Taxol-induced Bundling of Brain-derived Microtubules

PAMELA F. TURNER and ROBERT L. MARGOLIS
Department of Cell Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

ABSTRACT Taxol has two obvious effects in cells. It stabilizes microtubules and it induces microtubule bundling. We have duplicated the microtubule-bundling effect of taxol in vitro and report preliminary characterization of this bundling using electron microscopy, sedimentation, and electrophoretic analyses. Taxol-bundled microtubules from rat brain crude extracts were seen as massive bundles by electron microscopy. Bundled microtubules sedimented through sucrose five times faster than control microtubules. Electrophoretic analysis of control and taxol-bundled microtubules pelleted through sucrose revealed no striking differences between the two samples except for a protein doublet of ~100,000 daltons. Taxol-induced microtubule bundling was not produced by using pure tubulin or recycled microtubule protein; this suggested that taxol-induced microtubule bundling was mediated by a factor present in rat brain crude extracts. Taxol cross-linked rat brain crude extract microtubules were entirely labile to ATP in the millimolar range. This ATP-dependent relaxation was also demonstrated in a more purified system, using taxol-bundled microtubules pelleted through sucrose and gently resuspended. Although the bundling factor did not recycle with microtubule protein, it was apparently retained on isolated taxol-stabilized microtubules. The bundling factor was salt extracted from taxol-stabilized microtubules and its retained activity was demonstrated in an add-back experiment with assembled phophocellulose-purified tubulin.

Microtubules interact with intermediate filaments, microfilaments, membrane enclosed organelles, and neighboring microtubules to maintain cell architecture and to effect the movement of cells and of the organelles within them (for review see reference 6). In addition to numerous reports of microtubule associations with subcellular structures in vivo, in vitro interaction studies have shown that microtubules will bind to pituitary secretory granules and secretory granule membranes (34), actin filaments (8, 29, 33), skeletal muscle myosin (9), neurofilaments (28), pancreatic secretory granules (36), and other microtubules via linking proteins (13, 24, 36). These in vitro interactions require the presence of microtubule-associated proteins and many are inhibited by ATP.

Microtubules interact with each other to form structures whose degree of order ranges from unorganized bundles to highly ordered arrays. Microtubule bundles of relatively loose morphology are found as a marginal band in nucleated erythrocytes and in platelets, in the manchette surrounding and shaping the nucleus during spermatogenesis, and in the mitotic spindle and the telophase midbody (6). In the mitotic spindle, antiparallel microtubules from opposite poles preferentially interact and bridges are seen between them in cross-sectional analysis (21, 22, 27, 37). Highly ordered arrays of microtubules are seen in cytopharyngeal baskets in axopodia, nemadesmata, and axostyles of various protozoans (see reference 6 and 40 for reviews). Furthermore, precisely arrayed microtubule doublets determine the axoneme structure in cilia and flagella (6). Bundled microtubules are also seen in cells exposed to taxol (7, 25, 32).

Taxol, a tubulin-binding drug (14, 26) isolated from the bark of the western yew plant *Taxus brevifolia* (44), in addition to inducing extensive microtubule bundling in vivo also promotes microtubule assembly, shifting the dimer-polymer equilibrium both in vitro (31) and in vivo (7, 10). It stabilizes labile cytoplasmic microtubules against depolymerization by cold temperature and 4 mM Ca^{++} and lowers their sensitivity to other spindle poisons in vitro (14, 26, 31) and in vivo (5, 6, 32). The bundles observed with taxol in cultured mammalian interphase cells often occur at sites independent of microtubule-organizing centers (7, 32). In mitotic cells (the spindle of *Haemanthus* endosperm) taxol promotes lateral associations within and between antiparallel spindle microtubules (3, 23). In vivo, taxol not only reorders microtubules into bundles, but also alters their interactions with other cell components. In dorsal root ganglion spinal cord cultures, taxol causes microtubules to form concentric arrays alternating with layers of macromolecular material (20). When myoblasts in primary myogenic cultures from chicken are ex-

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posed to taxol, they form sarcomeres in which microtubules appear to substitute for actin (2, 39). In cultured chondroblasts treated with taxol, microtubules displace ribosomes from the surface of the rough endoplasmic reticulum and form highly ordered layers that alternate with and pack together adjacent endoplasmic reticulum sheets to form tiers of cisternae (38).

