Polarity Orientation of Axonal Microtubules

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ABSTRACT   The polarity orientation of cellular microtubules is widely regarded to be important in understanding the control of microtubule assembly and microtubule-based motility in vivo. We have used a modification of the method of Heidemann and McIntosh (*Nature* (*Lond.*). 286:517–519) to determine the polarity orientation of axonal microtubules in postganglionic sympathetic fibers of the cat. In fibers from three cats we were able to visualize the polarity of 68% of the axonal microtubules; of these, 96% showed the same polarity orientation. Our interpretation is that the rapidly growing end of all axonal microtubules is distal to the cell body. We support Kirschner's hypothesis on microtubule organizing centers (*J. Cell Biol.* 86: 330–334), although this interpretation raises questions about the continuity of axonal microtubules. Our results are inconsistent with a number of models for axonal transport based on force production on the surface of microtubules in which the direction of force is determined by the polarity of microtubules.

It seems likely that microtubules play an important role in determining cell morphology and in intracellular transport processes. However, the control of microtubule assembly to provide an organized cytoskeleton and the function of microtubules in many intracellular transport phenomena are poorly understood (9). The structural and growth polarity of microtubules has received considerable attention as a potential clue in understanding these microtubule functions (20, 25, 27, 36, 38, 42). Microtubule assembly occurs by a polar "head to tail" mechanism (2, 24). Kirschner (20) has proposed that this property could ensure organized assembly of microtubules if the concentration of tubulin in the cell were such that filaments with two free ends tend to depolymerize. Only filaments anchored in a microtubule organizing center at the slowly growing end, supressing depolymerization at that end, would be stable. Kirschner's hypothesis makes the strong prediction that all stable microtubules in the cell should have the same polarity orientation relative to their organizing center (20). Predictions of polarity orientation are also a testable feature of many models to explain microtubule-based motility. Models for motility based on microtubule sliding (27, 42), assembly/disassembly (25), rotation (14, 37), and microtubules acting as a chromatographic support (16), all make predictions of the polarity orientation of various populations of microtubules.

We have chosen to investigate the polarity orientation of axonal microtubules for the following reasons. Microtubules are an invarient and conspicuous component of axoplasm (51). These microtubules are responsible for the shape and possibly the growth of the axon (33, 35, 39, 52). The very anisotropic morphology of the axon indicates considerable spatial organi-

zation of microtubule assembly/disassembly in the neuron. Axonal microtubules are unusually stable (9, 31), thereby fulfilling the thermodynamic premise of Kirschner's proposal (20). The axon is also the site of intracellular transport both toward and away from the cell body (23, 49). Evidence from a variety of studies suggests that the same mechanism underlies transport in both directions (23). Many investigators believe that microtubules are important for this mechanism, although this is controversial (4, 7). At least four models to explain fast axonal transport based on microtubule function state or imply a specific polarity orientation of microtubules in the axoplasm for a given direction of transport (16, 30, 36, 38). We have determined the polarity of microtubules in axons of cat sympathetic nerve fibers using Euteneuer's modification (11) of the method of Heidemann and McIntosh (19). Microtubule polarity is visualized by this method in the electron microscope by the handedness of protofilament "hooks" that form on the walls of cellular microtubules during a microtubule assembly reaction in vitro (11–13, 18, 19).

MATERIALS AND METHODS

Cats (*Felis domesticus*) were anesthetized by intraperitoneal injection with sodium pentobarbitol (35 mg/Kg) or alpha chloralose (60 mg/Kg). Segments of sympathetic fibers arising from the inferior mesenteric ganglion that course to the colon (lumbar colonic nerves) as well as fibers from the celiac superior mesenteric ganglion to the kidney (renal nerves) were dissected free of adhering tissue *in situ*. Drs. J. Krier and L. C. Weaver of this department provided invaluable assistance in the dissection and identification of these fibers. The fibers were ligatured with suture at three or four places ~2 mm apart. Segments were then excised so that a final ligature marked the end of the segment proximal to the ganglion.

