Spatial Organization of Axonal Microtubules

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ABSTRACT Several workers have found that axonal microtubules have a uniform polarity orientation. It is the "+" end of the polymer that is distal to the cell body. The experiments reported here investigate whether this high degree of organization can be accounted for on the basis of structures or mechanisms within the axon. Substantial depolymerization of axonal microtubules was observed in isolated, postganglionic sympathetic nerve fibers of the cat subjected to cold treatment; generally <10% of the original number of microtubules/μm^2 remained in cross section. The number of cold stable MTs that remained was not correlated with axonal area and they were also found within Schwann cells. Microtubules were allowed to repolymerize and the polarity orientation of the reassembled microtubules was determined. In fibers from four cats, a majority of reassembled microtubules returned with the original polarity orientation. However, in no case was the polarity orientation as uniform as the original organization. The degree to which the original orientation returned in a fiber was correlated with the number of cold-stable microtubules in the fiber. We suggest that stable microtubule fragments serve as nucleating elements for microtubule assembly and play a role in the spatial organization of neuronal microtubules. The extremely rapid reassembly of microtubules that we observed, returning to near control levels within the first 5 min, supports microtubule elongation from a nucleus. However, in three of four fibers examined this initial assembly was followed by an equally rapid, but transient decline in microtubule number to a value that was significantly different than the initial peak. This observation is difficult to interpret; however, a similar transient peak has been reported upon repolymerization of spindle microtubules after pressure induced depolymerization.

Axonal growth and form are crucial to the formation of the appropriate connections within the nervous system. Microtubules (MTs) play a major role in the specification and maintenance of axonal shape (4, 10, 23). In view of this, it is not surprising that the MTs of neurons are highly organized in space. We became interested in the spatial organization of MTs when we found that the polarity orientation of axonal MTs is uniform (7, 13, 19). However, the mechanisms responsible for the organization of microtubules in the axon are very poorly understood.

In non-neuronal cells microtubule organization is generally thought to depend on microtubule organizing centers (31), the centrosome being the most familiar. Although the data of Spiegelman et al. (44), Gonatas and Robbins (18), and Tennyson (45) implicate the centrosome in the outgrowth of the axon, many experiments argue convincingly that axonal MTs are not organized by a centrosome or, indeed, any observable microtubule organizing center. Most recently, Sharp et al. (40) observed no consistent relationship between the location of centrioles and the position of the cell processes by serial section electron microscopy in four neuroblastoma clones and in primary dorsal root ganglion neurones. Similarly, observations by Lyser (28), and Zenker and Hohberg (51) do not support a role for the centriole/centrosome in the organization of axonal microtubules. On a more general level, the roles of the cell body and axon in the organization of the neuronal cytoskeleton are uncertain. The experiments of Solomon (43) showing recapitulation of detailed morphology by neuroblastoma cells after reversible microtubule disassembly and experiments on slow axonal transport by Lasek (26) suggest that the cell body specifies the geometry of the cytoskeleton and cell. In contrast, experiments on guidance of neurites and growth cones (8, 23, 27) and on the growth of isolated axons (30, 41) suggest that the form and growth of neuronal processes are independent of the cell body.

One common strategy for investigating MT (microtubule[1])

[1] *Abbreviation used in this paper:* MT, microtubule.

organization is to reversibly depolymerize cellular MTs then observe the reassembly process for evidence of organizing influences. Several studies showed that axonal MTs depolymerized upon cold treatment but subsequently returned upon warming (2, 6, 34, 37, 38). We extended this work using the cat lumbar colonic nerve as before (19) for three purposes. First, to determine the polarity orientation of the reassembled tubules as an assay for extent to which in situ MT organization is recapitulated in isolated axons. Second, to investigate more fully those microtubules not depolymerized by cold in light of recent studies on cold stable microtubules in vitro (22, 29, 49). Finally, we examined the time course of MT reassembly after rewarming.

