

Taxol-induced Rose Microtubule Polymerization In Vitro and Its Inhibition by Colchicine

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ABSTRACT Tubulin was isolated from cultured cells of rose (*Rosa*, sp. cv. Paul's scarlet) by DEAE-Sephadex A50 chromatography, and the taxol-induced polymerization of microtubules in vitro was characterized at 24°C by turbidity development, sedimentation analysis, and electron microscopy. Numerous, short microtubules were formed in the presence of taxol, and maximum levels of turbidity and polymer yield were obtained at ~2:1 molar ratios of taxol to tubulin. The critical concentration of rose tubulin for polymerization in saturating taxol was estimated to be 0.21 mg/ml. Colchicine inhibited the taxol-induced polymerization of tubulin as shown by sedimentation assays; however, much higher concentrations of colchicine were required for the inhibition of taxol-induced rose tubulin assembly than for inhibition of taxol-induced mammalian brain tubulin assembly. On the basis of the relative sensitivity of rose tubulin assembly to taxol and its insensitivity to colchicine, we propose that the taxol-binding site(s) on plant and animal tubulins have been more conserved over evolution than the colchicine-binding site(s).

Among those drugs causing rapid depolymerization of microtubules in cells, colchicine, an alkaloid from the meadow saffron (*Colchicum autumnale*), has been the most commonly used (10, 13). Studies on purified animal microtubule protein in vitro show that colchicine binds to soluble dimeric tubulin at a 1:1 molar ratio to form a tubulin-colchicine complex (6, 42, 58, 59). At concentrations substoichiometric to tubulin, the tubulin-colchicine complex co-assembles with tubulin to form co-polymers that have reduced dimer association rate constants at both ends (1, 4, 25, 33, 47, 48, 57, 58). Thus, addition of colchicine to tubulin solutions prevents the nucleation of new microtubules and causes rapid depolymerization of preformed microtubules. Whereas the rapid depolymerization of microtubules in animal cells occurs at or below micromolar colchicine concentrations, the depolymerization of microtubules in plant cells commonly requires 100- to 1,000-fold higher colchicine concentrations (10, 13, 15). To date, the biochemical basis for this resistance has not been determined.

Currently, the only antimicrotubule drug known to favor the polymeric state of tubulin (microtubules) is taxol, a taxane alkaloid from the western yew (*Taxus brevifolia*) (55). When applied to animal cells at micromolar concentrations, taxol promotes the assembly of new microtubules and stabilizes

microtubules to depolymerization by treatment with cold or nocodazole (3, 9, 16, 26, 37, 39). Although there have been fewer studies on the effects of taxol on microtubules in plant cells, similar microtubule promotion and stabilization properties have been reported for both the mitotic spindle and cytoplasmic microtubules of a higher plant and an alga (2, 17, 27).

Taxol promotes the in vitro assembly of mammalian brain tubulin into microtubules in the presence or absence of microtubule-associated proteins (MAPs),¹ guanosine triphosphate, and low temperature, and maximum microtubule assembly is observed at ~1:1 molar ratios of taxol and tubulin (8, 14, 21, 38, 40, 50). Apparently taxol slows the rate of exchange of tubulin dimers at the ends of steady-state microtubules through a decrease in the dissociation rate constants of dimers at both microtubule ends (7, 21). Studies with [³H]taxol have demonstrated specific binding to microtubules in cultured animal cells, sea urchin sperm flagellar outer doublet microtubules, and polymerized animal microtubules in vitro (24, 34, 35). Inasmuch as the molar binding stoichiometry of [³H]taxol and brain tubulin in microtubules is 0.78, it has

¹ Abbreviations used in this paper: MAP, microtubule-associated protein; SIB, sucrose isolation buffer.

been postulated that there is one taxol-binding site per tubulin dimer in microtubules (35). However, Carlier and Pantaloni (8) reported that the guanosine triphosphatase activity of unpolymerized brain tubulin-colchicine complex is enhanced by taxol suggesting that taxol binds also to the dimeric form of tubulin, but with a 10-fold lower affinity than its binding to tubulin in microtubules.

Taxol-stabilized brain microtubules are resistant *in vitro* to antimicrotubule drugs such as colchicine, podophyllotoxin, and vinblastine, which would ordinarily depolymerize microtubules completely (7, 21, 40). When these drugs and saturating amounts of taxol are added to tubulin solutions, the yield of assembled microtubules is diminished and much higher concentrations of depolymerizing drug are required for inhibition of assembly than are necessary for inhibition in the absence of taxol (7, 21, 40). Nevertheless, inhibition of taxol-induced assembly can be attained, with maximum inhibition produced at equimolar concentrations of colchicine or podophyllotoxin and tubulin (21, 40), rather than at substoichiometric ratios effective in the absence of taxol (33, 58). Apparently taxol and colchicine do not compete for binding to the same site on tubulin, but rather bind preferentially to different forms of tubulin, the dimer, or the polymer (21, 35).

