Inhibition of Neurite Initiation and Growth by Taxol

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ABSTRACT
We cultured sensory neurons from chick embryos in media containing the alkaloid taxol at concentrations from $7 \times 10^{-9}$ to $3.5 \times 10^{-6}$ M. When plated at taxol concentrations above $7 \times 10^{-8}$ M for 24 h, neurons have short broad extensions that do not elongate on the culture substratum. When actively growing neurites are exposed to these levels of taxol, neurite growth stops immediately and does not recommence. The broad processes of neurons cultured 24 h with taxol contain densely packed arrays of microtubules that loop back at the ends of the process. Neurofilaments are segregated from microtubules into bundles and tangled masses in these taxol-treated neurons. At the ends of neurites treated for 5 min with taxol, microtubules also turn and loop back abnormally toward the perikaryon. In the presence of $7 \times 10^{-9}$ M taxol neurites do grow, although they are broader and less branched than normally. The neurites of these cells appear to have normal structure except for a large number of microtubules.

Taxol probably stimulates microtubule polymerization in these cultured neurons. At high levels of the drug, this action inhibits neurite initiation and outgrowth by removing free tubulin from the cytoplasm and destroying the normal control of microtubule assembly in growing neurites. The rapid inhibition suggests that microtubule assembly may occur at neurite tips. At lower concentrations, taxol may slightly enhance the mechanisms of microtubule assembly in neurons, and this alteration of normal processes changes the morphogenetic properties of the growing neurites.

Many cellular microtubules are in a dynamic equilibrium with unpolymerized tubulin and associated proteins (1, 13, 17, 28, 40). Several drugs (e.g., colchicine) bind to tubulin, and promote the disassembly of these microtubules (25). This action has been used to probe the involvement of labile microtubules in several activities, including mitosis and cell motility (16, 42). Taxol, another plant alkaloid, binds to tubulin and lowers the critical concentration required for polymerization of purified tubulin (32, 35). The taxol-containing microtubules are resistant to cold temperature and concentrations of Ca$^{2+}$ that normally depolymerize microtubules. Addition of taxol to cells induces unusual arrays of microtubules, and produces effects that include abnormal myofibrillogenesis and inhibition of mitosis, cell proliferation, and cell migration (2, 3, 10, 29, 30, 33, 34, 39, 43). Thus, it appears that promotion and stabilization of microtubules can disrupt cell functions, just as microtubule depolymerization does.

Microtubules have several roles in neurite outgrowth. Sensitivity to colchicine has implicated microtubules in the outgrowth and stabilization of neurites, as well as in the normal suppression of filopodial activity along neurites, proximal to the growth cone (8, 9, 47). Taxol has been applied to explants of mouse sensory ganglia, and unusual arrays of microtubules were reported in neuronal somas and neurites (29). However, effects of taxol on neurite outgrowth were not mentioned in the paper.

We have found that neurite outgrowth in vitro from sensory neurons of chick embryos is inhibited by taxol in a dose-dependent manner. At high concentrations neurite production is limited to short stout meganeurites. At the low concentrations longer, thick neurites are formed, but they have abnormally few branches. Existing neurites cease growth immediately when exposed to high concentrations of taxol. Alteration in protrusive activity at neurite tips is also produced by taxol. Neurons treated from beginning of culture with higher concentrations of taxol contain bundles of closely packed microtubules that exclude neurofilaments and other organelles. These findings indicate that an abnormal enhancement of microtubule assembly and stability disrupts the mechanism of neurite elongation. We will discuss the relationships of these results to the dynamics of microtubules in growing neurites.

MATERIALS AND METHODS

Cell Culture: Dorsal root ganglia were dissected from 9-11-d-old chick embryos and dissociated with crude trypsin (20). The resultant cell suspension was placed into a culture medium containing 40% F12 nutrient mixture, 10%
calf serum, 50% heart conditioned medium (24) and 10 ng/ml nerve growth factor (a gift from Dr. Eric Shooter, Stanford University). 6-16 × 10^6 ganglion cells in 1.5 ml medium were put into 35-mm plastic dishes treated in several ways: for measurements of neurite outgrowth and for preparing thin sections, cells were plated onto polystyrene tissue culture dishes (Falcon 3001, Falcon Labs, Oxnard, CA); for immunocytochemistry, cells were plated onto 18-mm polystyrene-glass coverslips in petri dishes (Falcon 1008) and for whole mount transmission electron microscopy, cells were plated onto 75 mesh gold electron microscopy grids (Ted Pella, Inc. Tustin, CA), supporting a polystyrene-treated, carbon-coated Formvar film (21). For videomicroscopy, cells were plated into 50-mm plastic dishes (Falcon 1006), containing a polystyrene coverslip glued (Dow Corning Silicon cement (Dow Corning Corp., Midland, MI)) over a 22-mm diameter hole. The dishes were incubated at 37°C in a humidified 5% CO₂ incubator.