MATERIALS AND METHODS

Cats were anesthetized by intraperitoneal injection of a mixture of sodium diallylbarbiturate (70 mg/kg), urethane (280 mg/kg), and monoethyl urea (280 mg/kg) (48). Segments ~2–3 cm long of sympathetic nerve fibers coursing from the inferior mesenteric ganglion to the colon were dissected in situ in the region between the ganglion and the beginning of the arcade of fibers leading to the colon. During the dissection the nerves were occasionally rinsed with warm, oxygenated Krebs-Ringer's solution to eliminate blood and prevent the tissue from drying. These fibers were ligatured to mark the location of the ganglion then cut from the animal between the ganglion and the beginning of the arcade. Immediately after excision a segment of the isolated nerve was fixed for electron microscopy to serve as a control. The remainder was placed into a vented, stoppered tube containing Eagle's minimal essential medium buffered to pH 7.4 with 10 mM HEPES at 37°C. The medium was bubbled with 95 or 100% oxygen prior to and during all incubations. Nerve fibers were chilled by immersing the tube in a refrigerated circulator containing a 50% methanol solution at −2°C for 90 min. After cold treatment nerve fibers were warmed by immersing the tube in a 37°C water bath. In one experiment a fiber was placed after excision into medium containing 1 μg/ml nocodazole (Aldrich Chemical Co., Inc., Metuchen, NJ) at 37°C for 2 h. After 2 h of drug treatment the fiber was rinsed twice with warmed medium without nocodazole, then incubated at 37°C in fresh medium without nocodazole. At various times during the incubations segments were cut from the nerve, fixed for electron microscopy, and the remainder of the fiber replaced in the incubation medium. Determination of neurotubule polarity was assessed as described by Heidemann et al. (19). In brief, the fiber segment was incubated in buffer (0.5 M PIPES, pH 6.9, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM GTP, 5% dimethylsulfoxide, 1% Triton X-165, 0.5% sodium deoxycholate, 0.2% SDS) for 10 min at 37°C, followed by two changes of buffer containing 2 mg/ml microtubule protein from brain, each incubated for 10 min at 37°C. Rules for scoring the polarity of MTs based on hook formation have been described (20). The number density of microtubules, ie., no. MTs/μm^2, observed in cross section was determined from electron micrographs of random regions of the fiber. Micrographs had a final magnification of 39,500. All axons or Schwann cells in a given micrograph were cut from the micrograph, weighed, and the microtubules counted. The area of the cut-out was calculated from the weight by a proportionality constant derived from the weight of cut-outs of identically magnified vinyl beads (0.905 ± 0.006-μm diam; Sigma Chemical Co., St. Louis, MO).

RESULTS

Depolymerization of Axonal Microtubules

We confirmed the findings of Banks et al. (2) that MTs in sympathetic axons from the cat substantially depolymerize when subjected to cold treatment. Axons of lumbar colonic nerve bundles from five cats fixed immediately after excision had 80 or more MTs/μm^2, in one case as many as 170 MTs/μm^2 (Table I, Fig. 1). After incubation at −2°C for 90 min, <10% of the original number of MTs could be seen in cross-section electron micrographs in all but one sample (Fig. 2). In experiment C/1, a fiber that originally had 82 MT cross sections/μm^2 retained 21 MTs/μm^2 after cold depolymerization. Fiber D/1 was also exceptional in that a very small number of MTs remained after cold treatment, 0.14 ± 0.06 MTs/μm^2. This value is given as zero in Table I because the data in this table have been rounded off to the nearest integer. In one experiment, B/2, 1 μg/ml nocodazole for 2 h at 37°C was used as the depolymerizing agent instead of low temperature. Nocodazole and cold treatment had very similar effects on two fibers from the same cat. In both cases (B/1 and B/2) ~1% of the MTs remained after treatment. Although we were primarily interested in axonal microtubules, MT counts were made from the Schwann cells in fiber D/1. We found Schwann cells had a significantly lower density of MTs, 1/4 to 1/5, than axons from the same fiber. Schwann cell MTs appeared to be no more or less stable to cold depolymerization than axonal MTs; <5% of Schwann cell MTs remained after cold treatment.

