

# Temperature-dependent Reversible Assembly of Taxol-treated Microtubules

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**Abstract.** Taxol is a plant alkaloid that binds to and strongly stabilizes microtubules. Taxol-treated microtubules resist depolymerization under a variety of conditions that readily disassemble untreated microtubules. We report here that taxol-treated microtubules can be induced to disassemble by a combination of depolymerizing conditions. Reversible cycles of disassembly and reassembly were carried out using taxol-containing microtubules from calf brain and sea urchin eggs by shifting temperature in the presence of millimolar levels of  $\text{Ca}^{2+}$ . Microtubules depolymerized completely, yielding dimers and ring-shaped oligomers as revealed by negative stain electron microscopy and Bio-Gel A-15m chromatography, and reassembled into well-formed microtubule polymer structures. Microtubule-associated proteins (MAPs), including species previously identified only by taxol-based purification such

as MAP 1B and kinesin, were found to copurify with tubulin through reversible assembly cycles. To determine whether taxol remained bound to tubulin subunits, we subjected depolymerized taxol-treated microtubule protein to Sephadex G-25 chromatography, and the fractions were assayed for taxol content by reverse-phase HPLC. Taxol was found to be dissociated from the depolymerized microtubules. Protein treated in this way was found to be competent to reassemble, but now required conditions comparable with those for protein that had never been exposed to taxol. Thus, the binding of taxol to tubulin can be reversed. This has implications for the mechanism of taxol action and for the purification of microtubules from a wide variety of sources for use in self-assembly experiments.

**T**HE plant alkaloid, taxol, is unique in its effect on microtubules. In contrast to a large number of other microtubule-active drugs that disrupt or depolymerize microtubules, taxol promotes microtubule assembly and prevents microtubule disassembly, both *in vivo* and *in vitro* (28, 30). Like other microtubule-active drugs, taxol interferes with normal microtubule function. The drug blocks cell division at the  $G_2$  and  $M$  phases of the cell cycle (28). This appears to be the basis for its effectiveness as an antileukemic and antitumor agent (43). Because of its unique microtubule stabilizing effect, it has come into increasing use in cellular studies of microtubule function (8, 12, 46). Taxol has also proven to be a valuable tool for the purification of microtubules from many tissue and cell types (36, 40).

Some of the details of the mechanism of action of taxol are understood (25). Taxol binds to the microtubule polymer in a 1:1 stoichiometry with tubulin. Binding appears to be very strong, though the association constant has not been determined. The properties of taxol-stabilized microtubules are very different from those of untreated microtubules. The latter are unstable structures, and their assembly state is drastically affected by a number of conditions (see reference 11 for review). Assembly requires, or is strongly promoted by, elevated temperature, GTP hydrolysis, microtubule-stabiliz-

ing buffers, and microtubule-associated proteins (MAPs).<sup>1</sup> Depolymerization is favored in the cold by millimolar levels of calcium, by levels of salt that dissociate the MAPs, and by a variety of drugs, including colchicine, vinblastine, and nocodazole. Taxol-treated microtubules remain polymeric in the cold, in the presence of calcium and antimicrotubule drugs, and do not require GTP, MAPs, or organic buffers to assemble or remain assembled (29, 30). Even under conditions favorable for assembly, microtubules treated with taxol show an increased rate and extent of assembly without a lag period and a markedly decreased critical concentration of free subunits (30), indicative of a strongly enhanced subunit interaction.

The taxol binding site is accessible in the microtubule polymer and free and bound taxol are capable of rapid exchange (25). The binding site appears to be distinct from those for other microtubule-active drugs (18, 25, 29). It also appears to be distinct from the binding sites for the MAPs, because taxol and the MAPs can coexist on the microtubule polymer (36). In fact, all known MAPs bind to microtubules in the presence of taxol (36, 40).

1. *Abbreviation used in this paper:* MAP, microtubule-associated protein.

Purification schemes based on self assembly have been effective only in a limited number of cases. In systems where assembly could be achieved, such as brain tissue (1, 6, 9, 31) and HeLa cells (7, 44), the microtubules could subsequently be disassembled and reassembled. This formed the basis for preparative schemes in which repeated cycles of assembly and disassembly could be carried out, allowing for extensive microtubule purification. The major limitation of this method, in addition to the small number of applicable systems, is the highly restrictive conditions for assembly. Variations in temperature, buffer condition, and nucleotide composition, for example, are virtually impossible. Furthermore, because both assembly and disassembly of microtubules are relatively inefficient processes even in the best preparations, losses of microtubule components can occur during purification.

The use of taxol has allowed for microtubule purification and MAP identification in a wide variety of systems (36, 38, 40). The yield of MAPs appears to be higher than in the reversible assembly method, and purification can be conducted in the cold (36) and in the absence of GTP (34). Nucleotide and buffer composition can be varied, and this has led to the identification of novel MAPs (26, 35).

In the course of our studies on the effects of taxol we noticed that, under some conditions, taxol-treated microtubules showed evidence of partial depolymerization. We suspected that this might allow for the further experimental manipulation of microtubules purified with taxol, and, in addition, might shed further light on the mechanism of action of the drug. We report here conditions for the full disassembly and reassembly of taxol-treated microtubules. We use these conditions to further purify taxol-containing microtubules by reversible assembly cycling. We show that a number of proteins, previously identified as MAPs using only the taxol procedure, will copurify with tubulin by reversible assembly cycling. Finally, we find that taxol does not bind appreciably to the tubulin dimer under our conditions, and can be removed from soluble microtubule protein by gel filtration. This provides a means for obtaining fully functional tubulin from a wide variety of sources.

