Microtubule Reassembly from Nucleating Fragments during the Regrowth of Amputated Neurites

Peter W. Baas and Steven R. Heidemann

Department of Physiology, Michigan State University, East Lansing, Michigan 48824-1101

Abstract. We have proposed that stable microtubule (MT) fragments that resist depolymerization may serve as nucleating elements for the local control of MT dynamics in the axon (Heidemann, S. R., M. A. Hamborg, S. J. Thomas, B. Song, S. Lindley, and D. Chu, 1984, *J. Cell Biol.*, 99:1289–1295). Here we report evidence that supports this proposal in studies on the role of MTs in the regrowth of neurites from the distal segments of amputated chick sensory neurites. Amputated neurites collapse to "beads" of axoplasm that rapidly regrow (Shaw, G., and D. Bray, 1977, *Exp. Cell Res.*, 104:55–62). We examined both unarrested regrowth and regrowth after MT disassembly by either cold (−5°C for 2 h) or nocodazole (0.1 μg/ml for 15–20 min). In all these cases regrowth occurred at 3.5–4.5 μm/min with no delay times other than the times to reach 37°C or rinse out the nocodazole. Electron micrographs of untreated beads show many MTs of varying lengths, while those of cold- and nocodazole-treated beads show markedly shorter MTs. The robust regrowth of neurites from beads containing only very short MTs argues against unfurling of intact MTs from the bead into the growing neurite. Electron micrographs of cold-treated beads lysed under conditions that cause substantial MT depolymerization in untreated intact neurites show persistent MT fragments similar to those in unlysed cold-treated beads. We interpret this as evidence that the MT fragments in cold-treated beads are somehow distinct from the majority of the MT mass that had depolymerized. Collapsed neurites treated with a higher dose of nocodazole (1.0 μg/ml for 15–20 min) were completely devoid of MTs and regrew only after a 15–20 min delay in two cases but never regrew in 11 other cases. We found that MTs did not return in beads treated with 1.0 μg/ml nocodazole even 30 min after removal of the drug. It was unlikely that the inability of these beads to reassemble MTs was due to incomplete removal of nocodazole in that a much higher dose (20 μg/ml nocodazole) could be quickly rinsed from intact neurites. Beads treated with 1.0 μg/ml nocodazole could, however, be stimulated to reassemble MTs and regrow neurites by treatment with taxol. We conclude that the immediate, robust regrowth of neurites from collapsed beads of axoplasm requires MT nucleation sites to support MT reassembly. Our data suggest that tubulin within the bead can elongate existing MTs normally but has a limited capacity for self-nucleation relative to brain tubulin in vitro.

Microtubules (MTs)[1] are dynamic structures that are critical for the growth and maintenance of axons (7, 24, 47, 48). Axonal MTs enjoy a high degree of spatial organization (6, 14, 15, 36), yet are apparently not under the influence of a traditional MT-organizing center (29, 39, 49). Indeed, a growing body of evidence suggests that local and environmental cues play an important role in the control of the axonal cytoskeleton (5, 10, 20, 27). It has been speculated (see Morris and Lasek [31] and Brady et al. [4]) that cytoskeletal nucleation sites may be woven throughout the axon for the local maintenance of cytoskeletal morphology. We previously reported that MTs of cat sympathetic nerves are oriented in polar fashion (14), and are able to recapitulate their polar organization during recovery from cold- or drug-induced depolymerization (15). We proposed that MT fragments that resist depolymerization may play a role in MT organization by acting as nucleating "seeds" for MT elongation. Morris and Lasek (31) and Brady et al. (4) have similarly suggested that stable polymers in the cytoskeleton may play an important role in cytoskeletal organization. Biochemical evidence (18) suggesting that stable MTs exist in vitro as short, disassembly-resistant regions of longer, otherwise labile MTs has been confirmed by observations in vivo (22, 37). Stable MTs have been shown to be biochemically distinct from the labile polymer (2, 4, 16, 18, 21, 45). Nucleated assembly from stable fragments seems attractive in that elongation from an existing MT is faster and more energetically favorable than de novo initiation (1, 34, 35). A functional role for stable fragments as nucleation seeds would lend substance to speculations on individual

1. *Abbreviations used in this paper*: GEP buffer, 0.1 M Pipes, pH 6.9, with KOH, 1 mM EGTA, and 2.5 mM GTP; MT, microtubule.

© The Rockefeller University Press, 0021-9525/86/09/917/11 $1.00

nucleating elements for each MT (42, 44). Stable fragments may also provide the physical "cap" against treadmilling proposed by Kirschner (23), and may be of particular importance in MT organization if axonal MTs share the kind of "dynamic instability" recently reported of MTs in vitro (30).

Shaw and Bray reported that distal segments of amputated neurites from cultured chick sensory neurons collapse to shortened segments or "beads" of axoplasm that subsequently regrow neurites entirely similar to ordinary neurites on the light microscopic level (40). Wessells et al. (46) demonstrated the same phenomenon in ciliary ganglion neurons and provided evidence for the importance of growth cone motility in cytoskeletal organization during regrowth. George and Lasek have suggested that the collapse of amputated neurites may involve the same kinds of regulatory mechanisms that coordinate the cytoskeleton during slow transport (13). Although completely divorced from the cell body, the amputated neurites clearly contain all the informational cues required for cytoskeletal reorganization underlying collapse and regrowth. In an effort to understand this cytoskeletal reorganization we have examined the disassembly and reassembly of MTs during the collapse and regrowth of amputated neurites.

Materials and Methods

Cell Culture

Embryonic chick sensory neurons were cultured using a procedure slightly modified from that of Shaw and Bray (40). Dorsal root ganglia were dissected from the lumbosacral regions of 12-d embryos, and placed in L-15$^+$ medium (L-15 purchased from Gibco, Grand Island, NY; supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin). The ganglia were rinsed twice, then treated with 0.25% trypsin for 25 min at 37°C. The trypsin was removed, L-15$^+$ with 10% fetal calf serum (Hazleton Dutchland, Inc., Denver, PA) was added, and the ganglia were triturated with a pipette into a single cell dispersion. The cells were rinsed twice, and plated in L-15$^+$ with 10% fetal calf serum, 0.6% methyl cellulose (Methocel A4M, Dow Corning Co., Midland, MI), and 100 μg/ml nerve growth factor from mouse saliva (8). The cells were plated into 35-mm Corning tissue culture dishes at a density sufficiently low so as to render non-neuronal contamination inconsequential. Cultures were kept in a humid, 37°C incubator for 18-30 h before experimentation.

Amputation Experiments

Cultures were covered with a thin layer of mineral oil to prevent evaporation and maintain pH, and placed on a microscope stage warmed to 37°C with a Sage air curtain incubator. Temperatures of warming cultures were monitored by submerging the feedback thermister of the air curtain incubator directly into the culture medium, and taking the cooling mode as an indicator that the set temperature had been reached. Control experiments using larger volumes of fluid showed the air curtain incubator to be accurate within 1°C. Amputations were performed with a Leitz micromanipulator using glass needles made on a microelectrode puller. The needles were often broken to a diameter of ~10 μm, and neurites were cut from directly above to minimize lateral displacement. In an effort to maximize the number of amputated neurites that collapsed completely to beads of axoplasm, i.e., with no visible processes remaining after collapse, unbranched neurites with modest growth cones were selected for amputation and cut ~100 μm from the growth cone. Regrowth of collapsed neurites was arrested in three sets of experiments using conditions known to depolymerize MTs. Cultures were cold treated by incubating the dishes in a −5°C bath for 2 h, or treated with either 0.1 μg/ml or 1.0 μg/ml nocodazole (Aldrich Chemical Co., Milwaukee, WI) for 15-20 min at 37°C. After these treatments, regrowth properties were examined by rewarming the cold-treated beads on the microscope stage, or rinsing the nocodazole-treated beads twice with PBS then returning them to undrugged medium.