Those MTs that remained after cold treatment were of some interest to us given the recent results on cold stable MTs in vitro (22, 29, 49). In all cold treated fibers except C/1 ~80% of axonal cross sections were completely devoid of MTs. Although some axons contained a significant number of MTs after depolymerization, we could not discern a pattern among those axons that contained cold stable MTs. Axonal size is a variable that we could easily determine and that has long been known to be of functional significance (17). The correlation coefficient calculated for the number of cold stable microtubules as a function of axonal area for all axons containing cold stable MTs in experiments A/1, B/1, and E/1 was $r = -0.13$. Similarly, we found no evidence of proximo-distal differences in cold stable MTs. In one experiment a cold-treated segment was taken from both the proximal and

TABLE I

Depolymerization, Repolymerization, and Polarity Orientation of Neurotubules

Cat/fiber	Before disassembly*	After disassembly*	After reassembly*	Percentage of MTS with hooks‡	Percent of hooked MTs with right-handed hooks‡
				%	
A 1	84 ± 5	8 ± 3	73 ± 6	63	72
				41	67
B/1	170 ± 6	2 ± 1	192 ± 12	51	69
				37	68
B/2 (noc)	107 ± 6	1 ± 1	157 ± 10	—	—
C/1	82 ± 4	21 ± 3	96 ± 6	44	83
D/1	90 ± 6	0 (see text)	85 ± 5	46	57
D/1 Schwann	19 ± 2	1 ± 0.2	23 ± 3	—	—
E/1	152 ± 12	4 ± 1	168 ± 11	—	—

* Mean number of microtubules/μm^2 of cell ± SEM based on counts of 50 or more axons or Schwann cells.
‡ Based on counts of 1,000 or more microtubule cross sections.

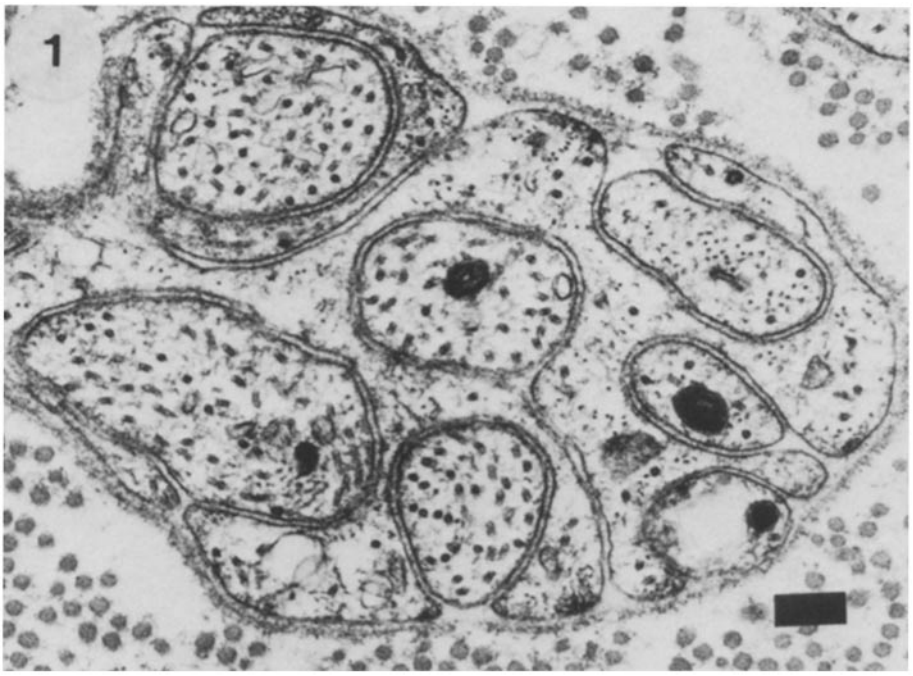

FIGURE 1 Electron micrograph from a cross-sectioned lumbar colonic nerve that was fixed immediately after excision. The microtubule density of such samples is reported in Table I under "Before Depolymerization." Bar, 0.24 μm. × 41,660.