Segments were transferred to 0.5 ml of a microtubule polymerization buffer at 37°C containing 0.5 M PIPES pH 6.9, 1 mM EDTA, 2 mM MgCl$_2$, 1 mM GTP, 5% dimethylsulfoxide, 1% Triton X-165, 0.5% sodium deoxycholate and 0.2% SDS with or without microtubule protein (MTP). The preparation of MTP buffer, and the polymerization properties are detailed elsewhere (18). A variety of protein concentrations and incubation conditions were tested. Our best results were obtained when nerve segments were incubated for 10 min in buffer without microtubule protein, then transferred into fresh buffer containing 2 mg/ml MTP for 10 min followed by a final 10 min with a fresh change of buffer and protein. We have previously shown that existing microtubules are stable in this buffer without added MTP (18).

After these incubations the assembly buffer was withdrawn and the segments were fixed by addition of 2% glutaraldehyde, 1% acrolein in 0.1 M PIPES pH 6.9, 2 mM MgCl$_2$ and 1 mM EDTA. Samples were osmicated, dehydrated, and embedded for electron microscopy by standard methods. Segments were thin-sectioned from the distal toward the proximal end. For this work, transverse sections of microtubules were required to assess hook handedness. We found that only a small fraction of axons had their long axis normal to the plane of sectioning at any given knife angle. Consequently, all samples were sectioned at several different knife angles. Care was taken to preserve the original handedness of the hooks during the procedures of sectioning, viewing in the Philips 300 electron microscope, and printing of negatives. Counts of microtubules and scoring of hooks were done on prints with a final magnification of 40,000 times. Various hook images and scoring categories are presented elsewhere (11).

RESULTS

Nerve segments from three cats were used to compile the data reported here. In the first cat, the renal nerve was excised, incubated in assembly buffer containing 1 mg/ml MTP for 15 min, then processed for electron microscopy. The lumbar colonic nerves were used from the remaining cats with the incubation procedure presented in Materials and Methods. Electron microscopy of thin sections perpendicular to the long axis of the nerve revealed protofilamentous hooks decorating the walls of the axonal microtubules (Figs. 1 and 2). Counts of microtubules from ~100 axons from each cat are summarized in Table I. In these axons, 68% of the microtubules displayed hooks and >95% of the hooked microtubules were observed to curve clockwise. Heidemann, Euteneuer, and McIntosh (11–13, 18, 19) have shown that such hooks reveal the structural polarity of microtubules. Hooks that curve clockwise are seen in transverse sections of microtubules whose rapid assembly or plus end (3) is pointed toward the observer. Our data therefore indicate that nearly all the microtubules that formed hooks have a single polarity orientation.

We noted that different regions along the long axis of each sample showed considerable variability in the percentage of microtubules with hooks. This is better understood by considering our observations in sample three. Sample three was a segment of nerve 1.55 mm long. 41% of the microtubules were found to be decorated in sections taken from the distal 8 μm (a good deal of material was wasted by the changing of knife angles) of this sample. The data reported in Table I were taken from the next 10 μm of the sample. In the region ~40 μm from the distal end of the nerve, 25% of the microtubules displayed hooks. Near the center of the segment, 12% of the microtubules were hooked. The other two samples showed similar variability. For this reason, the data reported here were taken from that region of each sample which showed a relatively large fraction of decorated microtubules.

DISCUSSION

Lumbar and thoracic sympathetic fibers of the cat were a favorable material for this study for a number of reasons. First, the cell bodies of all afferent and efferent neurons in these studies are known to be located central (proximal) to the

segment (10, 28). By marking the proximal end of each segment *in situ*, we have been able to interpret the polarity of the axonal microtubules relative to the cell nuclei. Second, these sympathetic fibers have been studied with respect to axonal transport; both anterograde (nucleus to terminal) and retrograde transport occur in these nerves (1, 10, 21). Third, the great majority of axons in these nerves are unmyelinated, allowing cell lysis and penetration of the hog brain tubulin to form hooks. Preliminary experiments with myelinated nerves were completely unsuccessful.

