were observed to become segregated in axon of IDPN-treated rats, with microtubules forming one or more bundles located centrally in the axon, and the neurofilaments being restricted to the periphery (11, 26). Using electron microscopic autoradiography, it was demonstrated that rapid axoplasmic transport was similarly restricted to the microtubule domains (11, 28).

Because the microtubule domain of the IDPN-treated axon represents a simplified cytoskeleton still containing all of the components necessary to support fast axonal transport, a detailed description of the structure and protein composition of this region should aid our understanding of the mechanism of fast transport. It seems reasonable to expect that among the protein components of the microtubule domain would be microtubule-associated proteins (MAPs) (14, 22, 25, 30, 36, 37). The two most abundant MAPs in brain tissue are high molecular weight proteins that have traditionally been classified into two groups referred to as MAP 1 and MAP 2. These proteins have the appearance of arms projecting from the outer surface of purified microtubules (14, 22, 35).

Both MAP 1 and MAP 2 are now known to consist of multiple distinct proteins (5). MAP 2 consists of two polypeptides (22) that are immunologically cross-reactive (34). MAP 1 consists of three proteins (5). The principal MAP 1 species in brain, MAP 1A, was found to be a widespread component of microtubules in many cell types and was clearly detectable in axons by immunofluorescence microscopy of neuronal tissue (4, 5). The second most abundant MAP 1 polypeptide, termed MAP 1B, has recently also been found to be present in axons, and to be immunologically distinct from MAP 1A (4a). In contrast to this work, it was found that MAP 2 was restricted in its distribution (5, 6, 24). In brain tissue, this protein was found by immunocytochemical means to be prominent only in the dendritic processes and perikarya of neurons (23). The low levels of MAP 2 found biochemically in white matter (33) were not detected by immunofluorescence microscopy in this study. More recent evidence by Papasozomenos and co-workers (27), however, indicated that MAP 2 could, in fact, be marginally detected in spinal nerve roots by immunocytochemical means.

The localization of MAP 1A, MAP 1B, MAP 2, and other MAPs in IDPN-treated axons should provide a means for assessing which of these proteins might be involved in fast transport.

In the study presented here, we have used three approaches to examine the structure of axons from IDPN-treated rats. First, we used the quick-freeze, deep-etch method of electron microscopy to determine whether microtubule-associated cross-links present in regions devoid of neurofilaments. Second, we performed immunofluorescence microscopy with antibodies to tubulin, MAP 2, MAP 1A, and MAP 1B to determine where these proteins are located in the IDPN-treated axon. Third, we observed the localization of MAP 1A and MAP 1B in the IDPN-treated axon by electron microscopic immunocytochemistry using colloidal gold second antibodies. We report here that in axons of IDPN-treated rats the microtubule domain is composed of an anastomosing network of fine strands, and that membranous organelles are associated with a structurally complex network of microtubule-associated cross-links. These cross-linking elements are likely to include MAP 1A and MAP 1B.

MATERIALS AND METHODS

IDPN Administration: IDPN (Eastman Kodak Co., Rochester, NY) in rat physiological saline (155 mM NaCl, 5 mM KCl, 5 mM HEPES, 0.18% glucose, 2 mM MgCl2, 4 mM CaCl2, 0.5 mM Na2HPO4 at pH 7.2) was injected into male Sprague-Dawley rats (100-150 g). Each animal received a total of 2 mg of diluted IDPN per gram body weight in four equal injections spaced at 3-d intervals (26). Saline was substituted for the IDPN solution in controls, and animals were sacrificed 3 wk after the final injection.

Quick-Freeze, Deep-Etch Electron Microscopy of Axons: Three types of tissue samples (sciatic nerves) were used: fresh, saponin-extracted, and fixed. Saponin-extracted samples were prepared by incubating unfixed tissue for 30 min at room temperature in 0.1% saponin, 70 mM KCl, 5 mM MgCl2, 3 mM EGTA, 30 mM HEPES pH 7.4, 10 mM taurine, and 0.1 mM phenylmethylsulfonyl fluoride. The fixed tissue was obtained from animals perfused with paraformaldehyde as described for immunofluorescence (see below), further fixed with 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, and was washed thoroughly with distilled water before freezing. In the cases of fresh tissues or saponin-extracted tissues, the sciatic nerves were dissected out, and the epineurial sheath was opened with a small scissors. Thus, the bundles of axons were exposed. They were quick-frozen freshly or after saponin treatment. The fixed nerves were cut by razor blades into half and frozen. Quick-freezing, deep-etching, and platinum replica formation were accomplished as described previously (16, 18). Replicas were examined on a JEOL 100CX or 1,200 EX electron microscope (JEOL, Tokyo, Japan) at 100 kV with a tilt of ± 10.