**Drug Treatments:** Taxol (obtained from the Natural Products Branch of the National Cancer Institute) was dissolved in dimethyl sulfoxide at concentrations from 0.002-2.0 mg/ml. The taxol solution was added to culture medium, so that the concentration of dimethyl sulfoxide did not exceed 0.3%. Dimethyl sulfoxide at 0.3% volume was added to the medium of control cultures. For study of recovery from taxol, dorsal root ganglion (DRG) cells were rinsed three times with F12 nutrient mixture and recultured in whole culture medium. The need for protein synthesis during recovery was examined by adding 10 μg/ml of cycloheximide (Sigma Chemical Co., St. Louis, MO) to culture medium. This concentration of cycloheximide inhibits protein synthesis in DRG cells by 95% (47).

**Videomicroscopy:** Motile behavior of neurites was recorded from the Zeiss IM microscope using a Model 65 SIT camera (Dage-MTI, Inc., Michigan City, IN), and a Panasonic Model NV-8300 timelapse videorecorder. Individual frames from a videotape were photographed from the videomonitor on Pan X film with a 35 mm SLR camera (Canon AE-1).

**Extraction and Fixation:** Cytoskeletons for immunocytochemistry and whole mount electron microscopy were prepared by incubation for 4 min in a buffer described by Schliwa and van Bleekom (36), containing 0.2% Triton X-100. Actin filaments were preserved during extraction by the addition of either 5 μg/ml phalloidin (Sigma Chemical Co., St. Louis, MO) or the S1 fragment of myosin (a gift from Dr. David Thomas, University of Minnesota). The cytoskeletons were fixed for immunocytochemical localization of actin, neurofilament protein and tubulin by exposure to 4% paraformaldehyde, 0.3% glutaraldehyde in the extraction buffer for 30 min. For whole mount electron microscopy, the cytoskeletons were fixed 30 min with 2% glutaraldehyde in the extraction buffer, rinsed, and stained with 0.4% tannic acid in water for 45 min (24). For thin sections of unextracted neurons, cells were fixed with 2% glutaraldehyde in 0.154 M cacodylate, pH 7.4, and postfixed in 1% osmium tetroxide on ice for 7 min (24).

**Immunocytochemistry:** Rabbit antiserum to chicken gizzard actin was generously provided by Dr. Judith Schollmeyer of the Roman Hruska Meat Science Center (22). A monoclonal antibody to the 210,000-mol-wt neurofilament protein of rats was a gift from Dr. John M. Wood of Burroughs-Wellcome (46). Sheep antibodies to tubulin purified from bovine brain was purchased from Caub Co. (Houston, TX).

After fixation and rinsing, the coverslips were incubated in 0.5 mg/ml sodium borohydride in Ca²⁺-Mg²⁺ free saline for 15 min. The primary antibodies were applied in phosphate-buffered saline, containing 0.2% Triton X-100 and 5 mg/ml bovine albumin (Sigma Chemical Co., St. Louis, MO). Actin antiserum was applied at 1/100 dilution, neurofilament antibody at 1/100 dilution of acetate fluid, and tubulin antibody at 66 μg/ml. Control coverslips were treated with actin antiserum presorbed with actin or with a wash solution lacking a primary antiserum for tubulin and neurofilament protein localization. Coverslips were rinsed and stained with 1/100 dilutions of appropriate fluorescent secondary antibodies purchased from Cappel Laboratories (West Chester, PA). After a final rinse, coverslips were mounted on slides in Eukane and viewed on a Zeiss IM microscope, with a 63X planapochromat objective illuminated by an HBO 100W lamp. Photography was done using Tri-X Pan film.

