Sketelj, J., & Brzin, M. (1979) *Histochemistry 61*, 239–248.
Stenman, S., & Vaheri, A. (1978) *J. Exp. Med. 147*, 1054–1064.
Vaheri, A., & Mosher, D. F. (1978) *Biochim. Biophys. Acta 516*, 1–25.
Vuento, M., & Vaheri, A. (1978) *Biochem. J. 175*, 333–336.
Yamada, K. M., & Olden, K. (1978) *Nature (London) 275*, 179–184.
Yamada, K. M., Kennedy, D. W., Kimata, K., & Pratt, R. M. (1980) *J. Biol. Chem. 255*, 6055–6063.

Taxol Assembles Tubulin in the Absence of Exogenous Guanosine 5′-Triphosphate or Microtubule-Associated Proteins†

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ABSTRACT: Taxol increases the rate and extent of microtubule assembly in vitro and stabilizes microtubules in vitro and in cells [Schiff, P. B., Fant, J., & Horwitz, S. B. (1979) *Nature (London) 277*, 665–667; Schiff, P. B., & Horwitz, S. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1561–1565]. We report herein that taxol has the ability to promote microtubule assembly in the absence of microtubule-associated proteins, rings, and added guanosine 5′-triphosphate (GTP) or organic buffer. The drug enhances additional microtubule assembly when added to microtubules at apparent steady state. This additional assembly can be attributed to both elongation of existing microtubules and spontaneous nucleation of new microtubules. Taxol-treated microtubules have depressed dissociation reactions as determined by dilution experiments. The drug does not inhibit the binding of GTP or the hydrolysis of GTP or guanosine 5′-diphosphate (GDP) in our microtubule protein preparations. Taxol does not competitively inhibit the binding of colchicine to tubulin.

Microtubules are an important component of the cytoskeleton of most eukaryotic cells. They are an integral part of the mitotic spindle, cilia, flagella, and cytoplasm of interphase cells. Drugs which interact with tubulin, the subunit of microtubules, have been useful tools in understanding the regulation of microtubule assembly in cells and the wide variety of cellular functions mediated by microtubules [see Dustin (1978) and Roberts & Hyams (1979)].

Our previous work (Schiff et al., 1979; Schiff & Horwitz, 1980, 1981) has demonstrated that taxol, an experimental antitumor drug (Wani et al., 1971), enhances microtubule assembly and stabilizes microtubules in vitro. Microtubules polymerized in the presence of taxol are resistant to depolymerization by cold (4 °C) or $CaCl_2$ (4 mM). The drug increases the rate, yield, and nucleation phase of the assembly reaction maximally when the tubulin dimer to taxol ratio is 1 and it decreases the critical concentration of microtubule protein (MTP)[1] required to initiate polymerization. The taxol binding site is present on the intact microtubule.

Our observations that the drug blocks cell replication predominantly in the mitotic phase of the cell cycle, inhibits fibroblast cell migration, and stabilizes microtubules in cells clearly relate the cytotoxic activity of the drug to our in vitro studies. Cytoplasmic microtubules in taxol-treated cells resist depolymerization by cold (4 °C) and by antimitotic drugs such as colchicine that normally disrupt microtubules. The drug may also promote microtubule assembly in cells.

We now report further in vitro studies to better characterize the microtubule assembly reaction in the presence of taxol. The effect of taxol on microtubules has been examined at apparent steady-state conditions, and dilution techniques have been used to investigate microtubule depolymerization in the presence of taxol. The relationship of microtubule-associated proteins (MAPs), rings, organic buffer, and colchicine to the effects of taxol on microtubule assembly is reported.

Experimental Procedures

Materials. Taxol and podophyllotoxin were obtained from the National Cancer Institute and dissolved in dimethyl sulfoxide (Me_2SO). [^{3}H]Colchicine (19.6 Ci mmol^{-1}) and Aquasol were from New England Nuclear, and Norit was from Eastman.

Preparation of Microtubule Protein. Calf brain MTP was prepared by two cycles of assembly–disassembly (Shelanski et al., 1973) and stored at −20 °C in Mes buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 6.6] containing 1 mM guanosine 5′-triphosphate (GTP) and 4 M glycerol. Prior to the onset of each experiment, the MTP was dialyzed for 3 h at 4 °C against 100 volumes of Mes buffer and centrifuged at 120000*g* for 20 min at 4 °C. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

MTP with a sedimentation coefficient of 6 s was prepared by ultracentrifugation (230000*g*) of MTP in Mes buffer for 90 min at 4 °C (Johnson & Borisy, 1977).

Microtubule seeds, which typically had an average length of 0.57 ± 0.34 μm, were prepared by passing a solution of polymerized microtubules (4 mg mL^{-1}) through a 22-gauge

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[1] Abbreviations used: MAP, microtubule-associated protein; MTP, tubulin plus microtubule-associated proteins; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid; Mes buffer, 0.1 M Mes, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 6.6; $NaDodSO_4$, sodium dodecyl sulfate; PBS, Dulbecco's phosphate-buffered saline; Me_2SO, dimethyl sulfoxide.