Taxol-induced bundling may represent a drug-induced stabilization of normally transitory microtubule linkage reactions. To investigate the nature of taxol-induced stable linkages, we have developed an in vitro system to form and quantitate taxol-induced microtubule bundling. We have found that taxol induces microtubule bundles through the mediation of a factor present in rat brain crude extracts. Although this factor was lost during recycling of microtubule protein, it was recovered from taxol-bundled microtubules isolated by centrifugation through a sucrose cushion. Using a sedimentation assay to quantitate the extent of microtubule bundling, we have found that purified microtubule bundles are labile to ATP in the millimolar range. They could also be dissociated by >0.4 M NaCl or by millimolar $CaCl_2$. A preliminary report of this work has been presented (41).

MATERIALS AND METHODS

Chemicals

Reagents and buffers were purchased from Sigma Chemical Co. (St. Louis, MO). The buffer designated MME,[1] used in all experiments unless noted otherwise, was composed of 100 mM 2-(*N*-morpholino)ethanesulfonic acid/1.0 mM $MgSO_4$/1.0 mM EGTA/0.02% NaN_3, pH 6.75. Taxol was obtained from the National Cancer Institute Drug Synthesis and Development Laboratory (Bethesda, MD). It was dissolved in DMSO to a stock concentration of 3.5 mM and stored at −20°C. GTP was from Boehringer Mannheim Biochemicals (Indianapolis, IN). PAGE materials were obtained from Bio-Rad Laboratories (Richmond, CA) and Eastman Kodak Co. (Rochester, NY). Phosphocellulose resin (P11) from Whatman Laboratory Products Inc. (Clifton, NJ) was prewashed in 0.5 N HCl and in 0.5 N NaOH according to manufacturer's instructions.

Methods of Microtubule Preparation[2]

RAT BRAIN CRUDE EXTRACT MICROTUBULE PREPARATION: A crude extract was prepared from adult rat brains (inbred strain W/FU and Sprague Dawley [Tyler Labs, Bellevue, WA]) as described by Margolis and Rauch (18) except that the rat brains were homogenized with MME containing 1.3 mM $CaCl_2$, after which the homogenate was placed into centrifuge tubes containing EGTA to yield a final concentration of 2.6 mM. After centrifugation, the rat brain crude extract supernatant was incubated at 30°C in MME containing 0.05 mM GTP for 30 min to assemble the microtubules (18). This microtubule preparation exhibited taxol-bundling activity and was used, after sucrose cushion sedimentation, as starting material for bundling-factor extraction.

TWICE-CYCLED RAT BRAIN MICROTUBULE PREPARATION: Rat brain crude extract microtubules were prepared as described above except that the assembly continued for 60 min. The preparation was then layered onto a 50% sucrose cushion (in MME) and centrifuged at 300,000 *g* for 90 min at 25°C in a Beckman L5-50 ultracentrifuge using a Beckman SW50.1 rotor (Beckman Instruments Inc., Palo Alto, CA). The pellets were resuspended in MME containing 2 mM $CaCl_2$, sheared by passage three times through a 25-gauge needle, and after 10 min on ice were centrifuged at 20,000 *g* for 30 min in a Sorvall RC-5B centrifuge using a Sorvall SS34 rotor (E. I. Dupont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT). The supernatant was brought to 2 mM EGTA and 0.5 mM GTP and was incubated for 30 min at 30°C to assemble the microtubules. These microtubules were used to test for taxol-induced bundling activity.

