

Individual Microtubules in the Axon Consist of Domains That Differ in Both Composition and Stability

Peter W. Baas and Mark M. Black

Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Abstract. We have explored the composition and stability properties of individual microtubules (MTs) in the axons of cultured sympathetic neurons. Using morphometric means to quantify the MT mass remaining in axons after various times in 2 $\mu\text{g/ml}$ nocodazole, we observed that $\sim 48\%$ of the MT mass in the axon is labile, depolymerizing with a $t_{1/2}$ of ~ 5 min, whereas the remaining 52% of the MT mass is stable, depolymerizing with a $t_{1/2}$ of ~ 240 min. Immunofluorescence analyses show that the labile MTs in the axon are rich in tyrosinated α -tubulin, whereas the stable MTs contain little or no tyrosinated α -tubulin and are instead rich in posttranslationally detyrosinated and acetylated α -tubulin. These results were confirmed quantitatively by immunoelectron microscopic analyses of the distribution of tyrosinated α -tubulin among axonal MTs. Individual MT profiles were typically either uniformly labeled for tyrosinated α -tubulin all along their length, or were completely unlabeled. Roughly 48% of the MT mass was tyrosinated, $\sim 52\%$ was detyrosinated, and $\sim 85\%$ of the tyrosinated MTs were depleted within 15 min of nocodazole treatment. Thus, the proportion of MT

profiles that were either tyrosinated or detyrosinated corresponded precisely with the proportion of MTs that were either labile or stable respectively. We also observed MT profiles that were densely labeled for tyrosinated α -tubulin at one end but completely unlabeled at the other end. In all of these latter cases, the tyrosinated, and therefore labile domain, was situated at the plus end of the MT, whereas the detyrosinated, and therefore stable domain was situated at the minus end of the MT, and in each case there was an abrupt transition between the two domains. Based on the frequency with which these latter MT profiles were observed, we estimate that minimally 40% of the MTs in the axon are composite, consisting of a stable detyrosinated domain in direct continuity with a labile tyrosinated domain. The extreme drug sensitivity of the labile domains suggests that they are very dynamic, turning over rapidly within the axon. The direct continuity between the labile and stable domains indicates that labile MTs assemble directly from stable MTs. We propose that stable MTs act as MT nucleating structures that spatially regulate MT dynamics in the axon.

MICROTUBULES (MTs)¹ are actively involved in the growth and maintenance of the axon. This was initially inferred from ultrastructural studies of MT organization in growing axons (Yamada et al., 1971; Peters et al., 1974), and from pharmacologic studies demonstrating that anti-MT drugs inhibit the growth of axons and can cause their retraction (Yamada et al., 1970; Daniels, 1973). Since these initial observations were made, considerable effort has been devoted toward defining in molecular terms the mechanisms by which MT assembly and organization are regulated in the axon (for reviews, see Lasek, 1988; Meininger and Binet, 1989; Diaz-Nido et al., 1990).

To date, most information on MT assembly and organization derives from *in vitro* studies on isolated MT proteins, or from studies on nonneuronal cells. These studies have established that MTs are assembled from tubulin subunits, and are intrinsically polar in their assembly kinetics (for review,

see Dustin, 1984). One end of the MT is favored for assembly over the other (for review, see Binder et al., 1975), and these two ends of the MT are referred to as the "plus" and "minus" end respectively (Bergen and Borisy, 1980). In the test tube, MT assembly can occur *de novo* by self-association of free subunits, but preferentially occurs by elongation from a MT template or nucleating structure (for review, see Kirschner, 1978). Studies on MT assembly in intact cells indicate that self-association is strongly suppressed in favor of nucleated assembly, and that MT nucleation is spatially regulated by discrete nucleating structures such as the centrosome (for review, see Brinkley, 1985). The minus ends of the MTs are directly associated with the nucleating structure, while the plus ends radiate away from the nucleating structure (Haimo et al., 1979; Heidemann and McIntosh, 1980; Euteneuer and McIntosh, 1981). Thus, a hallmark feature of MT nucleating structures is that they generate MT arrays of uniform polarity orientation.

It is now well established that MTs in the axon also have

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

a uniform polarity orientation; the plus ends of the MTs are directed away from the cell body toward the axon tip (Heidemann et al., 1981; Burton and Paige, 1981; Baas et al., 1987, 1988, 1989). Because axonal MTs are dynamic polymers, undergoing assembly and disassembly locally within the axon (Morris and Lasek, 1984; Okabe and Hirokawa, 1988; Black et al., 1989), mechanisms must exist within the axon to organize local MT dynamics so that uniform polarity orientation is maintained. This conclusion is reinforced by the observation that the uniform polarity orientation of axonal MTs is recapitulated after recovery from episodes of MT depolymerization (Heidemann et al., 1984; Baas et al., 1987). Thus, it is likely that nucleating structures organize MT dynamics locally in the axon. However, axons lack traditional MT nucleating structures such as the centrosome (Lyser, 1968; Sharp et al., 1982; Stevens et al., 1988; Okabe and Hirokawa, 1988), and there is no structural evidence of specializations at the minus ends of axonal MTs which could serve as nucleating structures (Chalfie and Thompson, 1979; Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). What are the structures that serve as templates for local MT dynamics in the axon?

It has been hypothesized by several authors that a unique class of especially stable MTs may act as the nucleating structures for MT dynamics in the axon (Brady et al., 1984; Morris and Lasek, 1984; Black et al., 1984; Heidemann et al., 1984; Baas and Heidemann, 1986). A key prediction of this model is that individual MTs in the axon will consist of a stable domain in direct continuity with a newly assembled domain. The newly assembled domain is presumably more labile than the stable domain in that MT stabilization is thought to occur only after a temporal delay after MT assembly (Kirschner and Mitchison, 1986; Khawaja et al., 1988). In support of the view that axonal MTs consist of stable and labile domains, stable and labile MTs have been identified in the axon (Brady et al., 1984; Morris and Lasek, 1982, 1984; Black et al., 1984, 1989), and it has been shown that the MTs remaining in the axon after episodes of MT depolymerization are generally shorter than the MTs present in the unperturbed axon (Baas and Heidemann, 1986; Joshi et al., 1986; Sahenk and Brady, 1987). However, to date, no evidence has been provided which directly demonstrates continuity between stable and labile MTs in the axon.

Here, we document a compositional difference between stable and labile MTs in the axon that permits us for the first time to simultaneously visualize and distinguish them at the electron microscopic level, and hence directly determine their spatial relationship relative to one another. In many types of MT arrays, posttranslational deetyrosination occurs on a portion of the α -tubulin after its incorporation into MTs (Gunderson et al., 1987), such that the more recently assembled, labile MTs are rich in the tyrosinated form of α -tubulin, whereas the older, more stable MTs are rich in the deetyrosinated form (Gunderson and Bulinski, 1986; Schulze et al., 1987; Kreis, 1987; Webster et al., 1987; Wheland and Weber, 1987; Gunderson et al., 1987; Bulinski et al., 1988). Our data indicate that this is also the case in the axon. Labile MTs label with the mAb YL 1/2, which recognizes the tyrosinated but not the deetyrosinated form of α -tubulin (Kilmartin et al., 1982; Wheland et al., 1983), whereas stable MTs do not label with this antibody. Using YL 1/2 in immunoelectron microscopic analyses, we demonstrate direct continuity be-

tween stable and labile MTs in the axon. Thus, individual MTs in the axon consist of distinct stable and labile domains.

Materials and Methods

Cell Culture

Rat sympathetic neurons were grown either as dissociated cultures or as explant cultures. For dissociated cultures, superior cervical ganglia were dissected from 1–5-d rat pups, treated with 0.25 mg/ml collagenase for 1 h followed by 0.25 mg/ml trypsin for 45 min, and then triturated with a Pasteur pipette into a single cell dispersion. The neurons were then plated onto collagen-coated 35-mm tissue culture dishes in N2 medium (Moya et al., 1980) supplemented with 2.5% FCS, 5% human placental serum, 50 ng/ml nerve growth factor, and 0.6% methyl cellulose. Cultures were fed the following morning with the same medium without the methyl cellulose, but containing 5 μ M cytosine arabinoside to reduce nonneuronal contamination. After 10–14 d in culture, the neurons were transplanted to glass chamber slides that had been coated with polyethylene amine and collagen as previously described (Black and Keyser, 1987). For transplantation, cultures were rinsed twice in PBS, and then treated with 0.25 mg/ml collagenase for 15 min to release the neurons from the substratum. The floating mass of neurons, held together by a dense network of neurites, was then lifted from the culture dish, treated for 15 min with 0.25% trypsin and triturated into a single cell dispersion. Cell bodies were separated from remnants of the neurites by centrifugation, and were then plated into the chamber slides. For most experiments reported here, the transplanted neurons were maintained for 7–10 d before experimentation, although comparable results were obtained with cultures ranging in age from 3–14 d. This procedure resulted in moderate to low density cultures in which individual neurons generated readily distinguishable axons and dendrites.

Explant cultures were prepared using a procedure slightly modified from Peng et al., 1986. Briefly, sympathetic ganglia were cut into 2–3 pieces/ganglion, and placed into collagen-coated 35-mm culture dishes containing N2 medium supplemented with 1% human placental serum, 50 ng/ml nerve growth factor, and 0.6% methyl cellulose. Only 3–4 explants were grown per dish so that the axonal networks of neighboring explants did not overlap. Cultures were fed the next morning with the same medium without the methyl cellulose and serum, but containing 10 μ M cytosine arabinoside, and were maintained for 7–10 d before experimentation. Explant cultures were a great advantage for electron microscopic analyses of axonal MTs because they consist of a centralized cell body mass that extends a dense halo of aligned axons, hundreds of which could be thin-sectioned simultaneously without contamination from cell bodies, dendrites, or nonneuronal cells (see Fig. 1).

Effects of Nocodazole on Axonal Microtubules

To quantify the effects of nocodazole on axonal MTs, explant cultures were treated for times ranging between 0 and 360 min with 2 μ g/ml nocodazole (Aldrich Chemical Co., Milwaukee, WI), and then prepared for electron microscopy as previously described (Baas and Heidemann, 1986). After embedment in resin, regions of the axonal halo were sectioned, viewed electron microscopically, and photographed. Total lengths of MT profiles were measured in the electron micrographs for each time point, and standardized to unit area of axoplasm. The latter was determined by trimming all empty space from the electron micrographs and weighing the micrographs as previously described (White et al., 1987). The MT mass remaining after drug treatment was then expressed as a function of the MT mass in control axons.