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1.5-in. syringe needle 5 times (Johnson & Borisy, 1977). Seeds were used within 30 min of preparation.

Further purification of MTP that had been prepared by two cycles of assembly–disassembly was achieved by phosphocellulose column chromatography (Weingarten et al., 1975; Sloboda et al., 1976). Columns (0.6 × 22 cm) were equilibrated with Mes buffer minus magnesium and were loaded with MTP (4 mg of protein mL^{-1} bed volume) that had been dialyzed against 100 volumes of this buffer and centrifuged at 120000*g* at 4 °C for 30 min. The tubulin was eluted with Mes buffer without magnesium and stored at 4 °C after magnesium was added back to a final concentration of 0.5 mM. Tubulin purified by this method was 98% pure as determined by scanning 3–27% $NaDodSO_4$–polyacrylamide gradient reducing slab gels (Swaney & Kuehl, 1976). This gel does not discriminate easily between α- and β-tubulin.

Turbidity Measurements. Microtubule assembly was monitored by the turbidity assay of Gaskin et al. (1974). Cuvettes (1 mg, 1-cm light path) containing Mes buffer, drug, and GTP were kept at room temperature prior to addition of MTP and shifting the temperature to 37 °C. Measurements were made every 20 s at 350 nm on a Gilford spectrophotometer equipped with an automatic recorder and a thermostatically regulated liquid circulator.

Sedimentation of Microtubules. Microtubules were separated from tubulin dimers by sedimentation. After the microtubule polymerization reaction reached an apparent steady state, as determined by turbidity measurements, the microtubule protein was either sedimented at 120000*g* for 30 min at 25 °C or layered onto a 50% sucrose cushion and sedimented at 230000*g* for 2 h at 25 °C. The amount of protein in the pellets and supernatants was determined, and the presence of microtubules in the pellet was ascertained by electron microscopy.

Electron Microscopy. Microtubule polymerization, depolymerization, and stability were confirmed by electron microscopy. A 20-μL sample was placed on carbon-stabilized Parlodion-coated grid for 15 s and displaced with 8 drops of 2% aqueous uranyl acetate. The excess uranyl acetate was removed from the grid with filter paper; the grid was air-dried and examined at 8000× and 40 000× magnifications with a Siemens Elmiskop 1A electron microscope at 80 kV.

Microtubule length measurements were made by using a Numonics digitizer on 8 × 10 electron micrographs printed at a final magnification of 6000×. Statistical treatment of these length measurements to determine the mean microtubule length took into account microtubules with one end off the electron micrograph (Johnson & Borisy, 1977). A minimum of 150 microtubules were measured for each histogram.

Colchicine Binding Assay. The binding of [^{3}H]colchicine to MTP was determined by the filter assay of Weisenberg et al. (1968). MTP in 1 mL of buffer was incubated at 37 °C with [^{3}H]colchicine (specific activity 0.1 Ci $mmol^{-1}$) for the desired amount of time. The binding reaction was stopped by the addition of 1 mL of 10^{-4} M colchicine. Bound colchicine was determined by filtration under gravity through Whatman DE-81 filter paper (2.4 cm in diameter). The filters were washed 8 times with 8-mL aliquots of cold, 10-fold-diluted Mes buffer and counted in 10 mL of Aquasol.

GTP Binding and GTPase Activity Assays. GTP was removed form MTP by charcoal treatment (Penningroth & Kirschner, 1977). MTP was incubated for 30 min at 37 °C to allow hydrolysis of GTP to GDP by the GTPase activity present in the preparation and was then mixed with charcoal (50 μL of a 100 mg mL^{-1} suspension of acid-washed Norit per

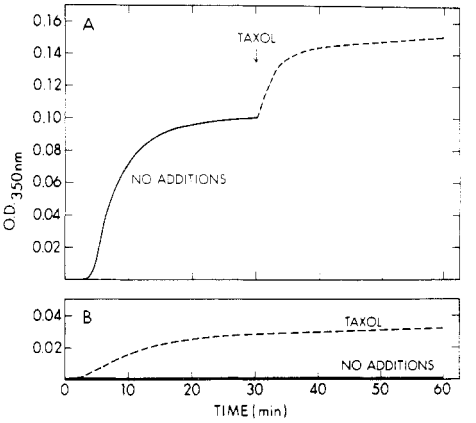

FIGURE 1: Addition of taxol to microtubules at apparent steady-state equilibrium. (A) MTP (1 mg mL^{-1}) was assembled in the absence of taxol in Mes buffer and 1 mM GTP at 37 °C (—). At 30 min, taxol (↓) was added to the reaction mixture at a final concentration of 5 μM. (B) MTP was assembled in the absence of taxol as described in (A). At 30 min, the reaction mixture was sedimented (120000*g* for 30 min at 25 °C) to remove microtubules. Taxol was added to the supernatant, which contained no microtubules: 5 μM taxol (---); untreated reaction (—). The absence or presence of microtubules was verified by electron microscopy.