Quick-Freeze, Deep-Etch Electron Microscopy of Purified Microtubules: Microtubules were prepared from calf brain tissue (whole cerebrum) by two cycles of assembly-disassembly purification (36) and stored frozen as the second microtubule pellet. The microtubules were thawed, resuspended to 2.5 mg/ml in 0.1 M PIPES, pH 6.6, containing 1.0 mM EGTA, 1.0 mM MgSO4, 0.1 M PIPES, pH 6.6, containing 1.0 mM EGTA, 1.0 mM MgSO4, 0.1 M PIPES, pH 6.6, containing 1.0 mM EGTA, 1.0 mM MgSO4, and stored at -20°C for up to 1 mo. The stock solution was diluted 1:1 with physiological saline containing 0.1 M PIPES, pH 6.6, and warmed to 37°C before use. The solutions were handled and dispensed under strict anaerobic conditions.
Suitable conditions for electron microscopy. Sections were assembled by incubation of the solution for 10 rain at 37°C. Taxol was then added to 20 mM to stabilize the microtubules, and the sample was split into two aliquots. To one, NaCl was added to 0.35 M to dissociate the MAPs (33), and both aliquots were then centrifuged through a layer of 10% sucrose in the respective buffers solutions. The two microtubule pellets were resuspended in PEMG buffer containing 40 μM taxol and were re-sedimented. The pellets were removed from the centrifuge tubes and subjected to rapid freezing as for the axonal tissue. All centrifugations were for 30 min at 37,000 g.

Electron Microscopic Immunocytochemistry: Rats were anesthetized with chloral hydrate and perfused transcardially with 1% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Segments of sciatic nerve and spinal cord were dissected, incubated an additional 4 h with fixative at room temperature, cut into small pieces, and incubated for 3-h periods in the phosphate buffer containing, sequentially, 5, 10, and 20% sucrose. The samples were then frozen in liquid freon and cut into 6-8 μm sections on a Darnon cryostat (Darnon Corp., Needham Heights, MA). Thorough washes with phosphate-buffered saline followed for the unextracted axons, the cross-linkers interconnecting neurofilaments were straight and more nearly uniform in length and width than were the cross-linkers interconnecting microtubules, which were relatively variable in length. The anastomosing character of much of the material interconnecting microtubules was much more obvious in saponin-treated axons (Fig. 5, A and B) than it was in unextracted axons (Fig. 2). In addition to material of this appearance, single, single-stranded cross-links between microtubules were also seen.

Cross-links between microtubules and membrane organelles were also preserved after saponin extraction (Fig. 6). As the microtubule-microtubule interconnections, these too were variable in length and consisted of a mixture of anastomosing and straight elements.

Axons from control and IDPN-treated rats were also frozen and deep-etched after prior aldehyde fixation and washing with distilled water (not shown). The appearance of this material was not significantly different from that of material frozen without prior treatment, except that the protofilamentous structure of the microtubules was no longer evident (17).

Quick-freeze, Deep-Etch Electron Microscopy of Purified Microtubules

To compare the appearance of the microtubule-associated microtubules, 25 nm in diameter, showed longitudinal arrays of protofilaments on their outer surface (15) and were easily discernible. Membranous organelles, including mitochrondria, smooth endoplasmic reticulum, and vesicles of various sizes were associated with the microtubule bundles (Figs. 1-3). Although some mitochondria and elements of the smooth endoplasmic reticulum were located in the boundary between the microtubule bundle and neurofilament domain (Fig. 1), in general, most vesicles existed very close to microtubules (Figs. 1-3).