In another set of experiments, cold-treated beads were permeabilized under conditions slightly modified from a procedure reported by Cande et al. (11) to depolymerize spindle MTs in PtK1 cells. In these experiments, cultures were gently lysed after cold treatment for 4-5 min at 37°C in a buffer containing 0.1 M Pipes adjusted to pH 6.9 with KOH, 1 mM EGTA, and 2.5 mM GTP (GEP buffer), with 0.08-0.1% Brij 58. Normal neurites were also lysed in this buffer with or without added 2 M glycerol. The latter conditions have been found to stabilize assembled MTs against dissassembly by dilution while allowing diffusion of free tubulin in a variety of cells (3, 43).

Regrowth properties of collapsed neurites treated with 1.0 μg/ml nocodazole were examined 15 and 30 min after rinsing by exposing them to either 2.3×10^{-5} M or 4.6×10^{-8} M taxol. Taxol, a drug reported to promote MT assembly (38), was a gift from the Developmental Therapeutics Program of the National Cancer Institute.

Electron Microscopy

Cultures were fixed for electron microscopy using one of two methods. Neurites and untreated collapsed neurites were fixed by replacing the medium with a solution containing 0.1 M cacodylate, 1 mM $MgCl_2$, and 2% glutaraldehyde for 20-30 min. Because collapsed neurites treated under MT depolymerizing or lysis conditions tended to lift from the dish surface during exchanges of fluid, these cultures were fixed by the addition of an equal amount of the same medium or buffer containing 4% glutaraldehyde. The cultures were then rinsed twice in 0.1 M cacodylate with 5% sucrose, treated with 0.15% tannic acid for 5 min, rinsed twice, postfixed in 1% OsO_4 for 5 min, dehydrated in ethanol series, and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA). Thin sections cut parallel to the substratum were stained with uranyl acetate and lead citrate, and observed with a Phillips 300 transmission electron microscope.

Serial Reconstruction of Beads

To determine whether the MTs left in collapsed neurites after cold treatment were in the form of fragments, a modification of the method of Nicklas et al. (33) was used for serial reconstruction. Outer boarders and MTs together with many membranous vesicles within the collapsed neurite were traced from the micrographs onto transparent plastic sheets. The tracings were aligned first by the membranous registration markers, then adjusted to maximize the matches of MT ends in consecutive sections by moving the tracings within a range of two MT diameters. MT length and orientation were then depicted in the form of a composite drawing.

Results

Collapse of Amputated Neurites

Our studies confirmed the observations of Shaw and Bray (40). Distal segments of amputated neurites collapsed to beads of axoplasm (hereafter "beads") which then regrew neurites almost immediately. Because we were interested in examining collapse and regrowth separately, we selected for amputation unbranched neurites with modest growth cones (Fig. 1 *a*), and amputated distal segments ~100 μm in length. We found such neurite segments most likely to completely collapse to beads before beginning regrowth. Fig. 1 shows a typical sequence of amputation, collapse, and regrowth. Fifty-three amputations were performed in a set of experiments to monitor regrowth. In 16 cases, regrowth began before the complete collapse of the neurite. In 37 cases, however, the amputated neurite collapsed to a flattened sphere or "bead" of axoplasm with no visible processes before the onset of regrowth. Initially, the growth cone flaired and began to "melt" almost immediately upon severing the neurite. Then, as reported by Shaw and Bray (40), the distal segment coiled (Fig. 1 *b*) as it collapsed to a bead (Fig. 1, *c* and *d*). Electron micrographs of distal segments at various times after amputation show varying degrees of MT depolymerization roughly correlated to the degree of coiling suffered by the neurite.

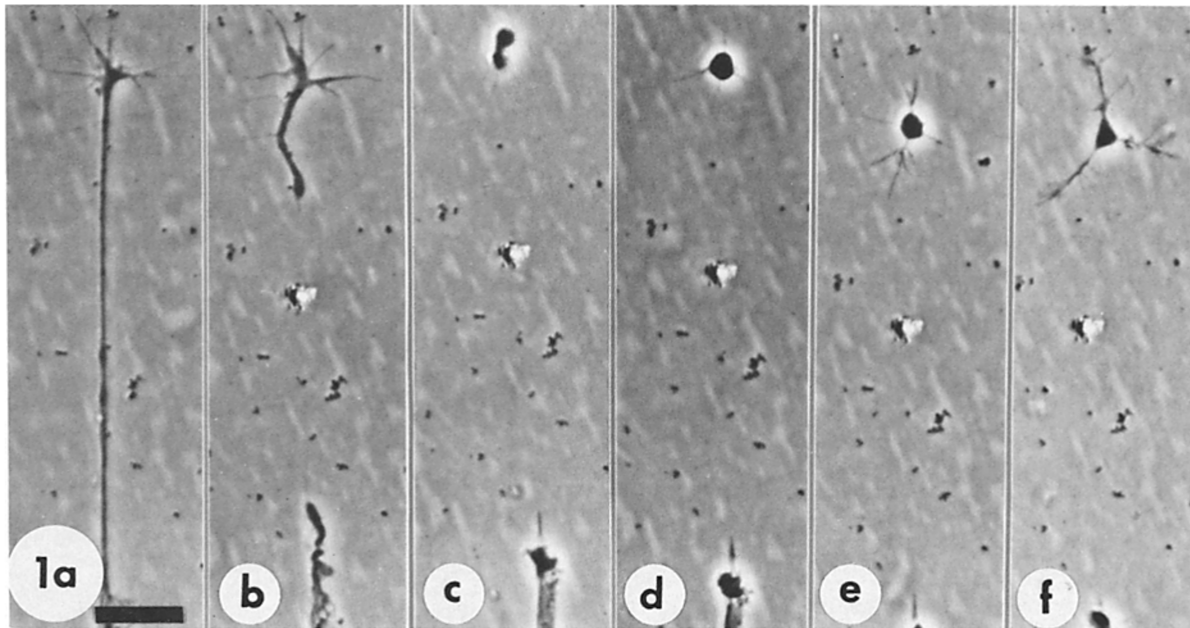


Figure 1. Phase-contrast micrographs of a sequence of collapse and regrowth of chick sensory neurons after amputation. (*a*) Neurite before amputation. (*b*) 3 min after amputation, growth cone is flaired and neurite is coiled and retracted. (*c*) 12 min after amputation, collapse to a bead is almost complete. (*d*) 13 min after amputation, collapse is complete and minor regrowth has begun. (*e*) 16 min after amputation, multipolar regrowths are apparent. (*f*) 18 min after amputation, regrowths have lengthened. Bar, 20 μm.

Ultrastructure of Collapsed Neurites with and without MT Depolymerization

We examined the ultrastructure of 10 amputated, untreated neurites collapsed to beads. All 10 clearly showed many long MTs in addition to multiple cross sections and apparent fragments (Fig. 2). Electron micrographs of four beads that were cold treated for 2 h in a −5°C bath (Fig. 3 *a*) show the marked absence of any long MTs similar to those found in the untreated beads. To confirm that the MTs left in the cold-treated bead were indeed short fragments, we reconstructed nine consecutive serial sections, ∼75%, of one typical bead. The reconstruction (Fig. 3 *b*) shows many cross sections of MTs and 158 longitudinal fragments. In this particular bead all MTs were found to be <0.5 μm in length while over 80% were <0.2 μm in length. Treatment of collapsed neurites with 0.1 μg/ml nocodazole for 15–20 min likewise resulted in substantial MT depolymerization. We examined four such beads and found short fragments as well as some longer MTs in the peripheral regions (Fig. 4 *a*). However, treatment with a higher dose of 1.0 μg/ml nocodazole appeared to completely depolymerize MTs. We examined sections from three such beads and could find no unambiguous MTs (Fig. 4 *b*).