4 mg of MTP) at 4 °C. The charcoal was immediately removed by sedimentation (12000*g*, 5 min, 4 °C) and the procedure repeated twice. The concentration of nucleotide associated with the MTP was determined (absorbance measurements at 260 nm) after removal of protein by precipitation with cold 5% perchloric acid. The charcoal-treated protein typically had a nucleotide/tubulin ratio of 1.3.

A sedimentation assay was used to measure GTP binding to MTP (Margolis & Wilson, 1978). Charcoal-treated MTP (1 mg mL^{-1}) was incubated with 1 mM [^{3}H]GTP (specific activity 8.18 Ci $mmol^{-1}$) for 30 min at 37 °C. At the end of the incubation period, 1 mL of the reaction mixture was layered onto 4.5 mL of 50% sucrose in Mes buffer and sedimented at 25 °C for 2 h at 230000*g* in an SW 50.1 rotor. The supernatant was removed by aspiration until approximately 0.5 mL remained. This was underlayered with 2 mL of Mes buffer containing 70% sucrose and the buffer removed by aspiration. Each tube was washed 3 times in this manner. The pellets were analyzed for protein and radioactivity, and structures were verified by electron microscopy.

GTPase activity of the MTP preparation was measured by a modified version of an assay developed by Sandoval et al. (1977). Charcoal-treated tubulin (1 mg) was incubated with 10 μM [^{3}H]GTP (10.8 Ci $mmol^{-1}$) in Mes buffer for 40 min at 37 °C in a final volume of 1 mL. The reaction was stopped by the addition of 1 mL of cold (4 °C) ethanol, kept in an ice bath for 10 min, and sedimented for 20 min at 120000*g*. A portion of the supernatant (4 μL) was spotted onto a flexible thin-layer sheet of PEI [poly(ethylenimine)]–cellulose and developed in 1.2 M $LiCl_2$ for 75 min at room temperature to separate nucleoside mono-, di-, and triphosphates (Randerath, 1966). Unlabeled GTP, GDP, GMP, and guanosine were used as standards. The chromatograms were cut into 0.5-cm pieces, and radioactivity was determined in Aquasol.

Results

Addition of Taxol to Microtubules at Apparent Steady State. Additional microtubule assembly occurred when taxol was added to a reaction mixture at a final concentration of 5 μM at 30 min, a time when apparent steady state is reached (Figure 1A). Sedimentation data demonstrated that the total yield (0.76 mg) of this reaction was equal to that of a reaction

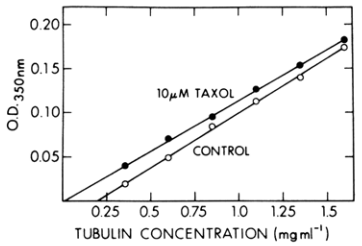

FIGURE 2: Dilution experiments with control and taxol-treated microtubules. MTP (1.5 mg mL^{-1}) was assembled in Mes buffer and 1 mM GTP at 37 °C in the absence and presence of 10 μM taxol. After 30 min, the samples were diluted in Mes buffer containing 1 mM GTP with or without 10 μM taxol at 37 °C, and new turbidity measurements were determined after 10 min. Only the protein concentration changed after dilution. Control (○); 10 μM taxol (●).

in which 5 μM taxol was present before the onset of assembly. In a separate experiment, microtubules were assembled in the absence of drug for 30 min at 37 °C and removed by sedimentation. Taxol was then added to the supernatant (0.25 mg mL^{-1} MTP) at a final concentrattion of 5 μM (Figure 1B). Turbidity and electron microscopy indicated that taxol had the ability to promote the assembly of microtubules from tubulin dimers that remained in the nonpolymerized form at apparent steady state.

Inhibition of Microtubule Depolymerization. Microtubule depolymerization was investigated by following the effect of dilution on preformed microtubules (Weisenberg & Deery, 1976). Microtubules polymerized in the presence of 10 μM taxol did not depolymerize when diluted, whereas untreated microtubules depolymerized rapidly to a new steady state (Figure 2). Microtubules could be observed with the electron microscope at 0.1 mg mL^{-1} MTP. By this technique, the critical concentration observed for the control reaction was 0.2 mg mL^{-1} MTP. The critical concentration decreased to 0.015 mg mL^{-1} protein in the presence of taxol. These values correspond well with those previously reported by sedimentation and turbidity measurements (Schiff et al., 1979).