## Materials and Methods

### Chemicals

Taxol was obtained from Dr. Matthew Suffness, National Products Branch, Division of Cancer Treatment, National Cancer Institute. It was dissolved in DMSO at a concentration of 10 mM and stored at  $-80^{\circ}\text{C}$ .

All experiments were conducted in 100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.6, containing 1 mM EGTA and 1 mM  $\text{MgSO}_4$  (PEM), plus additional constituents where noted.

### Protein Preparative Procedures

**Taxol-treated Bovine Brain Microtubules.** Microtubules were purified from whole calf brain cerebrum by the reversible assembly method (6, 37). Microtubule protein was stored at  $-80^{\circ}\text{C}$  after two assembly/disassembly cycles and carried through a third cycle just before use. A molar excess of taxol was added and the microtubules were incubated at  $37^{\circ}\text{C}$  for 10 min. Excess taxol was removed by sedimentation at 30,000 *g* for 30 min and the microtubules were suspended and resedimented in PEM buffer lacking taxol.

**Purification of Microtubules with Aid of Taxol.** Microtubules were prepared directly from calf brain white matter cytosol with the aid of taxol (36,

41). 5'-Adenylylimidodiphosphate (AMPPNP) at 1 mM was included during the taxol assembly step to promote binding of kinesin (35), and GTP was omitted. The taxol-purified microtubules were resuspended in PEM buffer in the absence of nucleotides or additional taxol for use in depolymerization experiments. Microtubules were also prepared from unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus* (38). Microtubule assembly in egg cytosol was induced with taxol in the absence of added nucleotides. Microtubules were collected by centrifugation and resuspended in PEM buffer containing no additional taxol. The taxol-purified egg microtubules were used immediately or after storage at  $-80^{\circ}\text{C}$ .

For some biological systems in which the microtubules are unusually stable, we find that depolymerization works best if taxol is omitted from the sucrose cushion and all subsequent steps and GTP is omitted from the entire procedure.

### Analytical Methods

**Light Scattering.** Microtubule depolymerization and assembly were monitored by turbidity measurement (14). Measurements for brain microtubules represent apparent absorbance at 350 nm using a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) equipped with a 1-cm light path water jacketed cuvette (NSG Precision Cells, Inc., Hicksville, NY). Light scattering of sea urchin microtubules was monitored at 500 nm due to the high absorbance of these samples at 350 nm. This effect was apparently due to bundling of the taxol-purified microtubules (C. A. Collins, unpublished observation). Nonetheless, we found that light scattering had a linear relationship with the amount of sedimentable polymer.

**Gel Electrophoresis.** SDS gel electrophoresis was carried out according to the method of Laemmli (19) using 7.5% polyacrylamide in the separating gel. For some experiments where greater resolution of high molecular weight proteins was desired, 4% polyacrylamide gels containing 2 M urea without SDS were used (4). Gels were stained with Coomassie Brilliant Blue R 250.

**Gel Filtration Chromatography.** Analysis of the assembly state of microtubule protein was accomplished using a Bio-Gel A-15m (Bio-Rad Laboratories, Richmond, CA) column (0.9  $\times$  55 cm) equilibrated in PEM buffer containing 2 mM  $\text{CaCl}_2$ . Fractions (0.45 ml) were collected using a flow rate of 0.15 ml/min. The column was calibrated using cold disassembled brain microtubule protein (37), as well as Sigma Chemical Co. (St. Louis, MO) molecular weight standards for gel filtration (apoferritin, thyroglobulin, catalase, and bovine serum albumin). To separate taxol from depolymerized taxol-treated microtubules, protein samples were applied to a Sephadex G-25 column (PD-10, Pharmacia Fine Chemicals, Piscataway, NJ) and eluted in PEM buffer.

**Determination of Taxol by Reverse-Phase HPLC.** Taxol was extracted from microtubule protein samples by addition of methanol to a final concentration of 80% (vol/vol). Precipitated protein was removed by centrifugation at 30,000 *g* for 30 min. Analysis of taxol in the methanol extract was performed on a reverse-phase column ( $\mu$ Bondapak  $\text{C}_{18}$ , 3.9  $\times$  300 mm, Waters Associates, Milford, MA) in methanol:water (70:30 vol/vol) at a flow rate of 1 ml/min (15). Taxol was detected by absorbance at 214 nm, and the concentration was determined by measurement of peak areas relative to a taxol standard.

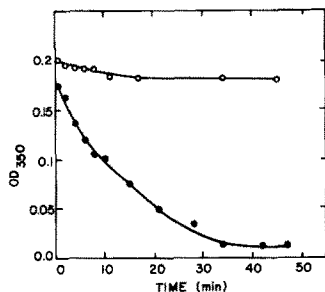
**Protein Determination.** Protein concentration was determined by the method of Lowry et al. (20) using bovine serum albumin as a standard.