In our initial report on the isolation and polymerization of higher plant tubulin *in vitro* (30), both glycerol and taxol were found to mediate microtubule assembly, and taxol-induced assembly yielded a ≈ 6 -fold greater amount of polymer than glycerol-induced assembly. Recently we have shown that the amounts of colchicine bound by tubulins from three higher plant species are much lower than that bound by bovine brain tubulin, suggesting that the resistance of plant microtubules to colchicine is due, at least in part, to a lower binding affinity of plant tubulin for colchicine (29). In the present study we have utilized the efficient, taxol-induced, rose microtubule assembly reaction to assess the effects of colchicine on plant microtubule assembly *in vitro*. Initially, the taxol-mediated polymerization reaction was characterized by turbidimetric and sedimentation assays. The results are compared with those obtained with parallel, bovine brain tubulin assembly experiments.

MATERIALS AND METHODS

Tubulin Isolation: Tubulin was isolated from suspension cultures of rose (*Rosa* sp. cv. Paul's scarlet) by DEAE-Sephadex A50 chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (30) with minor modifications (29). The tubulin-containing fraction was collected by ammonium sulfate precipitation and dialyzed at 4°C for 10 h against a sucrose isolation buffer (SIB) consisting of 1 M sucrose, 0.05 M PIPES-KOH (pH 6.9), 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM MgCl₂, 0.1 mM guanosine triphosphate (lithium salt), 1 μ M leupeptin hemisulfate, 1 μ M pepstatin A, and 0.02% NaN₃. The dialysate was clarified of large aggregates by centrifugation at 48,000 g for 1 h at 4°C, and the supernatant was frozen in small aliquots in a dry ice-chilled methanol bath and stored at -80°C.

The possibility that tubulin subunits may have been cleaved proteolytically during isolation (31) was routinely examined by immunoblotting of tubulin from gels (52). Blots were probed with polyclonal antibodies to either the α - or β -subunit (29) and incubated with ¹²⁵I-*Staphylococcus aureus* protein A. Autoradiographs showed that antibodies did not bind to minor, lower molecular weight polypeptides that co-purified with tubulin, indicating that tubulin subunits were uncleaved (31).

For parallel studies of brain microtubule assembly, tubulin from cow cerebra was purified directly from extracts by DEAE-Sephadex A50 chromatography (22) with modifications as described (29). This method is very similar to that used to isolate rose tubulin and utilizes the same buffer (SIB), but the proteolytic inhibitors leupeptin hemisulfate and pepstatin A were omitted. Dialysates were processed exactly as for rose tubulin. Brain tubulin was >98% pure and overloaded SDS slab gels revealed no high molecular weight MAPs.

Gel Electrophoresis, Quantitative Densitometry, and Protein Determination: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) was performed according to the method of Studier (49) with only minor modifications (30). Gels (7.5% polyacrylamide) were stained overnight in 0.25% Coomassie Brilliant Blue R-250 in 5% methanol/7.5% acetic acid.

Quantitative densitometry of tubulin samples in gels was used to estimate purity. Destained gels were scanned with an E-C densitometer (E-C Apparatus Corp., St. Petersburg, FL) equipped with a Varian G 2000 chart recorder (Varian Associates Inc., Palo Alto, CA), and tubulin subunit purity was determined by weighing peaks cut from chart paper tracings.

Determination of protein was made with the dye-binding method of Bio-Rad (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, MO) as a standard protein. Values for tubulin were corrected as previously described (29) and the molecular weight of tubulin was assumed to be 100,000 for calculation of amounts or concentrations of tubulin (20, 36, 53).

Turbidimetric and Sedimentation Assays: The assembly of microtubules *in vitro* was monitored by the change in turbidity (absorbance at 400 nm) in a Beckman Acta III recording spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) (5, 12, 19). Frozen tubulin samples were rapidly thawed to 0°C and added to SIB containing the appropriate drug(s) at 24°C. The mixture (100–150 μ l) was quickly added to a 400- μ l quartz cuvette (light path = 1 cm) maintained at 24 \pm 0.25°C and the change in absorbance was recorded continuously. It was estimated that the sample reached 24°C within 30 s after mixing.

The amount of polymer formed during each assembly experiment was determined by sedimentation assay (18, 19). After the assembly period the tubulin samples were centrifuged in cellulose nitrate tubes (5 \times 20 mm) with a Beckman airfuge (A-100/18 fixed-angle rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 48,000 g at 23–24°C for 1 h. Both the centrifuge tube walls and the exposed portion of the microtubule pellet were gently washed with 100 μ l of SIB and the pellets were resuspended in 100 μ l of SIB for protein determination.