Immunofluorescence Analyses

Cultures on chamber slides were prepared for immunofluorescence analyses using one of two extraction protocols, a harsher protocol for the moderate density cultures, and a milder protocol for the low density cultures. For the first, cultures were rinsed briefly in PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂ [pH 6.9]; Schliwa and van Blerkom, 1981), and then extracted for 10 min with 1% Triton X-100 in PHEM also containing 10 μ M taxol (gift from the National Cancer Institute) and 0.2 M NaCl to remove free tubulin but stabilize existing MTs (Black et al., 1986). For the second, cultures were rinsed briefly in PHEM, and extracted for 5 min with 0.2% Triton X-100 in PHEM containing 10 μ M taxol but no NaCl. For most experiments, extracted cultures were fixed by immersion in -20°C methanol for 10 min, rinsed with PBS, and incubated for 15 min

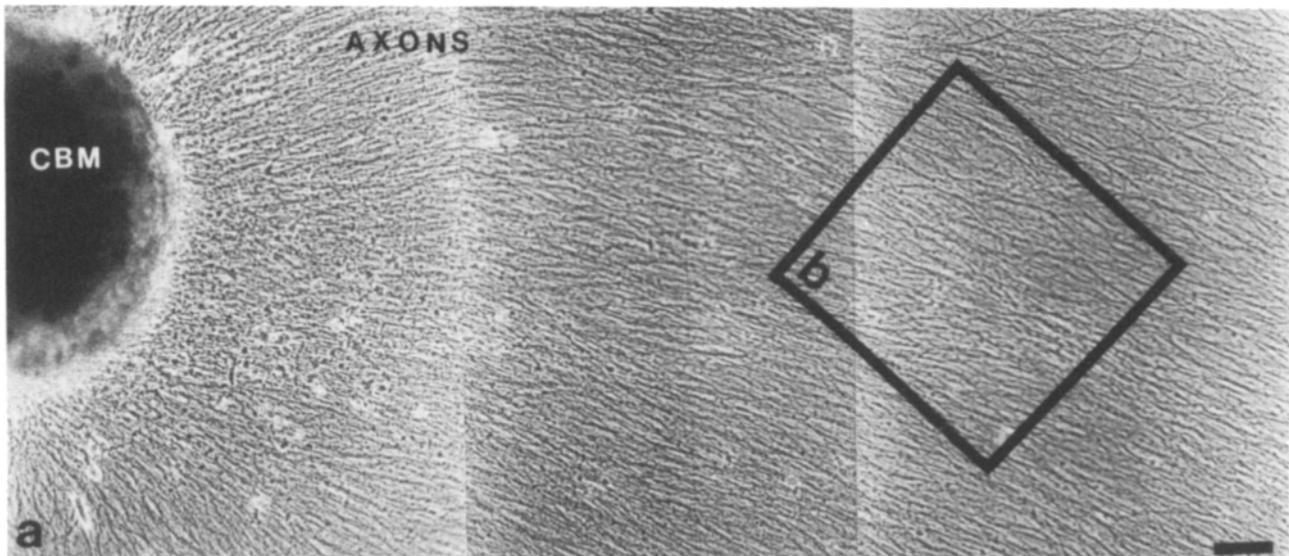


Figure 1. (a) Phase-contrast micrograph of an explant culture of sympathetic neurons showing the cell body mass (CBM) and the axonal halo. Insert (b) shows a typical region of pure axons used for electron microscopic analyses of axonal MTs. Bar, 100 μm .

in a blocking solution containing 2% normal goat serum and 1% BSA. Cultures were then incubated with two primary antibodies (see below) simultaneously for 1 h at 35°C, rinsed extensively, incubated again for 15 min in blocking solution, incubated with two appropriate fluorescently tagged second antibodies simultaneously for 1 h at 35°C, and rinsed extensively again. The slides were then removed from their chambers, mounted with a coverslip, and viewed with a Zeiss Axiovert 35 microscope using epifluorescence optics. In a limited number of experiments, extracted cultures were fixed with 0.5% glutaraldehyde in PHEM for 10 min at room temperature, rinsed with PBS, incubated with 2 mg/ml sodium borohydride for 15 min, and then blocked and incubated with primary and secondary antibodies as described above. Comparable results were obtained with either fixation protocol. The primary antibodies were YL 1/2, a rat mAb specific for tyrosinated α -tubulin (Kilmartin et al., 1982; Wheland et al., 1983), purchased from Accurate Chemical & Scientific Corp. (Westbury, NY), and used at 1:100 dilution, and 6-11B-1, a mouse mAb specific for acetylated α -tubulin (Piperno and Fuller, 1985), generously provided by Dr. G. Piperno of the Rockefeller University, and used as the undiluted culture supernatant. In some experiments, a mouse general α -tubulin mAb (purchased from Amersham Corp., Arlington Heights, IL) was substituted for the 6-11B-1. The second antibodies were a fluorescein-conjugated goat anti-rat adsorbed against mouse Ig, and a Texas red-conjugated goat anti-mouse adsorbed against rat Ig, both purchased from Accurate Chemical & Scientific Corp. and used at 1:100 dilution.

Immunoelectron Microscopy

For immunoelectron microscopic analyses of axonal MTs, explant cultures were rinsed briefly in PHEM, extracted for 10 min with 1% Triton X-100 in PHEM also containing 10 μM taxol and 0.2 M NaCl, and then fixed by the addition of an equal volume of PHEM containing 1% glutaraldehyde (Polysciences Inc., Warrington, PA) directly to the extraction buffer. After 10 min of fixation, the cultures were rinsed in PHEM, then treated with 2 mg/ml sodium borohydride in PHEM for 15 min. Subsequent steps in our protocol were adapted directly from the standard protocol described by Geuens et al. (1986). After sodium borohydride treatment, cultures were rinsed briefly in TBS₁ (10 mM Tris, 140 mM NaCl, pH 7.6), incubated with a blocking solution containing 5% normal goat serum in TBS₁, incubated overnight at 4°C with primary antibody (either YL 1/2 used at 1:200, or a mouse β -tubulin mAb purchased from Amersham Corp., and used at 1:50) diluted in TBS₁ containing 1% normal goat serum, rinsed extensively in TBS₂ (20 mM Tris, 140 mM NaCl, pH 8.2) containing 0.1% BSA, incubated for 3 h at 35°C with an appropriate second antibody conjugated to 5-nm gold particles (purchased from Janssen Life Sciences, Piscataway, NJ) diluted in TBS₂ containing 0.1% BSA, and rinsed extensively in TBS₂. The cultures were then fixed for 10 min with 1% glutaraldehyde

in 0.1 M cacodylate also containing 2 mg/ml tannic acid, rinsed in 0.1 M cacodylate, and postfixed in 2% OsO₄ for 10 min. Subsequent steps in electron microscopy were as previously described (Baas and Heidemann, 1986). In some experiments, the epon block was stained for 1 h at 60°C with 1% toluidine blue in 1% borax, buffed with lens tissue, and then covered with a thin layer of immersion oil. This procedure, modified from Bartlett and Banker (1984), enhanced the resolution of the axon as viewed by phase-contrast microscopy, and hence permitted us to readily identify the most distal regions of the extracted axons.

Results

Axons Contain Both Stable and Labile Microtubules

We previously reported that cultured sympathetic neurons contain two classes of MTs, termed stable and labile, that differ in their relative stability to MT depolymerizing drugs (Black et al., 1989). For the present studies, we wished to determine whether or not both classes of MTs are present specifically in the axons of these neurons. To address this issue, we used morphological means to quantify the MT mass in axons as a function of time in 2 $\mu\text{g}/\text{ml}$ nocodazole (see Materials and Methods). Fig. 2 shows the data derived from two separate studies. Fig. 2 (a–c) are representative electron micrographs of axons treated for 0, 15, and 120 min, showing a clear decrease in MT mass with increasing time in drug. Somewhat to our surprise, it appeared that a relatively substantial portion of the MT mass depolymerized after even fairly short treatments with drug. Quantitative analyses reveal that the decrease in MT mass is biphasic (Fig. 2 d), with $\sim 48\%$ of the MT mass depolymerizing with a $t_{1/2}$ of ~ 5 min, and the remaining polymer depolymerizing with a $t_{1/2}$ of ~ 240 min. These results clearly indicate that axons contain both stable and labile MTs.

Stable and Labile Microtubules in the Axon Differ in Their α -Tubulin Composition

We propose that stable and labile MTs in the axon exist as distinct domains on individual MTs rather than as separate

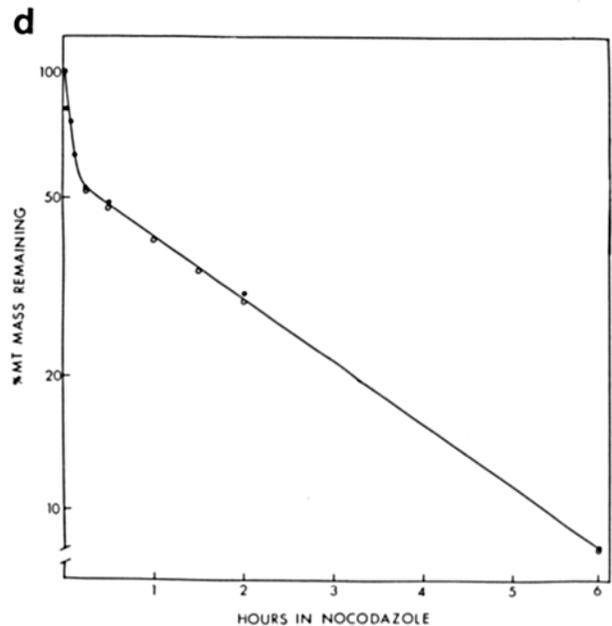
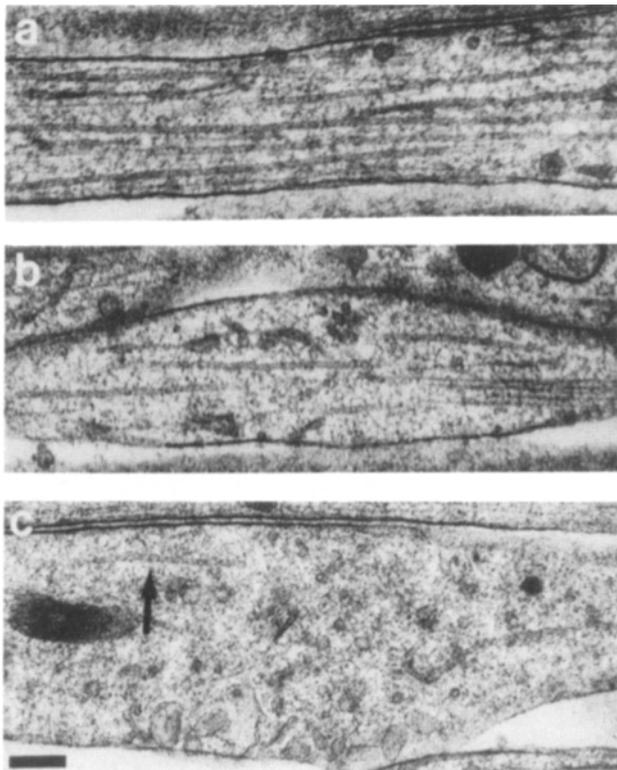


Figure 2. Effects of nocodazole on axonal MTs. *a-c* show examples of electron micrographs of axons treated with 2 $\mu\text{g/ml}$ nocodazole for 0, 15, and 120 min, respectively. Arrow (*c*) points to a MT left after drug treatment. *d* shows quantitative data on the effects of drug treatment on axonal MTs. The MT mass contained in drug-treated axons was quantified morphometrically as described in Materials and Methods, and expressed as a percentage of that in control axons. The open and closed circles represent the data from two separate sets of experiments. Nocodazole induced a biphasic decay in the MT mass, with $\sim 48\%$ depolymerizing with a $t_{1/2}$ of roughly 5 min, and $\sim 52\%$ depolymerizing with a $t_{1/2}$ of roughly 240 min. Bar, 0.2 μm .

populations of MTs. To test this hypothesis, we must be able to simultaneously visualize and distinguish stable and labile MTs, and thereby determine their spatial relationship relative to one another. In this section, we demonstrate that stable and labile MTs can be distinguished from one another. Then, in the following section, we use this information to show that stable and labile MTs exist as distinct domains on individual MTs.