**Electron Microscopy.**  $\text{Ca}^{2+}$ -dependent depolymerization and subsequent reassembly of taxol-containing brain microtubules were monitored by electron microscopy of negatively stained grids. Protein samples at 1.2 mg/ml were adsorbed for 10 s on carbon and Formvar-coated copper grids, fixed for 60 s with 0.5% glutaraldehyde in PEM, and rinsed with distilled water. The water was displaced with three drops of 2% aqueous uranyl acetate, and the grid was blotted dry and examined. Electron microscopy was performed using a 301 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

## Results

### Depolymerization and Repolymerization of Taxol-containing Microtubules

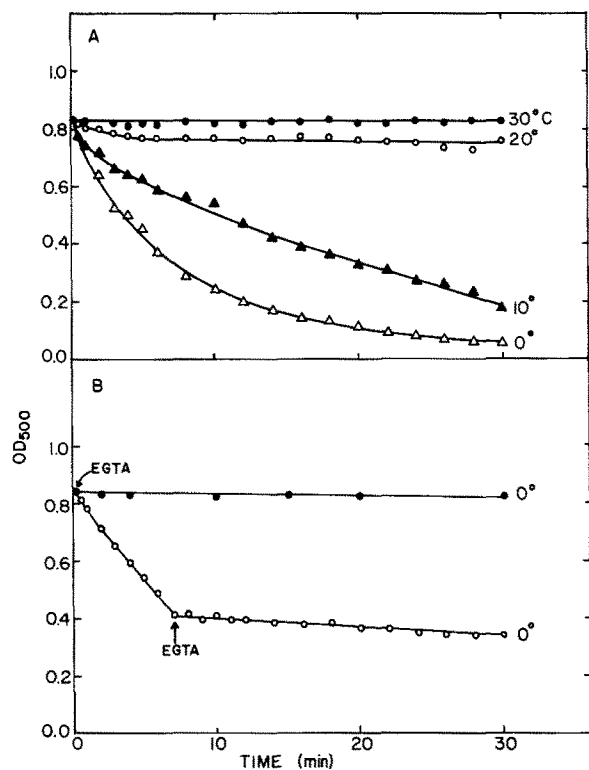
In the course of our work with taxol-containing microtubules, we have noticed that some tubulin becomes soluble at low temperatures either in the presence of high concentrations of  $\text{Ca}^{2+}$  or at elevated salt concentrations. To charac-



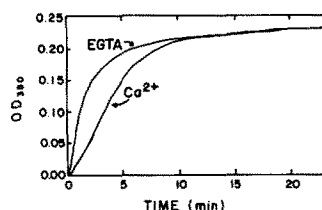
**Figure 1.** Temperature dependence of  $\text{Ca}^{2+}$ -induced depolymerization of taxol-treated microtubules from calf brain. Reversible assembly purified microtubule protein was exposed to taxol and then washed free of excess drug. The microtubules (final concentration, 0.66 mg/ml) were incubated at 20°C (○) or 0°C (●) in the presence of 3 mM  $\text{CaCl}_2$  in PEM buffer. Light scattering was monitored at 350 nm over the time-course indicated.

terize the extent and time course of disassembly, we monitored the assembly state of microtubule protein by light scattering. The effect of  $\text{Ca}^{2+}$  and temperature on the assembly state of taxol-treated calf brain microtubules is shown in Fig. 1. Electron microscopy of depolymerized microtubule samples was also performed.

Little microtubule disassembly occurred at 20°C (Fig. 1). However, at 0°C, extensive disassembly occurred. The time course was slow relative to the depolymerization of microtubules that have not been treated with taxol (cf. 14). None-



**Figure 2.** Temperature dependence of  $\text{Ca}^{2+}$ -induced depolymerization of taxol-purified microtubules from sea urchin eggs. Microtubules were prepared from unfertilized sea urchin egg cytosol using taxol and suspended in PEM buffer to 1 mg/ml. Depolymerization conditions were (A) 3 mM  $\text{CaCl}_2$  at the temperatures indicated, and (B) 0°C with (○) or without (●) 3 mM  $\text{CaCl}_2$ . An excess of EGTA (5 mM) was added where indicated by the arrow for the remaining incubation period.



**Figure 3.** Reassembly of depolymerized taxol-containing microtubules. Taxol-treated calf brain microtubules were depolymerized with 3 mM  $\text{CaCl}_2$  on ice for 30 min and centrifuged at 30,000 g for 30 min at 4°C. The supernate at 1.1 mg protein/ml was incubated at 37°C in the presence or absence of 5 mM EGTA as shown. Microtubule assembly was monitored at 350 nm.