THREE-TIMES-CYCLED BEEF BRAIN MICROTUBULE PREPARATION: Beef brain microtubules were purified by two cycles of assembly and disassembly according to a glycerol assembly procedure that is a substantial modification of that described by Shelanski et al. (35). Beef brain tissue, essentially free of blood clots and meninges, was homogenized with 1 ml MME/0.5 g of tissue in a Waring blender on the lowest speed for two 45-s periods with a 15-s rest. The homogenate was centrifuged at 32,000 *g* for 40 min in a Sorvall SS34 rotor and the resulting supernatant was brought to 2 mM GTP and centrifuged at 190,000 *g* for 30 min in a Beckman 50.2 rotor. This second supernatant was diluted with MME containing 8 M glycerol, 1:1 for a final GTP concentration of 1 mM and glycerol concentration of 4 M. Microtubules assembled for 30 min at 30°C were collected by centrifugation at 190,000 *g* for 45 min at 25°C in a Beckman 50.2 rotor. The pellets were Dounce homogenized in MME with a loose-fitting pestle and incubated on ice for 20 min. The microtubule protein in the supernatant of a 190,000 *g* spin for 30 min in a Beckman 60Ti rotor was diluted with MME containing 8 M glycerol (the same volume as used to resuspend the pellets). After the addition of 1 mM GTP, the microtubule protein was assembled during a 30-min incubation at 30°C and was collected and cleaned by sedimentation through a 35% sucrose cushion (in MME) at 190,000 *g* for 2 h at 25°C in a Beckman 50.2 rotor. The pelleted, twice-cycled microtubule protein was stored in liquid N_2 until use.

Three-times-cycled beef brain microtubules were then prepared from these stored microtubule pellets. After being thawed, the pellets were Dounce homogenized in MME, left on ice for 10 min, and cleared by centrifugation at 41,000 *g* for 10 min in a Sorvall SS34 rotor. The resulting supernatant was adjusted to an approximate tubulin concentration of 35 μM and the third cycle microtubules were assembled for 30 min at 30°C with 0.5 mM GTP. These microtubules were also tested for taxol-induced bundling activity.

PHOSPHOCELLULOSE-PURIFIED MICROTUBULE PREPARATION: Purified tubulin was prepared from microtubule protein essentially according to the procedure of Weingarten et al. (43). Three-times-cycled beef brain microtubules prepared as described above were collected by centrifugation (42,000 *g*, 25°C, 30 min in a Beckman 70.1Ti rotor), Dounce homogenized in MME with a loose-fitting pestle, kept on ice for 10 min, and cleared by centrifugation at 41,000 *g* for 10 min a a Sorvall SS34 rotor. The supernatant was placed on a phosphocellulose column equilibrated with MME (not >3.5 mg protein/ml column bed volume) and was eluted with MME. Pure tubulin was located in the elution profile by optical density readings at 280 nm using a Varian Cary 219 spectrophotometer (Varian Associates, Instrument Div., Palo Alto, CA). The most concentrated fractions were assembled in MME modified to contain 10% DMSO, 16 mM $MgCl_2$, and 1 mM GTP for 30 min at 30°C following on the procedures of Himes et al. (11) and Lee and Timasheff (15). This preparation was either tested for taxol-bundling capacity or was pelleted (42,000 *g*, 25°C, 30 min in a Beckman 70.1Ti rotor) and stored in liquid N_2 until use in experiments in which a salt-released fraction from taxol-bundled microtubules was assembled with pure tubulin.

Analytical Methods

Slab gel electrophoresis in an 8% polyacrylamide and 0.1% sodium lauryl sulfate system was performed as described by Margolis and Rauch (18) using the conditions described by Sheir-Neiss et al. (30). Microtubule preparations were examined using a JEOL JEM-100S electron microscope (Japanese Electronic Optics Limited, Tokyo, Japan) according to published procedures (19) except that the samples were resuspended in MME containing 50% sucrose. Total protein was determined in triplicate by the method of Lowry et al. (16) using BSA as a standard; optical densities were measured using a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA) with a 600-nm filter.