Stable and labile MTs have been identified in many non-neuronal cells on the basis of differences in their sensitivity to MTs depolymerizing drugs. These stable and labile MTs are also distinguishable on the basis of their α -tubulin composition; labile MTs are rich in tyrosinated α -tubulin, whereas stable MTs are rich in α -tubulin that has been post-translationally detyrosinated and/or acetylated (Gunderson

and Bulinski, 1986; Piperno et al., 1987; Schulze et al., 1987; Kreis, 1987; Webster et al., 1987; Wheland and Weber, 1987; Gunderson et al., 1987; Bulinski et al., 1988). If the same situation applies to the axon, then treatment of neurons with 2 $\mu\text{g/ml}$ nocodazole for a time sufficient to completely depolymerize the labile MTs, while only minimally affecting the stable MTs, should substantially reduce or completely eliminate tyrosinated MTs from the axon while having little or no effect on detyrosinated and acetylated MTs. To test this prediction, control and drug-treated neurons were extracted to remove unassembled tubulin, and then double-stained for immunofluorescence analyses of tyrosinated and acetylated MTs (see Materials and Methods).

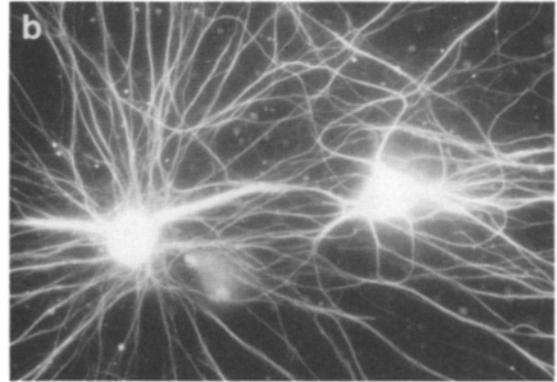
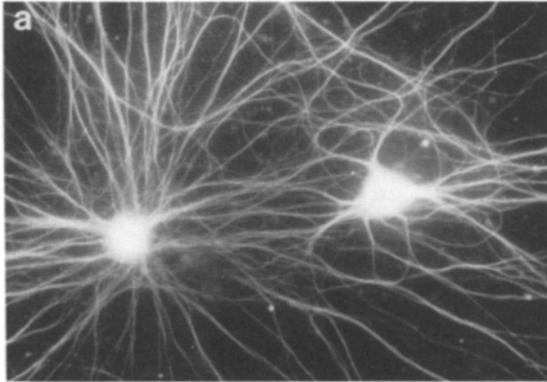
Fig. 3 shows the results from these analyses. Each micrograph in the figure shows one or two cell bodies, each having

Figure 3. Double-label immunofluorescence analyses using the 6-11B-1 mAb to localize MTs rich in acetylated α -tubulin (acetylated MTs), and the YL 1/2 mAb to localize MTs rich in tyrosinated α -tubulin (tyrosinated MTs) in neurons that had been treated for 0, 30, 60, or 120 min with 2 $\mu\text{g/ml}$ nocodazole (see Materials and Methods). Each micrograph shows one or two cell bodies, each having one or two dendrites that appear as relatively short processes that are broad at the base and taper with distance from the cell body. The remaining processes are axons. In control cultures, the staining patterns for tyrosinated and acetylated MTs are very similar, although lighter exposures reveal subtle differences in the staining patterns in the cell body (these will be discussed in a subsequent publication). Nocodazole treatment had minimal effect on the staining pattern for acetylated MTs throughout the neuron. Drug treatment also had minimal effect on the staining for tyrosinated MTs in cell bodies and dendrites. However, tyrosinated MTs in the axon were very labile to drug treatment, as shown by the near absence of staining for tyrosinated MTs after as little as 30 min in the drug. Bar, 40 μm .

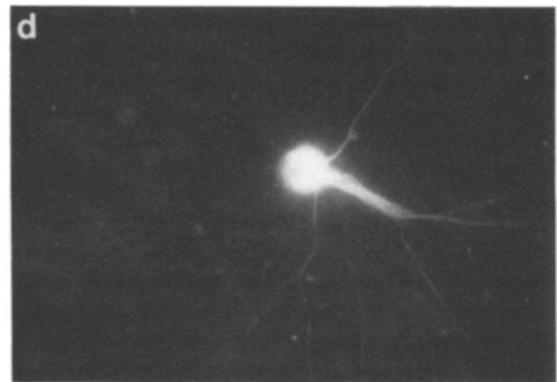
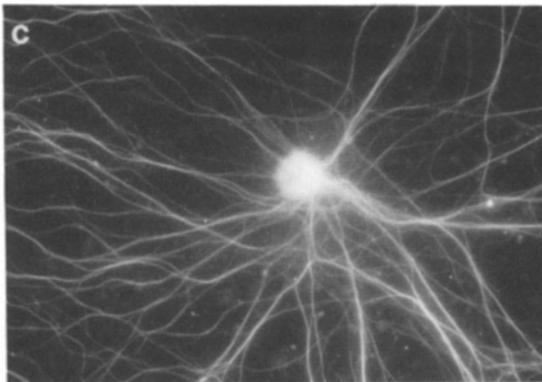
**acetylated
MT**

**tyrosinated
MT**

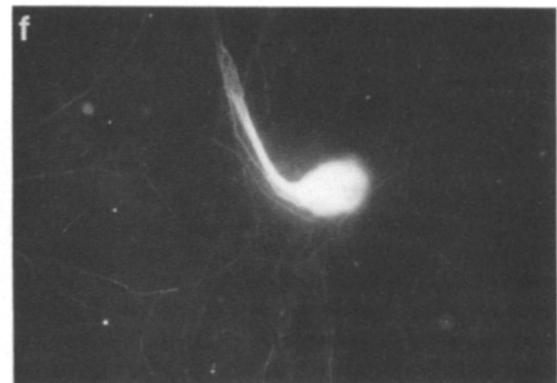
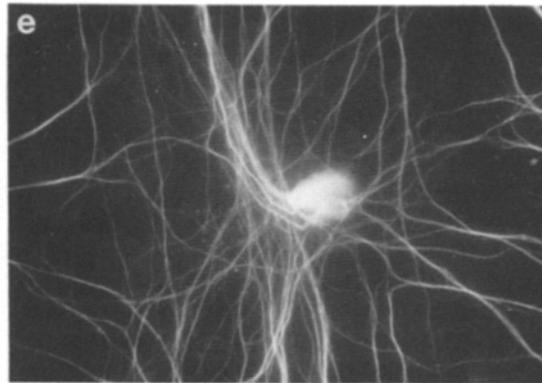
0 min



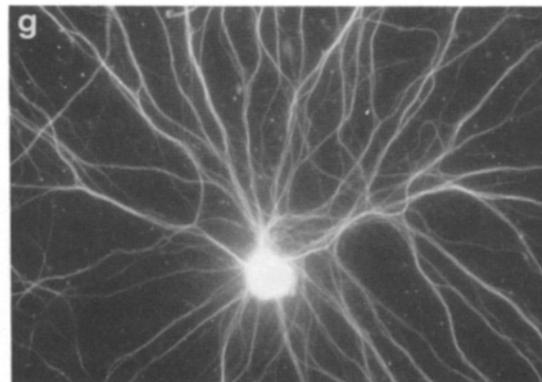
30 min



60 min



120 min



one or two dendrites that appear as relatively short processes that are broad at the base and taper with distance from the cell body. Processes with this appearance were confirmed to be dendrites by their staining for MAP-2, a somatodendritic-specific marker (data not shown; see also Peng et al., 1986). The remaining processes are axons, which typically run together in bundles containing varying numbers of axons. Because each neuron typically produces only a single axon (Bruckenstein and Higgins, 1988), the vast majority of axons appearing in the fields shown in these micrographs originate from cell bodies located outside of the field. In control cultures, both tyrosinated and acetylated MTs are clearly present throughout the cell bodies, dendrites, and axons (Fig. 3, *a* and *b*). Treatment with 2 $\mu\text{g/ml}$ nocodazole for 30, 60, or 120 min had no detectable effect on the staining pattern for acetylated α -tubulin (compare Fig. 3 *a* to *c*, *e*, and *g*). Similar results were obtained after an overnight (~ 15 -h) treatment with 2 $\mu\text{g/ml}$ nocodazole, although the intensity of staining was reduced considerably compared to controls or to cultures that had undergone short-term nocodazole treatment (data not shown). Likewise, the staining pattern obtained using a mAb against total α -tubulin was not detectably affected by nocodazole treatment, although the overall staining intensity was noticeably reduced at all times (30 min to overnight) examined (data not shown).

The staining pattern for tyrosinated MTs was dramatically affected by drug treatment, but in an unexpected manner. Treatment with 2 $\mu\text{g/ml}$ nocodazole for times ranging from 30 min to 15 h had minimal effect on the staining of the cell bodies and dendrites (Fig. 3 and data not shown), and immunoelectron microscopic studies confirmed that the staining remaining in the somatodendritic compartment after 2 h of drug treatment was in the form of MTs (data not shown). In contrast, tyrosinated MTs in the axon were very drug-labile, largely disappearing within as little as 30 min of drug treatment (Fig. 3, compare *b* with panels *d*, *f*, and *h*). Similar results were also obtained with neurons treated with 10 $\mu\text{g/ml}$ nocodazole. These results demonstrate that cell bodies and dendrites contain stable MTs, and that these MTs are rich in tyrosinated as well as acetylated α -tubulin. In contrast, in axons, labile MTs are rich in tyrosinated α -tubulin, whereas stable MTs contain little or no tyrosinated α -tubulin, but instead are rich in acetylated α -tubulin. Furthermore, because acetylated α -tubulin in these neurons is also detyrosinated (Baas and Black, 1989), stable MTs in the axon are also rich in detyrosinated α -tubulin.

The above results suggest a close correspondence between the relative stability of axonal MTs and their tyrosination state. This was confirmed quantitatively by immunoelectron microscopic analyses of the distribution of tyrosinated α -tubulin among axonal MTs. In these studies, control and drug-treated neurons were stained with the YL 1/2 mAb, which is specific for tyrosinated α -tubulin, and an appropriate second antibody conjugated to 5-nm gold particles (see Materials and Methods). Roughly half of the MT profiles were labeled all along their length with gold particles, indicating that these profiles were rich in tyrosinated α -tubulin, while roughly the other half were completely unlabeled, indicating that these profiles were deficient in tyrosinated α -tubulin, and hence rich in detyrosinated α -tubulin (Fig. 4, *b-e*). Typically tyrosinated MT profiles were densely and uniformly decorated with gold particles (see Fig. 4 *b*), but a fraction

of the MT profiles were noticeably less dense in their labeling (see Fig. 4 *c*). The significance of this difference in the density of labeling is unknown. The presence of labeled and unlabeled profiles in our preparations cannot be attributed to differences in the ability of the 5-nm gold particles to penetrate different regions of the axonal mat for two reasons. First, densely labeled and completely unlabeled MT profiles were frequently observed side-by-side. Second, axons immunolabeled with a mAb to β -tubulin known to label axonal MTs from other systems all along their length (Joshi and Cleveland, 1989) labeled all MT profiles in our preparation; most ($\geq 90\%$) profiles labeled for β -tubulin densely and uniformly, with only a very small number of profiles ($\leq 10\%$) showing short regions of somewhat sparser labeling (Fig. 4 *a*). Collectively, these observations indicate that the results obtained with the YL 1/2 mAb are not artifacts of penetration problems, but instead accurately reflect the existence of compositionally distinct MTs in the axon.