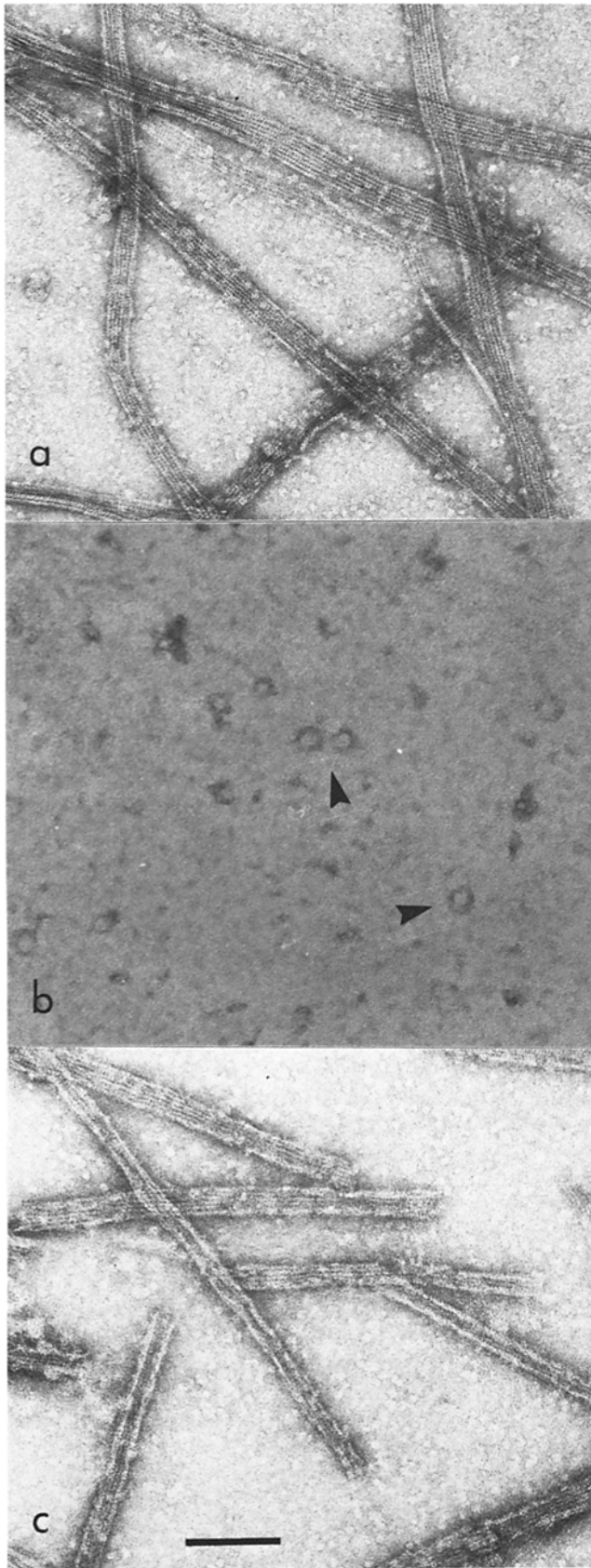
theless, after sufficient time at 0°C virtually complete disassembly occurred, with the turbidity level dropping almost to the value observed for unpolymerized microtubule protein after 30 min. We note that at high microtubule protein concentration (>2 mg/ml), disassembly was incomplete after 30 min, and longer incubation at 0° is necessary.

A similar effect of temperature in the presence of  $\text{Ca}^{2+}$  was observed for taxol-purified sea urchin microtubules (Fig. 2 A). Little or no depolymerization occurred at 20° or 30°C. At 10°C a gradual loss of microtubule polymer was observed, and at 0°C depolymerization was essentially complete after 30 min, again as judged by reduction of turbidity to the level seen for unpolymerized protein. Incubation of taxol-purified microtubules at 0°C for 30 min in the absence of  $\text{Ca}^{2+}$  did not result in depolymerization of sea urchin microtubules (Fig. 2 B). Thus,  $\text{Ca}^{2+}$  is required for depolymerization to occur. This is further indicated by the observation that addition of excess EGTA to partially depolymerized microtubules arrested disassembly at 0°C (Fig. 2 B).

Repolymerization of depolymerized taxol-containing microtubules was observed when the protein sample was incubated at elevated temperature (Fig. 3). Assembly occurred even in the presence of  $\text{Ca}^{2+}$ , in marked contrast to microtubule protein in the absence of taxol (23, 24). In fact, when  $\text{Ca}^{2+}$  was present, repeated cycles of reversible assembly and disassembly could be carried out simply by changing the temperature. We note that though the final extent of assembly was unaffected, reassembly was faster in the presence of EGTA (Fig. 3). EGTA was routinely added during warm reassembly steps to prevent  $\text{Ca}^{2+}$ -dependent proteolysis of the MAPs. In all of these experiments GTP was omitted and therefore was clearly not required for reassembly.

Microtubule disassembly and repolymerization were also monitored by electron microscopy (Fig. 4). The taxol-treated sample before depolymerization contained microtubules as well as partially unrolled microtubule sheets (Fig. 4 a). After 10 min of depolymerization with  $\text{Ca}^{2+}$ /cold treatment, only a few short microtubules remained. Ring-shaped oligomers were commonly observed (Fig. 4 b). Large numbers of well-formed microtubules were apparent in the repolymerized microtubule sample (Fig. 4 c).

Taxol-containing microtubules could also be disassembled at 0°C in the presence of 0.5 M NaCl (Table I). The disassembled microtubule protein repolymerized at 37°C after dialysis against PEM buffer to remove the salt. The extent of disassembly and reassembly were comparable with levels observed with  $\text{Ca}^{2+}$  treatment. The only reported effect of NaCl at the concentration used is to dissociate MAPs from



**Figure 4.** Negative stain electron microscopy of depolymerized and repolymerized microtubules. Taxol-treated calf brain microtubules at 1.2 mg protein/ml (a) were depolymerized by incubation with 3 mM  $\text{CaCl}_2$  on ice for 10 min (b). After 30 min on ice the mi-

**Table I. Comparison of Calcium and NaCl-induced Depolymerization and Reassembly of Taxol-containing Microtubules**

Treatment*	Polymer‡
	% total protein
Depolymerization, $\text{CaCl}_2$	10
Repolymerization	85
Depolymerization, NaCl	4
Repolymerization	75

\* Taxol-treated sea urchin microtubules were depolymerized with 3 mM  $\text{CaCl}_2$  or 0.5 M NaCl on ice for 45 min. After centrifugation at 30,000 g for 30 min at 4°C, the  $\text{Ca}^{2+}$  supernate was treated with 5 mM EGTA and the NaCl supernate was dialyzed against PEM buffer for 90 min. The supernates were then incubated at 37°C for 5 min and centrifuged at 37°C as above.