We observed that 96% of the axonal microtubules that formed hooks had a single polarity orientation. Their fast growing or plus end is distal to the cell body. Indeed, we believe that all of the axonal microtubules have this same polarity. Those few decorated microtubules that did not have clockwise hooks likely represent 'noise" in the technique. We found in earlier studies that as many as 10% of decorated microtubules of known polarity may display anomolous hooks (19). What of the microtubules that do not display hooks? Are these microtubules of opposite polarity orientation with respect to those that did form hooks? We think not. We believe that the microtubules that were undecorated represent variation of the technique and not an intrinsically different class of microtubules. We have found, as have Euteneuer and McIntosh (11–13), that the extent of microtubule decoration varies considerably with incubation conditions. The variability seen in the experiments reported here also supports the notion that the variation in hook formation is due to technical variability, not microtubule variation. We observed that hook formation varied among regions along the axis of the fiber but not within regions. If this variability was due to microtubule population differences, then these microtubule populations must be in register in each axon along the axis of the fiber. This seems very unlikely. Because the axons are known to be continuous, different axons cannot be present in different areas of the nerve. We are of the opinion that the variability in these experiments was due to problems of buffer penetration. Throughout these experiments, changes in incubation conditions that were consistent with better penetration, e.g., longer times of incubation, fresh changes of detergent mixture, increased tubulin concentration, etc., produced higher frequencies of hook formation. Moreover, we have no evidence that microtubules of opposite polarity have a lesser tendency to decorate. We previously showed that microtubules that were elongated from the proximal end of basal bodies had a similar frequency of hook formation as those elongated from the distal end (19). Therefore, we would suggest that the most likely interpretation of our data is that all of the microtubules in the nerve axon have the same polarity orientation. It is the fast growing or plus end that is distal to the cell nucleus. While this manuscript was in preparation, the same conclusion was reached by Burton and Paige using frog olfactory nerves (6). The similar findings in neurons that differ in embryological origin from organisms of different classes suggest that uniform polarity of axonal microtubules may be a general case.