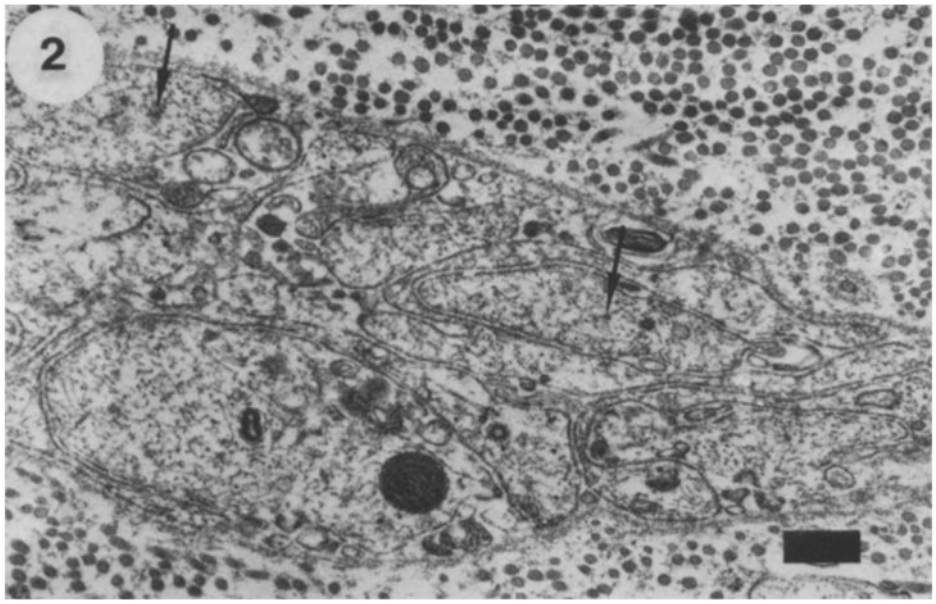

FIGURE 2 Cross section of a lumbar colonic nerve after 90 min at −2°C. The microtubule density of such samples is reported in Table I under "After Depolymerization." The arrows mark two remaining microtubules. Bar, 0.31 μm. × 32,258.

distal ends of the fiber. Both extremities showed identical numbers of cold stable MTs: 3.8 ± 1 and 3.7 ± 1 MTs/μm^2.

Repolymerization of Microtubules

As shown in Table I and Fig. 3, when lumbar colonic fibers were rewarmed to 37°C for 90 min, MTs returned in large numbers. In most instances statistical analysis indicated that the differences between MT number before disassembly and after reassembly were not significant. Only the increases in fibers B/2 and C/1 were found to be significant ($P = 0.05$) from the t-statistic calculated for a two-tailed ($\bar{x}_i \neq \bar{x}_j$) group comparison test. If a one-tailed test ($\bar{x}_j > \bar{x}_i$) was used, MT number before disassembly and after reassembly also differed significantly in fiber B/1, although just barely.

The time course of this reassembly was examined in fibers from cats other than those identified in Table I. As shown in Fig. 4, there is a rapid increase in the number of MT cross sections during the first 15 min of warm incubation which then levels off and remains rather stable for the next 2 h. We wished to examine this assembly at earlier times. Fig. 5 shows the reassembly of cold depolymerized MTs in four fibers from three cats during the first 30 min of reassembly. Three of the four fibers had re-established 75% or more of the initial (before depolymerization) density of MTs within the first 5 min. In the remaining fiber ~50% of the initial density of MTs was observed in the sample fixed after 5 min of incubation at 37°C. Temperature measurements of the incubation medium during this period showed that the temperature had reached 16°C at 1.5 min and 30°C at 5 min. One of the four fibers shown in Fig. 5 showed an increase in the number of