‡ Results are expressed as the percent of total protein which sediments under the centrifugation conditions employed.

microtubules (36). Thus, the observed depolymerization may reflect the properties of taxol-treated tubulin alone. In fact, we find that in the absence of MAPs, taxol-treated tubulin depolymerizes slowly at 0°C, whereas MAP-containing microtubules are stable indefinitely under these conditions.

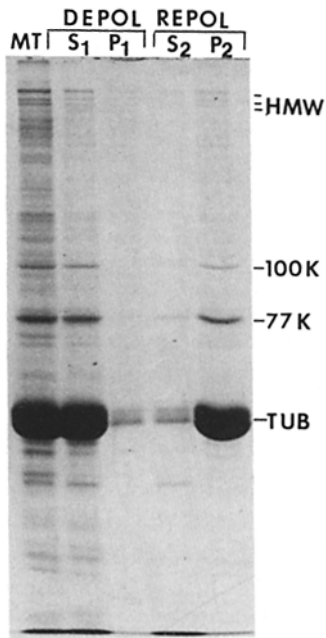
$\text{Ca}^{2+}$  rather than elevated NaCl was used for disassembly for the remainder of this study because reversible assembly could be achieved without the need for dialysis between steps.

#### **Protein Composition of Disassembled and Reassembled Taxol-containing Microtubules**

Fig. 5 shows the behavior of tubulin and nontubulin proteins during temperature-dependent disassembly and reassembly of taxol-purified microtubules in the presence of  $\text{Ca}^{2+}$ . The microtubules were obtained by addition of taxol to a cytosolic extract of sea urchin eggs, followed by centrifugation.

The microtubules (lane 1) showed tubulin as the major component. Numerous additional electrophoretic species were evident in this heavily loaded sample. The microtubules were disassembled by addition of  $\text{Ca}^{2+}$  and incubation for 45 min on ice. Centrifugation of the preparation revealed that most of the protein was solubilized by this procedure (lane  $S_1$ ). However, many of the less prominent bands were found in the pellet, suggesting that these proteins were particulate contaminants. The soluble material was recovered, incubated at 37°C to reassemble the microtubules, and recentrifuged. Almost all of the tubulin was found in the pellet (lane  $P_2$ ), confirming that good reassembly of the microtubules had occurred. A few additional nontubulin proteins remained in the supernate at this stage (lane  $S_2$ ), whereas most appeared along with tubulin in the pellet. Most prominent was a species of  $M_r$  77,000, which showed behavior similar to tubulin both in the depolymerization and

cro-tubule protein was centrifuged at 30,000 g for 30 min at 4°C to remove any residual polymer. EGTA was added to the supernate to a final concentration of 5 mM and the sample was warmed to 37°C for 10 min (c). Samples were processed for microscopy as described in Materials and Methods. Arrows indicate the presence of ring-shaped oligomers in the depolymerized microtubule sample. Bar, 0.1  $\mu\text{m}$ .

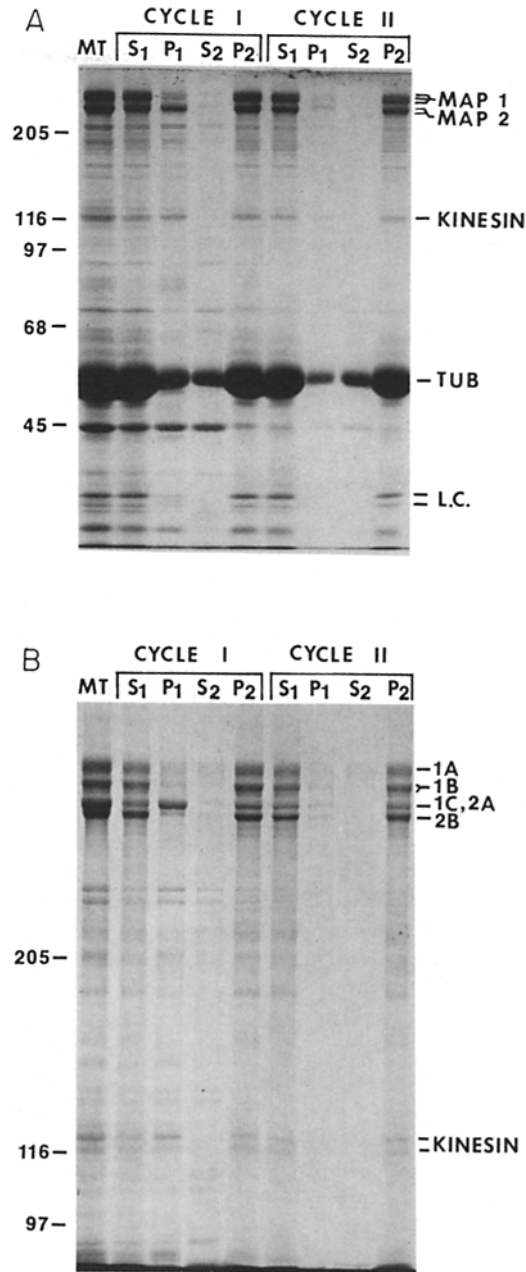


**Figure 5.** Electrophoretic analysis of depolymerized and repolymerized taxol-containing sea urchin egg microtubules. Microtubules (1.2 mg/ml) were depolymerized by treatment with 3 mM  $\text{CaCl}_2$  at 4°C for 45 min and then centrifuged at 30,000 g for 30 min at 4°C to remove residual polymer. The supernatant was recovered, warmed to 37°C for 5 min in the presence of 5 mM EGTA, and centrifuged at 37°C to sediment microtubule polymer. Samples of the initial microtubules (*MT*), the supernate (*S<sub>1</sub>*), and pellet (*P<sub>1</sub>*) after  $\text{Ca}^{2+}$  depolymerization and centrifugation, and the supernate (*S<sub>2</sub>*) and pellet (*P<sub>2</sub>*) after reassembly and sedimentation of the microtubule protein were analyzed by SDS-PAGE (7.5% polyacrylamide). The positions of previously identified MAPs and tubulin.