Taxol was obtained from Dr. Matthew Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. A stock 10 mM taxol solution was prepared in 100% dimethyl sulfoxide and stored at -20°C until use, whereupon desired substocks were prepared in the range of 1 \times 10⁻³ to 8 \times 10⁻⁴ M. All assembly mixtures contained a final dimethyl sulfoxide concentration of 0.5%. Fresh colchicine (Sigma Chemical Co.) stock solutions (1 \times 10⁻³ to 2 \times 10⁻² M) were prepared immediately before assembly experiments.

Electron Microscopy: Immediately after the assembly period (before centrifugation of polymers), 5 μ l of the sample was applied to Formvar- and carbon-coated, 200-mesh electron microscope grids and allowed to adsorb for 5 min. Grids were rinsed of excess sample with 10 drops of distilled water and stained with 5 μ l of 2% uranyl acetate. Uranyl acetate was withdrawn with filter paper, grids were air-dried, and structures were viewed and photographed on a Zeiss 9S-2 electron microscope at 60 KV (Carl Zeiss, Inc., New York).

RESULTS

Polypeptide Composition of Rose Tubulin and Microtubule Fractions

Tubulin was isolated from exponential phase-suspension cultures of rose cells by fractionation of whole-cell supernatant proteins on DEAE-Sephadex A50 columns (30) with minor modifications (29). Analysis of a typical tubulin-containing fraction by SDS PAGE is shown in Fig. 1. A series of increased loadings revealed prominent tubulin α - and β -subunits, a few high molecular weight and low molecular weight polypeptides and material running with the bromophenol blue dye front. A comparison by SDS PAGE of the polypeptide compositions of different tubulin preparations showed some variation in the abundance of some polypeptides which co-purified with tubulin. However, quantitative densitometry of stained gels containing these different preparations gave a consistent range of tubulin purity of 83–87%.

Because plant cells grow and assemble microtubules at lower temperatures than do mammalian cells and because rose cells were grown at room temperature (22–25°C), we

have examined the *in vitro* assembly of rose tubulin at 24°C. Polymeric structures formed by 8.7 μM rose tubulin after 1 h at 24°C in the presence of 20 μM taxol were negatively stained

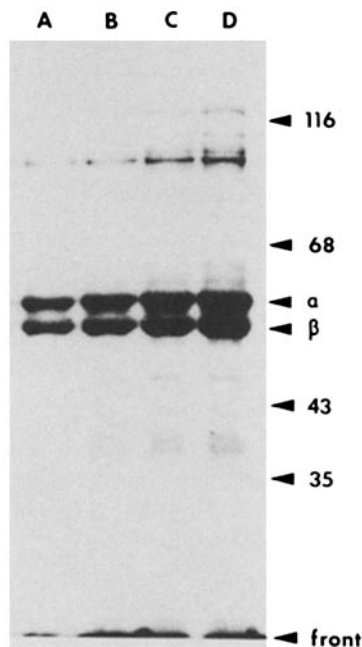


FIGURE 1 SDS PAGE of rose cell tubulin isolated by DEAE-Sephadex A50 chromatography. Lanes A, B, C, and D contain 3, 6, 9, and 12 μg of protein, respectively. Position of tubulin α - and β -subunits, molecular weight protein standards, and the bromophenol blue tracking-dye front are indicated with arrowheads. Protein standards were *Escherichia coli* β -galactosidase (116,000), bovine serum albumin (68,000), ovalbumin (43,000), and bovine heart lactate dehydrogenase (35,000) and are indicated $\times 10^3$.

and viewed on the electron microscope. Numerous short microtubules ($0.49 \pm 0.38 \mu\text{m}$) were observed with fairly uniform widths of $235 \pm 12 \text{ \AA}$ (Fig. 2). Short constricted areas containing fewer protofilaments were occasionally seen, and many microtubule ends were frayed with either free protofilaments or ribbon structures. (Fig. 2, *inset*).

The protein composition of sedimented microtubules was analyzed by SDS PAGE. A typical microtubule pellet was composed mainly of tubulin and sometimes a small amount of a low molecular weight polypeptide (M_r 41,000) and material running at the dye front (Fig. 3). Quantitative densitometry of stained gels indicated >95% purity of tubulin in microtubule pellets. The possibility that tubulin was cleaved during isolation (31) in the presence of protease inhibitors was examined by immunoblotting, and no cleavage products were detected in the batches of tubulin used in these studies on microtubule assembly (refer to Materials and Methods).