We wished to determine if the MT fragments that persisted in the cold-treated bead were merely "mass action fragments" that could not depolymerize in the high tubulin concentration of the cold treated bead, or if they were in some way distinct

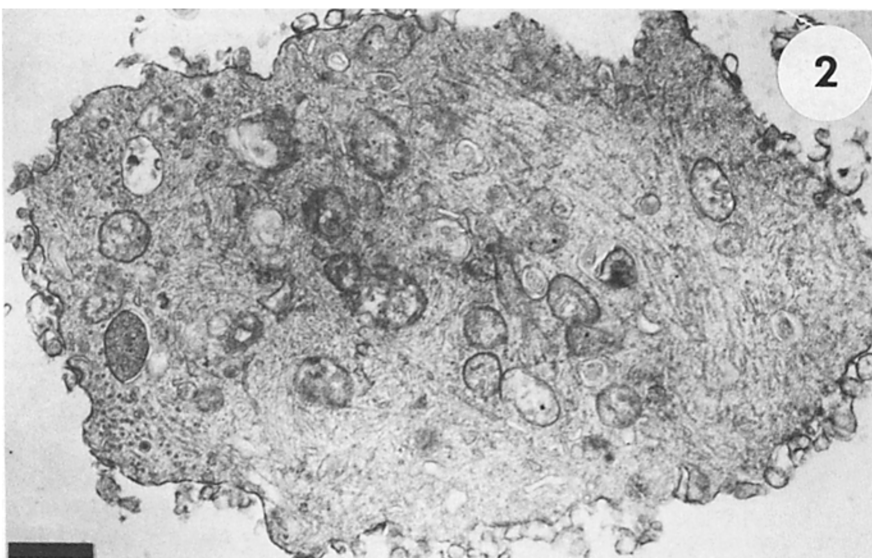
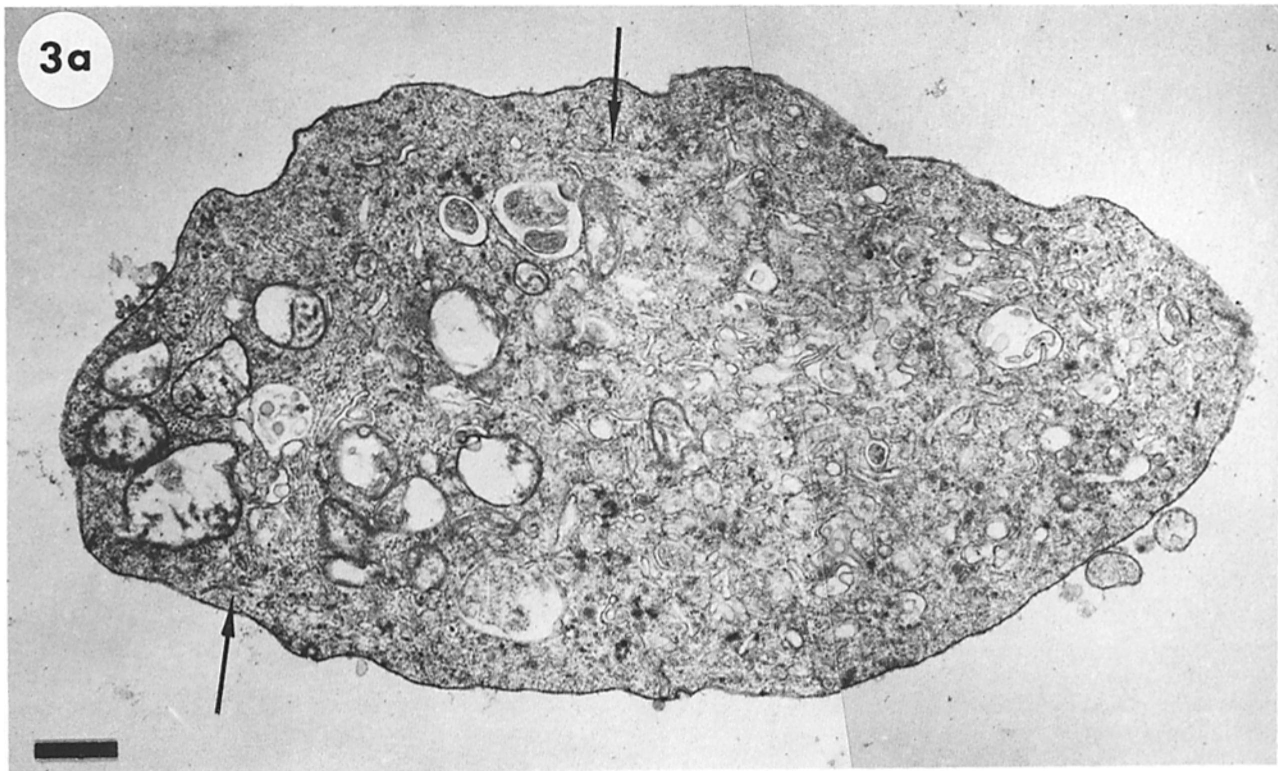


Figure 2. Transmission electron micrograph of a distal portion of an amputated neurite collapsed to a bead and fixed before regrowth. Many MTs of varying lengths are apparent. Bar, 0.5 μm.



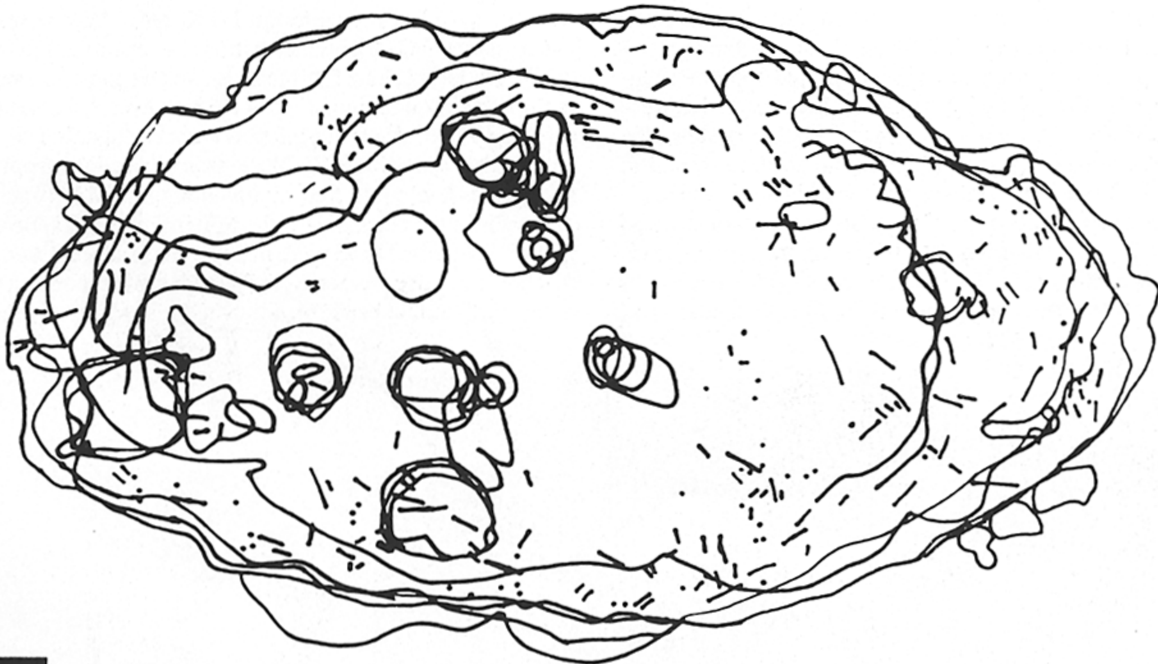


Figure 3. (*a*) Transmission electron micrograph of a collapsed neurite fixed after 2 h at −5°C showing many randomly arranged MT cross sections and short longitudinal sections (*arrows*). (*b*) Serial reconstruction of nine consecutive sections through the cold-treated bead showing MT longitudinal sections as line segments, and cross sections as dots. Bar, 0.5 μm.