Kirschner's (20) view of microtubule organizing centers (MTOCs, reference 34) has received considerable experimental support based on its prediction of microtubule polarity. Microtubules associated with easily visualized organizing centers were of uniform polarity, their plus end distal to an organizing center (11–13, 19, 43). Microtubule organization in the axon is not well understood. However, interpreting our results according to Kirschner's analysis would indicate that all axonal
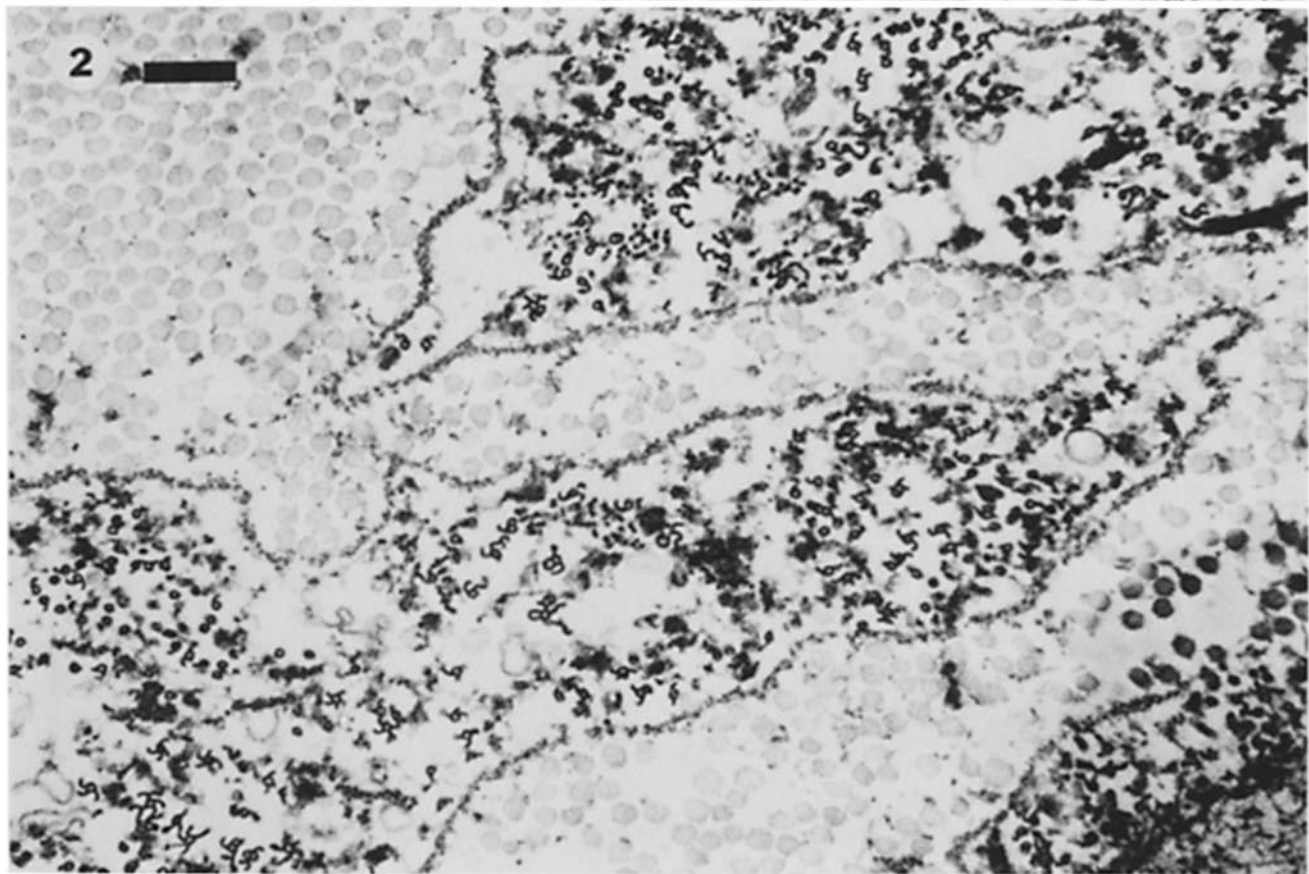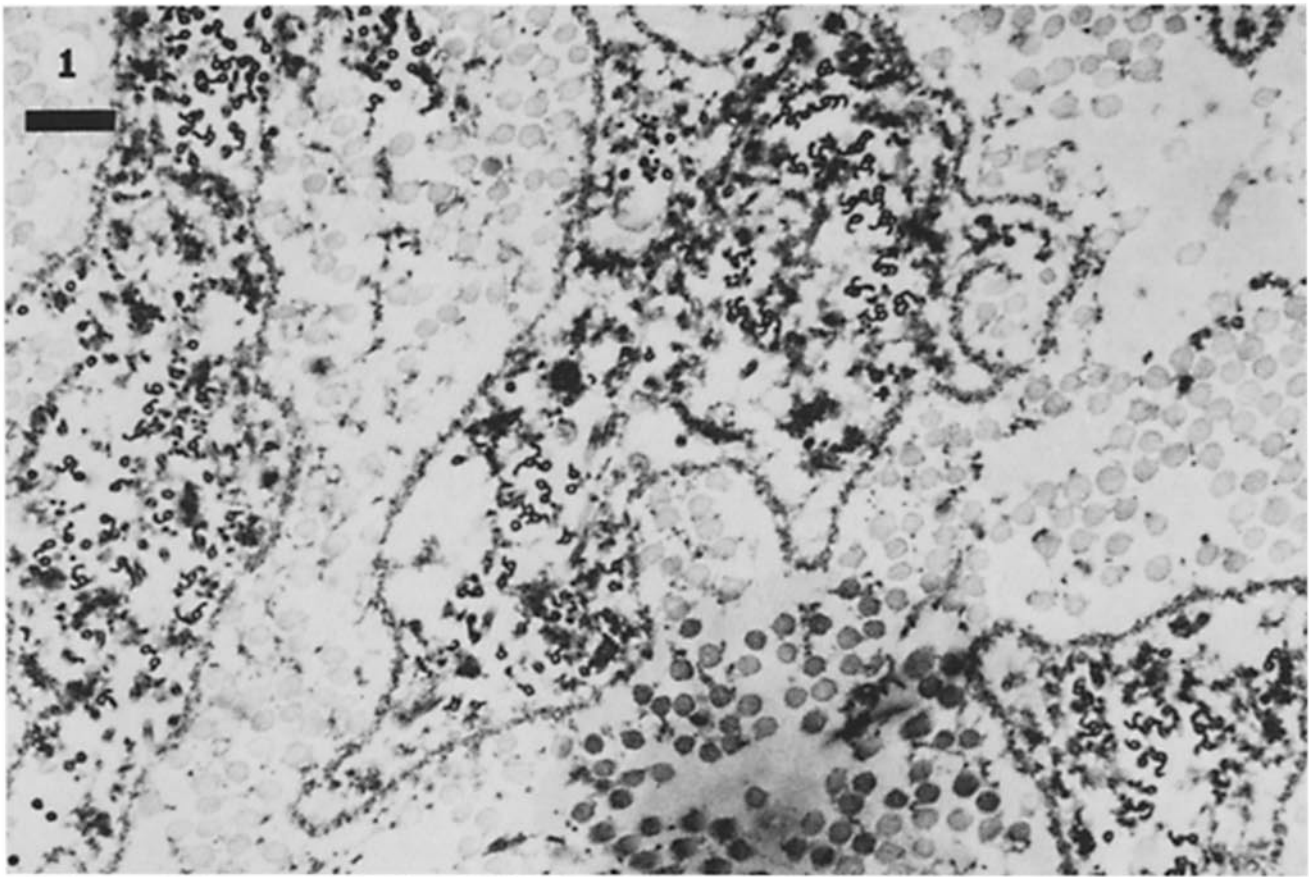
FIGURES 1 and 2 Electron micrographs of sections of cat lumbar colonic nerves that had been incubated, as described in Materials and Methods, in a microtubule reassembly buffer containing 2 mg/ml porcine brain microtubule protein. The clockwise hooks seen decorating the walls of microtubules display the structural polarity of the microtubule. Bar, 0.19 µm.