Table I shows the results of quantitative analyses of the effects of nocodazole treatment on relative proportions of tyrosinated and detyrosinated MTs in the axon. In control cultures, 48% of the MT mass was tyrosinated, while the remaining 52% was detyrosinated. After 15 min in nocodazole, roughly the amount of time needed to depolymerize the labile MTs without affecting the stable MTs (see Fig. 2 *d*), only 7% of the MT mass was tyrosinated (Fig. 5), thus decreasing the tyrosinated MT mass in the axon by $\sim 85\%$. After 30 min, 4% of the MT mass was tyrosinated, and after 60 min, no tyrosinated MTs remained. These data indicate that the tyrosinated MTs in the axon represent essentially the same subset of axonal MTs that are labile, and that the detyrosinated MTs in the axon represent the same subset of axonal MTs that are stable. Thus, tyrosinated α -tubulin can be used as a reliable and highly specific marker that distinguishes the labile MTs from the stable MTs in the axon.

We note that the tyrosinated MT profiles remaining after drug treatment were entirely similar in appearance to the tyrosinated MT profiles in control cultures, and were not preferentially of either the more or less densely labeled type. The low levels of tyrosinated MTs in the axon remaining after drug treatment may reflect recently stabilized MTs that have not yet undergone a substantial degree of detyrosination. This interpretation is consistent with the temporal delay known to occur between MT stabilization and detyrosination (Khawaja et al., 1988).

Stable and Labile Microtubules in the Axon Represent Distinct Domains on Individual Microtubules

If stable and labile MTs in the axon exist as distinct domains on individual MTs, then it should be possible to identify composite MTs in the axon that label for tyrosinated α -tubulin at one end but not at the other. Indeed, several examples of MT profiles labeled in this manner were observed (Fig. 6). In all these cases, there was an abrupt transition between the labeled and unlabeled regions. The existence of these composite profiles indicates that at least some MTs in the axon consist of both a stable and a labile domain. We estimated the frequency of this type of MT taking into account the following considerations. The average length of an MT profile in our electron micrographs is $\sim 1 \mu\text{m}$, while the average length of an entire MT in the axons of cultured neurons

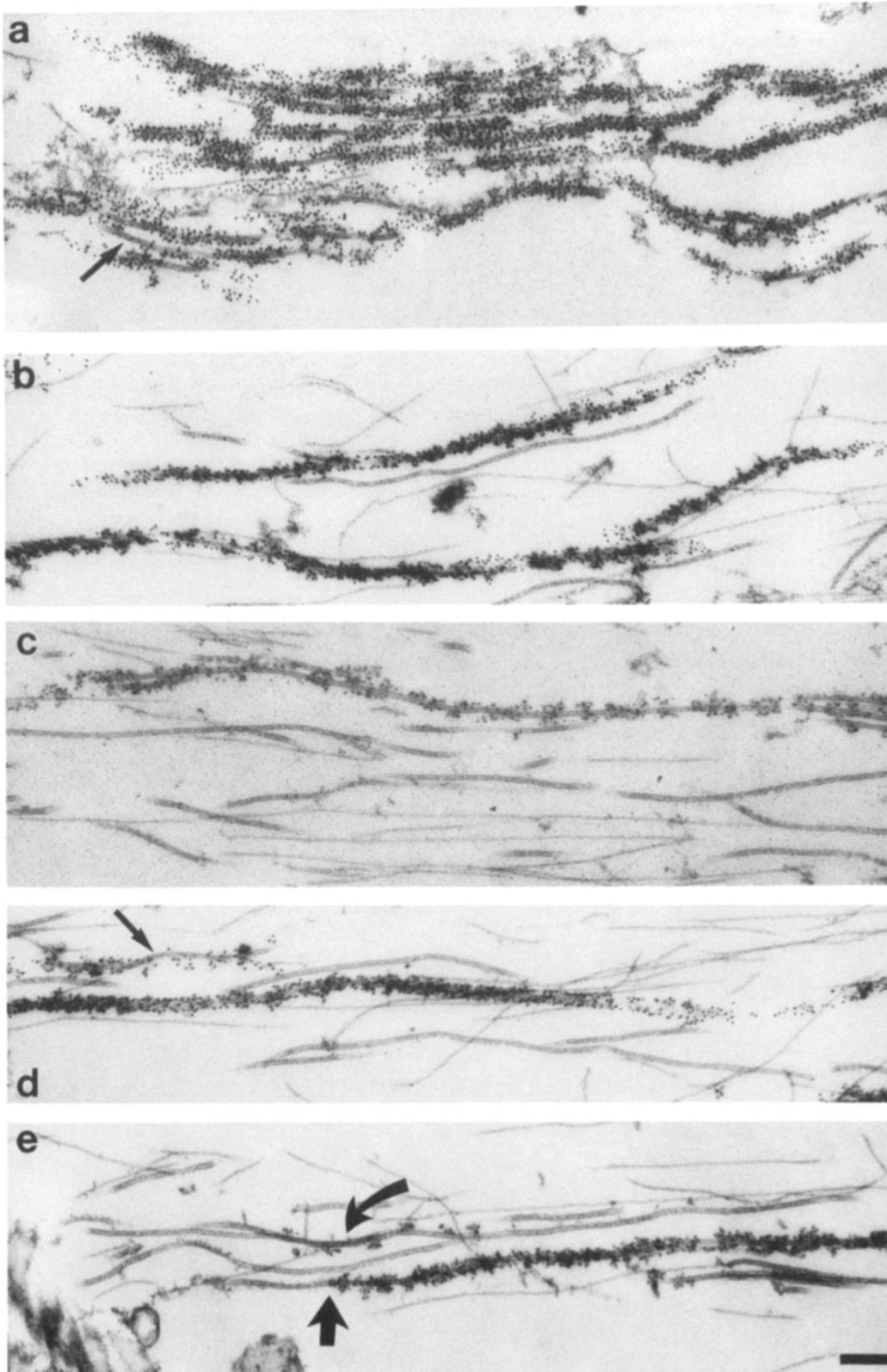


Figure 4. Electron micrographs of axonal MTs immunolabeled with a mAb against either total β -tubulin (*a*) or with the YL 1/2 mAb against tyrosinated α -tubulin (*b-e*), and appropriate second antibodies conjugated to 5-nm gold particles. (*a*) In samples stained with the β -tubulin antibody, all MTs were labeled, and most were heavily labeled all along their length. On rare occasions, short segments of MTs were observed that were labeled somewhat more sparsely than the more typical heavily labeled profiles (*arrow*). (*b-e*) In samples stained with the YL 1/2 mAb, the vast majority of MT profiles either were labeled more or less uniformly all along their lengths, or were completely unlabeled. Some of the labeled profiles were less densely labeled than others; *arrow* (*d*) shows a less densely labeled profile situated above a more densely labeled profile. In addition, some MT profiles showed regions of dense labeling in direct continuity with unlabeled regions (*e*, *straight arrow*). *e* also shows an example of overlapping MTs decorated with gold particles in which it was not possible to determine which MT was specifically associated with the gold particles (*curved arrow*). Bar, 0.2 μ m.

Table I. Proportion of Axonal Microtubule Mass That Labels with YL 1/2 in Immunoelectron Microscopic Analyses

Time in drug (min)	Labeled (μm)	Unlabeled (μm)	Percent labeled
0	482	522	48
15	71	933	7
30	41	961	4
60	0	1,000	0

Axons treated for 0–60 min with 2 $\mu\text{g}/\text{ml}$ nocodazole were immunolabeled for tyrosinated α -tubulin with the mAb YL 1/2 (see Materials and Methods). For each time point, a total of $\sim 1,000$ μm of MT profiles on randomly selected micrographs were scored as either labeled or unlabeled (see Results). A small number of profiles on some micrographs were not scored because of ambiguities resulting from closely spaced MT, one or more of which may have been labeled. The total lengths of the unlabeled and labeled profiles for each time point were then summed separately and recorded in the table.

is ~ 100 μm (Bray and Bunge, 1981). Thus, if all MTs in the axon consist of one stable domain and one labile domain, we would expect 1% of the profiles to show the transition region between these domains. In our studies, 21 of 5,000 MT profiles, or $\sim 0.4\%$, showed both domains, suggesting that $\sim 40\%$ of the MTs in the axon consist of both domains. The true proportion is probably higher in that we used very stringent criteria in scoring MT profiles consisting of both domains, eliminating several possible candidates because of neighboring MTs to which the gold particles might have alternately been associated (Fig. 4 *e*). Collectively, these observations indicate that a sizable proportion of MTs in the axon consist of a relatively stable domain in direct continuity with a more labile domain.

Which end of the composite MT is stable and which end is labile? Explant cultures were especially useful in addressing this question because all of the axons from an explant culture grow in the same direction relative to the cell body mass

(Fig. 1). Thus, all of the MTs in a region of axons selected for thin-sectioning have the same polarity orientation, plus ends distal to the cell body mass, permitting ready determination of the plus and minus ends of each MT profile observed in our electron micrographs. In all 21 of the MT profiles we observed showing both stable and labile domains, the labile domain was always situated at the plus end of the stable domain (Fig. 6).

Distribution of Stable and Labile Microtubules in the Axon

The immunofluorescence experiments shown in Fig. 3 indicate that stable and labile MTs are distributed throughout all regions of the axon discernable in moderate density cultures. However, the high degree of axonal bundling in these cultures precluded detailed analyses of the labeling patterns all along the length of the axon. Therefore, in a separate set of experiments, we analyzed the distribution of stable and labile MTs in the axons of lower density cultures. In these cultures, individual axons were minimally 2-mm in length, and stained for tyrosinated MTs all along their length. However, there was a clear diminution in the intensity of staining for tyrosinated MTs moving from the distal region of the axon into its main shaft. The most distal 25–75 μm of the axon stained brightly for tyrosinated MTs (Fig. 7), with the intensity of staining gradually decreasing over the next 15–100 μm , reaching a relatively constant level of staining over the remaining portion of the axon (not shown). The same axons stained more or less uniformly for acetylated MTs all along their length, except at their most distal region contiguous with the growth cone, which stained poorly or not at all (Fig. 7). Thus, the most distal region of the axon stained for tyrosinated MTs but not for acetylated MTs. The length of this distal region varied among different axons, ranging from ~ 10 μm (Fig. 7, *c* and *e*) to ~ 1 –2 μm (Fig. 7, *d* and *f*). These observations were confirmed by immunoelectron microscopic analy-