from the majority of MT mass that had depolymerized. We reasoned that detergent lysis would release soluble tubulin dimer allowing depolymerization of any "mass action fragments." The efficacy of this treatment in depolymerizing MTs in neurites was examined by comparing the MT array left in intact neurites lysed in an MT reassembly buffer (GEP buffer; see Materials and Methods) with the array left in neurites lysed in the same buffer supplemented with 2 M

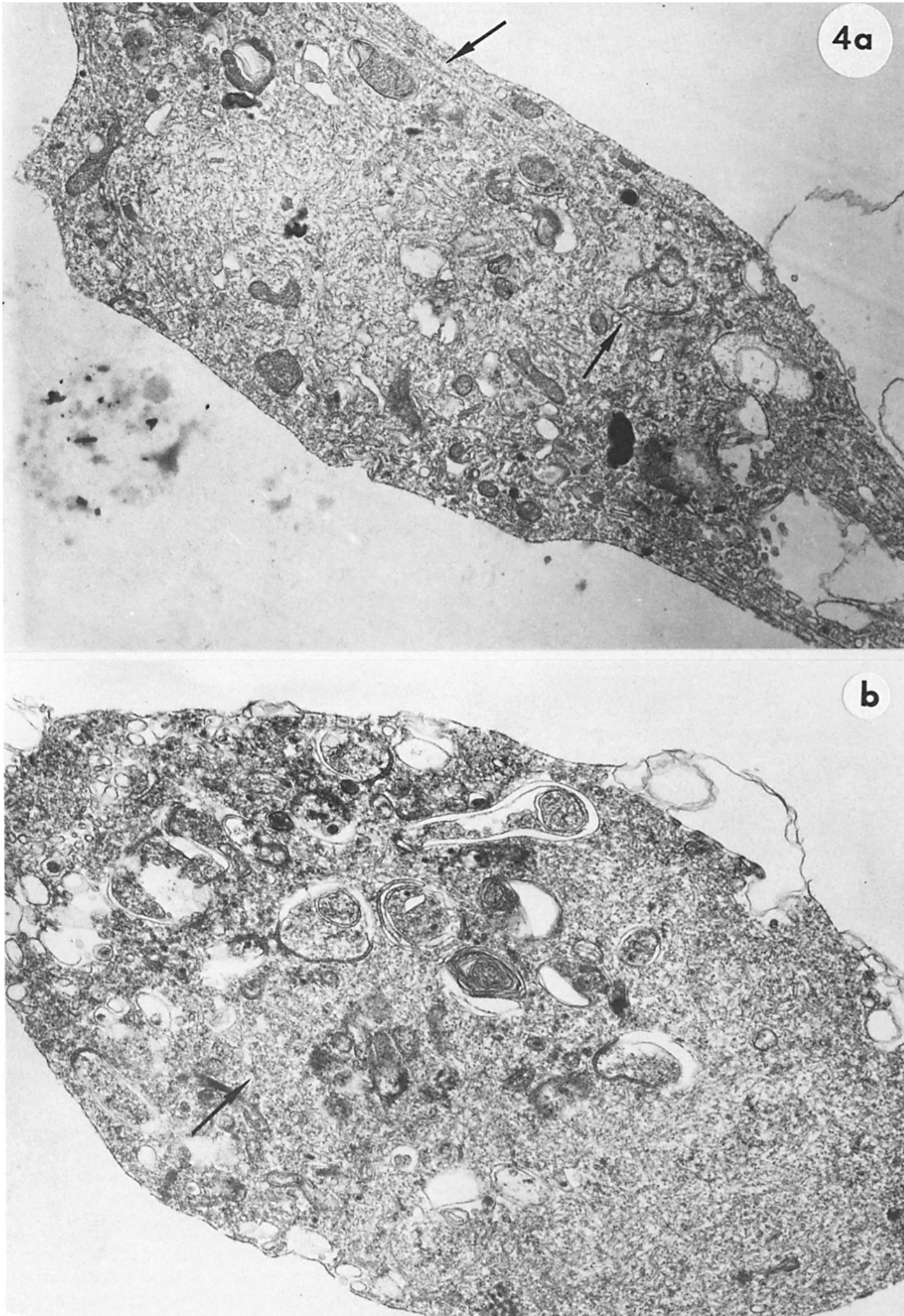


Figure 4. (*a*) Transmission electron micrograph of a collapsed neurite fixed after 15–20 min of treatment with 0.1 μg/ml nocodazole. Partial MT depolymerization is apparent, although MTs of varying lengths persist (*arrows*). (*b*) Transmission electron micrograph of a collapsed neurite fixed after 15–20 min of treatment with 1.0 μg/ml nocodazole. MT depolymerization is complete. Arrow marks an ambiguous vesicular element. Bar, 0.5 μm.