Extensive cross-links were observed between microtubules, between neurofilaments, and between microtubules and membranous organelles (Figs. 1-3). In addition, bridges were observed between microtubules and neurofilaments at the boundaries of the microtubule and neurofilament domains. The microtubule-associated cross-links were more granular in appearance than were the cross-links between neurofilaments. In addition, the microtubule-associated structures branched and appeared in many areas to form an anastomosing network in contrast to the simpler interneurofilament bridges. The cross-links were best seen in saponin-extracted myelinized axons from control and IDPN-treated rats and IDPN-extracted myelinated axons of a sciatic nerve from an IDPN-treated rat. Saponin extraction preserved both types of cross-bridges. The cross-bridges were considerably less granular in appearance than they were in unextracted axons (Figs. 1-3), and their detailed structure could be more readily examined. As noted above for the unextracted axons, the cross-linkers interconnecting neurofilaments were straight and more nearly uniform in length and width than were the cross-linkers interconnecting microtubules, which were relatively variable in length. The anastomosing character of much of the material interconnecting microtubules was much more obvious in saponin-treated axons (Fig. 5, A) than it was in unextracted axons (Fig. 2). In addition to material of this appearance, simple, single-stranded cross-links between microtubules were also seen.
Figure 1. Quick frozen, deep etched fresh myelinated axon in the sciatic nerve from IDPN-treated rat. Microtubules (MT) form a large bundle in the center of the axoplasm. Neurofilaments (NF) become redistributed to the peripheral portion of the axon and cross-linked by numerous bridges. Membrane bounded organelles presumably conveyed by fast axonal transport (long arrows) exist in channels surrounded with microtubules. Mitochondria (M) and smooth endoplasmic reticulum (short arrows) also tend to localized in microtubule channels or at the boundary between microtubule domain and the neurofilament lattice. Note that the microtubules are linked with each other and with membranous organelles by cross-bridges that are granular in appearance. Bar, 0.1 μm. × 50,000. (Inset) A high magnification view of a membrane-bounded organelle cross-linked with microtubules. Bar, 0.1 μm. × 164,000.
material observed in axons with microtubule-associated proteins in vitro, microtubule proteins purified from brain tissue were assembled, centrifuged, and examined by quick-freeze, deep-etch electron microscopy (Fig. 7). Microtubules containing MAPs were covered by a filamentous network strikingly similar to that which we observed in axons (Fig. 7A). The
filaments contained both anastomosing and simple elements, as observed in the microtubule domains of axons from IDPN-treated rats. In samples exposed to elevated ionic strength conditions, which dissociates the MAPs from the microtubule surface (33), the filamentous network associated with the microtubules was absent (Fig. 7 B).

**Immunocytochemical Localization of the Component Proteins of Microtubules in IDPN-treated Axons**

To identify the component proteins of the microtubule domain of axons from IDPN-treated rats, we stained sections of neuronal tissue with antibodies to tubulin and to three different high molecular weight MAPs found in brain tissue: MAP 1A, MAP 1B, and MAP 2.

In control rats that had not been exposed to IDPN, anti-tubulin stained the cytoplasm of both central and peripheral nervous system axons uniformly, as did anti–MAP 1A (5). Anti–MAP 1B also stained the cytoplasm of axons uniformly (Fig. 8 B). Anti–MAP 2 staining (not shown) was undetectable in white matter in general (5, 6, 24), though marginal staining of some peripheral axons could be seen as reported by Papasozomenos et al. (27).

IDPN caused a redistribution of the microtubule protein components (Fig. 8). In IDPN-treated axons, anti-tubulin staining appeared as a bright spot (or sometimes several closely spaced smaller bright spots) located centrally in the axon (Fig. 8 D and G), consistent with the location of microtubules seen by electron microscopy (Figs. 1 and 2; reference 26). Double labeling with anti-tubulin and anti–MAP 1A (Fig. 8 E) revealed that the distribution of tubulin and MAP 1A were identical. Similarly, double-labeling with anti-tubulin and anti–MAP 1B (Fig. 8 H) revealed that the distributions of these two proteins were identical. In contrast, anti–MAP 2 showed two staining patterns, both marginally detectable relative to the patterns observed with the other antibodies. Anti–MAP 2 was sometimes observed to stain weakly the marginal portion of axons in IDPN-treated rats (data not shown). This confirms a similar observation of Papasozomenos et al. (27) and is consistent with the observation of Bloom and Vallee (3) that MAP 2 could be found associated with both microtubules and intermediate filaments in cultured brain cells. We did not consistently observe staining of the peripheral portion of the axon in IDPN-treated rats, but also observed weak, uniform staining throughout the axonal cross-section (data not shown). We do not know the basis for the variability in these results. However, at no time did we observe co-localization of MAP 2 with the microtubule domain in the IDPN-treated axon.