RESULTS

Taxol Bundles Rat Brain Crude Extract Microtubules In Vitro

Rat brain crude extract microtubules, treated in vitro with taxol, become stabilized against depolymerizing conditions. However, they are more than merely locked in their polymerized state. When centrifuged through a sucrose cushion, these microtubules sediment at a much faster rate than untreated microtubules.

To precisely determine the differences in the sedimentation rates, we assembled rat brain crude extract microtubules to equilibrium (18) and then incubated half the sample with 35 μM taxol in 1% DMSO, and the control half in 1% DMSO alone. As shown in Fig. 1, *top*, the $t_{1/2}$ for pelleting the taxol-

[1] *Abbreviations used in this paper:* MME, a buffer composed of 100 mM 2-(*N*-morpholino)ethanesulfonic acid/1.0 mM $MgSO_4$/1.0 mM EGTA/0.02% NaN_3, pH 6.75.

[2] All procedures in the following section were carried out at 0–4°C unless otherwise noted.

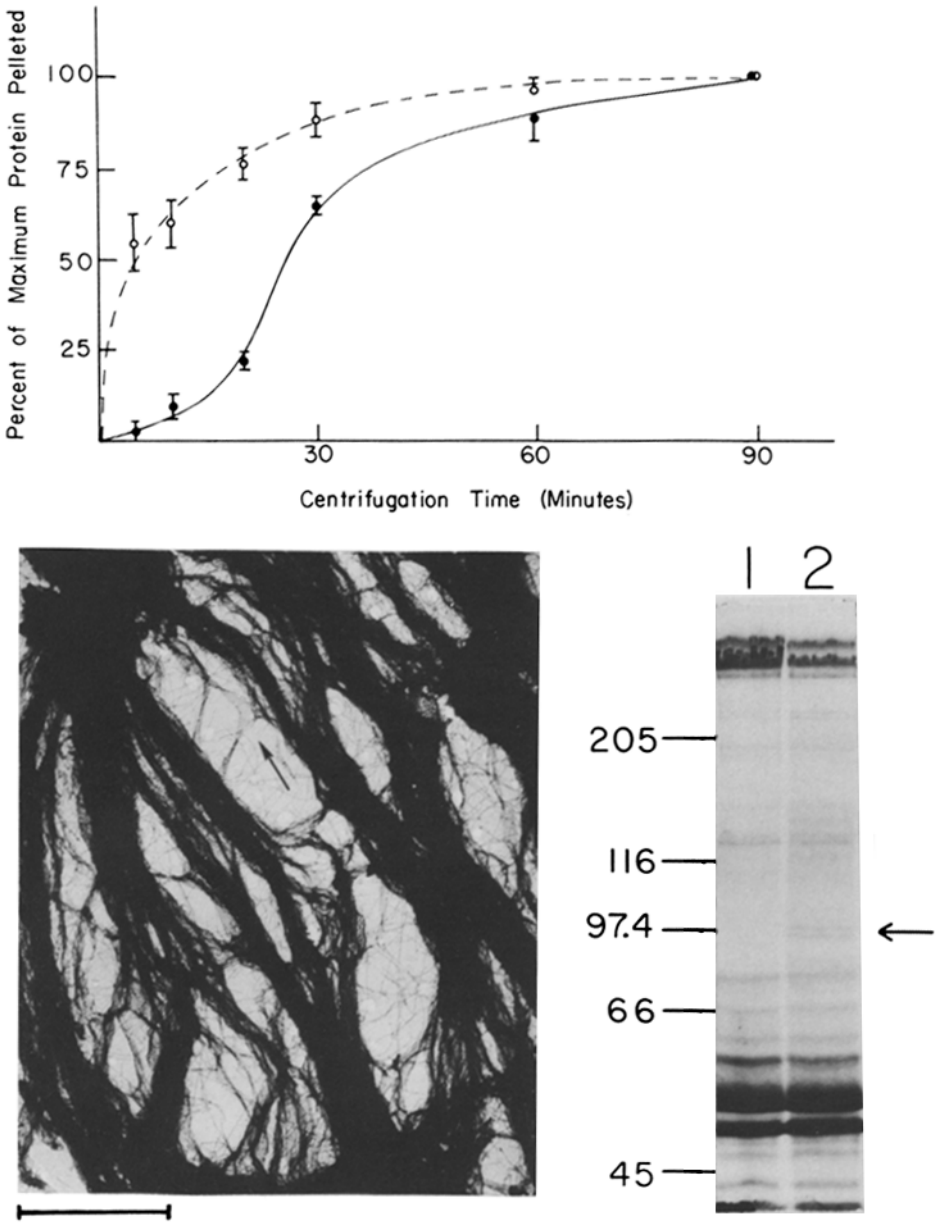

FIGURE 1 Effect of taxol on rat brain crude extract microtubules. (*top*) Rat brain crude extract microtubules prepared as described in Materials and Methods were incubated for 20 min at 30°C with 35 μM taxol (equimolar to tubulin) in 1% DMSO (○) or with 1% DMSO alone (●). Each sample was centrifuged through a 35% sucrose cushion at 150,000 *g*, 25°C, for the times indicated in a SW50.1 Beckman rotor. The pelleted microtubules were resuspended in 2% sodium lauryl sulfate and the total protein in each pellet was determined. Results expressed as a percent of the amount of protein pelleted in 90 min represent $\bar{\chi} \pm$ SEM for three experiments. (*lower left*) Electron microscopy was performed on each sample before the centrifugation through sucrose described above. The result of the taxol-treated sample is shown. The arrow points to a single microtubule. Bar, 10 μm. (*lower right*) PAGE was performed on the control (lane *1*) and taxol-treated microtubules (lane *2*) after pelleting through sucrose as described above. 150 μg protein was loaded onto each lane to increase the intensity of the bands between the high molecular weight microtubule-associated proteins and α- and β-tubulin. The arrow points to a doublet band which appeared specific to the taxol-bundled microtubules. Scale at the left indicates approximate molecular weights × 10^{-3}.