Qualitative and Quantitative Analysis of Microtubule Polymerization

The taxol-induced assembly of rose tubulin was studied by monitoring the change in turbidity (absorbancy at 400 nm). For each concentration of taxol tested, tubulin solutions (8.7 μM) showed increased turbidity over time with the kinetics of assembly being sigmoidal (Fig. 4). Each assembly assay was

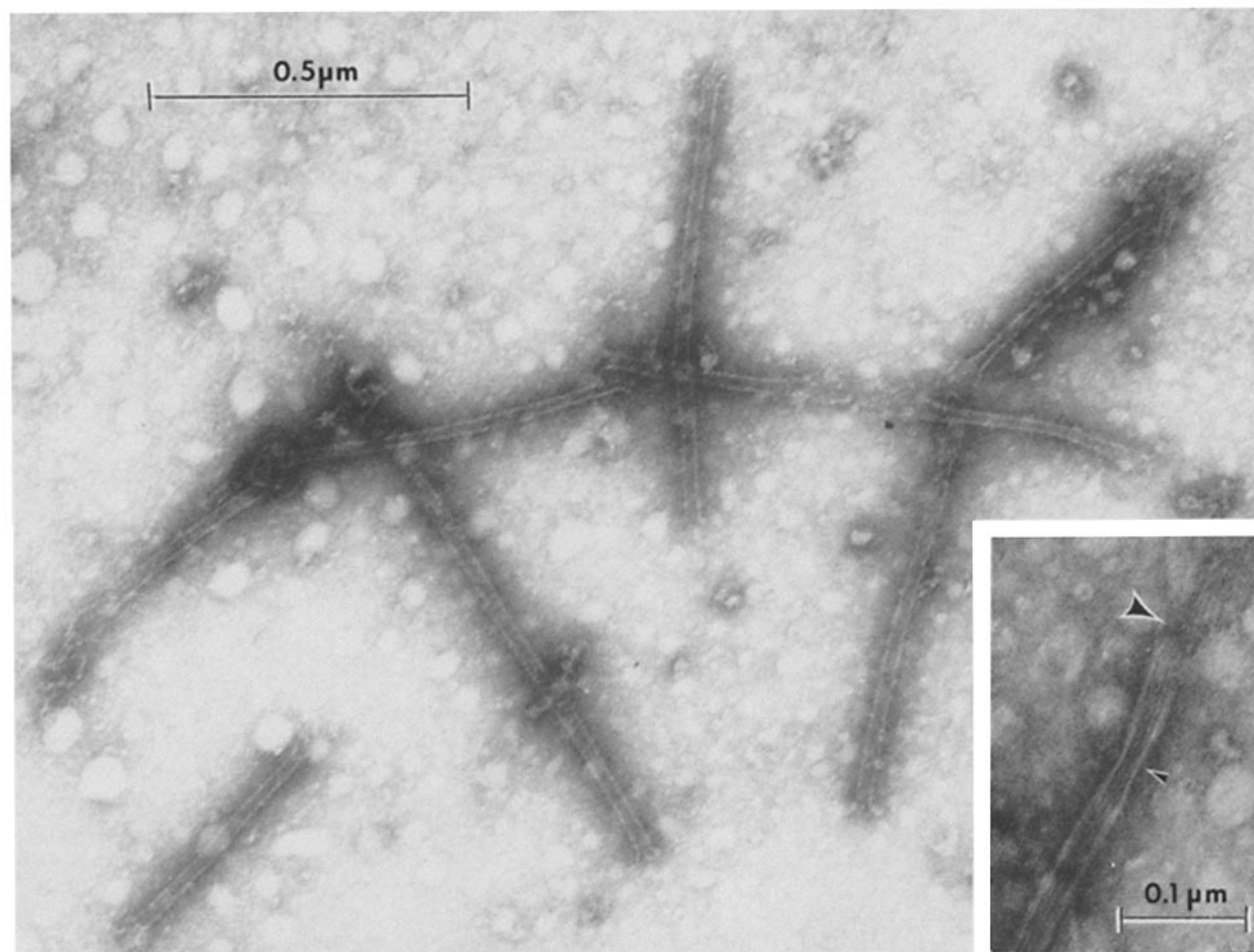


FIGURE 2 Electron micrograph of negatively stained taxol-induced microtubules. The products formed after incubation of 8.7 μM tubulin with 20 μM taxol were applied to grids and negatively stained with uranyl acetate. (*inset*) End of negatively stained microtubule with obvious protofilament substructure, constricted area (small arrowhead), and ribbon (large arrowhead). Bar, 0.5 μm . 0.1 μm (*inset*).

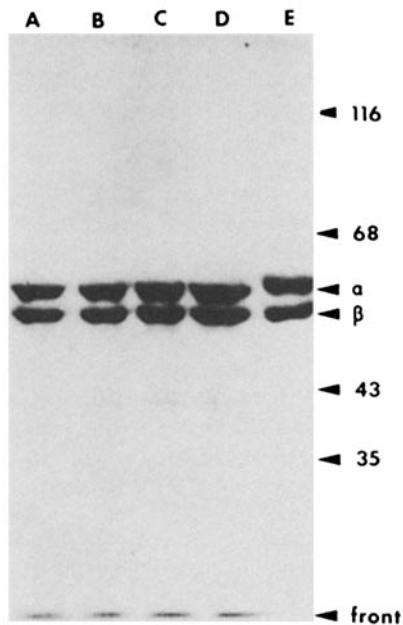


FIGURE 3 SDS PAGE of microtubule pellet. Microtubules formed after incubation of 8.7 μM tubulin with 20 μM taxol for 1 h at 24°C were sedimented at 48,000 g for 1 h at 23°C. The washed microtubule pellet was boiled in SDS sample buffer and run in a series of increased loadings. Lanes A, B, C, and D contain 3, 6, 9, and 12 μg of protein, respectively. Lane E contains 3 μg of DEAE-purified brain tubulin. Molecular weight markers are as in Fig. 1.