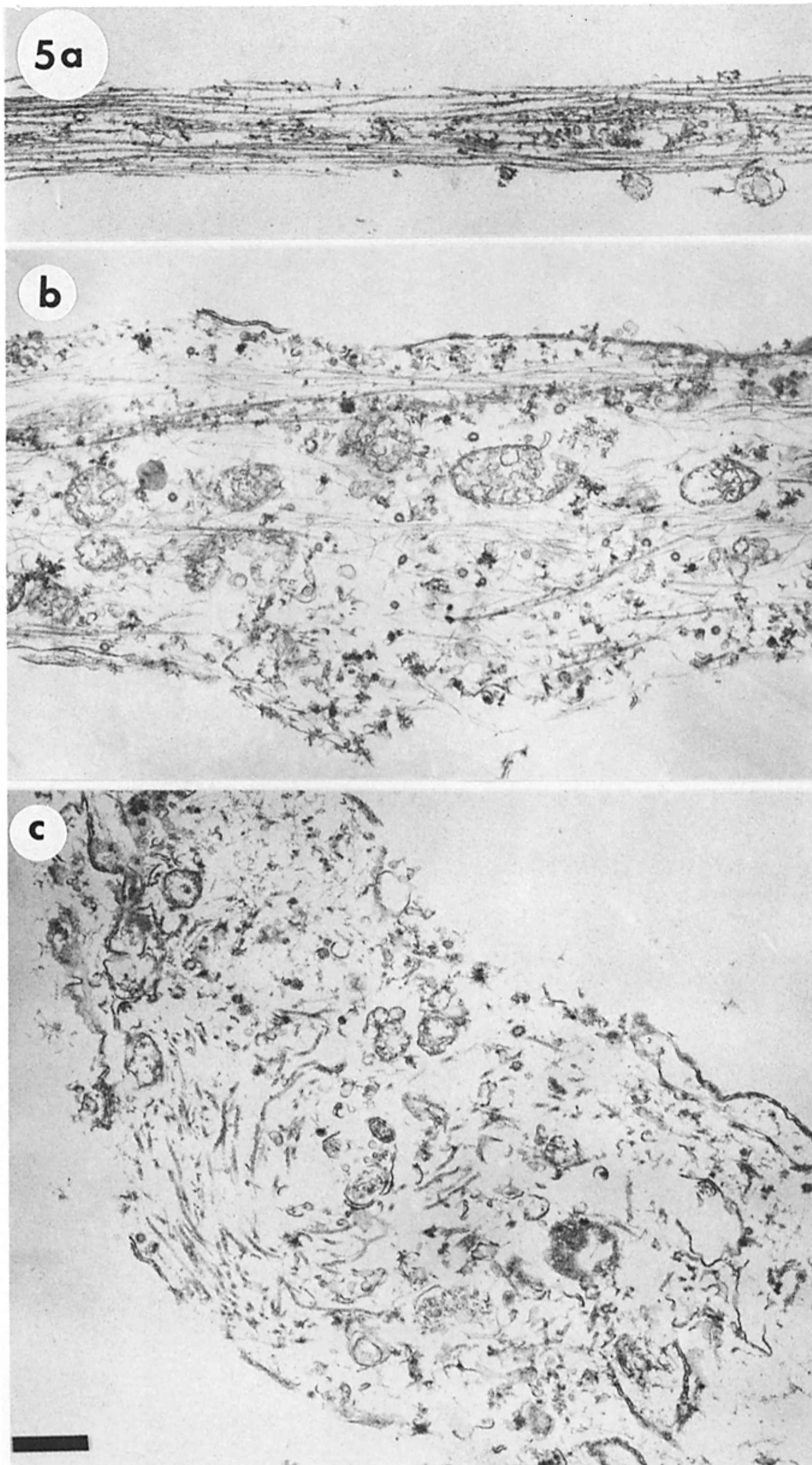


Figure 5. (*a*) Transmission electron micrograph of a neurite lysed in GEP buffer supplemented with 2 M glycerol. The MT array is intact. (*b*) Transmission electron micrograph of a neurite bundle lysed in GEP alone. Substantial MT disassembly is apparent. (*c*) Transmission electron micrograph of a cold-treated, collapsed neurite lysed in GEP alone. MT fragments similar to those found in unlysed cold-treated beads persist. Bar, 0.5 μm.

glycerol. The latter conditions have been found to stabilize assembled MTs against dissassembly by dilution while allowing diffusion of free tubulin in a variety of cells (3, 43). Light microscope observations of neurites lysed in GEP without glycerol showed a transient beading within 1 min of adding lysis buffer, an effect symptomatic of MT depolymerization in neurites (17, 22). Neurites lysed in GEP with glycerol showed no such beading. Electron micrographs of neurites lysed in GEP with glycerol show a dense array of long MTs (Fig. 5 *a*), while electron micrographs of neurites lysed in GEP alone show substantial MT depolymerization (Fig. 5 *b*). Electron micrographs of cold-treated beads lysed in GEP without glycerol (Fig. 5 *c*) show MT fragments similar to those MT fragments in unlysed, cold-treated beads.

Table I. Summary of Regrowth Data

	Unarrested	Cold arrested	0.1 μg/ml Nocodazole arrested
Number of collapse/regrowth events observed	53	10	6
Numbers of events in which regrowth occurred from a completely collapsed bead	37	10	6
Rate of regrowth for regrown neurites averaged per bead (μm/min)	3.5–4.5	3.5–4.5	3.5–4.5
Numbers of beads sectioned and observed under the electron microscope	10	4	4

That is, lysis of cold-treated beads caused no major additional MT disassembly.

Regrowth from Collapsed Neurites

Regrowth of neurites from beads began either within the first 30 s after collapse or often before collapse was complete. We followed regrowth in some cases for 45–60 min after collapse. In these cases the total length of the regrown neurites approximated that of the original distal segment, although the bead never entirely disappeared. The fastest and most robust regrowth typically occurred over the first 15 min. Table I summarizes the regrowth data. Out of 53 amputations, 16 began regrowth before the complete collapse of the neurite. In these cases, the remnants of the growth cone or neurite elongated to form the regrown neurite(s). In the 37 cases in which the amputated neurite collapsed completely, there was no relationship between the number and/or orientation of regrown neurites with the original neurite/growth cone. In all cases regrowth ensued at rates of 3.5–4.5 μm/min per regrown neurite averaged per bead. These rates were at least double those reported by Shaw and Bray (40), perhaps due to our use of plastic instead of glass substrata. Electron micrographs of regrown beads show paraxial MT arrays in the regrown neurites (Fig. 6, *a* and *b*) entirely similar to those in ordinary neurites, and fewer MTs in the bead than before regrowth (Fig. 6 *a*).

After rewarming, regrowth from cold treated beads was very similar to regrowth from untreated beads. Cold arrested regrowth resumed within 30 seconds of the cultures reaching a narrow temperature range within 1–2°C of 37°C. The rates of regrowth from cold treated beads were in the same range of 3.5–4.5 microns/minute as regrowth from untreated beads.

The regrowth of nocodazole treated beads was more difficult to study in that the rinsing required to remove the nocodazole often caused detachment of the bead from the dish surface. Both cold and nocodazole treated beads were less adherent to the substratum than untreated beads. However, cold treated beads did not require a medium change and

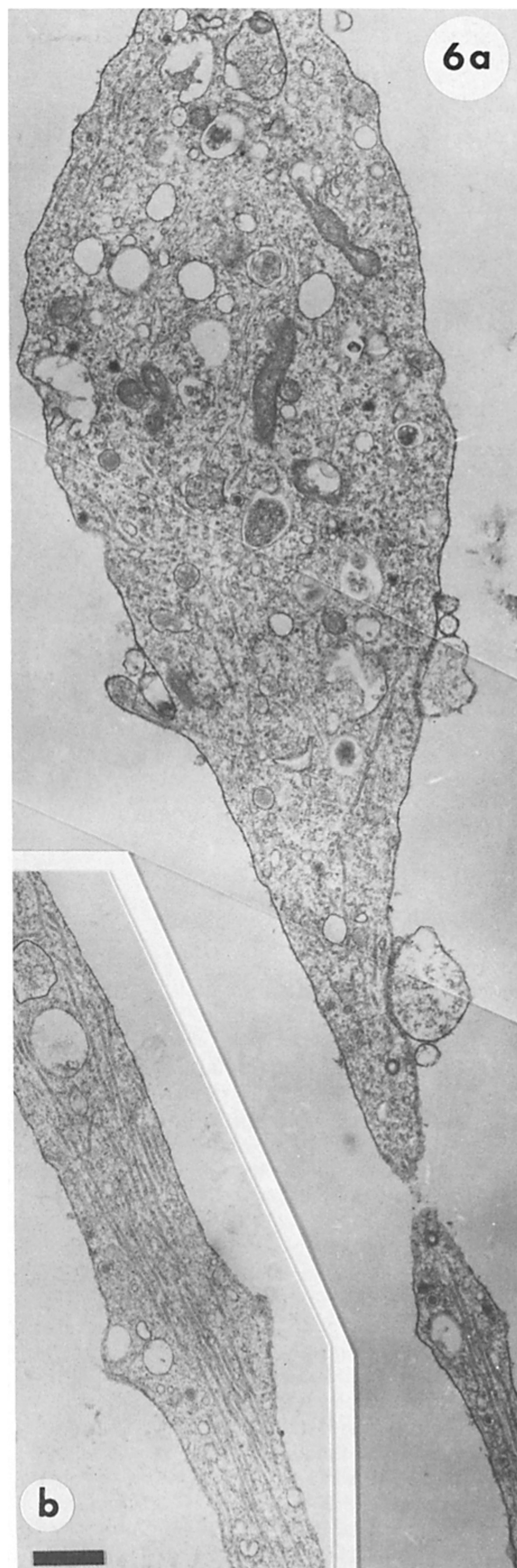


Figure 6. Transmission electron micrographs of an amputated neurite that collapsed to a bead then regrew neurites (*a*) in the region of the bead and (*b*) in the region of the regrown neurite. MTs are randomly oriented and of varying lengths in the bead, but are paraxially oriented and entirely similar to arrays in ordinary neurites in the regrown neurites. Bar, 0.5 μm.