TABLE I

Hook Formations on Axonal Microtubules

Sample	No. of Axons	No. of tubules showing no hooks	No. of tubules showing clockwise hooks	No. of tubules showing counter-clockwise hooks	No. of tubules with both hooks
Renal nerve, Cat 1	105	605 (33.5%)	1,243 (66.1%)	4 (0.2%)	3 (0.2%)
Colonic nerve, Cat 2	80	617 (33.7%)	1,173 (64.0%)	19 (1%)	23 (1.2%)
Colonic nerve, Cat 3	133	1,028 (30.0%)	2,270 (66.4%)	94 (2.8%)	45 (1.4%)
Total	315	2,250 (31.6%)	4,686 (65.8%)	117 (1.6%)	71 (1%)

microtubules grow out distally from a proximal organizing center. One possibility is that the neuron, like other interphase cells (5, 15, 32, 41, 45), has a perinuclear organizing center. Immunofluorescent visualization of microtubules during neurite outgrowth of neuroblastoma suggests just that (40). This implies that microtubules are continuous through the axon. Continuity of axonal microtubules is supported by some ultrastructural studies (46, 47) but not by others (8, 29). Chalfie and Thomsen reported convincing evidence for discontinuous axonal microtubules but these appear to be an unusual subpopulation of microtubules (8). According to Kirschner's model, a population of uniformly oriented, discontinuous axonal microtubules would require multiple, oriented MTOCs from which microtubules grow in one direction. If all stable microtubules must have an MTOC (20), discontinuous microtubules can arise only from many MTOCs. Microtubules that have their minus end associated with intra-axonal MTOCs (20) will show as many absolute orientations relative to the soma as there are directions of growth from the MTOCs. Therefore, only one direction of growth from an MTOC and the same orientation of growth among the MTOCs can account for our results in the postulated circumstances. It appears that the question of microtubule continuity will be important in assessing the mechanism and cytoskeletal role of microtubule organizing centers.

Some of the working models for microtubule function in axonal transport predict that a nerve fiber capable of bidirectional transport should have an antiparallel population of microtubules. The models of Gross (16), Ochs (30), Schmitt (36), and Schwartz et al. (38) all postulate vectorial force-generating reactions on the surface of the microtubule. The direction of force is determined by a microtubule-associated ATPase that is oriented in the sense of the microtubule helix. Our finding of a single polarity orientation of microtubules in fibers that manifest retrograde and anterograde transport is inconsistent with these hypotheses. Our inability to interpret our results more generally with respect to axonal transport reflects the lack of understanding of this process at the cellular level. We would like to draw attention to three major gaps. A number of transport 'types" have been reported for whole nerve fibers (48); however, the universality of these transport processes among different axons in the fiber is unknown. For example, retrograde transport may be confined to a special class of neuron (22). The role of microtubules in axonal transport is rather controversial. Are microtubules the engine, the tracks (16, 30, 36, 38), the ties (49), or superfluous (4, 7), for axonal transport? Of particular relevance to our results, the general relationship of microtubule polarity to force production has been questioned. Euteneuer and McIntosh found a uniform polarity orientation of microtubules associated with bidirectional transport in heliozooans and fish melanophores (12). Warner and Mitchell (44) have shown that ciliary dynein, which clearly recognizes the polarity of the microtubule (17),

is able to crossbridge with parallel or antiparallel microtubules. Other recent evidence also suggests that microtubules are involved in both antiparallel (26) and parallel (50) sliding mechanisms. A clearer understanding of the relationship of microtubules to transport mechanisms will be a major goal of our future research.