characterized by an obvious lag period with a decrease in turbidity (from the initial value) followed by an increase in turbidity. The duration of the lag period decreased with increased taxol concentration. After the lag period, both the rate and extent of turbidity development increased as functions of increased taxol concentration, with maximum turbidity levels achieved at $\geq 20 \mu\text{M}$ taxol and apparent steady state reached within 60 min. Tubulin in 0.5% dimethyl sulfoxide showed no turbidity change in the absence of taxol. Nearly identical kinetic patterns were observed with rose tubulin (8.7 μM) from different preparations.

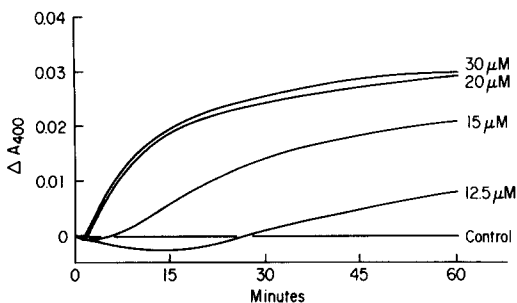


FIGURE 4 Dependence of microtubule assembly on taxol concentration. Tubulin at 8.7 μM was polymerized at 24°C by the addition of increased concentrations of taxol as indicated (12.5–30 μM). The control sample contained no taxol. All samples contained 0.5% dimethyl sulfoxide. Turbidity was recorded continuously for 60 min and is expressed as the change in absorbancy at 400 nm (ΔA_{400}).

It is known that maximum levels of assembly of pure mammalian brain tubulin are obtained at nearly 1:1 molar ratios of taxol and tubulin at 32–37°C (21, 38, 40). To determine whether the apparent 2:1 ratio of taxol to rose tubulin required for maximum turbidity development may

have been related to the use of a suboptimal assembly temperature (24°C), parallel taxol-induced assemblies of rose tubulin and DEAE-purified bovine brain tubulin were performed at 24 and 37°C.

The results of this experiment are summarized in Table I. After 1 h in the presence of 40 μM taxol no significant difference in the level of turbidity or amount of sedimented polymer was observed between 24 and 37°C for either rose tubulin or brain tubulin. Furthermore, at the two concentrations of tubulin used (8.7 and 10 μM), maximum turbidity levels produced at 24°C by rose tubulin assembly were 15–17% lower than those produced by brain tubulin assembly and the yield of polymeric rose tubulin was 22–25% lower than that of polymeric brain tubulin. Typically, 50–60% of rose tubulin formed polymers, whereas 70–80% of brain tubulin polymerized. The turbidity kinetics of brain tubulin assembly were hyperbolic rather than sigmoidal and contained very short or undetectable lag periods (data not shown). These results suggested that the greater requirement of rose tubulin assembly for taxol was related to some difference between rose and brain tubulins and was not due to the use of a suboptimal temperature for the interaction of taxol with tubulin.

TABLE I
Effect of Temperature on Turbidity Development and Polymer Yield

Source of tubulin	Assembly temperature °C	Tubulin concentration μM	ΔA_{400}	Amount of sedimented polymer μg	Total tubulin polymerized %
Rose	24	8.7	0.028	49	56
		10.0	0.033	59	57
	37	10.0	0.033	60	58
Brain	24	8.7	0.033	65	75
		10.0	0.040	76	77
	37	10.0	0.039	72	73

Reaction mixtures containing either 8.7 or 10 μM rose tubulin or brain tubulin were assembled for 1 h either at 24 or 37°C in the presence of 40 μM taxol. Turbidity was monitored at absorbancy = 400 nm (A_{400}) and the amount of polymer formed was assayed by sedimentation as described in Materials and Methods.

Quantitation of the assembly requirements of rose and brain tubulins for taxol at 24°C was made by sedimentation analysis. After 1 h of assembly of rose tubulin (8.7 and 12.5 μM) and brain tubulin (8.7 μM) in the presence of different concentrations of taxol, microtubules were sedimented and pellets were assayed for protein. Fig. 5 relates the yield of sedimented tubulin (polymer) to the concentration of taxol used in the assembly mixture. Maximum polymerization of 8.7 μM rose tubulin was achieved at $\geq 18 \mu\text{M}$ taxol, whereas the maximum level of 8.7 μM brain tubulin assembly was obtained at about half as much taxol ($\geq 9.4 \mu\text{M}$). Furthermore, less rose tubulin polymer was formed than brain tubulin polymer at saturating levels of taxol. Rose tubulin (12.5 μM) isolated in a different preparation showed maximum microtubule assembly at $\geq 21 \mu\text{M}$ taxol, further indicating that nearly 2:1 ratios of taxol to rose tubulin were necessary for maximum polymerization.