Taxol Induces Assembly in the Absence of Rings and MAPs. Centrifugation of MTP at 230000*g* for 1.5 h at 4 °C sediments structures with a sedimentation coefficient of 36 S. MTP remaining in the supernatant after such sedimentation will not assemble in the presence of GTP (Borisy & Olmsted, 1972). Taxol will promote the assembly of tubulin that is depleted of 36S structures as measured by turbidity, sedimentation, and electron microscopy. These microtubules were resistant to depolymerization by 4 mM $CaCl_2$. Addition of 0.22 mg of microtubule seeds (average length 0.57 ± 0.34 μm) to 6S tubulin immediately initiated elongation of the microtubule seeds. An assembly mixture containing 6S tubulin, microtubule seeds, and 20 μM taxol attained the greatest turbidity of the four reaction mixtures examined (Figure 3). An analysis of the mean microtubule lengths in the individual reaction mixtures at 40 min (Figure 4) demonstrated that microtubule seeds could be elongated in the presence of taxol. However, taxol initiated new nucleation events in the reaction mixture containing 6S tubulin and seeds since the yield of this reaction was greater than that of the 6S tubulin plus taxol reaction. This was true in the absence of a significant change in the mean length of the microtubules les in the two assembly reactions. Addition of tubulin rings (0.2 mg of MTP) to 6S tubulin induced microtubule assembly with a characteristic lag time (data not shown). The mean length of these microtubules (3.42 ± 1.68 μm) was less than the mean length of those assembled with microtubule seeds (4.4 ± 2.63 μm). Sedimentation analysis confirmed the turbidity measurements:

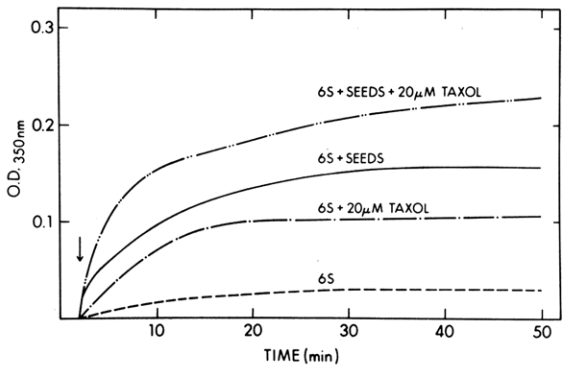

FIGURE 3: Assembly of 6S tubulin in the presence of microtubule seeds and/or taxol. The assembly reaction contained Mes buffer, 1 mM GTP, and 3.1 mg mL^{-1} 6S tubulin (prepared as described under Experimental Procedures) in the absence or presence of microtubule seeds (0.22 mg, average length 0.57 ± 0.34 μm) and 20 μM taxol in a final volume of 1 mL. Assembly was initiated by addition of seeds and/or taxol at 2 min (↓). 6S tubulin (---), 6S tubulin plus 20 μM taxol (--), 6S tubulin plus microtubule seeds (—), and 6S tubulin, microtubule seeds, and 20 μM taxol (-·--).

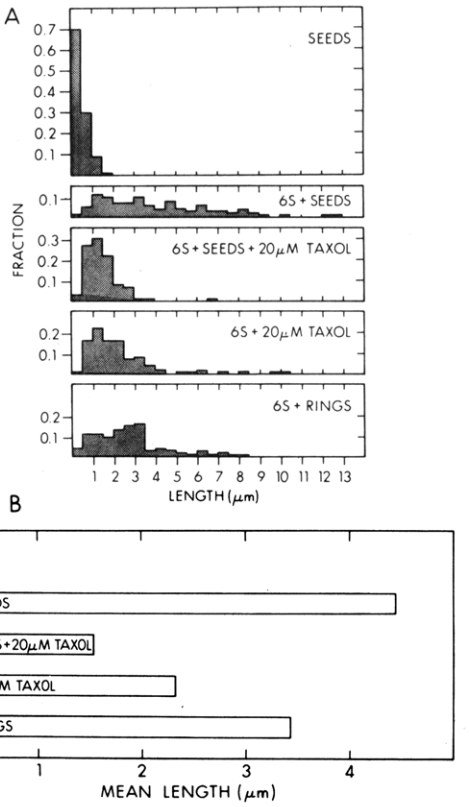

FIGURE 4: Histogram analysis of microtubules assembled from 6S tubulin, microtubule seeds, and/or taxol. The assembly reaction is as described in Figure 3; determinations were made at 40 min. (A) The fraction of microtubules in each 0.5-μm length range for (from top to bottom) microtubule seeds alone, 6S tubulin plus microtubule seeds, 6S tubulin plus seeds plus 20 μM taxol, 6S tubulin plus 20 μM taxol, and 6S tubulin plus rings (0.22 mg of MTP from microtubules depolymerized in the cold, 4 °C). (B) A bar graph showing mean microtubule lengths for the above assembly reactions: seeds, 0.57 ± 0.34 μm; 6S tubulin plus seeds, 4.44 ± 2.63 μm; 6S tubulin plus seeds plus 20 μM taxol, 1.54 ± 0.86 μm; 6S tubulin plus 20 μM taxol, 2.33 ± 1.80 μm; and 6S tubulin plus rings, 3.42 ± 1.68 μm.

in reaction mixtures containing 3.1 mg of protein, 2.33 mg of protein was pelleted (30 min at 120000*g*) from the reaction containing 6S tubulin, seeds, and taxol; 1.49 mg of protein was pelleted from the reaction containing 6S tubulin and seeds; and 1.10 mg of protein was pelleted from the reaction containing 6S tubulin and 20 μM taxol.