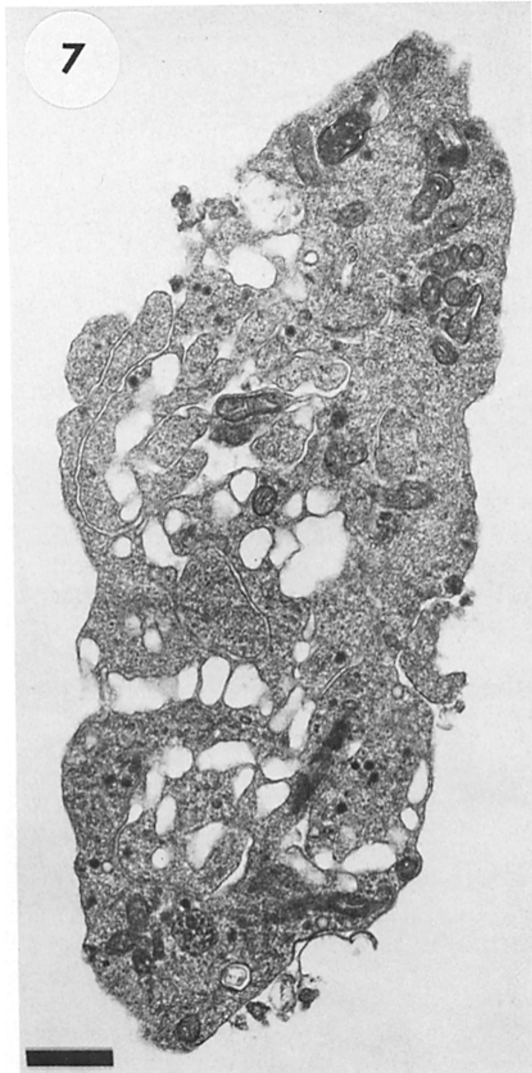


Figure 7. Transmission electron micrograph of a collapsed neurite treated with 1.0 μg/ml nocodazole for 15–20 min, rinsed twice with PBS, and returned to undrugged medium for 30 min before fixation. No MT reassembly has occurred. Bar, 0.5 μm.

thus could be regrown with minimal disturbance. Only 6 of 40 beads remained attached after rinsing of the lower dose (0.1 μg/ml) of nocodazole and only 5 of 50 beads survived rinsing of the higher dose (1.0 μg/ml). All 6 of the beads treated with the lower dose began regrowth within 30 seconds of their return to undrugged medium at a rate of 3.5–4.5 microns/minute. However, the beads treated with the higher dose of nocodazole showed very poor regrowth. Only 1 of the 5 beads recovering from the higher dose regrew and this was only after a 20 minute delay. The other 4 never regrew even after 4 hours of observation.

We wished to determine whether MT assembly was occurring without regrowth in beads rinsed after treatment with 1.0 μg/ml nocodazole. Eight beads were treated with 1.0 μg/ml nocodazole, rinsed twice, and returned to undrugged medium. Seven of the 8 failed to regrow. Electron micrographs of two such beads fixed after 15 min in undrugged medium and three such beads fixed after 30 min in undrugged medium show no MTs (Fig. 7). In two of the 30-min cases, we were able to obtain sections covering ~90% of the bead and all were devoid of MTs. The single bead that regrew did so after a 15-min delay, its regrown neurite contained MTs as expected (not shown), and the bead was on the same plate as a non-regrowing bead found to be devoid of MTs. To determine whether the apparent inability of such beads to initiate MT assembly was due to the incomplete removal of nocodazole by our rinsing method, we examined the recovery of normal neurites treated with a 20-fold higher dose (20 μg/ml) of nocodazole. These unamputated neurites retained a very few MT fragments (Fig. 8 *a*) and reassembled a dense array of MTs after 15 minutes in undrugged medium (Fig. 8 *b*).

To determine whether beads treated with 1.0 μg/ml nocodazole were inviable and/or whether MT reassembly was no longer possible, we treated such beads with taxol. All four beads treated with 1.0 μg/ml nocodazole for 15 min, then rinsed and treated with 4.6×10^{-8} M taxol, rapidly regrew after a delay of ~3 min (Fig. 9). Such regrown neurites contained an array of MTs similar to those of unarrested regrown beads (not shown). Six of 6 beads treated with nocodazole, rinsed, and treated with 2.3×10^{-5} M taxol failed to regrow but showed substantial MT reassembly in the beads (Fig. 10).

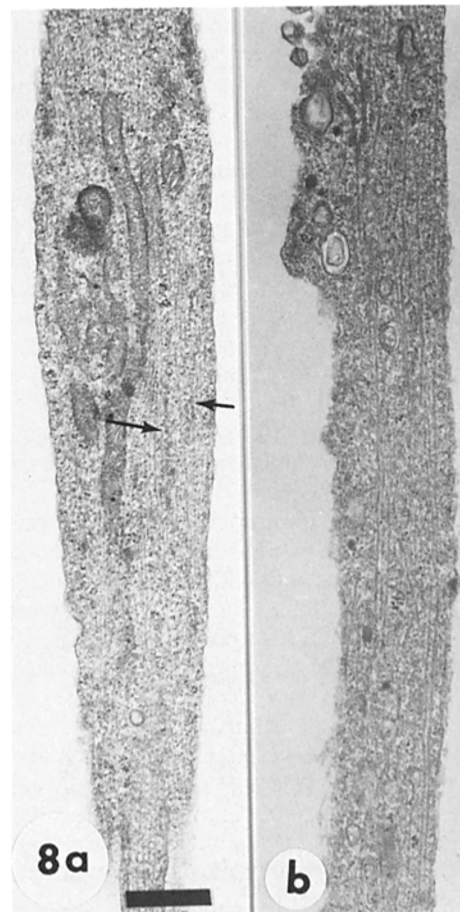


Figure 8. Transmission electron micrographs of (*a*) an intact neurite treated with 20 μg/ml nocodazole for 15–20 min showing persistent MT fragments (*arrows*), and (*b*) an intact neurite treated with 20 μg/ml nocodazole, rinsed twice with PBS, and returned to undrugged medium for 15 min before fixation. Such neurites rapidly recover a normal array of MTs. Bar, 0.5 μm.

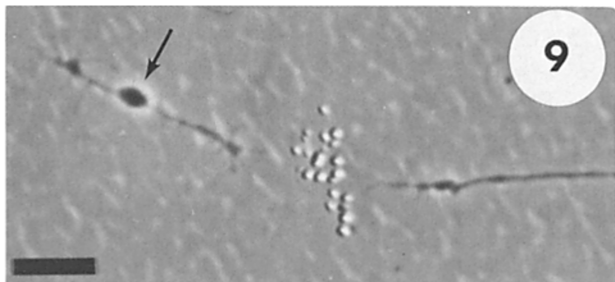


Figure 9. Phase-contrast micrograph of a collapsed neurite (*arrow*) treated with 1.0 μg/ml nocodazole for 15–20 min, rinsed twice with PBS, then treated with fresh medium containing 4.6×10^{-8} M taxol showing robust regrowth that began 3 min after adding taxol. All four neurites treated in this manner regrew neurites after a short delay. Bar, 20 μm.

Discussion

We report here an investigation of the role of MT assembly and disassembly in the regrowth of neurites from the collapsed, distal segments of amputated neurites of chick sensory neurons. Fully collapsed beads contained many long MTs (Fig. 2). One possibility was that these MTs might simply "unfurl" into the axon during regrowth in a manner roughly analogous to Lasek's proposal for slow axonal transport of MTs in the form of assembled polymers (26). However, beads treated at −5°C for 2 h (Fig. 3) or with 0.1 μg/ml nocodazole for 15–20 min (Fig. 4 *a*) contained only short fragments of MTs, and yet regrew with no delay after reaching 37°C or rinsing out the nocodazole in the absence of MTs long enough to "unfurl." The rates of regrowth in all three cases were indistinguishable and in the range of 3.5–4.5 μm/min. The rapidity of regrowth and lack of a delay before the onset of regrowth argue in favor of nucleated reassembly of MTs and against the possibility of de novo initiation (1, 19, 34, 35). Indeed, beads treated with a higher dose of 1.0 μg/ml nocodazole were devoid of MTs (Fig. 4 *b*), and failed to regrow in 11 out of 13 cases. Although the poor adhesion of the treated beads to the substrata might also disable regrowth by not allowing a growth cone to form, beads treated with cold or with the lower dose of nocodazole were equally precariously attached yet regrew without delay. Taxol, not known to increase cell adhesion, enabled beads treated with 1.0 μg/ml nocodazole to regrow. Incomplete removal of nocodazole by our rinsing method also seems an unlikely factor in that a very high dose of 20 μg/ml was rapidly reversible in normal neurites (Fig. 8). Further, neurites treated with the lower dose of nocodazole began regrowth within 30 s of rinsing. The rapidity of this reversal would not be expected if nocodazole was sequestered by some peculiarity of the bead. Nocodazole poisoning has been found to be immediately reversible in a variety of cell types (12, 41). We conclude that rapid regrowth of axonal fragments is dependent upon the presence of MTs or MT fragments that serve as nucleating elements for MT elongation.