In a separate experiment (data not shown) designed to reproduce the conditions used for electron microscopy (Figs. 1, 5, and 6), frozen sections were prepared from fresh tissue extracted with saponin. The pattern of staining observed in the axons of IDPN-treated rats using all four of the antibodies was identical to that observed without saponin extraction (see preceding paragraph).

**Electron Microscopic Immunocytochemical Localization of Anti–MAP 1A and Anti–MAP 1B in the IDPN-treated Axons**

To further clarify where the MAP 1A and MAP 1B localize in the microtubule domain, we performed electron microscopic immunocytochemistry. As shown in Fig. 9, gold particles were found mostly on the microtubule domain in the sections incubated with anti–MAP 1A or anti–MAP 1B. In both cases, most of the gold particles tended to localize on the fuzzy filamentous structures or spaces between microtubules, whereas some localized on the microtubules (Fig. 9). In control sections, gold particles were rarely found in the axon (Fig. 10).
FIGURE 5 A high magnification comparison between microtubule domain (A) and neurofilament lattice (B) from an IDPN-intoxicated myelinated axon that was extracted with saponin. Cross-bridges are still seen between microtubules and are less granular in appearance than without saponin extraction. The cross-bridges tend to form a branching and anastomosing network (long arrows in A). In contrast, the cross-links between neurofilaments tend to be straight. Cross-connections between microtubules and neurofilaments can also be seen (short arrows in A). Bar, 0.1 μm. × 147,000.

DISCUSSION

We now report the existence of a network of cross-linking elements between microtubules, between neurofilaments, and membranous organelles, using the quick-freeze, deep-etch method of electron microscopy in the axons of IDPN-treated rats. The characteristics of these structural elements are somewhat obscured in the normal axon because of the intermin-
FIGURE 6 A high magnification view of microtubules domain in a saponin-treated myelinated axon from IDPN-treated rat. Although the membrane is somewhat affected, cross-bridges between a membrane organelle (thick arrow) and microtubules are clearly preserved (short arrow). Arrowheads, a part of a membrane organelle affected by saponin. Bar, 0.1 μm. x 159,000.

Gling of microtubules and neurofilaments (17, 29). However, because of the spatial segregation of organelles in the axon of IDPN-treated rats, the existence of bridges connecting microtubules with each other and with membrane-bounded organelles has now been clearly revealed.

We found, in addition, that the high molecular weight microtubule-associated proteins MAP 1A and MAP 1B (5) were associated with the microtubule domains in the axons of IDPN-treated rats, in contrast to MAP 2. The co-localization of MAP 1A and MAP 1B with tubulin at the light microscope level, and electron microscopic immunocytochemical data showing that the anti-MAP 1A and anti-MAP 1B stained fuzzy filamentous structures between microtubules in the present study suggest that both of these proteins are part of the elaborate cross-bridging system observed in the microtubule domain of the IDPN-treated axon. Our observations with MAP 2 are consistent with earlier work indicating that this MAP alone is diminished in amount, though not completely absent (33), in axons relative to dendrites (6, 24, 33). In addition, the co-localization of MAP 2 with neurofilaments that we observed using a polyclonal antibody to this protein, appears to confirm an earlier report using a monoclonal anti-MAP 2 (27). Although we have not consistently obtained this result and have also seen uniform staining of axonal cross-sections with anti-MAP 2, we have never observed staining limited to the microtubule domain of the IDPN-treated axon. Thus, we conclude that MAP 2 is, at most, part of the system of cross-bridges observed in the microtubule domain of the IDPN-treated axon.