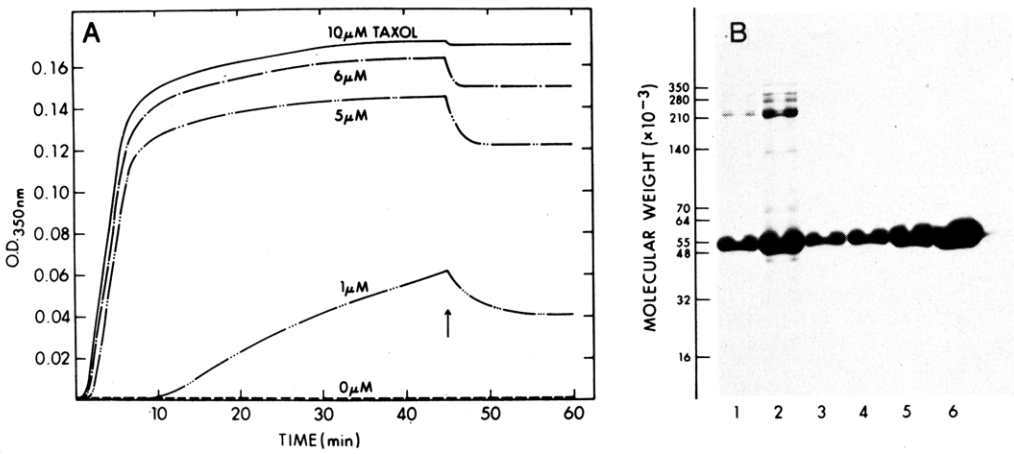

FIGURE 5: Assembly of phosphocellulose-purified tubulin in the presence of taxol. (A) The assembly reaction contained Mes buffer, 1 mM GTP, and 1.5 mg mL^{-1} tubulin: no additions (---), 1.0 (·····), 5.0 (·-·-), 6.0 (-··-), and 10 (—) μM taxol. $CaCl_2$ is added at a final concentration of 4 mM at 45 min (↑). (B) Sodium dodecyl sulfate (3–27%)–polyacrylamide gradient reducing slab gel stained with Coomassie blue. Tubulin before phosphocellulose chromatography (lane 1, 20 μg; lane 2, 40 μg of protein). Tubulin after phosphocellulose chromatography (lane 3, 10 μg; lane 4, 20 μg; lane 5, 40 μg; lane 6, 80 μg of protein). Cross-linked hemoglobin, cross-linked albumin, and cross-linked hemocyanin were used as molecular weight markers.

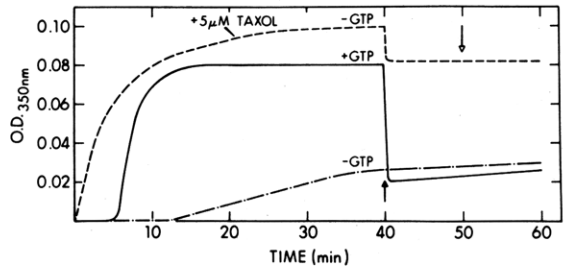

FIGURE 6: MTP polymerization with substoichiometric concentrations of GTP. Tubulin dimers with substoichiometric concentrations of GTP at their exchangeable site were prepared as described under Experimental Procedures. The assembly reaction contained Mes buffer and 0.8 mg mL^{-1} MTP: no additions (---), 1 mM GTP (—), and 5 μM taxol in the absence of added GTP (---). Addition of 4 mM $CaCl_2$ at 40 min (↑). Addition of 1 mM GTP at 50 min (↓). Only protein aggregates were present in the minus GTP assembly reaction on examination by electron microscopy.

Table I: GTP Binding to Tubulin in the Absence and Presence of Taxol[a]

	tubulin (nmol)	[³H]GDP (nmol)	[³H]GDP/ tubulin dimer
control	3.11	1.86	0.60
10 μM taxol	4.14	2.40	0.58

[a] Polymerization products of reactions with and without 10 μM taxol were analyzed for [³H]GDP content as described under Experimental Procedures. Charcoal-treated tubulin was polymerized in the presence of 1 mM [³H]GTP in the absence or presence of 10 μM taxol for 30 min at 37 °C. The microtubules were sedimented through a 50% sucrose cushion to separate microtubules from tubulin dimers. The ratio of [³H]GDP to tubulin dimer in the microtubules was determined in the pellets.