When different concentrations of rose tubulin (0.5, 0.58, 0.65, 1.0, 1.25 mg/ml) were assembled at 24°C in the presence of saturating taxol (40 μM) and the polymerization was monitored by turbidimetry, a concentration-dependent increase

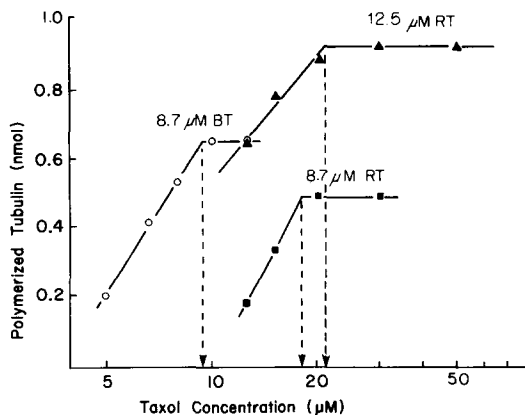


FIGURE 5 Dependence of microtubule yield on taxol concentration. Rose tubulin and brain tubulin were assembled after addition of increased concentrations of taxol (as in Fig. 4). Microtubules were sedimented and processed as described in Materials and Methods. Purity of tubulin in pellets was 95 and 99% for rose and brain microtubules, respectively. The amounts of rose tubulin (RT) polymer formed by $8.7 \mu\text{M}$ tubulin (■) and $12.5 \mu\text{M}$ tubulin (▲), and brain tubulin (BT) polymer formed by $8.7 \mu\text{M}$ tubulin (○) are expressed in nanomoles. Each broken line and arrow designates the estimated minimum concentration of taxol required for maximum polymer yield.

in the rate and extent of turbidity development was seen (data not shown). Estimation of the critical concentration (C_c) for taxol-induced rose tubulin assembly at 24°C was made by plotting the amount of sedimented tubulin polymer formed after 1 h of assembly versus the tubulin concentration (12, 19) and is shown in Fig. 6. The apparent C_c for rose tubulin polymerization was 0.21 mg/ml and for parallel bovine brain tubulin polymerization was 0.33 mg/ml . The lower slope of the line derived from the rose tubulin polymerizations reflects the greater amount of unassembled dimer as represented in Table I.

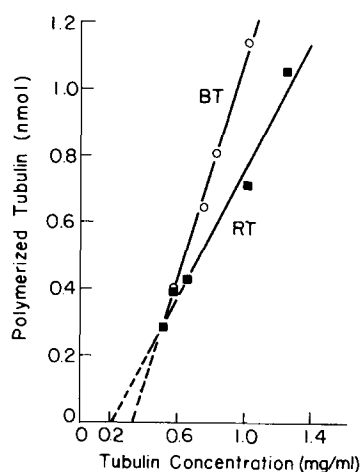


FIGURE 6 Estimation of the critical concentration of tubulin assembly in saturating taxol by sedimentation assays. For each concentration of tubulin assembled (0.5, 0.58, 0.65, 1.0, 1.25 ml/ml), the amount of polymer sedimented is expressed in nanomoles. Lines and extrapolated abscissa intercepts were derived by the method of least squares linear regression. Closed squares and line represent rose tubulin (RT) polymer (slope = 0.980; correlation

coefficient = 0.984) and open circles and line represent brain tubulin (BT) polymer (slope = 1.428; correlation coefficient = 0.991).

Effect of Colchicine on Taxol-induced Polymerization

In a previous paper (29) we compared the colchicine-binding activities of the tubulins from cultured cells of rose, hibiscus, and carrot and bovine brain. Colchicine-binding

TABLE II
Quantitation of Colchicine Inhibition of Taxol-induced Microtubule Assembly by Sedimentation Assays

Source of tubulin	Colchicine concentration	Amount of sedimented polymer	Amount of control
	<i>mM</i>	μg	%
Rose	0	71	100
	0.10	66	93
	0.50	45	63
	1.00	38	53
Brain	0	84	100
	0.01	8	10
	0.05	9	11

Tubulin ($10 \mu\text{M}$) was added to SIB containing a saturating concentration of taxol (for rose, $40 \mu\text{M}$; for brain, $27 \mu\text{M}$) and a given concentration of colchicine at 24°C and was assembled for 1 h. Polymerized tubulin was sedimented at $48,000 \text{ g}$ for 1 h and pellets were assayed for protein content. Data are expressed as percent of control (100% = amount of polymer sedimented in the absence of colchicine).