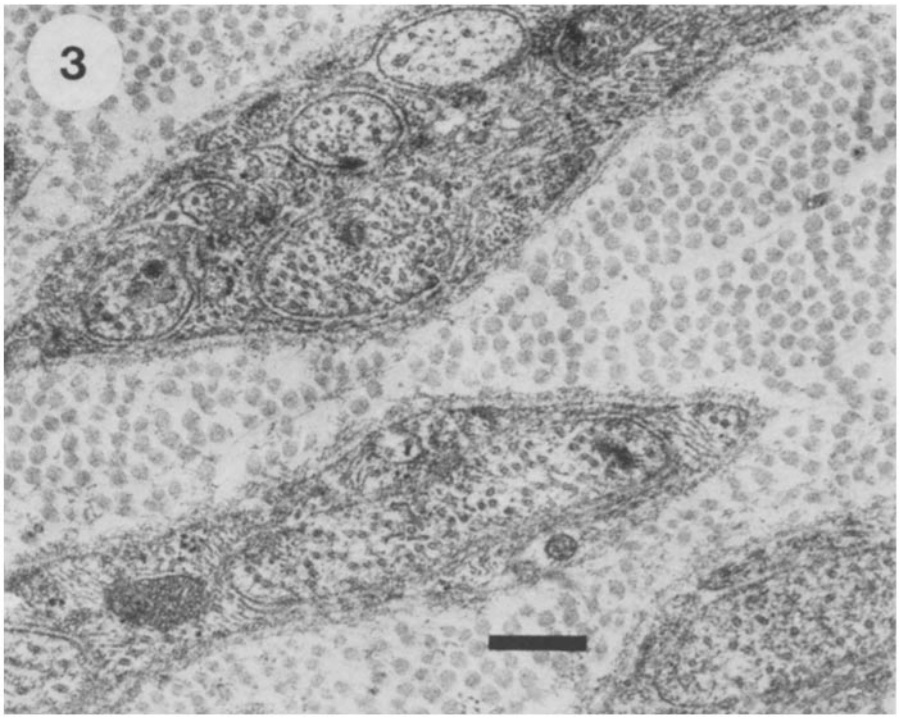

FIGURE 3 Cross section of a lumbar colonic fiber after cold depolymerization and after 90 minutes at 37°. Clearly, microtubules have returned in large number. Microtubule counts from such samples are reported in Table 1 under "After Repolymerization." Bar, 0.50 μm. × 24,000.

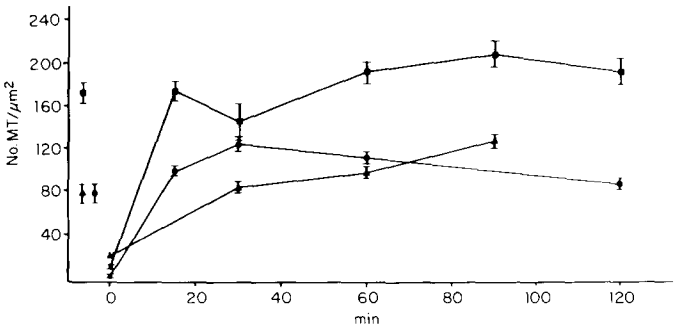

FIGURE 4 Number of microtubules/μm^2 seen in cross sections as a function of time of incubation at 37° after cold depolymerization in fibers from 3 cats. Each data point is based on counts of 50 or more axons, the error bar represents the standard error of the mean. The points reported at the extreme left represent the microtubule density seen in the respective fibers before depolymerization, ie. control values.

MTs through the first 20 min and then leveled off. However, in three of the four fibers the density of MTs reached a peak value within the first 5 or 10 min and then declined sharply several minutes later. Comparing the "peak" and "valley" value in each of these fibers by a two tailed, group comparison, t-statistic indicated that these points differed very significantly ($P = 0.01$) in all three fibers. However, in only one case (cross hatched line) was the peak value greater than the initial density of MTs in the fiber.

Polarity Orientation of Repolymerized Microtubules

We previously concluded that MTs within the lumbar colonic nerve of cats had a uniform polarity orientation, the "+" end distal to the cell body (19). In those experiments we were able to decorate 2/3 of the axonal microtubules with polarity revealing protofilament ribbons and 96% of these had the same handedness of hook, right handed as seen looking toward the cell body. We employed the same experimental protocol to determine the polarity of the repolymerized MTs in these experiments (Fig. 6). Table I summarizes these data. Counts of 1,000 or more MTs in each of four fibers examined indicated a majority of those repolymerized microtubules that displayed hooks returned with the polarity characteristic of axonal MTs before depolymerization, ie., displayed right-handed hooks as seen looking toward the ganglion. However, in no fiber were the MTs as uniformly oriented as they had been before depolymerization. There was also much greater variability of MT polarity among fibers after depolymerization/repolymerization. The most homogeneous orientation was observed in fiber C/1 in which 83% of the decorated MTs displayed the original orientation. This same fiber retained an exceptionally large number of MTs after cold treatment. Conversely, fiber D/1 which had the smallest number of cold stable microtubules had only 57% of the repolymerized MTs with the original orientation. Fibers A/1 and B/1 which were more typical in terms of the number of MTs remaining after cold treatment had ~70% of the decorated, repolymerized MTs with right-handed hooks. In all cases, 4–6% of the decorated MTs displayed hooks of both handedness; the remainder of the decorated MTs displayed only left handed hooks as seen looking toward the cell body.