In further support of our contention that the MAPs represent the microtubule-associated cross-bridges observed in the IDPN axon are our results obtained with quick-freeze, deep-etch electron microscopy of purified brain microtubules (Fig. 6). The purified microtubules were coated with an elaborate network of fine strands that could be removed from the microtubules under conditions that were previously shown to dissociate MAPs from microtubules (33). The ultrastructural appearance of the microtubule-associated fibers in the purified microtubules was remarkably similar to that of the microtubule-associated system of cross-links in the IDPN-treated axon. Both the simple, straight cross-links and the anastomosing cross-links of variable length observed in situ were observed in the purified microtubules, suggesting that both types of structure were composed of proteins present in the purified microtubules. Since the purified microtubules contain MAP 2 as the most abundant nontubulin species (see, for example, reference 33), while this protein was undetectable in the microtubule domain of the IDPN axon, the comparison between the two systems is not exact. However, it is known that the MAP 1 polypeptides represent arms on the microtubule surface as observed by thin section electron microscopy (35). Presumably, then it is MAP 1A and MAP 1B that are observed to be associated with microtubules in the IDPN-treated axon.

Ultrastructure of Cross-Linking Network

Because of the spatial segregation of microtubules and neurofilaments in the axons of IDPN-treated rats, we have been able to distinguish different classes of cross-linking elements between microtubules and between neurofilaments. The neurofilament–neurofilament cross-links appeared to be uniform in length and to be more periodic in their association with the neurofilament surface. Branching of these elements was rare. Such elements had been obvious from earlier work (17, 20), owing to the existence of neurofilament-rich regions even in normal axons. Microtubule-associated cross-bridges were also observed (17). However, as a result of the small size of the microtubule bundles found in the normal axon and the close proximity of other axoplasmic organelles, the detailed structure of the cross-bridges could not readily be evaluated.
In the present study, we have been able to characterize the microtubule-associated material in more detail. We have found that much of the material interconnecting microtubules and linking these structures to membrane-bounded organelles was represented by a rather complex, anastomosing network of cross-bridges, though a fraction of these elements had the...
simpler, straighter appearance of the interneurofilament crossbridges. The microtubule-associated strands had a relatively thick, somewhat granular appearance compared with the neurofilament-associated strands, even in axons extracted with saponin to remove soluble proteins.

Whereas the interneurofilament cross-bridges have also been seen by Schnapp and Reese (29) in their investigation of normal axons, these workers observed what they referred to as a loose granular matrix associated with microtubules. Cross-bridges between microtubules and mitochondria were observed, but the existence of such structures between microtubules and other membrane organelles was questioned (29). We cannot fully account for the disparate descriptions of axonal ultrastructure in the different studies. However, the present study indicates the microtubule cross-bridging material to be quite reproducible in appearance. In addition, it is quite similar to what we observe in purified microtubule preparations which contain only MAPs plus tubulin, and, because of the use of taxol in our preparations, virtually no soluble protein. These observations support our contention

**FIGURE 8** Immunofluorescence microscopy of cross-sections of rat sciatic nerves double-labeled with antibodies to tubulin and MAPs. Rats were perfused with paraformaldehyde, and pieces of tissue were further fixed before preparation of frozen sections. (A) Nonintoxicated axons stained with guinea pig anti-tubulin. (B) Same section stained with mouse monoclonal anti-MAP IB. (C) Same section, phase contrast image. (D) IDPN-intoxicated axons stained with anti-tubulin. (E) Same section stained with anti-MAP 1A. (F) Same section, phase contrast image. (G) IDPN-intoxicated axons stained with anti-tubulin. (H) Same section stained with mouse monoclonal anti-MAP 1B. (I) Same section, phase contrast image. (J) IDPN-intoxicated axon stained with preimmune guinea pig IgG. (K) Same section stained with unconditioned hybridoma medium. (L) Same section, phase contrast image.
that the intermicrotubule cross-links that we observed exist in the microtubule domain in the axon. As we observed in this study, the cross-bridges were frequently decorated by granular materials most of which may be soluble proteins in fresh axons. Therefore, shallow etching (29) may not lower water table deep enough to reveal the true anastomosing and cross-linking nature of the microtubule-associated network so that it probably picks up only the granular nature of cross-
bridges covered with some soluble proteins.