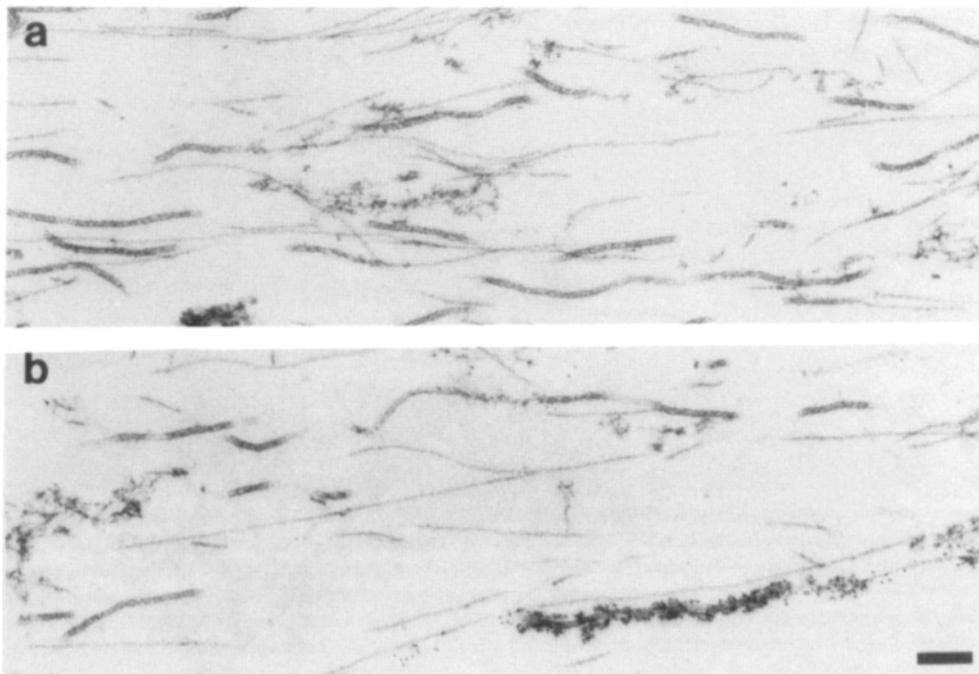


Figure 5. Electron micrographs of MT profiles in axons that were treated for 15 min with 2 $\mu\text{g}/\text{ml}$ nocodazole, and immunolabeled to reveal tyrosinated α -tubulin. *a* is a typical region, showing many unlabeled but no labeled MT profiles. *b* is a much less typical region, showing many unlabeled MT profiles and one labeled profile. Bar, 0.2 μm .

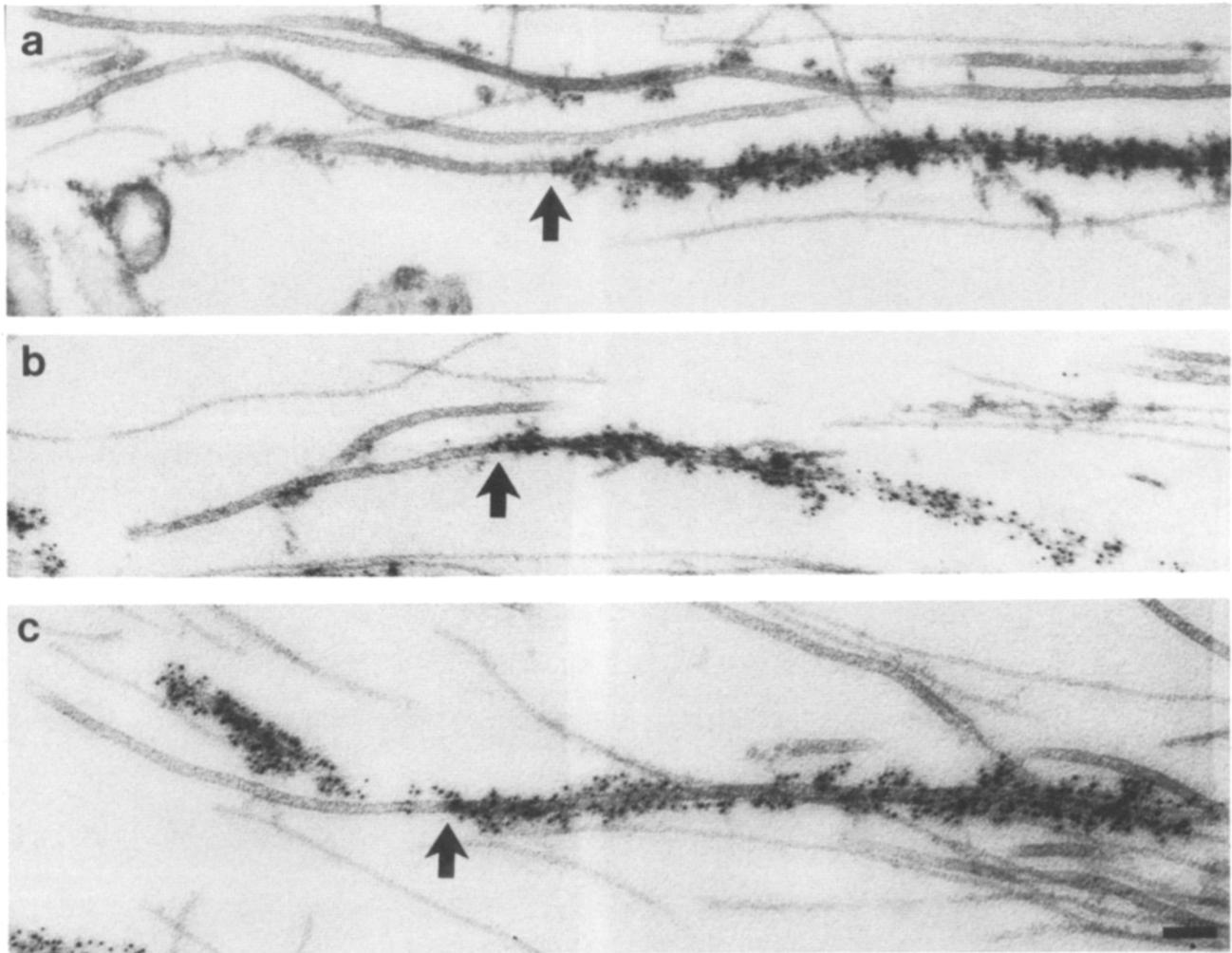


Figure 6. Higher magnification electron micrographs revealing composite MT profiles that were labeled at one end for tyrosinated α -tubulin but not at the other end. In the fields shown, the axons were growing from the left to the right. Axonal MTs are oriented with their plus ends pointing away from the cell body and toward the axon tip (Heidemann et al., 1981). Relative to the axonal fields illustrated in this figure, the cell bodies are situated toward the left and the axon tips are situated toward the right (the cell bodies and axon tips are located outside the fields shown). Thus, the MTs in each field have their minus ends toward the left and their plus ends toward the right. Note that for all composite MT profiles, the regions labeled for tyrosinated α -tubulin are toward the right of the panels. Hence, the tyrosinated domains of composite MTs are situated at the plus ends of the detyrosinated domains. In addition, in all cases there is an abrupt transition between the labeled and unlabeled regions (*arrows*). *a* is a higher magnification photograph of the MT profiles shown in *e* of Fig. 4. Bar, 0.1 μ m.

ses; in three different axons examined, all of the MT profiles in the most distal 1–2 μ m were labeled densely for tyrosinated α -tubulin, while none of the MT profiles were unlabeled (Fig. 8). Since tyrosinated MTs correspond to labile MTs in the axon, these results indicate that the distal region of the axon contains only labile MTs. Indeed, in five different axons treated with 2 μ g/ml nocodazole for 15 min, time sufficient to completely depolymerize labile MTs in the axon (Fig. 2), the most distal 1–2 μ m contained no MTs, differing from control axons that contained several MTs in their distal regions (Fig. 9). This contrasts with the situation in the main shaft of the axon, which contains roughly equal amounts of stable and labile MTs (see above, Fig. 4, *b–e*). Thus, both stable and labile MTs are present throughout the length of the axon, with the exception of its most distal region contiguous with the growth cone, which contains only labile MTs.

Discussion

Our studies reveal a strong correlation between the stability properties of axonal MTs and their tyrosination state. Roughly 48% of the MT mass in the axons of cultured sympathetic neurons is labile, depolymerizing with a $t_{1/2}$ of \sim 5 min in the presence of 2 μ g/ml nocodazole, while the remaining 52% of the MT mass is stable, depolymerizing with a $t_{1/2}$ of \sim 240 min under these conditions (Fig. 2). Immunofluorescence analyses indicate that labile MTs in the axon are rich in tyrosinated α -tubulin, whereas stable MTs contain little or no tyrosinated α -tubulin, but instead are rich in acetylated and detyrosinated α -tubulin (Fig. 3). Quantitative analyses performed at the immunoelectron microscopic level confirmed this result, indicating that \sim 85% of the tyrosinated MTs in the axon are depolymerized within 15 min of nocoda-

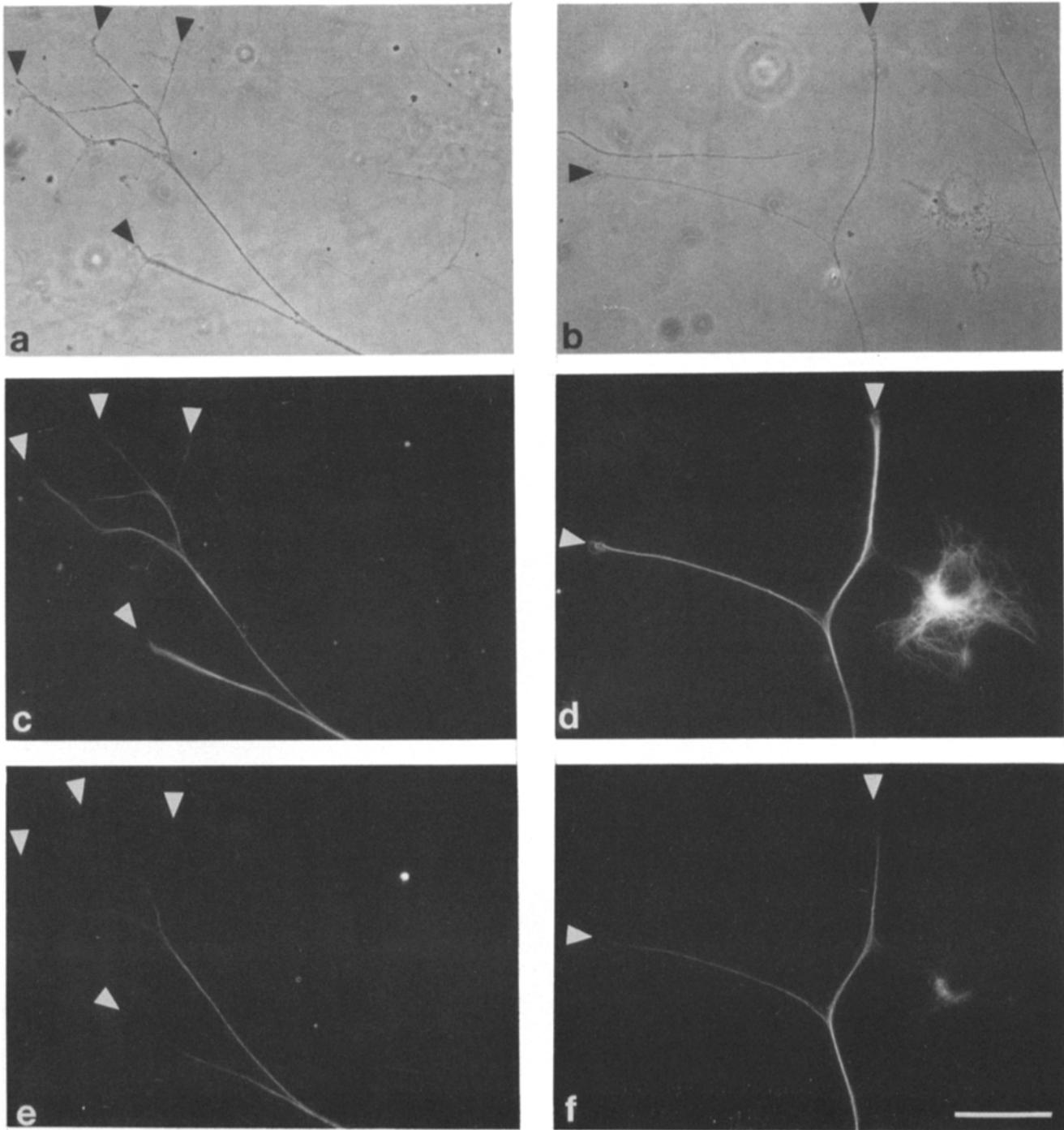


Figure 7. Phase-contrast (*a* and *b*) and immunofluorescence (*c-f*) images of the distal regions of two different axons double stained to reveal labile and stable MTs. Labile MTs were visualized with the mAb YL 1/2 against tyrosinated α -tubulin (*c* and *d*), while stable MTs were visualized with the mAb 6-11B-1 against acetylated α -tubulin (*e* and *f*). Labile and stable MTs have similar distributions along the length of the axon, except in the most distal region, contiguous with the growth cone, which contains only labile MTs. Arrowheads mark sites where growth cones end. Unlabeled structures in *b* (above left arrowhead, and parallel to the axon in the middle) are collagen fibers. Bar, 30 μ m.