**Electron Microscopy:** After tannic acid treatment the grids with cytoskeletons were transferred to a grid holder, stained with aqueous 2% uranyl acetate for 30 min, dehydrated through a graded series of ethanol and critical point dried, and stabilized by deposition of a thin layer of carbon.

For preparation of thin sections of unextracted neurons, the fixed cells were stained with aqueous 2% uranyl acetate, dehydrated through ethanol and embedded by addition of plastic resin to the tissue culture dishes. Blocks containing single cells were mounted on larger plastic bases and thin sections were cut parallel to the substratum.

**Measurements:** Neurite outgrowth was measured after 24-h culture.

DRG cells in polylysine-treated dishes were fixed with 2% glutaraldehyde in 0.154 M cacodylate, rinsed in phosphate-buffered saline, and stained with 1% osmium tetroxide on ice for 7 min. Percent of DRG neurons with neurites, mean neurite length and width, and number of filopodia per growth cone were calculated from counts of randomly selected microscopic fields representative of all areas of the culture substratum.

**Microtubule-Microtubule Separation:** Measurements of microtubule diameter and intermicrotubular distance were taken from electron micrograph negatives (magnification of 70,000) using a 10X ocular positioned above an x-ray viewing box. Each observation was recorded when the following criteria were met: (a) microtubule diameter <300 nm; diameters greater than this were found to be adjacent microtubules in tangential plane; (b) intermicrotubular distance may include organelles such as a mitochondrion or filaments, but was calculated as the total distance between two microtubules. Measurements were made only in the center of the negatives, where the microtubules could be followed for a greater length on the negative. Data were standardized against calibration negatives.

**RESULTS**

**Effects of Taxol Concentration on Neurite Initiation and Growth**

Dissociated cells from dorsal root ganglia were cultured 24 h in media containing 3.5 × 10⁻⁶ - 7 × 10⁻⁸ M taxol (Table I). The frequency of neurites was lowest at concentrations of taxol greater than 7 × 10⁻⁸ M and was limited to short, broad extensions, which we called meganeurites (Fig. 1b; also see Fig. 14a). Several hours of videotapes showed that filopodia but not broader lamellipodia or veils were protruded along the meganeurites, but protrusive activity was not concentrated at the neurite tip to form a growth cone. In addition to protrusion of filopodia, movements of mitochondria and other particles were also visible in meganeurites. However, despite these two types of motility characteristic of growing neurites, meganeurites did not elongate during our videotaping. Neurons with short, broad meganeurites were still present after 5 d in taxol-containing medium.

At the lowest concentration of taxol used in these experiments, 7 × 10⁻⁹ M, the frequency of neurite outgrowth approached normal levels (Table I). These neurites were much less branched than control neurites and often ran hundreds of micrometers without one branch point (compare Fig. 1a and c). The difference between untreated neurites and neurites formed in the presence of 7 × 10⁻⁹ M taxol was quantified in other ways. The mean width of neurites in a sample of 20 neurons from each situation was three times greater in the presence of 7 × 10⁻⁹ M taxol than in the same sample the mean number of filopodia per growth cone (12.3 ± 5 vs. 7.7 ± 4) was also greater with 7 × 10⁻⁹ M taxol than for untreated cells. If the neurites are

### Table 1

**Effects of Taxol on Neurite Initiation and Outgrowth**

| Concentration of taxol | % cells with neurites (n) | Mean length ± SD of neurites formed (n) |
|------------------------|--------------------------|---------------------------------------|
| 0                      | 36 (549)                 | 607 ± 424 (54)                        |
| 7 × 10⁻⁹               | 32 (281)                 | 156 ± 123 (28)                        |
| 2.3 × 10⁻⁸             | 22 (276)                 | 145 ± 119 (27)                        |
| 7 × 10⁻⁸               | 17 (507)                 | 58 ± 47 (44)                          |
| 2.3 × 10⁻⁷             | 18 (543)                 | 52 ± 32 (52)                          |
| 7 × 10⁻⁷               | 18 (313)                 | 35 ± 22 (26)                          |
| 3.5 × 10⁻⁶             | 15 (284)                 | 39 ± 21 (24)                          |

DRG cells were cultured 24 h in medium containing indicated concentrations of taxol. Cells were fixed, then rinsed, and values reported are gathered from two or four dishes at each concentration. n is sample total size.