levels were lower with the plant tubulins than with brain tubulin suggesting that the resistance of plant cell microtubules to colchicine is due to a lower affinity of tubulin for colchicine. The effects of colchicine on the taxol-induced formation of rose and brain microtubules at 24°C were compared by electron microscopy and sedimentation assays. Addition of colchicine to the assembly mixtures at the initiation of polymerization resulted in shorter and fewer microtubules (data not shown) and decreased yields of polymer for both rose and brain tubulins (Table II). However, much higher concentrations of colchicine were required to inhibit the assembly of rose microtubules than of brain microtubules. At the highest concentration of colchicine tested (1 mM) rose tubulin polymer yield was 53% of the control value, whereas at a 100-fold lower concentration of colchicine (0.01 mM) brain tubulin polymer yield was 10% of the control level.

DISCUSSION

Taxol-induced assembly at 24°C produced numerous, short, rose microtubules, which resembled those described in brain tubulin studies (40, 50). SDS PAGE showed that microtubule pellets contained 95% tubulin and a small amount of low molecular weight polypeptides. Although the high molecular weight polypeptides that co-elute with tubulin from DEAE columns might at first be construed to be MAPs, this is very unlikely because MAPs generally behave as polycations and would not be expected to elute with such a strong polyanion as tubulin, and because they did not co-assemble with tubulin into microtubules and remained in the supernatant fraction (30). The possibility that taxol may have displaced the presumptive MAPs during assembly is also remote because it has been established with brain microtubule protein assembly in vitro that the presence of taxol does not alter either the amount or spectrum of MAPs that co-assembles with tubulin into microtubules (7, 21, 54). The identities of low molecular weight polypeptides occasionally found in the polymerized tubulin were not established, but they appear not to be proteolytic fragments of tubulin subunits (31).

The sigmoidal kinetics of turbidity development derived from taxol-induced rose tubulin assembly were clearly different from those of parallel assemblies of DEAE-purified brain tubulin at 24°C and from those of phosphocellulose-purified brain tubulin at 37°C published by other laboratories (7, 8, 14, 38, 40). The lag period of rose tubulin assembly contained

a decrease in turbidity indicating that taxol mediated the decrease by formation of structures having lower light scattering properties. It is possible also that the rose tubulin solutions used in these studies contained small tubulin aggregates formed during the isolation dialysis step that were not sedimented at 48,000 g. Warming of such solutions in the presence of taxol would be expected to result in the reorganization of the existing small aggregates into smaller nucleating species of polymer, inasmuch as microtubule assembly has been proposed to proceed via a cooperative process of nucleation and condensation-polymerization (12, 19). It is very unlikely that these aggregates represent MAP-containing rings seen in other microtubule preparations (45, 50), because no MAP-like polypeptide species were identified in these experiments and no rings were observed in electron micrographs.

Because rose microtubule assembly at 24°C showed saturable extents of turbidity increase and polymer yield in the presence of taxol, it is likely that taxol binds to rose microtubules and/or tubulin (8) in a manner similar to its binding to mammalian microtubules. However, maximum assembly and yield of mammalian brain microtubules occur at equimolar ratios of taxol and tubulin (21, 40), and [³H]taxol binds to brain microtubules at 0.78 mol of taxol/mol of tubulin suggesting that there is one binding site per tubulin dimer (35). More recently Parness et al. (34) have observed binding of [³H]taxol to reassembled flagellar microtubules from sea urchin at ratios of 1.32 mol of taxol/mol of tubulin. They postulated that nonmammalian microtubules may contain more than one taxol-binding site per tubulin dimer and predicted that the degree of response of different tubulins to taxol-induced assembly may be different. Indeed, while we have not examined the binding of taxol to rose microtubules directly, the nearly 2:1 molar ratio of taxol to tubulin required for assembly saturation suggests some combination of the following possibilities: (a) more than one taxol-binding site exists on rose tubulin in microtubules and saturation of these sites with taxol is required for maximum assembly; (b) the taxol-binding site(s) on rose microtubules has a normally lower affinity for taxol than that on brain microtubules so that higher concentrations of taxol are necessary to overcome the low affinity and to "drive" the polymerization reaction; and (c) the rate of decay of the taxol-binding site(s) or dimer-dimer-binding sites of rose tubulin is faster than that of brain tubulin and this decay is reflected by inefficient promotion of assembly by taxol. To distinguish conclusively between these possibilities taxol-binding studies would have to be performed directly, but radiolabeled taxol is not yet commercially available.