zole treatment, time sufficient to completely depolymerize the labile MTs in the axon while only minimally affecting the stable MTs (Fig. 5, Table I). These results demonstrate that tyrosinated α -tubulin is a reliable and highly specific marker for labile MTs in the axon, and can be used to distinguish

stable and labile MTs in microscopic analyses of the axon. We have taken advantage of these findings to simultaneously visualize and distinguish stable and labile MTs in the axon, and hence determine their distribution and spatial relationship relative to one another.

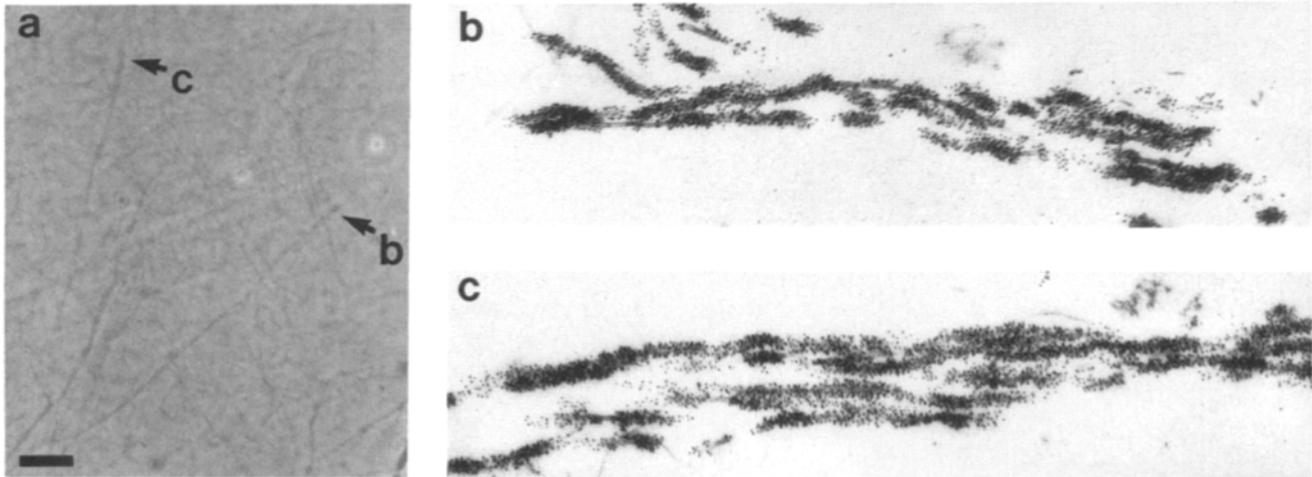


Figure 8. Phase-contrast (*a*) and immunoelectron (*b* and *c*) micrographs of the distal regions of axons immunolabeled to reveal tyrosinated α -tubulin. The phase-contrast image of the extracted axons was enhanced by staining of the epon block with toluidine blue (see Materials and Methods). In the most distal 1–2 μm of the axon (*a*, arrows), all of the MTs are densely labeled for tyrosinated α -tubulin (*b* and *c*). Bar, (*a*) 10 μm ; (*b*) 0.2 μm .

Stable and Labile Microtubules Coexist along the Length of the Axon

Qualitative immunofluorescence analyses localizing tyrosinated and acetylated MTs in a variety of neuronal systems indicate that the main shaft of the axon contains both stable and labile MTs, whereas the most distal region of the axon contiguous with the growth cone contains predominantly labile MTs (Figs. 3 and 7; see also Wheland and Weber, 1987; Robson and Burgoyne, 1988; Lim et al., 1989). We have confirmed and expanded upon these results quantitatively by showing that similar levels of stable and labile MTs are present, and situated side-by-side with one another, along the

main shaft of the axon (Figs. 2 and 4, *b–e*), whereas exclusively labile MTs are present in the distal region of the axon contiguous with the growth cone (Figs. 8 and 9). Collectively, these results indicate that both stable and labile MTs coexist throughout the axon, except in its most distal region, which contains only labile MTs.

An initially surprising aspect of our data concerned the relative proportions of stable and labile MTs in the axon. Many studies on the properties of MTs in the axon have focused on the greater stability of axonal MTs compared to the rapidly turning-over MTs characteristic of proliferating cell types (Morris and Lasek, 1982; Black and Greene, 1982; Black et al., 1984, 1989; Brady et al., 1984; White et al.,

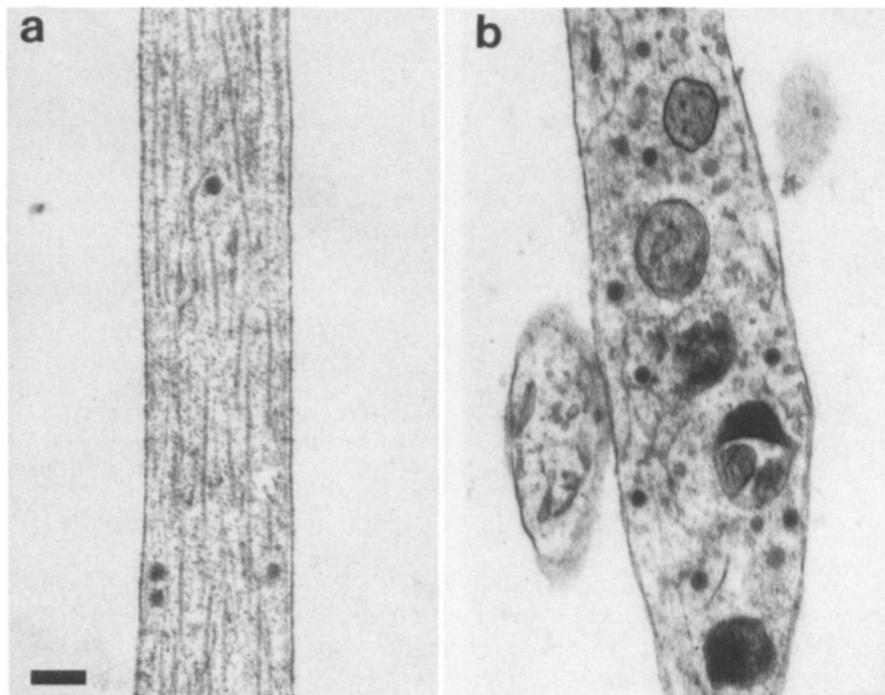


Figure 9. Electron micrographs of the most distal 1–2 μm of the axon. *a* is an axon from a control culture, whereas *b* is an axon from a culture treated for 15 min with nocodazole to depolymerize labile MTs. MTs extend into the most distal 1–2 μm of the control axon, but are completely absent from the same region of the nocodazole-treated axon. Note that as expected (Joshi et al., 1986), the drug-treated axon contains many more membranous profiles than the control axon. Bar, 0.2 μm .

1987). In addition to documenting the presence of high levels of stable MTs in the axons of cultured sympathetic neurons, our studies also indicate that a sizable proportion (~48%) of the MT mass in these axons is labile, completely depolymerizing within the first 15 min in nocodazole (Fig. 2). Because a high sensitivity to MT depolymerizing drugs generally indicates a rapid rate of subunit exchange, these results suggest that roughly half of the MT mass in the axon is very dynamic. Other studies also suggest substantial MT dynamics in axons. For example, 50% or more of the MT mass in diverse systems such as the squid giant axon (Morris and Lasek, 1984), mature mammalian axons (Heidemann et al., 1984), and the axons of cultured chick sensory neurons (Baas and Heidemann, 1986) rapidly disassemble in response to MT depolymerizing conditions. Also, haptenized-tubulin microinjected into PC12 cells and cultured neurons rapidly incorporates into the MTs of their axons (Okabe and Hirokawa, 1988, 1990), suggesting relatively rapid subunit exchange between axonal MTs and the soluble tubulin pool. These findings suggest that MT dynamics comparable in degree to those observed in the axons of cultured sympathetic neurons may be a general feature of axons.

It has been proposed that local assembly of MTs in the axon and particularly at the axon tip contributes to the net addition of MTs to the axon during axon growth (Bamburg et al., 1986; Robson and Burgoyne, 1988; Mitchison and Kirschner, 1988). This proposal derives from evidence suggesting that MTs in the distal region of the axon undergo rapid assembly/disassembly dynamics. As indicated above, our studies confirm such dynamics at the axon tip, and also demonstrate substantial assembly/disassembly dynamics throughout the length of the axon. Unfortunately, the demonstration of local MT dynamics in the axon does not directly address the contribution of local MT assembly to the net addition of MTs to the growing axon. While local assembly may increase the MT mass of the axon, it is also possible the assembly events are perfectly balanced by disassembly events within the MT array of the axon. New information will be required to resolve this issue.

A somewhat unexpected finding from our studies was that while tyrosinated α -tubulin is a reliable marker for labile MTs in the axon, it is not a reliable marker for labile MTs in the somatodendritic compartment of the neuron. MTs in cell bodies and dendrites contain high levels of tyrosinated α -tubulin, but as shown in Fig. 3, some portion of these tyrosinated MTs are stable to drug treatments that completely depolymerize tyrosinated MTs in the axon. Thus, stable MTs are present in the somatodendritic compartment of the neuron as well as in the axon, but stable MTs in cell bodies and dendrites, as opposed to those in the axon, contain tyrosinated α -tubulin. Furthermore, based on the relative intensity of MT staining in control and drug-treated neurons, these stable but tyrosinated MTs represent a substantial fraction of the total MT mass present in cell bodies and dendrites.