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1 **Abbreviations used in this paper:** DRG, dorsal root ganglion.
FIGURES 1-5

Fig. 1: Comparison of morphology of neurons cultured with low and high concentrations of taxol. (a) Control neuron cultured 24 h without taxol. (b) Neurons cultured 24 h with $7 \times 10^{-7}$ M taxol. Short broad meganeurites are present (m). (c) Neuron cultured 24 h with $7 \times 10^{-9}$ M taxol. Single long neurite is unbranched. Compare with degree of neurite branching of neuron in a. x 150.

Fig. 2: Whole mount of an extracted cytoskeleton of the perikaryon of a neuron cultured 24 h with $7 \times 10^{-7}$ M taxol. Bundles (B) of microtubules loop around perimeter of neuron, and many microtubules extend from perikaryon into the meganeurites (M). Part of a satellite cell is at left border. x 2,800.

Fig. 3: Distal tip (T) of a cytoskeleton of a meganeurite. Dense bundle of microtubules terminates in a tightly looped mass. No ends of microtubules are readily visible at the tip of meganeurite. x 4,600.

Fig. 4: A sheet of regularly spaced microtubules in a meganeurite cultured in $7 \times 10^{-7}$ M taxol. There is little material evident in the spaces between microtubules (parentheses), except for short filamentous segments that may interconnect the microtubules. x 100,000.

Fig. 5: Portion of a meganeurite that illustrates three unusual morphological features of taxol-treated cells. Array of dense, regularly packed microtubules is at A. Thin, long membrane sacs (S) are oriented along microtubules. Tangled masses of neurofilaments (N) are segregated from microtubular arrays. x 53,000.
roughly cylindrical, then neurites formed in $7 \times 10^{-9}$ M taxol have a mean cross-sectional area approximately nine times greater than untreated neurites. On videotapes, the activity of neurites treated with the low concentration of taxol was generally similar to normal growth cone behavior, though the formation of lamellipodia and veil-like expansions of filopodia at the growth cones was less frequent.

**Ultrastructure of Taxol-treated Neurons**

As reported for studies of other cell types (2, 10, 29, 30, 33, 34, 39, 43), higher concentrations of taxol induce unusual arrays of microtubules in DRG neurons. Microtubules circled the perikaryon in bundles and passed into the meganeurites (Fig. 2). At the distal ends of meganeurites, most microtubules looped back without terminating (Fig. 3), as occurs in untreated neurites (24). Along the meganeurites, microtubules were often closely packed into bundles or sheets (Figs. 4 and 5) of microtubules separated by narrow regular spaces that eliminate other structures except for short fuzzy segments of material (Figs. 4 and 5). The mean intermicrotubule distance in taxol-treated meganeurites was significantly less ($12.0 \pm 10.6 \text{ nm}$, $n = 200$) than the mean distance between microtubules in control neurites (32.5 ± 31.7 nm, $n = 155$). Another feature previously observed in taxol-treated cells was the close association of microtubules with narrow sacs or tubes of smooth endoplasmic reticulum (Fig. 5), compressed into narrow spaces between the nearly parallel microtubules.

Neurofilaments were excluded from the bundles of microtubules, and were frequently grouped into fascicles within meganeurites (Fig. 5). Occasionally, a knot of neurofilaments was present at the distal end of a meganeurite. As in the arrays of microtubules, these neurofilament tangles contained few other organelles except for some filamentous cross-bridges and small vesicles. It is interesting, then, that microtubules and neurofilaments, which are intermixed in untreated neurites were segregated in taxol-treated meganeurites into separate arrays.

The ultrastructure of neurites exposed to $7 \times 10^{-9}$ M taxol was much more normal in that microtubules were separated by variable distances and were interspersed with other organelles along the length of neurites (Figs. 6 and 7). When studying whole mounted specimens, we were impressed by the large numbers of microtubules in the cytoskeletons of neurites exposed to $7 \times 10^{-9}$ M taxol (Fig. 8). This observation is consistent with the larger caliber of these neurites. These cytoskeletal preparations also showed that many microtubules ends were present at the neurite tips, as normally occurs (24).