treated samples through a sucrose cushion was ~20% of the control. To account for this change in the sedimentation profile, taxol must have induced a dramatic increase in the mass of the microtubule structure. Electron microscopic examination of a sample of these microtubules taken before pelleting through sucrose showed that taxol caused these microtubules to form massive bundles and interlinking cables of bundles (Fig. 1, *lower left*). If taxol induced unique protein associations with microtubules to form these bundles, they might appear as an extra band or an intensified band in a polyacrylamide gel. Electrophoretic analysis of the control and taxol-treated microtubules, pelleted through sucrose cushions, on 8% polyacrylamide slab gels revealed no striking differences between the two samples (Fig. 1, *lower right*). However, a few minor bands (arrow in Fig. 1, *lower right*) appeared specific to the taxol-bundled microtubules. Since linkers need not be abundant to be effective, these minor changes may be meaningful. The most abundant unique polypeptides were two bands migrating at ~100,000 daltons. It should be noted that, as judged by gel analysis, taxol-induced cross-linking did not involve changes in actin or intermediate filament associations with microtubules.

The observation that taxol-bundled microtubules sediment five times faster through a sucrose cushion than control microtubules suggested a simple procedure to assay the extent of microtubule bundling. It was apparent (Fig. 1, *top*) that taxol induces a large difference in the percent of microtubule protein pelleted after 10 min of centrifugation, compared with that pelleted at 90 min. This difference in sedimentation at 10 min was reproducible. Unbundled microtubules sedimented 0.14 ± 0.03 ($\bar{\chi} \pm$ SEM, $n = 7$) of the 90-min value within 10 min, whereas bundled microtubules were 0.74 ± 0.02 ($n = 32$) pelleted by 10 min. Use of a 10 min/90 min pelleting ratio thus yielded a convenient sedimentation assay for the extent of bundling, and this assay was employed where appropriate.