In the absence of nucleating elements, the expectation on the basis of MT assembly in vitro would be a delay in regrowth while initial nucleation occurred, after which assembly would occur rapidly (1, 19, 34, 35). In 2 out of 13 cases, beads treated with the higher dose of nocodazole apparently sufficient to completely depolymerize MTs indeed responded in this fashion, while the majority, 11 of 13, did not. One possibility is that in the two cases of regrowth, the 1.0 μg/ml nocodazole was not sufficient to completely depolymerize bead MTs. Given the 15–20 min delay before regrowth, it is more likely that the bead tubulin managed self-nucleated MT assembly after complete depolymerization. However, the failure of 11 out of 13 to regrow even after extended periods suggests a limited capacity of bead tubulin for self-nucleation. This suggestion is supported by the observation that beads that failed to regrow after 15 or 30 min in undrugged medium were still devoid of MTs (Fig. 7). Beads rinsed of 1.0 μg/ml nocodazole and then treated with taxol, a drug that promotes MT assembly (38), was sufficient to stimulate bead tubulin into initiating assembly de novo. In 4 out of 4 cases, beads treated with 4.6×10^{-8} M taxol after being rinsed of 1.0 μg/ml nocodazole regrew after a 3-min delay (Fig. 9). In six other cases, a higher dose of 2.3×10^{-5} M taxol failed to stimulate regrowth, but did cause substantial MT reassembly in the beads (Fig. 10). Letourneau and Ressler had previously reported that neurite outgrowth from chick sensory neurons was limited to low levels of taxol and that higher doses inhibit neurite outgrowth (28). The ready regrowth when MT assembly is stimulated by taxol suggests that it was indeed the lack of MT assembly that inhibited regrowth previously, rather than inviability of the bead or loss of competent tubulin. It is unclear from our experiments whether the limited capacity of tubulin to initiate assembly is characteristic of neuronal tubulin or of the bead only. Morris and Lasek (32) found in squid axoplasm that the amount of tubulin in subunit form is higher than what would be predicted by in vitro studies given the total tubulin

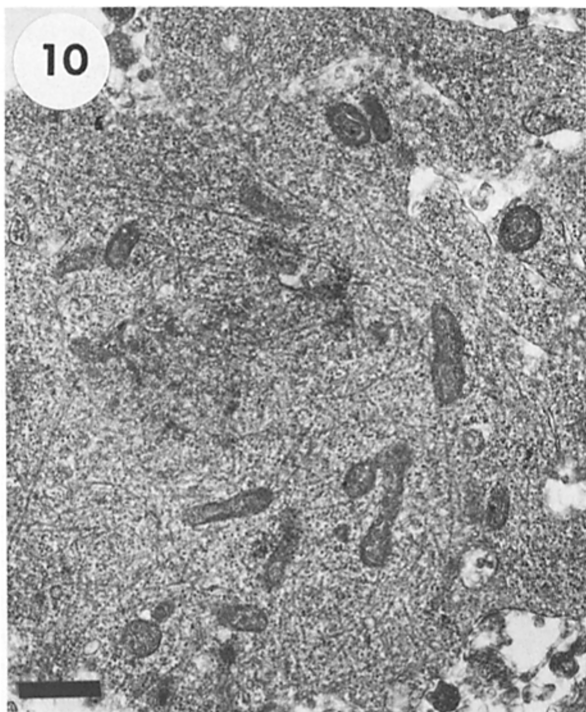


Figure 10. Transmission electron micrograph of a collapsed neurite treated with 1.0 μg/ml nocodazole for 15–20 min, rinsed twice with PBS, then treated with fresh medium containing 2.3×10^{-5} M taxol for 30 min. No regrowth ensued, but substantial MT reassembly is apparent. Bar, 0.5 μm.

concentration. They concluded that there are factors in the cell that inhibit the polymerization of tubulin. Our evidence suggests that such factors may function in part by inhibiting de novo initiation. On the other hand, we found that the bead had some MT disassembly properties not shared by the intact neurite. Nocodazole was more effective at completely depolymerizing MTs in beads than in intact neurites (Figs. 4 *b* and 8 *a*). Lasek's model for the slow transport of cytoskeletal elements maintains that MTs are selectively degraded in axon terminals (25). Burton has recently reported evidence of MT disassembly at the distal ends of intact frog axons (9). During collapse of the neurite, its terminal growth cone becomes incorporated into the bead. If a degradation factor similar to that in synaptic terminals exists in the growth cones of advancing neurites, it might account for both the increased sensitivity of bead MTs to nocodazole and their limited ability to self-nucleate.

If neuronal tubulin has only a limited capacity for self-nucleation, the importance of MT nucleating elements would be paramount for cytoskeletal reorganizations occurring during axonal growth. Abundant evidence suggests that certain neuronal MTs are especially stable (2, 4, 16, 18, 21, 45), and that these MTs exist in the form of short regions of otherwise labile MTs (18, 22, 37). We wondered whether the fragments left in the cold-treated bead were merely "mass action fragments" maintained because of a high tubulin concentration in the bead, or if they were intrinsically stable regions of longer MTs. We reasoned that if the fragments were "mass action fragments" they would depolymerize if the free tubulin were allowed to escape from the bead by lysing. If, however, the fragments were intrinsically stable, they would not depolymerize upon subunit dilution. Indeed, as shown in Fig. 5, MT fragments persisted in cold-treated beads lysed under conditions that caused substantial MT depolymerization in normal neurites. All available evidence indicates that the lysis buffer we used causes MT disassembly by dilution since it supports MT assembly in vitro while allowing soluble tubulin to diffuse from lysed cells (3, 11, 43). We interpret this as indirect evidence that the fragments in the cold-treated beads were different from the majority of the MT mass. Job et al. (18), Brady et al. (4), and others have provided evidence that cold stable MTs differ in either MAP or tubulin composition. The fragments left in the cold-treated beads might similarly vary from the soluble MT fraction for these reasons.

We wish to thank Mike McKinney and Bruce Buckmaster of the Animal Science Department at Michigan State University (MSU) for their expert assistance in the handling of eggs, and Dr. Jacob Krier of the Department of Physiology, MSU, for allowing us to use his microelectrode puller. We are also indebted to the Department of Microbiology, MSU, for the use of their electron microscope facility. We especially wish to thank H. Stuart Pankratz for his assistance and encouragement in all phases of this work. We are also indebted to the reviewers of this paper for their stimulating comments.

This work was supported by National Science Foundation grant BNS 8401904. S. R. Heidemann is a recipient of a Research Career Development Award from the National Institutes of Health.

Received for publication 10 March 1986.

References

1. Allen, C., and G. G. Borisy. 1974. Structural polarity and directional growth of microtubules of *Chlamydomonas* flagella. *J. Mol. Biol.* 90:381–402.

2. Black, M. M., J. M. Cochran, and J. T. Kurdyla. 1984. Solubility properties of neuronal tubulin: evidence for labile and stable microtubules. *Brain Res.* 295:255–265.