**Immunocytochemistry**

The distribution of three cytoskeletal proteins, tubulin, actin, and the 210,000-mol-wt subunit of neurofilaments, in taxol-treated neurons was assessed by fluorescence immunocytochemistry. As expected, cells treated with $7 \times 10^{-7}$ M taxol contained large fluorescent masses of bound antitubulin, that mimicked the distribution of microtubules (Figs. 2, 3, and 10). Antiactin was diffusely distributed along meganeurites with peripheral concentrations at sites of filopodia (Fig. 11), but no large accumulations at the tips of meganeurites, as at a growth cone (24). The distribution of antibody to the 210,000-mol-wt neurofilament subunit was generally similar to the distribution of tubulin (Fig. 12). Some cells contained bright immunofluorescence for the 210,000-mol-wt protein at the tips of meganeurites, corresponding to the knots of neurofilaments at the ends of some meganeurites (Fig. 5).

Cells stained with preabsorbed antibody to actin or without any primary antibody did not show the concentrations of staining observed with these cytoskeletal antibodies. Thus, each of the antibodies stains the meganeurites of taxol-treated cells in a manner consistent with the distribution of morphologically identifiable structures, and the bundles of microtubules do not contain these cytoskeletal proteins in unusual associations that could be related to the inhibition of neurite outgrowth by taxol.

**Effects of Taxol on Previously Formed Neurites**

Growth cones of DRG cells that had been cultured for 24 h in control medium were videotaped before and after the addition of taxol to a concentration of $7 \times 10^{-7}$ M. Within 90 s, as soon as the growth cone could be refocused after adding the drug, a response was evident (Fig. 13, a–c). The neurite retracted for several micrometers and become broader at the tip. Lamellipodia from the neurite tip continued, but lamellipodia and veil-like expansions of filopodia were no longer produced. These growth cones did not elongate further over 1 h of recording. The location of several neurites was marked on the dish, and after 24 h of further culture with taxol these neurites had not grown. In fact, some of the neurites had retracted their distal branches for 30–50 μm.

**Figs. 6–13**  Figs. 6 and 7: Comparison of ultrastructure of neurites cultured for 24 h in $7 \times 10^{-9}$ M (Fig. 6), or no (Fig. 7) taxol. Microtubules are irregularly oriented along the neurite in the presence of $7 \times 10^{-9}$ M taxol (Fig. 6), as in control neurites. Many microtubules are packed into this neurite, but not with the regularity displayed at higher concentrations of taxol. × 53,000. Fig. 8: Whole mount of the detergent-extracted cytoskeleton of a neurite tip cultured in $7 \times 10^{-9}$ M taxol. Neurite contains many microtubules, and the microtubules do not loop back at the tip (T) as they do at the end of meganeurites. Filamentous material (f) at periphery of neurite tip is S1 decorated-actin filaments (see Letourneau [24]). × 5,100. Fig. 9: Whole mount of the cytoskeleton at the tip of a neurite, exposed for 5 min to $7 \times 10^{-7}$ M taxol, before extraction in a buffer lacking taxol. In rapid response to the drug, microtubules are looped and tangled at the neurite tip. × 5,700. Fig. 10: Localization of tubulin in a neuron cultured for 24 h with $7 \times 10^{-7}$ M taxol. Soma (S) is at left, tip (T) of meganeurite is at right. Intense immunofluorescence for tubulin can sometimes be resolved into fibrous structures that may represent microtubules or bundles of microtubules. × 900. Fig. 11: Localization of actin in a neuron cultured for 24 h with $7 \times 10^{-7}$ M taxol. Soma (S) is at left, Punctate and diffuse staining are present in soma and meganeurite. Linear staining at tip (arrows) are actin-containing filopodia. × 900. Fig. 12: Localization of neurofilament protein in a neuron cultured for 24 h with $7 \times 10^{-7}$ M taxol. Soma (S) is at left. Bright staining at tip of meganeurite (T) may represent tangles of neurofilaments as seen in Fig. 5. × 900. Fig. 13: Morphology of a neurite tip just before and after addition of $7 \times 10^{-7}$ M taxol at 14:37:30. Lamellipodia or veils (V) are present in a before taxol is added. Note in b that neurite tip has already begun to retract within 2 min of adding taxol. Neurite tip has stabilized in c, and filopodia (f), but not lamellipodia continue to be protruded. × 850.
Neurites that were cultured 24 h in regular medium and then treated with $7 \times 10^{-7}$ M taxol for 5–20 min were briefly extracted in detergent-containing buffer without taxol and examined by whole mount transmission electron microscopy. At the ends of these neurites microtubules were not longitudinally oriented as in untreated neurites, rather the microtubules looped back or circled in a disordered group (Fig. 9). It did not appear that taxol had induced the formation, as occurs in preparations of purified tubulin (35), of many short microtubules at the neurite tip. The actin filament network was noticeably reduced at the tips of these neurites. This is probably related to the rapid reduction in protrusive activity when taxol is added.