repolymerization steps. This protein was previously identified in our laboratory as a MAP using our standard taxol procedure (38) as well as by immunofluorescence microscopy using mAb's (2). What was probably the same protein was previously identified as an  $M_r$  80,000 species by Rebhun et al. (27), who observed it to copurify with tubulin by reversible assembly cycling of microtubules from sea urchin egg mitotic spindles. The same protein has recently been identified as the major MAP in reversibly assembled microtubules from whole sea urchin eggs (32).

Additional proteins were also found to persist though the cycle of reversible assembly of taxol-containing microtubules. These included a band at  $M_r$  100,000 and several high-molecular weight species previously identified as MAPs by the biochemical criteria inherent to the standard taxol preparative procedure (36, 38, 40). In fact, the overall composition of the repolymerized pellet was very similar to the composition of the purest microtubules prepared by the standard taxol procedure (2, 38). A band at the position of actin was specifically depleted during the repolymerization step. This species is virtually always a contaminant in the standard taxol procedure.

Fig. 6 shows the behavior of microtubule proteins prepared using taxol from calf brain white matter. Unlike the usual preparation, GTP was omitted and AMPPNP was added to induce the binding of kinesin (35). Two consecutive cycles of temperature-dependent reversible assembly in the presence of  $\text{Ca}^{2+}$  were examined. Complete disassembly of microtubules was not achieved in this particular experiment (see *S<sub>1</sub>* and *P<sub>1</sub>* of cycle I). This was most likely due to the high protein concentration in the resuspended microtubules during the  $\text{Ca}^{2+}$ /cold treatment (over 3 mg/ml; see Discus-



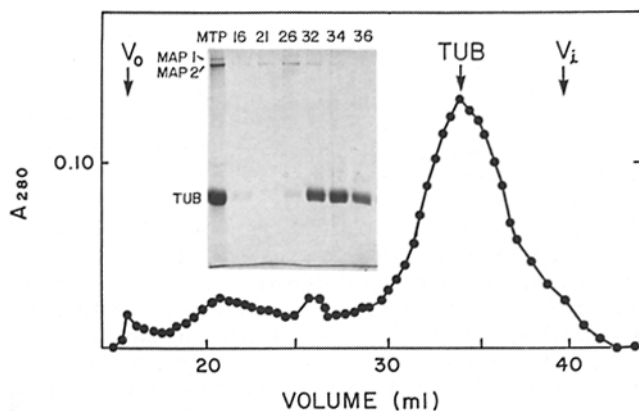
**Figure 6.** Electrophoretic analysis of depolymerized and repolymerized taxol-containing calf brain microtubules. Two disassembly and reassembly cycles are shown. Microtubules were prepared directly from calf brain white matter cytosol with the aid of taxol. AMPPNP (1 mM) was included during the taxol assembly step to promote binding of kinesin. Microtubules (3.2 mg/ml) were depolymerized by treatment with 3 mM  $\text{CaCl}_2$  at 4°C for 30 min and then centrifuged at 30,000 g for 30 min at 4°C to remove residual polymer. The supernate was recovered, warmed to 37°C for 10 min in the presence of 5 mM EGTA, and centrifuged at 37°C to sediment reassembled polymer. The depolymerization and reassembly steps were repeated for the second cycle. The initial microtubule sample (*MT*) and supernate and pellet samples after each disassembly (*S<sub>1</sub>* and *P<sub>1</sub>*) and assembly (*S<sub>2</sub>* and *P<sub>2</sub>*) step were analyzed by SDS gel electrophoresis. (A) 7.5% polyacrylamide gel. (B) 4% polyacrylamide/2 M urea gel showing the same samples to resolve the high molecular weight proteins. The positions of previously identified MAPs, tubulin, and molecular weight standards (in kD) are indicated.

sion). However, disassembly was virtually complete at this protein concentration in cycle II. Most of the nontubulin proteins in the preparation exhibited the behavior expected for MAPs. They became soluble along with tubulin upon  $\text{Ca}^{2+}$ /cold treatment ( $P_1$  of cycles I and II), and reassembled with tubulin at  $30^\circ\text{C}$  ( $P_2$  of cycles I and II). This class of protein included MAP 1A (3, 5), MAP 1B (4, 42), MAP 2A, MAP 2B, kinesin (35), and MAP 1 light chains LC 1 and LC 2 (41). A low-molecular weight polypeptide migrating faster than LC 2 was also observed to copurify with microtubules. It has not yet been further characterized. MAP 1B and kinesin have not previously been observed to copurify with microtubules through reversible assembly cycles. We note that kinesin appears as a distinct doublet in these preparations. This is particularly obvious in the high resolution polyacrylamide gel shown in Fig. 6 B. Actin and some other minor species were eliminated during the preparative procedure.