The estimated value of C_c for the assembly of MAP-containing brain tubulin solutions is reduced by the addition of taxol from ≈ 0.2 mg/ml to 0.015 mg/ml (38, 40). Addition of taxol to brain tubulin solutions that have been purified of MAPs by chromatographic methods reduces the C_c for polymerization from ≈ 4.0 mg/ml (45) to 0.3–0.4 mg/ml (21). Our estimations of C_c for the assembly of MAP-free rose tubulin (0.21 mg/ml) and brain tubulin (0.33 mg/ml) in saturating taxol are surprisingly similar when the diverse phylogenetic relationship and different physiological conditions of rose and cow are considered. Moreover, this similarity intimates that in the absence of taxol, more efficient plant tubulin polymerization may be obtained in the presence of MAP-like factors analogous to those which are known to co-polymerize with animal tubulin both in vitro and in vivo (43, 45).

Our finding that rose tubulin formed less polymer than

brain tubulin at saturating taxol concentrations (Table I and Figs. 5 and 6) may be indicative of more decay of the taxol-binding site(s) and/or the dimer-dimer-binding sites on rose tubulin. Parallel assembly experiments with DEAE-purified brain tubulin demonstrated that several well-documented characteristics of taxol-induced polymerization could be reproduced without prior enrichment for assembly-competent dimers. These characteristics included hyperbolic turbidity kinetics, maximum polymerization levels at approximately 1:1 molar ratios of taxol to tubulin, 70–80% polymerizable tubulin, a C_c of 0.33 mg/ml, and maximum inhibition of assembly at 1:1 ratios of colchicine and tubulin (7, 21, 38, 40). These findings indicate that neither the sucrose-containing buffer, the 24°C assembly temperature, nor the DEAE-chromatographic procedure per se was responsible for the distinct features of taxol-induced rose microtubule assembly. That all tubulin dimers in cells are assembly-competent has not been shown and seems unlikely. It is conceivable that individual cell types could contain different and particular amounts of assembly-competent dimer, and that the different yields of polymeric rose and brain tubulins observed herein reflect such differences *pari passu*.

A method commonly employed for the estimation of the proportion of "native" dimers in a tubulin solution has been the colchicine-binding assay (57). We have shown previously (29) that the colchicine-binding activities of tubulins from rose, hibiscus, and carrot are low even in the presence of stabilizing agents such as dithiothreitol, GTP, and sucrose which are known to slow the rate of decay of the colchicine-binding site (11, 46, 57). However, the rates of decay of the colchicine site and polymerization are not necessarily the same (e.g., see reference 56). Nevertheless, we chose to include sucrose in the assembly buffer because the colchicine-binding site on animal tubulin is stabilized by sucrose (11, 46), and because sucrose preserves the native state of tubulin (51) and is compatible with microtubule assembly (41). We reasoned that any decay of the low-affinity colchicine-binding site(s) on rose tubulin would be maintained at a minimum rate by the presence of sucrose during taxol-induced assembly experiments.

As expected, much higher concentrations of colchicine were required for the inhibition of taxol-mediated rose microtubule assembly when compared with those inhibiting taxol-induced brain microtubule assembly. Although it is clear that the taxol-induced assembly system used in these studies is not physiological, the relative resistance of plant microtubule assembly to colchicine when compared with brain microtubule assembly under identical conditions indicates that plant tubulin has a lower affinity for colchicine. It is likely that the high concentrations of colchicine commonly used to disrupt microtubules in plant cells are necessary to overcome the low affinity and to produce tubulin-colchicine complexes that poison microtubule dynamics. The effect of colchicine on the depolymerization of taxol-stabilized microtubules was not studied because the assembly buffer contained sucrose which is known to stabilize preformed microtubules to depolymerization (41). It would be impossible, therefore, to distinguish between the individual contributions made to microtubule stability by taxol and sucrose.

Sherline et al. (44) and Lockwood (23) have found endogenous inhibitors of colchicine binding to brain tubulin in brain extracts and have suggested that such factors could act as allosteric inhibitors of assembly in vivo. Similarly, Schiff et al. (38), Thompson et al. (50) and Parness et al. (34) have

suggested that the taxol-binding site may represent a binding site for some as yet undiscovered factor which could act as an allosteric regulator of microtubule assembly in cells. Interestingly, Heidemann and Gallas (16) found that new microtubules were assembled after taxol injection into *Xenopus laevis* eggs, and yet, taxol injection into tubulin-rich immature oocytes failed to produce new microtubules. This observation suggests masking of the taxol site by some endogenous factor and emphasizes the possibility that this site may also serve as a site for the binding of an allosteric regulator of microtubule dynamics in cells. On the basis of our observations on the effects of taxol and colchicine on the biological activity (polymerization) of rose tubulin, we propose that the taxol-binding sites of plant and animal tubulins have been more conserved over evolution than the colchicine-binding sites. These results along with our recent findings that the antimicrotubule herbicides oryzalin and amiprophos-methyl bind to rose tubulin and inhibit taxol-induced microtubule assembly *in vitro* at micromolar concentrations (28, 32) have shown that these drugs recognize sites on plant tubulin that are analogous to, but distinct from the colchicine site on animal tubulins, and that plant tubulin is pharmacologically different from animal tubulin.

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