In our previous study of axonal MT polarity we drew attention to the variability in the frequency of hook decoration of axonal MTs (19). We observed similar variability of decoration in these experiments. We sought to determine if this variability had any effect on the proportion of decorated MTs that displayed hooks of a given handedness. After 90 min of warm treatment (repolymerization) two small, ligatured segments were removed from fibers A/1 and B/1. Each segment

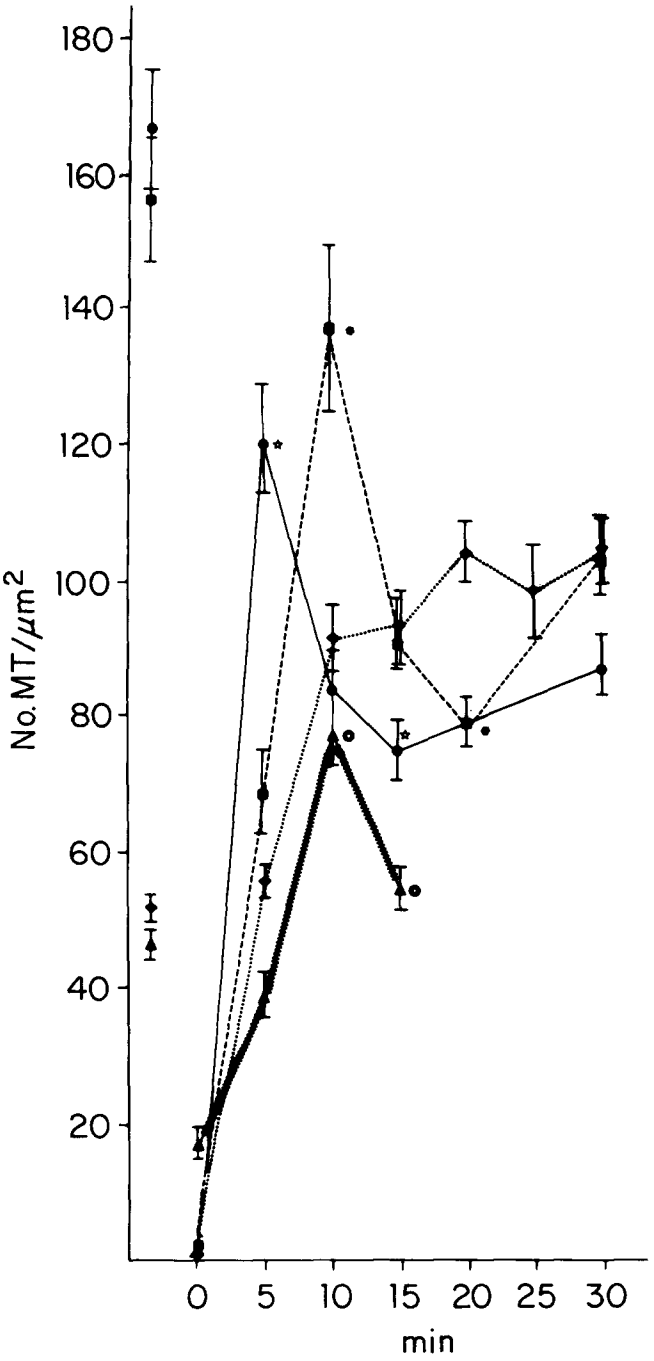

FIGURE 5 Number of microtubules/μm^2 seen in cross sections as a function of time of incubation at 37° after cold depolymerization in 4 fibers from 3 cats. Data points marked by (■) and (●) are from the same cat. Each data point is based on counts of 50 or more axons, the error bar represents the standard error of the mean. The points reported at the extreme left represent the microtubule density seen in the respective fibers before depolymerization, ie. control values. In three of the fibers an intial peak value within the first 10 minutes (marked by a star, asterisk, or torus) rapidly declined to a significantly different low point value (also marked).