Taxol Does Not Bundle Microtubules Assembled from Purified or Recycled Microtubule Protein

Having found that taxol-bundled microtubules assembled from a crude extract of rat brain, we proceeded to determine whether bundling could be induced in more purified microtubule systems. Pure tubulin, isolated from recycled microtubule protein by phosphocellulose column chromatography (43), was assembled into microtubules and then tested for taxol-induced bundling by the sedimentation assay. The centrifugation profile of microtubules composed of pure tubulin (Fig. 2, the dashed line represents the sedimentation profile of taxol-bundled rat brain crude extract microtubules) showed clearly that taxol-induced bundling required a factor not associated with pure tubulin. An assay with beef brain microtubule protein, purified by three cycles of assembly and disassembly (35), showed (Fig. 2) that bundling did not occur in recycled microtubules. To test whether bundling required a microtubule-associated protein unique to rat brain, twice-cycled rat brain microtubules were also tested, and also failed to produce taxol-induced bundling (Fig. 2). Therefore, a factor was present in the rat brain crude extract that enabled taxol to bundle the microtubules, and this factor was lost on recycling of the microtubule protein.

Effect of ATP on Taxol-induced Microtubule Bundling

Since many interactions between microtubules and organelles (34, 36), cytoskeletal components (8, 28, 29, 33), or other microtubules (36) are affected by ATP, we tested for the effects of ATP on taxol-bundled rat brain crude extract microtubules. To do this, microtubules were assembled, exposed to taxol, and incubated with increasing concentrations of ATP for 30 min. As shown in Fig. 3*a*, the microtubule sedimentation profile changes on exposure to ATP in the millimolar

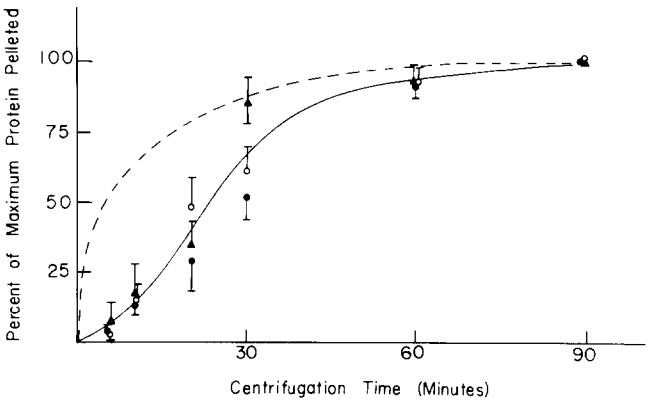

FIGURE 2 Effect of taxol on microtubules prepared from purified or recycled microtubule protein. Phosphocellulose purified microtubules (▲), three-times-cycled beef brain microtubules (○), and twice-cycled rat brain microtubules (●) were prepared as described in Materials and Methods. Each preparation was tested for the capacity to bundle with taxol as described in Fig. 1. The results of the taxol-treated samples ($\bar{x}$ ± SEM for three experiments) are compared with the sedimentation profile of taxol-bundled rat brain crude extract microtubules from Fig. 1, *top* (dashed line).