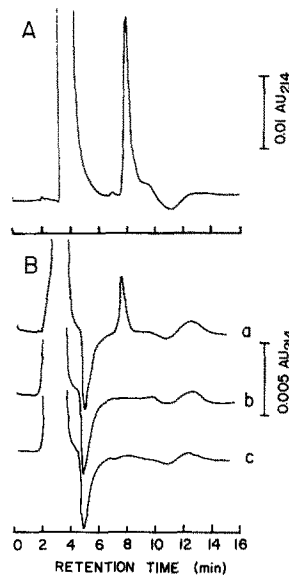
A substantial fraction of kinesin and virtually all of MAP 1C, a protein previously identified in this laboratory (5), were partitioned into the pellet after the first microtubule depolymerization step (Fig. 6 B,  $P_1$  of cycle I). We believe that this indicates a higher affinity for residual microtubules than that of the other MAPs in the preparation (see Discussion).

### Characterization of the Depolymerized Microtubule Protein

Electron microscopy of calf brain microtubules under depolymerizing conditions showed few short microtubules. Some ring-shaped structures characteristic of depolymerized brain microtubules that have not been exposed to taxol were observed (Fig. 4), but most of the protein appeared to be un-assembled. To characterize the depolymerized tubulin and MAPs further, gel filtration chromatography was used (Fig. 7). The MAPs were found in high-molecular weight fractions. Tubulin eluted as a single species, with the peak fraction in the same position as an unpolymerized brain tubulin



**Figure 7.** Gel filtration of depolymerized taxol-treated microtubule protein. Taxol-treated calf brain microtubules (MTP) were depolymerized with 3 mM  $\text{CaCl}_2$  at  $4^\circ\text{C}$  for 2 h. After centrifugation at 30,000 g for 30 min at  $4^\circ\text{C}$ , the supernate (1.5 ml of 0.5 mg protein/ml) was applied to a Bio-Gel A-15m column equilibrated in PEM buffer containing 2 mM  $\text{CaCl}_2$ . Fractions were collected and monitored for protein by absorbance at 280 nm, and selected fractions were analyzed by SDS gel electrophoresis on 7% polyacrylamide (*inset*).



**Figure 8.** Dissociation of taxol from depolymerized microtubules. Taxol-treated calf brain microtubules (0.95 mg/ml) were depolymerized with 3 mM  $\text{CaCl}_2$  for 90 min on ice and centrifuged at 30,000 g for 30 min at  $4^\circ\text{C}$ . The supernate was applied to a Sephadex G-25 column (PD-10; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in PEM buffer. Samples (10  $\mu\text{l}$ ) of the protein peak (0.9 mg/ml) and the included volume were extracted with methanol and analyzed for taxol content by reverse-phase HPLC. (A) Pure taxol standard (1 nmol) in 70% methanol. (B) (a) Initial microtubule sample; (b) gel filtered protein peak; and (c) included volume peak.

standard. Although we observed numerous ring-shaped structures in depolymerized microtubules (Fig. 4), a tubulin oligomer peak (39) was not seen by gel filtration. We attribute this to the extreme concentration dependence of ring formation (21), and the low concentration of protein used in the gel filtration experiment.

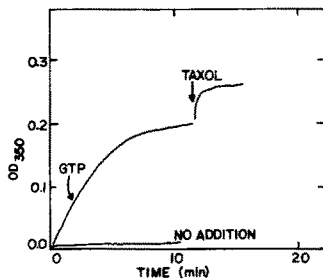
To determine whether taxol remained bound to the soluble tubulin, samples of depolymerized taxol-treated microtubules were subjected to gel filtration on a desalting column. Microtubule protein samples before and after gel filtration were extracted with cold methanol (80% final concentration) to precipitate protein and extract taxol, and the amount of taxol was determined by reverse-phase chromatography (Fig. 8). The level of taxol in the initial microtubule sample was determined to be 1.5 mol/mol tubulin dimer. Passage of the depolymerized protein over Sephadex G-25 resulted in the removal of the taxol from the microtubule protein. The level of taxol in the protein fraction was  $<0.05$  mol/mol tubulin, based on the estimated sensitivity of the reverse-phase chromatographic assay for taxol. The taxol was not recovered in the included volume. This apparently indicates that taxol, like other heterocyclic aromatic compounds, can bind to Sephadex (16). In a similar experiment using Bio-Gel P-6, taxol was again undetectable in the protein fraction but could be observed in the included volume fractions (data not shown).

Using G-25 for efficient removal of taxol from depolymerized microtubule protein, we assayed the ability of the protein to reassemble (Fig. 9). Almost no assembly was observed at  $37^\circ\text{C}$ . Addition of GTP strongly promoted assembly. These microtubules could be depolymerized by cold and were sensitive to 3 mM  $\text{Ca}^{2+}$  and 0.35 M NaCl (not shown). This behavior is characteristic of microtubule protein that has never been exposed to taxol. It confirmed that the taxol was efficiently removed by G-25 chromatography and also indicated that the tubulin was still assembly-competent.