Phosphocellulose-purified tubulin, which is depleted of MAPs, will polymerize in the presence of stoichiometric concentrations of taxol (Figure 5). Normally, 1.5 mg mL^{-1} phosphocellulose-purified tubulin will not assemble under these experimental conditions. However, addition of 1, 5, 6, or 10 μM taxol resulted in assembly of such tubulin. These microtubules formed were resistant to depolymerization by 4 mM $CaCl_2$. When observed at 30 min by electron microscopy, the microtubules appeared to have more frayed ends than those assembled in the presence of taxol and MAPs. In addition, some ribbon-like structures were observed.

The productes of a taxol assembly reaction were examined to determine if the drug displaced any of the MAPs that are normally associated with microtubules. Microtubules assembled in the presence of taxol were sedimented (120000*g* at 25 °C for 30 min), resuspended in Mes buffer, and analyzed by electrophoresis on 3–27% polyacrylamide slab gels. Taxol did not appear to have displaced any of the MAPs that cosediment with the microtubules.

Taxol Eliminates the GTP and Organic Buffer Requirement for Microtubule Assembly. Taxol will promote the assembly of microtubules in the absence of added GTP. When microtubule protein is treated to remove GTP bound to the exchangeable site on the tubulin dimer, MTP (0.8 mg mL^{-1}) will not assemble unless 1 mM GTP or 5 μM taxol is added to the reaction mixture (Figure 6). Even in the absence of GTP, taxol decreases the lag time required for assembly compared to the reaction containing GTP but no taxol. The microtubules assembled with 5 μM taxol but no GTP were resistant to depolymerization by 4 mM $CaCl_2$, and the subsequent addition of GTP did not restore any detectable sensitivity to calcium.

Taxol does not inhibit the binding of GTP to MTP or the hydrolysis of GTP or GDP. Charcoal-treated MTP (1 mg mL^{-1}) was incubated with 10 nmol of [³H]GTP with or without 10 μM taxol for 30 min at 37 °C, layered onto a 50% sucrose cushion, and sedimented at 230000*g* for 2 h at 25 °C. The pellets, which contained microtubules (confirmed by electron microscopy), were analyzed for microtubule protein and ³H-labeled nucleotide concentration (Table I). The ³H-labeled nucleotide to tubulin ratio was calculated to be 0.6 for both the untreated and taxol-treated reactions. The effect of taxol on the total GTPase activity of the tubulin preparation was examined by incubating charcoal-treated MTP (1 mg mL^{-1}) with 10 nmol of [³H]GTP for 40 min at 37 °C in the absence and presence of various concentrations of taxol. The reaction was stopped, and nucleotide products were analyzed by thin-layer chromatography. Taxol did not inhibit the hydrolysis of GTP or GDP (Table II). In fact, there was an enhanced yield of GDP in the presence of taxol that correlated with the increased yield of the assembly reaction.

Taxol (10 μM) promotes the assembly of microtubules in PBS without added GTP (Figure 7). These microtubules were resistant to depolymerization by 4 mM $CaCl_2$, and the assembly reaction was totally dependent on the presence of taxol.

Table II: Effect of Taxol on Total GTPase Activity Associated with Microtubule Protein[a]

reaction	GTP nmol	GTP %[c]	GDP nmol	GDP %	GMP nmol	GMP %
blank (buffer, [^{3}H]GTP)	9.5	95.3	0.5	4.7	0.0	0.0
control (charcoal-treated tubulin, buffer, and [^{3}H]GTP)[b]	2.6	25.5	6.5	64.5	1.0	10.0
0.5 μM taxol	2.0	20.3	6.8	68.4	1.1	11.3
5 μM taxol	1.2	12.3	7.7	76.7	1.1	11.0
50 μM taxol	1.4	13.5	7.6	75.5	1.1	11.0

[a] Values are expressed as a yield after 10 nmol of [^{3}H]GTP is incubated with 7.7 nmol (1 mg mL^{-1}) of charcoal-treated MTP for 40 min at 37 °C. [b] Contents of all reaction mixtures except the blank. [c] Percent of total guanine nucleotide after 40 min.

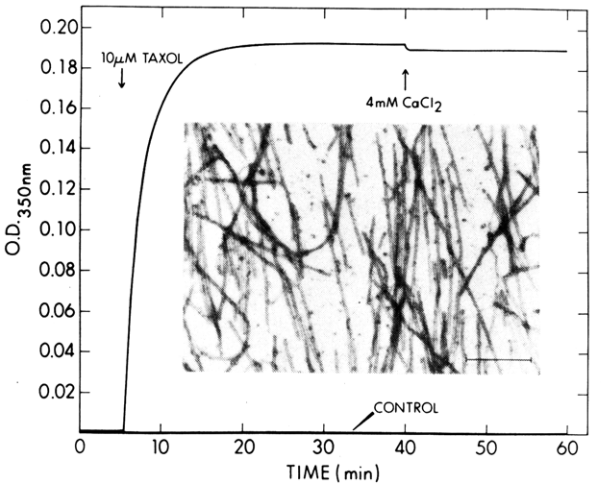

FIGURE 7: MTP assembly in PBS in the presence of taxol. Prior to the assembly reaction, MTP in Mes buffer was dialyzed against 100 volumes of PBS for 3 h at 4 °C and sedimented at 120000g for 30 min at 4 °C. MTP remaining in the supernatant (1.2 mg mL^{-1}) in PBS was shifted to 37 °C. Taxol was added to a final concentration of 10 μM at 5 min (↓). $CaCl_2$ (4 mM) addition (↑) was at 40 min. No assembly could be detected in the absence of taxol. Inset: Electron micrograph of microtubules which were assembled in PBS and persisted in the presence of 4 mM $CaCl_2$ for 20 min. Bar, 1.0 μm.