Stable and Labile Microtubules Represent Distinct Domains on Individual Microtubules in the Axon

In addition to MT profiles that were either labeled for tyrosinated α -tubulin all along their length or were completely unlabeled, immunoelectron microscopic analyses revealed a third type of MT profile that was densely labeled for tyrosi-

nated α -tubulin at one end, but was completely unlabeled at the other end. In all of these cases, the tyrosinated domain of these profiles was situated at the plus end of the detyrosinated domain, and there was an abrupt transition between the two domains (Fig. 6). The existence of this third type of profile indicates that at least some MTs in the axon are composite, consisting of a detyrosinated domain in direct continuity with a tyrosinated domain. Additional considerations provide information on the number of distinct tyrosinated and detyrosinated domains comprising these composite MTs. First, the fact that in any given section most MT profiles, regardless of their length, were either completely and uniformly labeled for tyrosinated α -tubulin or were completely unlabeled suggests that axonal MTs have relatively infrequent transitions from one type of domain to the other. Indeed, even the longest MT profiles captured in our sections (~4 μ m) contained no more than two domains. Second, the finding that tyrosinated MTs in the axon are depolymerized by ~85% within the first 15 min in nocodazole, and are completely depolymerized by 1 h (Fig. 5; Table I), indicates that only a very small portion of the tyrosinated MTs in the axon could be buried within the more stable detyrosinated domains. Finally, if a tyrosinated domain were flanked by two detyrosinated domains, or vice versa, we would expect to capture detyrosinated domains at the plus ends of tyrosinated domains equally as often as the reverse orientation. However, in all 21 composite profiles we observed, the tyrosinated domain was always situated at the plus end of the detyrosinated domain. Collectively, these observations suggest that composite MTs in the axon consist of one tyrosinated domain situated toward the plus end of the MT, and one detyrosinated domain situated toward the minus end of the MT. Furthermore, because tyrosinated and detyrosinated MTs in the axon correspond to labile and stable MTs respectively, we conclude that composite MTs consist of a single labile domain in direct continuity with a single stable domain, with the labile domain always situated at the plus end of the stable domain.

We tentatively estimate that 40% of the MTs in the axon are composite. This estimate is based on the observed frequency of MT profiles showing a transition region between the labile and stable domains, on the assumption that MTs in the axons studied average ~100 μ m in length, and on the probability of capturing the transition region between the labile and stable domains of individual MTs in our sections (see Results). Because very strict criteria were used in scoring MT profiles consisting of both domains, the actual proportion of composite MTs in the axon may be much higher (see Results). Thus, the results of our studies clearly indicate that a sizable proportion of MTs in the axon are composite, consisting of one stable domain in direct continuity with one labile domain.

Both the extreme drug sensitivity of the labile domains and the abrupt transition in tyrosination state between the stable and labile domains on composite MTs suggests that the labile domains turnover very rapidly, more rapidly than the rates at which they can be detyrosinated. Conversely, the relative insensitivity of the stable domains to drug-induced depolymerization and their high levels of detyrosinated and acetylated α -tubulin indicate that these MTs turnover quite slowly relative to the labile domains. The direct continuity of the rapidly turning over labile domains with the slowly turning

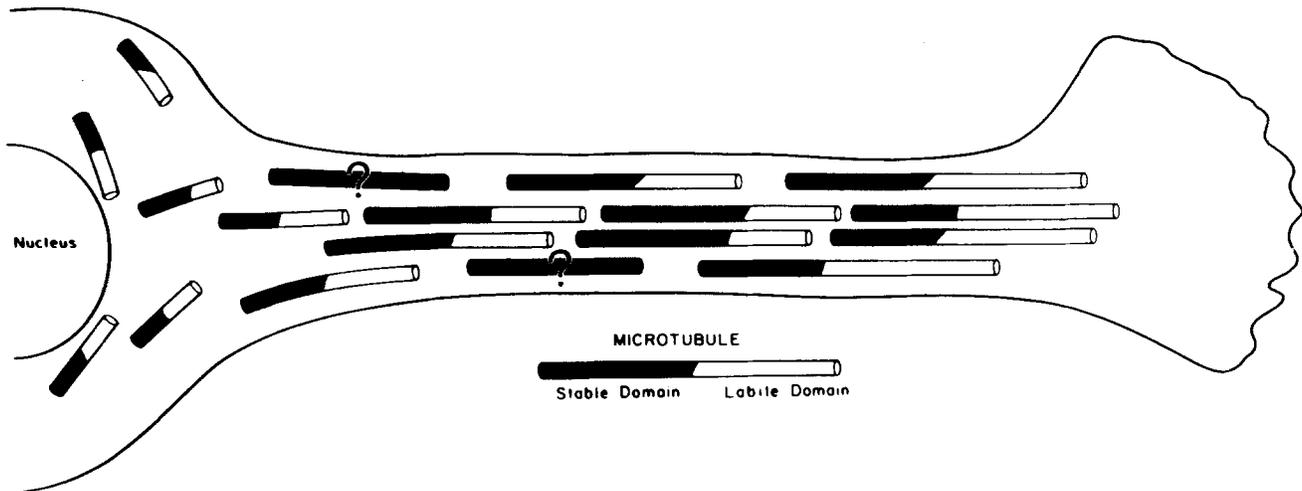


Figure 10. Schematic depicting the spatial organization of labile and stable MTs in the axon. The plus ends of the MTs are directed away from the cell body, while the minus ends of the MTs are directed toward the cell body. A sizable proportion of the MTs in the axon consist of two domains, a relatively stable domain at the minus end, and a more labile domain at the plus end. In addition, the axon may contain some MTs that are entirely stable (indicated with question marks). These MTs may be entirely stable because they are capped against assembly at both ends, or because their labile domains have recently undergone catastrophic disassembly (see text). The existence of entirely labile MTs in the axon is doubtful in that new MT assembly in the axon is apparently restricted to elongation from preexisting MTs (see text). Both labile and stable MT domains are present all along the length of the axon, except in the most distal region of the axon contiguous with the growth cone, into which extend only the labile domains of MTs originating more proximally in the axon. The schematic also depicts MTs in the cell body as consisting of both domains. At present, this is hypothetical; we have identified stable and labile MTs in the cell body, but have not explored their spatial relationship relative to one another.

over stable domains thus indicates that the labile MTs assemble directly from the stable MTs. It therefore follows that the plus ends of stable MTs are assembly competent, and nucleate MT assembly in the axon. Recent studies on MT dynamics in isolated segments of the axon (Baas and Heidemann, 1986) further suggest that stable MTs represent the only long-lived templates for MT assembly in the axon. In these studies, isolated segments of the axon were treated with varying doses of nocodazole, rinsed free of the drug, and then evaluated for MT reassembly by electron microscopy. MT reassembly occurred rapidly during recovery from drug treatments that depleted labile but not stable MTs, but did not occur during recovery from drug treatments that depleted the stable as well as the labile MTs. These results indicate that MT assembly in the axon occurs by elongation from preexisting MTs rather than by self-association of tubulin subunits into new MTs or by elongation from non-MT nucleating structures. Thus, stable MTs are not only assembly competent, but also apparently represent the preferred and perhaps exclusive templates for local MT dynamics in the axon.

The mechanisms that underlie the rapid turnover of labile MTs in the axon are a matter of speculation at present. In many cell types, the rapid turnover of MTs can be explained by the dynamic instability model (for review, see Kirschner and Mitchison, 1986). Assuming that dynamic instability accounts for aspects of MT turnover in the axon (Mitchison and Kirschner, 1988), it seems reasonable to suggest that the labile domains of axonal MTs may be dynamically unstable, undergoing rapid phases of elongation from the plus ends of stable MTs, followed by catastrophic disassembly of the labile MTs either partially or completely back to the plus ends of the stable domains. In this view, the stable MTs not only serve as templates for local MT dynamics in the axon, but also rescue catastrophic disassembly of the labile domains

and thereby ensure the continued existence of the MTs. Because axons apparently lack the ability to generate MTs de novo (Baas and Heidemann, 1986), this rescue function may be particularly important for maintaining the MT array of the axon.

Stable Microtubules Organize Microtubule Assembly in the Axon

Fig. 10 schematically summarizes the contribution of the present study to current understanding of the organization of MTs in the axon. Previous studies have shown that axonal MTs do not radiate from a single, centrally situated nucleating structure (Lyser, 1968; Sharp et al., 1982; Stevens et al., 1988; Okabe and Hirokawa, 1988), but rather start and stop at multiple sites along the length of the axon (Chalfie and Thompson, 1979; Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). Our studies suggest that most MTs in the axon are composite, consisting of a labile domain in direct continuity with the plus end of a stable domain. Entirely stable MTs are depicted with question marks; while we have no direct evidence that such MTs exist, they may in principle be generated from composite MTs after complete catastrophic disassembly of their labile domains. No entirely labile MTs are depicted because labile MTs are proposed to elongate specifically from the plus ends of stable MTs (see above). Furthermore, any spontaneously formed labile MTs would be at a kinetic disadvantage compared to composite MTs, and so would be expected to depolymerize in favor of elongation from composite MTs (Kirschner and Mitchison, 1986). The plus ends of stable MTs are distributed all along the length of the axon, indicating that stable templates for local MT dynamics exist throughout the axon. Labile MTs that grow from these templates will have the same polarity orientation as the templates themselves, which is plus end distal

to the cell body (Heidemann et al., 1984). Furthermore, because assembly occurs preferentially from the plus ends of the stable MTs, MT assembly in the axon will occur in a direction away from the cell body toward the axon tip. Thus, stable MTs organize the assembly of MTs in a common direction in the axon, and with a common polarity orientation. These observations indicate that stable MTs spatially organize MT assembly in the axon in much the same way that traditional MT nucleating structures such as the centrosome organize MT assembly in other cell types. However, unlike the centrosome, which organizes MT assembly from a discrete, often centralized location in the cell, stable MTs in the axon organize assembly from multiple sites that are widely distributed along the length of the axon.

The preceding discussion has emphasized the potential role of stable MTs as nucleating structures for local MT assembly/disassembly dynamics in the axon. In particular, we have suggested that stable MTs may be the only long-lived templates for MT assembly in the axon, and, as such, they will help ensure the continued existence of the axonal MT array. An issue not addressed by the present studies but of obvious relevance concerns the generation of the stable MT array of the axon. This issue encompasses many questions, including how and where stable MTs are generated, and how stable MTs obtain their uniformly plus-end-distal polarity orientation. Studies are in progress to begin answering these questions.

We wish to thank Sue Humphries and Theresa Slaughter for expert technical assistance, Dr. Giani Piperno for his kind gift of the 6-11B-1 mAb, and Drs. Ray Lasek, Anthony Brown, and Harish Joshi for helpful discussions. We also thank Dr. Anthony Brown for his critical comments on the manuscript.

This work was supported by National Institutes of Health grants NS 17681 and NS 23530. P. W. Baas is funded by a postdoctoral grant from the National Institutes of Health.

Received for publication 28 March 1990 and in revised form 16 April 1990.