The anastomosing character of the microtubule-associated material in our preparations has not been previously observed in rapidly frozen, deep-etched preparations of mammalian neurons. However, quite similar images have been obtained with rapidly frozen, deep-etched preparations of crayfish giant axons (19). Microtubules are prominent in these axons, whereas neurofilaments appear to be entirely absent. In the IDPN-treated axons, it was possible to observe the microtubule-associated material with some clarity. An anastomosing network of somewhat granular strands was observed to cross-link microtubules with each other and with membranous organelles in the crayfish axon, much as in the axon of the IDPN-treated rat. Because of the large phylogenetic gap between crayfish and mammalian systems and because the giant axons conceivably have some unique properties, the finding of a similar microtubule-associated cross-linking system in rats is of considerable importance and interest.

The existence of an anastomosing microtrabecular system throughout the axon (8) as well as in other cells (38) has been reported. How this system of fibers is related to those reported here is not entirely clear because of the radical differences in electron microscopic technique that are involved. In the earlier work of Porter and co-workers (8, 38), chemical fixation was used. In contrast, the meshwork of anastomosing material observed by us in the microtubule domain of the IDPN-treated axon was found even in unfixed and unextracted tissue. The cross-bridges were also observed in extracted tissues or fixed tissues washed with distilled water before freezing. This indicates that the meshwork is not simply an artifact of fixation or of the condensation of salts or soluble proteins onto the microtubule surface during sample preparation. It is equally interesting that no such meshwork was observed in the neurofilament domain of the IDPN-treated axon. This argues that a microtrabecular lattice is not a uniform entity distributed throughout the axoplasm. Furthermore, it argues against an artifactual origin for the microtubule-associated cross-bridges.

It also seems to be worth noting that not all of the interconnections between microtubules were of the anastomosing type. Simple bridges were also seen. Whether these are chemically different or represent a different functional state of the anastomosing meshwork cannot be stated as yet.

Implications for the Mechanism of Axonal Transport

Although the question is not completely settled, much recent information suggests that it is microtubules rather than intermediate filaments that are involved in rapid axonal transport. As noted above, active vesicle transport occurs in the crayfish giant axon, which contains no neurofilaments (9, 10). Direct evidence for the involvement of microtubules in vesicle transport in non-neuronal cells was obtained by Hayden et al. (13), who were able to observe particle movement in direct association with individual cytoplasmic filaments which were identified as microtubules using immunofluorescence microscopy. Finally, as noted throughout this paper, it has been found using electron microscopic autoradiography that rapid axonal transport occurs in association with the microtubule domain of IDPN-treated axons (11, 28).

While these studies strongly implicate microtubules in axonal transport, the mechanism of this phenomenon is little understood beyond that point. In the present study, as in that of Papasozomenos et al. (26), it was observed that membranous organelles were localized with the microtubule domain of the IDPN-treated axon. While some of these organelles were found in the region between the microtubule and neurofilament domains, it seems likely, in view of the earlier work discussed in the previous paragraph, that it is the association with microtubules that is relevant to the question of the mechanism of transport.

Membranous organelles were associated with microtubules via cross-bridges of similar appearance to those observed to interconnect microtubules. Presumably these cross-links are involved in the movement of the membranous organelles. We cannot say whether the cross-links that were observed were involved in force production or, conversely, whether they represented associations formed with immobile particles.

Some recent evidence (21) has implicated actin in axonal transport, and an association between actin filaments and MAPs in vitro has also been reported (12). Thus, there is some suggestion that acto-myosin in association with microtubules could be involved in transport. In the present report, ultrastructural evidence for typical F-actin in the microtubule domain of the IDPN axon was not obtained. Although actin-like filaments could be readily identified subjacent to the plasma membrane in axons (17, 29), we failed to see actin filaments in the meshwork of interconnecting fibers in the microtubule domain of the IDPN-treated axon. It is conceivable that the observed meshwork contains a form of actin that has not been previously described. Further experiments will be required to test this possibility.

It should also be mentioned that at least two laboratories have suggested on the basis of work with a pharmacological agent specific for the ciliary and flagellar enzyme dynein, that a dynein-related molecule could be involved in rapid organelle transport.
transport (10, 32). We have seen no obvious evidence for regular dynein arms in the microtubule domain of the IDPN-treated axon similar to those seen in cilia and flagella. However, we cannot rule out the possibility that a dynein-like molecule, perhaps of different morphology from the axonemal protein, is present at low abundance in the axon.

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