Taxol Is Not a Competitive Inhibitor of Colchicine Binding to Tubulin. Colchicine binding has been investigated in assembly reactions containing taxol. The binding of [^{3}H]-colchicine to MTP in the presence of taxol or podophyllotoxin was determined in three experimental conditions (Table III): (1) in assembly buffer, (2) in nonassembly buffer (100-fold-diluted Mes buffer), and (3) by preincubation with either taxol or podophyllotoxin prior to addition of [^{3}H]colchicine in assembly buffer. The results of these experiments demonstrted that (a) [^{3}H]colchicine bound equally well to tubulin in assembly and nonassembly conditions, (b) podophyllotoxin significantly inhibited colchicine binding (~90%) in all three experimental conditions, and (c) taxol inhibited colchicine binding slightly in assembly conditions, not at all in nonassembly conditions, and significantly if the reaction mixture was preincubated with taxol. These experiments demonstrated that the inhibition of colchicine binding by taxol most likely resulted from colchicine being unable to bind to microtubules assembled in the presence of taxol. It is known that colchicine binds to the tubulin dimer but not the intact microtubule (Wilson & Meza, 1973).

Preliminary experiments indicate that taxol will promote microtubule assembly in the presence of a concentration of podophyllotoxin that completely inhibits assembly. When taxol (5 μM) was added to reaction mixtures that had been inhibited with 10 μM podophyllotoxin for 30 min, assembly of short microtubules and ribbon structures was promoted. It is not known at this time whether these structures contain podophyllotoxin.

Table III: Effect of Taxol on [^{3}H]Colchicine Binding to Tubulin

	nmol of [^{3}H]colchicine mg^{-1} of tubulin	% inhibition of control
(A) Assembly Conditions[a]		
control	3.10[b]	
0.5 μM taxol	3.07	1.0
5.0 μM taxol	2.87	7.4
50 μM taxol	2.08	33.0
50 μM podophyllotoxin	0.37	88.0
(B) Nonassembly Conditions[c]		
control	3.11[b]	
0.5 μM taxol	3.06	1.6
5.0 μM taxol	3.07	1.2
50 μM taxol	3.08	1.1
50 μM podophyllotoxin	0.36	88.3
(C) Preincubation Experiment[d]		
no addition	1.40	56.4
0.5 μM taxol	0.79	75.4
5.0 μM taxol	0.33	89.7
50 μM taxol	0.20	93.5
50 μM podophyllotoxin	0.11	96.6

[a] Assembly conditions: Mes buffer, 1 mM GTP, and 1 mg mL^{-1} MTP, 37 °C. At zero time, 50 μM [^{3}H]colchicine plus drug was added. Bound [^{3}H]colchicine was determined at 60 min. [b] Colchicine/tubulin dimer ratio is 0.4. [c] 100-fold-diluted Mes buffer. [d] Preincubation for 30 min with either taxol or podophyllotoxin followed by incubation with 50 μM [^{3}H]colchicine for 60 min. A 90-min incubation with [^{3}H]colchicine yields 3.21 nmol bound (equals 100%).

Discussion

The experiments in this paper were directed at acquiring a better understanding of in vitro microtubule polymerization and depolymerization reactions in the presence of taxol. Efficient microtubule polymerization in vitro requires a sufficient concentration of tubulin, magnesium, GTP, MAPs, organic buffer, and heat and an absence of calcium (see Roberts & Hyams, 1979). It is known that a dynamic equilibrium exists between microtubules and tubulin dimers in vitro (Gaskin et al., 1974; Lee & Timasheff, 1975; Johnson & Borisy, 1977).

The addition of taxol to microtubules at apparent steady state results in a rapid increase of microtubule polymerization. This result was expected since it has been demonstrated that taxol decreases the critical concentration of microtubule protein required to achieve microtubule polymerization in vitro (Schiff et al., 1979). This additional polymerization most likely results from a combination of elongation of existing microtubules and spontaneous nucleations of new microtubules after addition of the drug.

It is now known that there are two distinct association–dissociation reactions taking place at the opposite ends of microtubules (Margolis & Wilson, 1978; Bergen & Borisy, 1980). The method of microtubule dilution (Weisenberg &

Deery, 1976) was used to examine depolymerization of microtubules assembled in the presence of taxol. This technique has been helpful in studying the individual association and dissociation reactions at opposite ends of microtubules (Bergen & Borisy, 1980). Since no significant depolymerization of taxol-treated microtubules could be detected after a 5-fold protein dilution, we have concluded that taxol depresses the dissociation reaction at both ends of the microtubules.