References

- Baas, P. W., and S. R. Heidemann. 1986. Microtubule reassembly from nucleating fragments during the regrowth of amputated neurites. *J. Cell Biol.* 103:917-927.
- Baas, P. W., and M. M. Black. 1989. Compartmentation of alpha tubulin in cultured neurons: identification of a somatodendritic-specific variant of alpha-tubulin. *Neuroscience*. 30:795-803.
- Baas, P. W., L. A. White, and S. R. Heidemann. 1987. Microtubule polarity reversal accompanies regrowth of amputated neurites. *Proc. Natl. Acad. Sci. USA*. 84:5272-5276.
- Baas, P. W., J. S. Deitch, M. M. Black, and G. A. Banker. 1988. Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc. Natl. Acad. Sci. USA*. 85:8335-8339.
- Baas, P. W., M. M. Black, and G. A. Banker. 1989. Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. *J. Cell Biol.* 109:3085-3094.
- Bamburg, J. R., D. Bray, and K. Chapman. 1986. Assembly of microtubules at the tip of growing axons. *Nature (Lond.)*. 321:788-790.
- Bartlett, W. P., and G. A. Banker. 1984. An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. *J. Neurosci.* 4:1944-1953.
- Binder, L. I., W. Dentler, and J. L. Rosenbaum. 1975. Assembly of chick brain tubulin onto flagellar microtubules from *Chlamydomonas* and sea urchin sperm. *Proc. Natl. Acad. Sci. USA*. 72:1122-1126.
- Bergen, L., and G. G. Borisy. 1980. Head-to-tail polymerization of microtubules in vitro. Electron microscope analysis of seeded assembly. *J. Cell Biol.* 84:141-150.
- Black, M. M., and L. A. Greene. 1982. Changes in colchicine susceptibility of microtubules associated with neurite outgrowth: studies with nerve growth factor-responsive PC12 pheochromocytoma cells. *J. Cell Biol.* 95:379-386.
- Black, M. M., and P. Keyser. 1987. Acetylation of alpha-tubulin in cultured neurons and the induction of alpha-tubulin acetylation in PC12 cells by treatment with NGF. *J. Neurosci.* 7:1833-1842.
- 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.
- Black, M. M., P. Keyser, and E. Sobel. 1986. Interval between the synthesis and assembly of cytoskeletal proteins in cultured neurons. *J. Neurosci.* 6:1004-1012.
- Black, M. M., P. W. Baas, and S. Humphries. 1989. Dynamics of alpha-tubulin deacetylation in intact neurons. *J. Neurosci.* 9:358-368.
- Brady, S. T., M. Tytell, and R. J. Lasek. 1984. Axonal transport and axonal tubulin: biochemical evidence for cold-stability. *J. Cell Biol.* 99:1716-1724.
- Bray, D., and M. B. Bunge. 1981. Serial analysis of microtubules of cultured rat sensory neurons. *J. Neurocytol.* 10:589-605.
- Brinkley, B. R. 1985. Microtubule organizing centers. *Annu. Rev. Cell Biol.* 1:145-172.
- Bruckenstein, D. A., and D. Higgins. 1988. Morphological differentiation of embryonic rat sympathetic neurons in tissue culture. II. Serum promotes dendritic growth. *Dev. Biol.* 128:337-348.
- Bulinski, J. C., J. E. Richards, and G. Piperno. 1988. Posttranslational modifications of alpha-tubulin: deetyrosination and acetylation differentiate populations of interphase microtubules in cultured cells. *J. Cell Biol.* 106:1213-1220.
- Burton, P. R., and J. L. Paige. 1981. Polarity of axoplasmic microtubules in the olfactory nerve of the frog. *Proc. Natl. Acad. Sci. USA*. 78:3269-3273.
- Chalfie, M., and J. N. Thompson. 1979. Organization of neuronal microtubules in the nematode *Caenorhabditis elegans*. *J. Cell Biol.* 82:278-289.
- Daniels, M. P. 1973. Fine structural changes in neurons associated with colchicine inhibition of nerve fiber formation in vitro. *J. Cell Biol.* 58:463-470.
- Diaz-Nido, J., M. A. Hernandez, and J. Avila. 1990. Microtubule proteins in neuronal cells. In *Microtubule Proteins*. J. Avila, editor. CRC Press, Inc., Boca Raton. 193-257.
- Dustin, P. 1984. *Microtubules*. 2nd edition. Springer-Verlag New York Inc., New York.
- Euteneuer, U., and J. R. McIntosh. 1981. Structural polarity of kinetochore microtubules in PTK2 cells. *J. Cell Biol.* 98:338-345.
- Geuens, G., G. G. Gunderson, R. Nuydens, F. Cornelissen, J. C. Bulinski, and M. De Brabander. 1986. Ultrastructural colocalization of tyrosinated and deetyrosinated alpha-tubulin in interphase and mitotic cells. *J. Cell Biol.* 103:1883-1893.
- Gunderson, G. G., and J. C. Bulinski. 1986. Microtubule arrays in differentiated cells contain elevated levels of a post-translationally modified form of tubulin. *Eur. J. Cell Biol.* 42:288-294.
- Gunderson, G. G., S. Khawaja, and J. C. Bulinski. 1987. Postpolymerization deetyrosination of alpha-tubulin: a mechanism for subcellular differentiation of microtubules. *J. Cell Biol.* 105:251-264.
- Haimo, L. T., B. R. Telzer, and J. L. Rosenbaum. 1979. Dynein binds to and cross-bridges cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA*. 76:5759-5763.
- Heidemann, S. R., and J. R. McIntosh. 1980. Visualization of the structural polarity of microtubules. *Nature (Lond.)*. 286:517-519.
- Heidemann, S. R., J. M. Landers, and M. A. Hamburg. 1981. Polarity orientation of axonal microtubules. *J. Cell Biol.* 91:661-665.
- Heidemann, S. R., M. A. Hamburg, S. J. Thomas, B. Song, S. Lindley, and D. Chu. 1984. Spatial organization of axonal microtubules. *J. Cell Biol.* 99:1289-1295.
- Joshi, H. C., and D. W. Cleveland. 1989. Differential utilization of beta-tubulin isotypes in differentiating neurites. *J. Cell Biol.* 109:663-673.
- Joshi, H. C., P. Baas, D. T. Chu, and S. R. Heidemann. 1986. The cytoskeleton of neurites after microtubule depolymerization. *Exp. Cell Res.* 163:233-245.
- Khawaja, S., G. G. Gunderson, and J. C. Bulinski. 1988. Enhanced stability of microtubules enriched in deetyrosinated tubulin is not a direct function of deetyrosination level. *J. Cell Biol.* 106:141-150.
- Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.
- Kirschner, M. W. 1978. Microtubule assembly and nucleation. *Int. Rev. Cytol.* 54:1-71.
- Kirschner, M., and T. Mitchison. 1986. Beyond self-assembly: from microtubules to morphogenesis. *Cell*. 45:329-342.
- Kreis, T. E. 1987. Microtubules containing deetyrosinated tubulin are less dynamic. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2597-2606.
- Lasek, R. J. 1988. Studying the intrinsic determinants of neuronal form and function. In *Intrinsic Determinants of Neuronal Form and Function*. R. J. Lasek and M. M. Black, editors. Alan R. Liss, New York. 1-58.
- Lim, S.-S., P. J. Sarnack, and G. G. Borisy. 1989. Progressive and spatially differentiated stability of microtubules in developing neuronal cells. *J. Cell Biol.* 109:253-264.
- Lyster, K. M. 1968. An electron microscopic study of centrioles in differentiating neuroblasts. *J. Embryol. Exp. Morphol.* 20:343-354.
- Meininger, V., and S. Binet. 1989. Characteristics of microtubules at the different stages of neuronal differentiation and maturation. *Int. Rev. Cytol.* 114:21-79.
- Mitchison, T., and M. Kirschner. 1988. Cytoskeletal dynamics and nerve

- growth. *Neuron*. 1:761-772.
- Morris, J. R., and R. J. Lasek. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192-198.
- Morris, J. R., and R. J. Lasek. 1984. Monomer-polymer equilibria in the axon: direct measurement of tubulin and actin as polymer and monomer in axoplasm. *J. Cell Biol.* 98:2064-2076.
- Moya, F., M. B. Bunge, and R. P. Bunge. 1980. Schwann cells proliferate but fail to differentiate in defined medium. *Proc. Natl. Acad. Sci. USA.* 77:6902-6906.
- Okabe, S., and N. Hirokawa. 1988. Microtubule dynamics in nerve cells: analysis using microinjection of biotinylated tubulin into PC12 cells. *J. Cell Biol.* 107:651-664.
- Okabe, S., and N. Hirokawa. 1980. Turnover of fluorescently labeled tubulin and actin in the axon. *Nature (Lond.)*. 343:479-482.
- Peng, I., L. I. Binder, and M. M. Black. 1986. Biochemical and immunological analyses of cytoskeletal domains of neurons. *J. Cell Biol.* 102:252-262.
- Peters, A., S. L. Palay, and H. de Webster. 1974. *The Fine Structure of the Nervous System*. W. B. Saunders Company, Philadelphia.
- Piperno, G., and M. T. Fuller. 1985. Monoclonal antibodies specific for an acetylated form of α -tubulin recognize antigens in cilia and flagella from a variety of organisms. *J. Cell Biol.* 101:2085-2094.
- Piperno, G., M. LeDizet, and X. Chang. 1987. Microtubules containing acetylated α -tubulin in mammalian cells in culture. *J. Cell Biol.* 104:289-302.
- Robson, S. J., and R. D. Burgoyne. 1988. Differential levels of tyrosinated, detyrosinated, and acetylated alpha-tubulins in neurites and growth cones of dorsal-root ganglion neurons. *Cell Motil. Cytoskeleton.* 12:273-282.
- Sahenk, Z., and S. T. Brady. 1987. Axonal tubulin and microtubules: morphologic evidence for stable regions on axonal microtubules. *Cell Motil. Cytoskeleton.* 8:155-164.
- Schliwa, M., and J. J. van Blerkom. 1981. Structural interaction of cytoskeletal components. *J. Cell Biol.* 90:222-235.
- Schulze, E., D. J. Asai, J. C. Bulinski, and M. Kirschner. 1987. Posttranslational modification and microtubule stability. *J. Cell Biol.* 105:2167-2177.
- 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.
- Sherwin, T., and K. Gull. 1989. Visualization of detyrosination along single microtubules reveals novel mechanisms of assembly during cytoskeletal duplication in trypanosomes. *Cell.* 57:211-221.
- Stevens, J. K., J. Trogadis, and J. R. Jacobs. 1988. Development and control of axial neurite form: a serial electron microscopic analysis. In *Intrinsic Determinants of Neuronal Form and Function*. R. J. Lasek and M. M. Black, editors. Alan R. Liss, New York. 115-146.
- Tsukita, S., and H. Ishikawa. 1981. The cytoskeleton in myelinated axons: serial section study. *Biomed. Res.* 2:424-437.
- Webster, D. R., G. G. Gunderson, J. C. Bulinski, and G. G. Borisy. 1987. Differential turnover of tyrosinated and detyrosinated microtubules. *Proc. Natl. Acad. Sci. USA.* 84:9040-9044.
- Wheland, J., and K. Weber. 1987. Turnover of the carboxy-terminal tyrosine of alpha-tubulin and means of reaching elevated levels of detyrosination in living cells. *J. Cell Sci.* 88:185-203.
- Wheland, J., M. C. Willingham, and I. V. Sandoval. 1983. A rat monoclonal antibody reacting specifically with the tyrosinated form of α -tubulin. Biochemical characterization. Effects on microtubule polymerization *in vitro* and microtubule polymerization and organization *in vivo*. *J. Cell Biol.* 97:1476-1490.
- White, L. A., P. W. Baas, and S. R. Heidemann. 1987. Microtubule stability in severed axons. *J. Neurocytol.* 16:775-784.
- 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.
- 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.