### Discussion

Taxol-treated microtubules have been reported to be stable





**Figure 9.** Assembly characteristics of calf brain microtubule protein from which taxol had been removed. Taxol-treated calf brain microtubules were depolymerized with 3 mM  $\text{CaCl}_2$  on ice for 100 min and centrifuged at 30,000  $g$  for 30 min at 4°C. The supernate was applied to a Sephadex G-25 column equilibrated in

PEM. The protein peak was pooled (1.1 mg protein/ml) and used for assembly experiments at 37°C in the presence and absence of 1 mM GTP as indicated. Taxol (20  $\mu\text{M}$ ) was added to the GTP-containing sample where indicated by the arrow to determine the maximum amount of assembly-competent microtubule protein. Assembly was monitored by light scattering at 350 nm.

under a variety of conditions that lead to the disassembly of untreated microtubules. We find in the present study that taxol-containing microtubules will depolymerize using a combination of destabilizing conditions, either  $\text{Ca}^{2+}$  and low temperature, or elevated ionic strength and low temperature. It is thought that  $\text{Ca}^{2+}$  at millimolar levels directly affects tubulin (17, 47), and elevated NaCl concentrations dissociate MAPs (36). Whether these agents also affect binding of taxol to tubulin is not known. We believe this to be unlikely because the stabilizing effects of taxol can still be seen in the presence of either  $\text{Ca}^{2+}$  or NaCl. Examples of this are shown in Figs. 1 and 2 where taxol-treated microtubules are very stable to  $\text{Ca}^{2+}$  at 20° or 30°C. Even at 0°C disassembly is slower in the presence of taxol (Figs. 1 and 2) than in its absence (14). Finally, reassembly of tubulin proceeds in the presence of  $\text{Ca}^{2+}$  and taxol, indicating that  $\text{Ca}^{2+}$  does not block taxol binding (Fig. 3).

We have followed the fate of taxol through the steps of calcium-induced depolymerization and reassembly. Although taxol in solution has been reported to exchange with taxol bound to tubulin polymer (25), we found little or no apparent loss of polymer after repeated washes of microtubules with buffer in the absence of taxol. However, we found that taxol had a much reduced affinity for tubulin solubilized by  $\text{Ca}^{2+}$ /cold treatment and could be removed by gel filtration of depolymerized microtubule samples. These results suggest a model for taxol-induced microtubule assembly in which taxol has a low affinity for dimeric tubulin, but instead binds to and stabilizes tubulin polymer, thereby shifting the assembly equilibrium in the direction of polymerization. Such a mechanism has been proposed for the interaction of phalloidin with actin (10), based on the effects of that drug on the kinetics of actin assembly and disassembly.

Examination of the composition of microtubules during cycles of  $\text{Ca}^{2+}$ /cold disassembly and warm reassembly revealed that proteins identified as MAPs by other criteria copurified with tubulin as expected. MAP 1B (4) and kinesin (35), which had been previously identified only in taxol-purified microtubule preparations, were also found to have this behavior (Fig. 6). It is important to note that some MAPs can be lost during cycling if depolymerization is incomplete. MAP 1C, which has been extensively characterized as a microtubule-interacting protein (26), provides a good example of this behavior (Fig. 6 B). The high-molecular weight

MAPs from sea urchin eggs also fall into this category. We believe that this behavior results simply from the binding of these MAPs to the small amount of residual polymer that may persist after depolymerization.

This observation has important implications for the purification of MAPs in general. Some classes of MAP could bind to residual microtubules in cell or tissue homogenates and therefore fail to appear in the cytosolic extracts used to prepare microtubules. In addition, MAPs that are extracted initially may be lost during the microtubule depolymerization stages of the traditional reversible assembly preparation because of the well-documented "cold-stable" microtubules in these preparations (45). We note that it is more difficult to disassemble taxol-purified microtubules prepared directly from tissue extracts (Fig. 6) than those first purified by reversible assembly and subsequently exposed to taxol. This may reflect the presence of the same factors that render normal microtubules cold-stable *in vitro* (22).

$\text{Ca}^{2+}$  cycling can be used as a means of further purification of taxol-containing microtubule preparations, particularly from cells and tissues in which tubulin is a minor component. The conditions required for complete disassembly of taxol-treated microtubules may vary from one preparation to another. However, the most important experimental variables have proven to be depolymerization time (Figs. 1 and 2) and protein concentration. The latter variable is most important in microtubules prepared directly from tissue extracts using taxol. At concentrations  $> \sim 2$  mg/ml of microtubules, disassembly was incomplete even at very long times of incubation at 0°C. As discussed in the preceding paragraph, complete disassembly at this stage is an important factor influencing the ultimate composition and recovery of MAPs. In our experience, protein concentration has not been a factor in the depolymerization of microtubules during subsequent cycles. Even tubulin from poikilotherms such as sea urchin, which is inherently more stable than mammalian tubulin (13, 33), will depolymerize and repolymerize using our protocol. We do find in some cases, such as dogfish brain and clam egg preparations, that it is necessary to eliminate excess free taxol and GTP to obtain good disassembly (see Methods).

After depolymerization, taxol can be removed from the soluble microtubule protein by gel filtration. This should provide a means for the preparation of tubulin and whole microtubule protein from a wide variety of sources for characterization of microtubule self-assembly.

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*Note Added in Proof.* A modification of the method described in Fig. 8 has been useful for recovering taxol from microtubule preparations. We extracted protein samples with chloroform or methanol (80% final concentration). The organic phase was air dried (after centrifugation in the case of methanol extraction), the sample was redissolved in 80% methanol, and the taxol was further purified by reverse-phase HPLC. Taxol was recovered in high yield and was found to be fully active in promoting tubulin assembly.

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