Taxol induces formation of microtubules from MTP depleted of rings. In addition, when microtubule seeds are added to this preparation in the presence of taxol, both elongation of seeds and spontaneous nucleation of new microtubules appear to take place simultaneously. The taxol assembly reaction is also sensitive to the presence of microtubule seeds. The addition of seeds (nucleation sites) to a 6S tubulin and taxol reaction increases the yield of the assembly reaction and decreases the mean length of the microtubules when compared to a reaction without seeds. Taxol will also promote the polymerization of microtubules and tubulin ribbon structures from a tubulin preparation depleted in MAPs. Many of these microtubules appear to have frayed ends, unlike the majority of microtubules polymerized in the presence of taxol, tubulin, and MAPs. There is no evidence, as determined by gel electrophoresis, that taxol displaces any of the MAPs on microtubules when assembly takes place in the presence of MAPs and taxol.

Since it has been reported (Arai & Kaziro, 1976; Weisenberg & Deery, 1976; Sandoval et al., 1977) that several nonhydrolyzable analogues of GTP induce assembly of microtubules that are relatively stable compared to those polymerized with GTP, we examined taxol for its ability to affect GTP binding or hydrolysis. In our conditions, taxol did not inhibit GTP binding to microtubules nor did it inhibit the hydrolysis of GTP or GDP in our MTP preparations.

Taxol does not competitively inhibit the binding of colchicine to tubulin. We know from our previous work that taxol confers calcium resistance to microtubules (Schiff et al., 1979), suggesting that taxol binding sites are available on microtubules. This contrasts with the binding sites for colchicine (Wilson & Meza, 1973) and GTP (Weisenberg et al., 1976), which are not available on intact microtubules.

We have demonstrated previously that taxol inhibits cell replication predominantly in mitosis and stabilizes microtubules in cells against depolymerization by cold (4 °C) and antimitotic agents, such as steganacin (Schiff & Horwitz, 1980). It is not known if there is spontaneous nucleation of microtubules in taxol-treated cells. It is clear, however, that in vitro taxol enhances the assembly of stable microtubules in the absence of rings, GTP, organic buffer, and MAPs and confers stability on existing microtubules. This may be accomplished by depressing the dissociation reaction at both ends of the microtubules. The unusual capacity of taxol to shift the equilibrium between dimer and polymer in favor of the microtubule can be observed even in the presence of podophyllotoxin, a potent inhibitor of microtubule assembly, although the mechanism by which taxol confers stability and promotes assembly is not clear.

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References

Arai, T., & Kaziro, Y. (1976) *Biochem. Biophys. Res. Commun. 69*, 369–376.

Bergen, L. G., & Borisy, G. G. (1980) *J. Cell Biol. 84*, 141–150.

Borisy, G. G., & Olmsted, J. B. (1972) *Science (Washington, D.C.) 177*, 1196–1197.

Dustin, P. (1978) *Microtubules*, Springer-Verlag, New York.

Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.

Johnson, K. A., & Borisy, G. G. (1977) *J. Mol. Biol. 117*, 1–31.

Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183–5187.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Margolis, R. L., & Wilson, L. (1978) *Cell (Cambridge, Mass.) 13*, 1–8.

Penningroth, S. M., & Kirschner, M. W. (1977) *J. Mol. Biol. 115*, 643–673.

Randerath, K. (1966) *Thin Layer Chromatography*, 2nd ed., (Libmann, D. D., Transl.) Verlag Chemie, Academic Press, New York.

Roberts, K., & Hyams, J. S. (1979) *Microtubules*, Academic Press, London.

Sandoval, I. V., MacDonald, E., Jameson, J. L., & Cuatrecassas, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4881–4885.

Schiff, P. B., & Horwitz, S. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1561–1565.

Schiff, P. B., & Horwitz, S. B. (1981) in *Second Annual Bristol-Myers Symposium in Cancer Research* (Sartorelli, A., Ed.) pp 483–507, Academic Press, New York.

Schiff, P. B., Fant, J., & Horwitz, S. B. (1979) *Nature (London) 277*, 665–667.

Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.

Sloboda, R. D., Dentler, W. L., & Rosenbaum, J. L. (1976) *Biochemistry 15*, 4497–4505.

Swaney, J. B., & Kuehl, K. S. (1976) *Biochim. Biophys. Acta 446*, 561–565.

Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., & McPhail, A. T. (1971) *J. Am. Chem. Soc. 93*, 2325–2327.

Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.

Weisenberg, R. C., & Deery, W. J. (1976) *Nature (London) 263*, 792–793.

Weisenberg, R. C., Borisy, G. G., & Taylor, E. W. (1968) *Biochemistry 7*, 4466–4479.

Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15*, 4248–4254.

Wilson, L., & Meza, I. (1973) *J. Cell Biol. 58*, 709–719.