was treated independently but identically to produce hooks. The extent of decoration and proportion of decorated MTs displaying only right handed hooks for each segment of each fiber is reported in Table I. One segment of A/1 had a relatively high proportion of MTs with hooks, 63%, while only 41% of the MTs in the other segment of the same fiber were decorated. Despite this difference the polarity orientation of MTs was very similar in both segments, 72 and 68% with right handed hooks, respectively. Similarly, the segments of fiber B/1 show different degrees of decoration but very similar proportions of MTs with the orginal polarity orientation. This evidence indicates that the degree of decoration does not much affect the conclusion drawn as to polarity orientation. Nevertheless, a limitation of the polarity data reported here, as well as previous reports (7, 13, 19), is the large number of axonal MTs that do not display hooks. Our data and experience to date continues to suggest to us that the undecorated MTs are the result of technical problems, probably the inability of exogenous tubulin to easily penetrate into the solid, very collagenous tissue of the nerve fiber. These data also indicate that the hook-forming method of polarity orientation does reveal non-uniform polarity orientation of MTs. The uniform polarity of MT orientation found in a number of systems (11, 12, 20) caused us some concern that the hook method might not be decorating MTs of both polarities. Our results here argue against this possibility.

DISCUSSION

The postganglionic, sympathetic nerve fibers used in these experiments can be considered a bundle of axons isolated from their cell bodies. The tissue remained alive despite the length of treatment times. After 90 min of cold treatment and 90 min at 37°C the tissue appeared normal ultrastructurally and in some nerve fibers more than the initial number of MTs was observed. We found that the majority of MTs that repolymerized in these axons after cold depolymerization returned with their initial polarity orientation. This suggests that the axon contains MT organizing structures within the axon. Clear candidates for these organizing structures are the cold stable microtubules that we observed in these axons. Our data is most easily interpreted by assuming that these cold stable microtubules are short fragments within larger, primarily cold-labile MTs as has been shown in vitro (22). Preliminary serial section data from our laboratory indicates that neurites of cold treated PC 12 cells contain large numbers of short MT fragments. Such fragments would serve as nucleating sites for the addition of free tubulin dimer after cold depolymerization, imparting to the elongated MT the same polarity orientation as the fragment. We speculate that these fragments are prevented from turning end for end as a result of cross bridging with cold stable cytoskeletal elements such as neurofilaments (42). If cold stable fragments serve as nucleating sites for axonal MT assembly and organization, the degree to which repolymerized MTs recapitulate the original uniform orientation should depend on the extent to which cold stable fragments remain to serve as "seeds". Data of fiber B/1 fulfill this expectation in that this fiber had the largest number of cold stable fragments and most nearly recapitulated the original, uniform MT polarity orientation. Similarly, fiber C/1 that had the fewest MTs remaining after cold treatment had the lowest proportion of the repolymerized, decorated MTs return with the + end distal to the cell body. The very rapid repolymerization of MTs reported in Fig. 4 is also consistent with an elongation mechanism. In vitro studies have shown that elongation from existing MTs occurs more rapidly than does initiation de novo (35) in agreement with theoretical studies that indicate that nucleation, not elongation, is the rate limiting step in protein polymer formation (36). Indeed, such studies make it difficult to avoid postulating some elongation of MTs from cold stable fragments in these experiments.