Recovery from Taxol Treatment

It was of interest to ask whether the effects of taxol on neurite growth are readily reversible by removing the drug. Within 30 min of rinsing $7 \times 10^{-7}$ M taxol from the culture medium, growth cones with many filopodia emerged from meganeurites and from the perikarya of neurons. This was reminiscent of the initiation of neurites from untreated neurons in the first hours of normal culture procedures. By 2 h after removing taxol, numerous cells had sprouted one or more actively growing neurites (Fig. 14). The need for protein synthesis during recovery was assessed by the addition of the inhibitor cycloheximide (47). Exposure to 10 $\mu$g/ml cycloheximide for up to 4 h before removal of taxol and continuing with cycloheximide after removal of taxol did not stop the rapid formation of growth cones and neurites in the first 3–5 h of recovery. These data indicate that taxol binding to tubulin is reversible, and suggests that the large arrays of microtubules may be depolymerized and reutilized when the cells are returned to normal conditions.

DISCUSSION

The assembly and disassembly of cytoplasmic microtubules is controlled by a diverse group of components that determine where microtubule polymerization begins, as well as the length, number, and stability of microtubules (1, 13, 17, 28, 40). These interactions orchestrate intricate changes in cytoplasmic microtubules during dynamic processes such as mitosis (16), cell elongation (7), and chemotaxis (26). Taxol probably disrupts the control of microtubular organization by sharply lowering the critical concentration of tubulin for assembly of microtubules (35). Thus, microtubules may form at unusual sites, and normal signals for disassembly of microtubules may not work. This is suggested by the presence in taxol-treated cells of large arrays of closely packed microtubules (29, 30, 33, 34, 39, 43), but an absence of microtubules associated with centrioles or kinetochores of cells entering mitosis (10). The inhibition of neurite outgrowth by taxol may result, then, from two primary drug actions; uncontrolled microtubule assembly and enhanced stability of microtubules (3, 27, 32, 35, 38). The actions of taxol will eventually be better understood from quantitative analyses of the effects of taxol on total amount of cellular tubulin, pools of unpolymerized tubulin, and number of microtubules per cell.

There is general agreement that intact microtubules are required for the initiation and maintenance of neurite growth (8, 9, 47). However, there is not strong support for a single model explaining how microtubules accumulate in growing neurites (14, 18, 23, 37). Possible mechanisms are that stable microtubules are polymerized in the perikaryon before transport into the neurites or that tubulin is transported and polymerized within neurites. Of course, both processes may go on, as suggested from studies of microtubule dynamics in mature axons (18). It is not clear, however, what is the precise relationship of microtubule assembly, stability, and transport to the immediate events of neurite elongation.

When cultured for 24 h in taxol concentrations $>7 \times 10^{-8}$ M, some neurons have short processes that we called meganeurites. However, these processes do not resemble growing neurites in several important respects, and we propose that they are not really neurites in a morphogenetic sense. Evidence to indicate that meganeurites do not grow includes the fact that (a) there is no concentrated protrusive activity, resembling a growth cone, at the end of meganeurites, that (b) individual meganeurites do not elongate during prolonged videotaping, and that (c) microtubules do not terminate at the ends of meganeurites, as in a growing neurite (24). These meganeurites were probably formed from the proximal stumps of neurites that were severed when the sensory ganglia were dissected and dissociated. Normally, most of these stumps are resorbed into the perikaryon soon after plating, though the stumps can attach to a highly adhesive substratum, regenerate growth cones and initiate outgrowth (6, 45). In the presence of taxol, the stable microtubules may have stabilized the stumps against collapse, and as the pool of tubulin in the neurons polymerized into more microtubules, the stumps widened into meganeurites. However, these microtubules apparently were not used for neurite outgrowth.