REFERENCES

1. Anderson, P. N., J. Mitchell, and D. Mayor. 1980. The uptake of horseradish peroxidase by neuronal elements within the guinea-pig distal colon and its subsequent retrograde transport to the inferior mesenteric ganglion. *J. Anat.* 130:153–157.
2. Bergen, L. A., and G. G. Borisy. 1980. Head to tail polymerization of microtubules in vitro. Electron microscopic analysis of seeded assembly. *J. Cell Biol.* 84:141–150.
3. Borisy, G. G. 1978. Polarity of microtubules in the mitotic spindle. *J. Mol. Biol.* 124:565–570.
4. Brady, S., S. D. Crothers, C. Nosal, and W. O. McClure. 1980. Fast axonal transport in the presence of high Ca^{++}: evidence that microtubules are not required. *Proc. Natl. Acad. Sci. U. S. A.* 77:5909–5913.
5. Brinkley, B. R., S. M. Cox, and S. H. Fistel. 1981. Organizing centers for cell processes. *Neuro. Res. Prog. Bull.* 19:108–124.
6. Burton, P. R., and J. L. Paige. 1981. Polarity of axoplasmic microtubules in the olfactory nerve of the frog. *Proc. Natl. Acad. Sci. U. S. A.* 78:3269–3273.
7. Byers, M. R. 1974. Structural correlates of rapid axonal transport: evidence that microtubules may not be directly involved. *Brain Res.* 75:97–113.
8. Chalfie, M., and J. N. Thomsen. 1979. Organization of neuronal microtubules in the nematode *Caenorhabditis elegans. J. Cell Biol.* 82:278–289.
9. Dustin, P. 1978. Microtubules. Springer-Verlag, New York.
10. Elfvin, C. G., and C. J. Dalsgaard. 1977. Retrograde axonal transport of horseradish peroxidase in afferent fibers of the inferior mesenteric ganglion of the guinea pig. Identification of the cells of origin in dorsal root ganglion. *Brain Res.* 126:149–153.
11. Euteneuer U., and J. R. McIntosh. 1980. Polarity of midbody and phragmoplast microtubules *J. Cell Biol.* 87:509–515.
12. Euteneuer, U., and J. R. McIntosh. 1981. Polarity of some motility related microtubules. *Proc. Natl. Acad. Sci. U. S. A.* 78:372–376.
13. Euteneuer, U., and J. R. McIntosh. 1981. Structual polarity of kinetochore microtubules in PtK₁ cells. *J. Cell Biol.* 89:338–345.
14. Filner, P., and N. S. Yadav 1979. Role of microtubules in intracellular movements. Encyc. Plant Physiol. (New series) 7:95–113.
15. Frankel, F. R. 1976. Organization and energy dependent growth of microtubules in cells. *Proc. Natl. Acad. Sci. U. S. A.* 73:2798–2802.
16. Gross, G. W. 1975. The microstream concept of axoplasmic and dendritic transport. Adv. Neurology 12:283–296.
17. Haimo, L. T., B. R. Telzer, and J. R. Rosenbaum 1979. Dynein binds to and crossbridges cytoplasmic microtubules. *Proc. Natl. Acad. Sci. U. S. A.* 76:5759–5763.
18. Heidemann, S. R. 1980. Visualization of microtubule polarity. *In:* Microtubules and Microtubule Inhibitors 1980. M. DeBrabander and J. DeMey, editors. North Holland/Elsevier, Amsterdam.
19. Heidemann, S. R., and J. R. McIntosh. 1980. Visualization of the structural polarity of microtubules. *Nature (Lond.).* 286:517–519.
20. Kirschner, M. W. 1980. Implications of treadmilling for the stability and polarity of actin and tubulin polymers in vivo. *J. Cell Biol.* 86:330–334.
21. Kreuler, D. L., and J. H. Szurszewski. 1979. Electrophysiological and morphological basis for organization of prevertebral ganglia. *In:* Frontiers of Knowledge in the Diarrheal Diseases. H. D. Janowitz and D. B. Sachar, Eds. Projects in Health, Inc., N.Y.
22. LaVail, J. H. 1975. The retrograde transport method. *Fed. Proc.* 34:1618–1624.
23. Lubinska, L. 1975. On axoplasmic flow. *Int. Rev. Neurobiol.* 17:241–296.
24. Margolis, R., and L. Wilson. 1978. Opposite end assembly and disassembly of microtubules at steady state in vitro. *Cell.* 13:1–8.
25. Margolis, R. L., L. Wilson, and B. J. Kiefer. 1978. Mitotic mechanism based on intrinsic microtubule behavior. *Nature (Lond.).* 272:450–452.
26. McDonald, K. L., M. K. Edwards, and J. R. McIntosh. 1979. Cross sectional structure of the central spindle of *Diatoma vulgare.* Evidence for specific interactions between antiparallel microtubules. *J. Cell Biol.* 83:443–461.