The variability of MT density we observed in these experi-

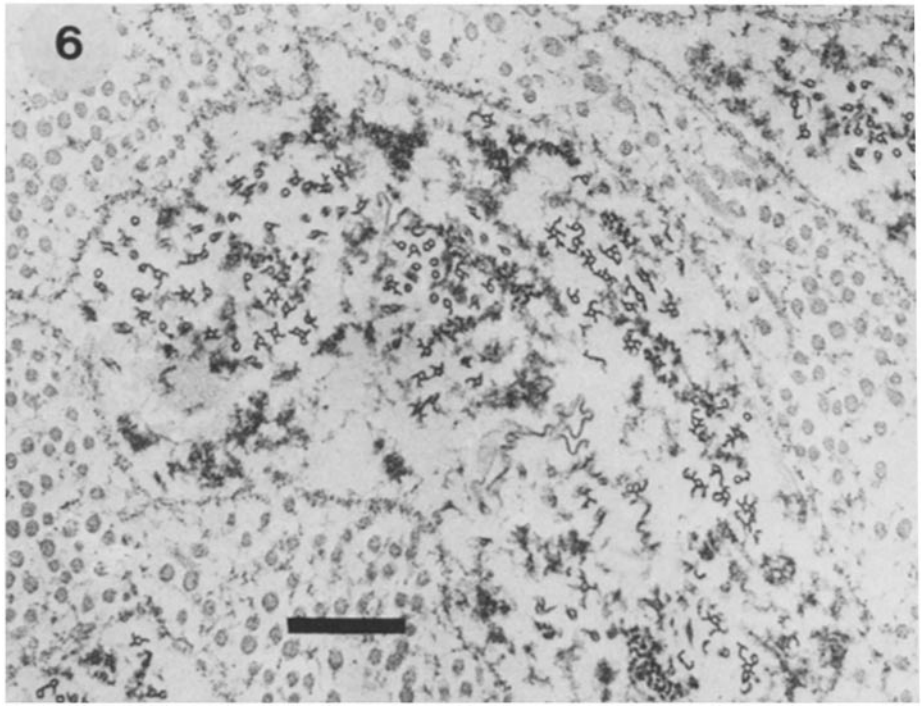

FIGURE 6 Cross section of a lumbar colonic nerve after repolymerization of microtubules which had been subjected to "hook" forming conditions. Bar, 0.50 μm. × 32,000.

ments is typical of a variety of measurements made on intact nerve fibers (1, 14, 15, 16, 33). We found no evidence of any regional variation in MT density, cold stable MTs, MT polarity, etc. Numbers of cold stable MTs were not correlated with axonal area nor with the proximo-distal location of the segment. Cold stable MTs also occurred in Schwann cells (Table I). Our findings suggest that cold stable MT fragments are randomly dispersed among all neuronal cells. The polarity of MTs was similar in two different, albeit adjacent, segments of both fibers A/1 and B/1 (Table I). Our data are consistent with earlier studies that found uniformity of microtubule distribution along the length of unmyelinated nerve fibers (14, 25, 50). Indeed, a large body of work indicates that unmyelinated nerves are quite homogeneous throughout the axonal tract (1, 14–16, 25, 33, 50). For example, the distribution of axonal diameters in unmyelinated fibers is unimodal with a narrow range (15, 33). Likewise, we found that the average axonal area was very similar for all our data points. In view of the homogeneity within unmyelinated tracts, our procedure of assaying random regions across the face of the nerve is unlikely to have introduced a sampling error into our results.

Cold stability of MTs is a manifestation of the general thermodynamic stability of these polymers. Our data on such stable fragments are in good agreement with the study of Morris and Lasek (32) which showed that some 15% of squid axon tubulin remains as MTs when extracted with physiological saline. Stable MT fragments serving as assembly seeds to locally control MT organization would give substance to Tucker's (47) speculations on "free nucleating elements" which contrast with microtubule organizing centers. A role for local elements in the organization of axonal MTs is particularly attractive for a number of reasons. Axonal MTs are known to be discontinuous (5, 9, 46). Axons isolated from their cell body can retract to a small fraction of their original length then regrow substantially (41). Normal branching of axons also appears to involve initial depolymerization of MTs (3, 23) with subsequent MT assembly for the growth of the axon.

The changes in the number of MT cross sections observed during the first 20 min of repolymerization is particularly intriguing (Fig. 5). In three of four fibers examined an initial very rapid rise in the number of MTs quickly gave way to a statistically significant decline. These data are difficult to interpret unambiguously because we measured only numbers of MTs, which may not be an accurate measure of polymer mass, particularly during a kinetic transient. Nevertheless, two points seem of interest in this regard. Salmon (39) observed a transient "overshoot" of spindle birefringence during recovery from an extended period of pressure-induced MT depolymerization. This qualitative pattern of a rapid peak followed by a decline is consistent with Kirschner's (24) proposal for MT organization based on "capped" MTs in the case of a depolymerization repolymerization process according to the argument given by Hill and Kirschner (21). However, we note that the quantitative aspects of our data do not fit this model; the transient peak we observe is not greater than the initial number of MTs before treatment.

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