A more revealing picture of microtubule metabolism during neurite outgrowth is taxol’s immediate arrest of actively growing neurites. The response is so fast, it seems likely due to a rapid assembly of tubulin at the neurite tip. There was not a generation of many short microtubules, but instead tubulin has added onto the many microtubule ends projecting into a growth cone (24). Because, few microtubule ends occur proximal to the neurite tip (23), the rapidly elongating ends looped back towards the perikaryon, depleting the free tubulin in the

![Figure 14](image-url) Recovery of neurite outgrowth after removing taxol from culture medium. Neuron in (a) was cultured 24 h with $7 \times 10^{-7}$ M taxol. Same cell in (b) is shown 2.5 h after washing taxol out of culture medium. Five neurites have been initiated from the tip of the meganeurite (A) and from the perikaryon. x 300.
neurite. These proposed events following addition of taxol would occur only if tubulin subunits are present in neurites, and is consistent with the view that microtubules are dynamic structures and normally undergo assembly in growing neurites.

Our use of taxol at $7 \times 10^{-6} \text{M}$ is the lowest concentration that has been reported to produce a cellular response (Table I and Fig. 1c). It is a 100-fold lower than the association constant for taxol binding to tubulin from calf brain (32), so the effect of this concentration may be to slightly stimulate microtubule assembly in neurites. Stimulation of microtubule assembly might be expected to trigger neurite initiation and branching, but instead we observed shorter, broader neurites that were less branched than normally. Apparently, the mechanisms of neurite outgrowth are not related in a simple way to microtubule assembly. Perhaps, the events of neurite initiation are not disturbed by this low concentration, but the drug enhances production of microtubules at the initiation site, and the neurites maintain an increased number of microtubules by continued transport of many microtubules or free tubulin into the neurites. The infrequent branching of neurites at this concentration of taxol may be related to the large diameter and microtubule numbers of these neurites. The branching of a neurite tip is thought to involve contractile forces in the growth cone margins that separate the neurite contents into several portions (5, 23, 44). Perhaps these forces are insufficient to subdivide the extraordinarily large complement of microtubules and associated structures in such large caliber neurites.

Though not fully understood, these effects of low concentrations of taxol are interesting, because they show that major features of neuronal morphology, axon caliber, and branching, could be regulated by a single factor that influences the equilibrium of the tubulin in neurites. It may turn out that different morphological classes of neurons are distinguished by simple differences in molecular components, such as a microtubule-associated protein that acts like taxol.

We also noted that protrusion of the neurite surface is disturbed by taxol. Certainly, this might reduce neurite outgrowth, and this response, too, can be related to taxol's effects on microtubules. Numerous studies, using colchicine primarily, have indicated that microtubules regulate the shape and motility of cell surfaces (4, 6, 31, 42), perhaps through an influence on the shape and associations of actin filaments. The evidence of interactions between microtubules and microfilaments is sparse (12, 36). Alternatively, Berlin et al. (4) and Oliver et al. (31) have suggested that it is membrane-bound tubulin that influences cell surface activity, and the stimulation of microtubule assembly by taxol may substantially reduce the availability of tubulin to associate with membranes. At present, however, there is no clear mechanism of how microtubules and/or tubulin influence surface motility.

It is not known how the large regular arrays of microtubules in taxol-treated cells or in naturally occurring situations are organized or maintained (2, 10, 29, 33, 34, 39, 40). Taxol does not inhibit binding of the protein, microtubule-associated protein 2, to tubulin (41), so this molecule may interconnect microtubules with each other and with the elongated membrane sacs (11, 13, 15, 40). Neurofilament bundling and tangles were another unusual feature of taxol-treated neurons. Interestingly, these arrays of neurofilaments resemble the masses of neurofilaments in colchicine-treated neurons (47).

Perhaps, in both situations there is an enhanced self-association of neurofilaments when they do not interact with microtubules. Colchicine, of course, destroys microtubules, while the regular packing of microtubules in taxol-treated neurons may be energetically favored and exclude interactions with neurofilaments. Microtubule-associated protein 2 binds to neurofilaments and thus may interconnect the neurofilaments in these bundles and tangles (19).

In summary, taxol is a useful probe of neurite growth. Our findings indicate that microtubules are dynamic structures in growing neurites. The rapid inhibition of growth when taxol is added to neurites is consistent with the proposal that a controlled assembly of microtubules occurs at the neurite tip. Morphological alterations induced by lower concentrations of taxol suggest that factors that modulate the equilibrium of microtubule assembly and disassembly can regulate neuronal shape.

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