27. McIntosh, J. R., P. K. Hepler, and D. G. VanWie. 1969. A model for mitosis. *Nature (Lond.).* 224:659–663.
28. Mitchell, G. A. G. 1953. Anatomy of the Autonomic Nervous System. Livingstone, Edinburgh.
29. Nadelhaft, I. 1974. Microtubule densities and total numbers in selected axons of the crayfish abdominal nerve cord. *J. Neurocytol.* 3:73–86.
30. Ochs, S. 1972. Fast transport of materials in mammalian nerve fibers. *Science (Wash. D. C.).* 176:252–260.
31. O'Connor, T. M., C. C. Houston, and F. Samson. 1974. Stability of neuronal microtubules to high pressure in vivo and in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 71:4198–4202.
32. Osborn, M., and K. Weber. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing center towards the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* 73:867–871
33. Peterson, E. R. 1969. Neurofibrillar alterations in cord ganglion culture exposed to spindle inhibitors. *J. Neurpathol. Exp. Neurol.* 28:168. (Abstr.).
34. Pickett-Heaps, J. D. 1969. The evolution of the mitotic apparatus: an attempt at comparative ultrastructural cytology in dividing plant cells. *Cytobios.* 1(3):257–280.
35. Roisen, F. J., W. G. Braden, and J. Friedman. 1975. Neurite development in vitro. III. The effects of several derivatives of cyclic AMP, colchicine and colcemid. *Ann. N. Y. Acad. Sci.* 253:545–561.
36. Schmitt, F. O. 1968. Fibrous proteins - neuronal organelles. *Proc. Natl. Acad. Sci. U. S. A.* 60:1092–1101.
37. Schulz, D., and R. Jarosch. 1980. Rotating microtubules as a basis for anaphase spindle elongation in diatoms. *Eur. J. Cell Biol.* 20:249–253.
38. Schwartz, J. H., J. E. Goldman, R. Aubron, and D. J. Goldberg. 1976. Axonal transport of vesicles carrying [³H]serotonin in the metacerebral ganglion of *Aplysia californica. Cold Spring Harbor Symp. Quant. Biol.* 40:83–92.
39. Solomon, F., and A. Zurn. 1981. The cytoskeleton and specification of neuronal morphology. *Neurosci. Res. Program Bull.* 19:100–107.
40. Spiegelman, B. M., M. Lopata, and M. W. Kirschner. 1979. Aggregation of microtubule initiation sites preceding neurite outgrowth in mouse neuroblastoma cells. *Cell.* 16:253–263.
41. Spiegelman, B. M., M. A. Lopata, and M. W. Kirschner. 1979. Multiple sites for initiation of microtubule assembly in mammalian cells. *Cell.* 16:239–252.
42. Subirana, J. A. 1968. Role of spindle microtubules in mitosis. *J. Theor. Biol.* 20:117–123.
43. Telzer, B. R., and L. T. Haimo. 1981. Decoration of spindle microtubules with dynein: Evidence for uniform polarity. *J. Cell Biol.* 89:373–378.
44. Warner, F. D., and D. R. Mitchell. 1981. Polarity of dynein-microtubule interactions in vitro: cross bridging between parallel and anti-parallel microtubules. *J. Cell Biol.* 89:35–44.
45. Watt, F. M., and H. Harris. 1980. Microtubule organizing centers in mammalian cells in culture. *J. Cell Sci.* 44:103–121.
46. Weiss, P., and R. Mayr. 1971. Neuronal organelles in neuroplasmic (axonal) flow. II. Neurotubules. *Acta Neuropathol. Suppl.* 5:198–206.
47. Weiss, P., and R. Mayr. 1971. Organelles in neuroplasmic (axonal) flow: neurofilaments. *Proc. Natl. Acad. Sci. U. S. A.* 68:846–850.
48. Willard, M., W. M. Cowan, and P. R. Vagelos. 1974. The polypeptide composition of intraaxonally transported proteins: evidence for four transport velocities. *Proc. Natl. Acad. Sci. U. S. A.* 71:2183–2187.
49. Wilson, D. C., and G. C. Stone. 1979. Axoplasmic transport of proteins. *Annu. Rev. Biophys. Bioeng.* 8:27–45.
50. Woodrum, D. T., and R. W. Linck. 1980. Structural basis of motility in the microtubular axostyle: implications for cytoplasmic microtubule structure and function. *J. Cell Biol.* 87:404–414.
51. Wuerker, R. B., and J. B. Kirkpatrick. 1972. Neuronal microtubules, neurofilaments, and microfilaments. *Int. Rev. Cytol.* 33:45–75.
52. Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1970. Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. U. S. A.* 66:1206–1212.