3. Black, M. M., and L. A. Greene. 1982. Changes in the colchicine susceptibility of microtubules associated with neurite outgrowth: studies with nerve growth factor-responsive PC12 pheochromocytoma cells. *J. Cell Biol.* 95: 379–386.

4. Brady, S. T., M. Tytell, and R. J. Lasek. 1984. Axonal tubulin and axonal microtubules: biochemical evidence for cold stability. *J. Cell Biol.* 99:1716–1724.

5. Bray, D. 1973. Branching patterns of individual sympathetic neurons in culture. *J. Cell Biol.* 56:702–712.

6. Bray, D., and D. Gilbert. 1981. Cytoskeletal elements in neurons. *Annu. Rev. Neurosci.* 4:505–523.

7. Bunge, M. B. 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* 56:713–735.

8. Burton, L. E., W. H. Wilson, and E. M. Shooter. 1978. Nerve growth factor from mouse saliva. *J. Biol. Chem.* 253:7807–7812.

9. Burton, P. R. 1985. Microtubules of the frog olfactory axons: their length, number and the apparent fate of their distal ends. *J. Cell Biol.* 101(5, Pt. 2):125a. (Abstr.)

10. Campenot, R. B. 1977. Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 74:4516–4519.

11. Cande, W. Z., J. Snyder, D. Smith, K. Summers, and J. R. McIntosh. 1974. A functional mitotic spindle prepared from mammalian cells in culture. *Proc. Natl. Acad. Sci. USA.* 71:1559–1563.

12. DeBrabander, M., G. Geuens, R. Van de Veire, F. Thone, F. Aerts, L. Desplanter, J. De Cree, and M. Borgers. 1977. The effects of R 17934 a new antimicrotubular substance, on the ultrastructure of neoplastic cells in vivo. *Eur. J. Cancer.* 13:511–528.

13. George, E. B., and R. J. Lasek. 1983. Contraction of isolated neural processes: a model for studying cytoskeletal translocation in neurons. *J. Cell Biol.* 97 (5, Pt. 2):267 a. (Abstr.)

14. Heidemann, S. R., J. M. Landers, and M. A. Hamborg. 1981. Polarity orientation of axonal microtubules. *J. Cell Biol.* 91:661–665.

15. Heidemann, S. R., M. A. Hamborg, S. J. Thomas, B. Song, S. Lindley, and D. Chu. 1984. Spatial organization of axonal microtubules. *J. Cell Biol.* 99:1289–1295.

16. Hesketh, J. E. 1984. Differences in polypeptide composition and enzyme activity between cold-stable and cold-labile microtubules and study of microtubule alkaline phosphatase activity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 169:313–318.

17. Horie, H., T. Takenaka, and M. Kaiho. 1983. Effects of disruption of microtubules on translocation of particles and morphology in tissue cultured neurites. *Brain Res.* 288:85–93.

18. Job, D., C. T. Rauch, E. H. Fischer, and R. L. Margolis. 1982. Recycling of cold stable microtubules: evidence that cold stability is due to substoichiometric polymer blocks. *Biochemistry.* 21:509–515.

19. Johnson, K. A., and G. G. Borisy. 1977. Kinetic analysis of microtubule self-assembly *in vitro*. *J. Mol. Biol.* 117:1–32.

20. Johnston, R. N., and N. K. Wessells. 1980. Regulation of the elongation of nerve fibers. *Curr. Top. Dev. Biol.* 16:165–206.

21. Jones, D. H., E. G. Gray, and J. Barron. 1980. Cold stable microtubules in brain studied in fractions and slices. *J. Neurocytol.* 9:493–504.

22. Joshi, H. C., P. Baas, D. T. Chu, and S. R. Heidemann. 1986. The cytoskeleton of neurites after microtubule depolymerization. *Exp. Cell Res.* 163:233–244.

23. 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.

24. Landis, S. C. 1983. Neuronal growth cones. *Annu. Rev. Physiol.* 45: 567–580.

25. Lasek, R. J. 1981. The dynamic ordering of the neuronal cytoskeleton. *Neurosci. Res. Program Bull.* 19:7–32.

26. Lasek, R. J. 1982. Translocation of the neuronal cytoskeleton and axonal locomotion. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:313–327.

27. Letourneau, P. C. 1975. Cell-to-substratum adhesion and guidance of axonal elongation. *Dev. Biol.* 44:92–101.

28. Letourneau, P. C., and A. H. Ressler. 1984. Inhibition of neurite initiation and growth by taxol. *J. Cell Biol.* 98:1355–1362.

29. Lyser, K. M. 1968. An electron microscopic study of centrioles in differentiating neuroblasts. *J. Embryol. Exp. Morphol.* 20:343–354.

30. Mitchison, T., and M. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature (Lond.).* 312:237–242.

31. Morris, J. R., and R. J. Lasek. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192–198.

32. Morris, J. R., and R. J. Lasek. 1984. Monomer-polymer equilibria in the axon: direct measurement of tubulin and actin a spolymer and monomer in axoplasm. *J. Cell Biol.* 98:2064–2076.

33. Nicklas, R. B., D. F. Kubai, and T. S. Hays. 1982. Spindle microtubules and their mechanical associations in anaphase. *J. Cell Biol.* 95:91–104.

34. Olmsted, J. B., J. M. Marcum, K. A. Johnson, C. Allen, and G. C. Borisy. 1974. Microtubule assembly: some possible regulatory mechanisms. *J. Supramol. Struct.* 2:429–450.

35. Oosawa, F., and S. Asakura. 1975. Thermodynamics of the Polymerization of Protein. Academic Press, Inc. New York. 41–47.

36. Pachter, J. S., R. K. H. Liem, and M. L. Shelanski. 1984. The neuronal cytoskeleton. *Adv. Cell. Neurobiol.* 5:113–142.
37. Sahenk, Z., and S. Brady. 1983. Morphological evidence for stable regions in axonal microtubules. *J. Cell Biol.* 97(5, pt. 2):210a. (Abstr.)
38. Schiff, P. B., J. Fant, and S. B. Horwitz. 1979. Promotion of microtubule assembly *in vitro* by taxol. *Nature (Lond.).* 277:665–667.
39. Sharp, G. A., K. Weber, and M. Osborn. 1982. Centriole number and process formation in established neuroblastoma cells and primary dorsal root ganglion neurones. *Eur. J. Cell Biol.* 29:97–103.
40. Shaw, G., and D. Bray. 1977. Movement and extension of isolated growth cones. *Exp. Cell Res.* 104:55–62.
41. Snyder, J. A., and B. T. Hamilton. 1982. Loss of mitotic centrosomal microtubule initiation capacity at the metaphase-anaphase transition. *Eur. J. Cell Biol.* 27:191–199.
42. Solomon, F. 1980. Organizing microtubules in the cytoplasm. *Cell.* 22:331–332.
43. Solomon, F., M. Magendantz, and A. Salzman. 1979. Identification with microtubules of one of the microtubule-associated proteins. *Cell.* 18:431–438.
44. Tucker, J. B. 1979. Spatial organization of microtubules. *In* Microtubules. K. Roberts and J. S. Hyams, editors. Academic Press, Ltd. London. 315–357.
45. Webb, B. C., and L. Wilson. 1980. Cold stable microtubules from brain. *Biochemistry.* 19:1991–2001.
46. Wessells, N. K., S. R. Johnson, and R. P. Nutall. 1978. Axon initiation and growth cone regeneration in cultured motor neurons. *Exp. Cell Res.* 117:335–345.
47. Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1970. Axon growth: role of microfilaments and microtubules. *Proc. Natl. Acad. Sci. USA.* 66:1206–1212.
48. Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* 49:614–635.
49. Zenker, W., and E. Hohberg. 1973. An alpha-nerve fiber: number of neurotubules in the stem fiber and in the terminal branches. *J. Neurocytol.* 2:143–148.