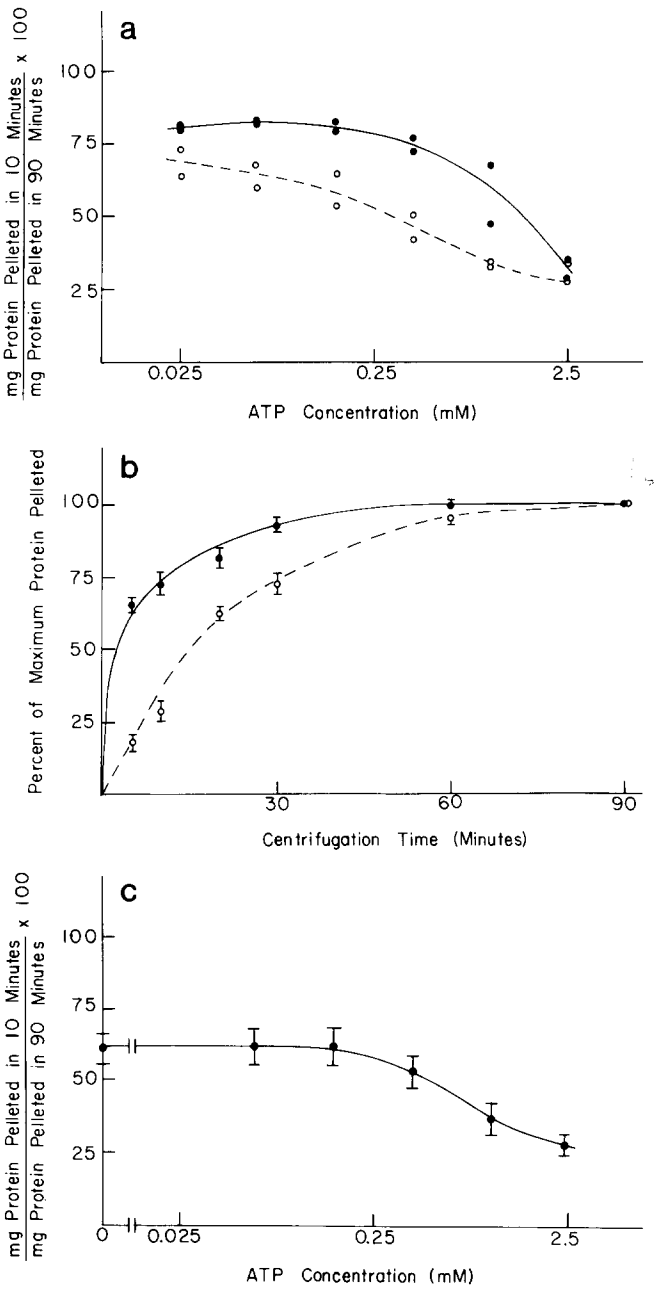

FIGURE 3 Effect of ATP on taxol-bundled rat brain crude extract microtubules. Rat brain crude extract microtubules were prepared as described in Materials and Methods and bundled with 35 μM taxol as in Fig. 1. (*a*) Taxol-bundled microtubules were incubated in increasing concentrations of ATP as indicated for 30 min (●) or 10 min (○) at 30°C. The extent of microtubule bundling remaining after the incubation was gauged by comparing the protein pelleted through a 35% sucrose cushion in 10 min with that pelleted in 90 min at 25°C and 150,000 *g* in a Beckman SW50.1 rotor (see text for discussion of this comparative sedimentation assay). Two experiments were performed with each ATP incubation period. (*b*) Taxol-bundled microtubules were incubated with (○) or without (●) 2.5 mM ATP for 30 min at 30°C and were centrifuged through sucrose as described in Fig. 1. Results expressed as in Fig. 1, *top* represent $\bar{x}$ ± SEM for three experiments. (*c*) Taxol-bundled microtubules were isolated through a 35% sucrose cushion at 150,000 *g* for 10 min at 25°C in a Beckman SW50.1 rotor. The pelleted microtubules were resuspended in MME containing 35 μM taxol and increasing concentrations of ATP as indicated for 30 min at 30°C. After this incubation, the samples were pelleted through 35% sucrose at 150,000 *g* for 10 and 90 min at 25°C in a Beckman SW50.1 rotor. Results expressed as the percent protein pelleted in 10 min compared with that pelleted in 90 min represent $\bar{x}$ ± SEM for four experiments.

range; this indicated a loosening of the bundled structure. The effective ATP concentration was surprinsingly high. However, a repeat of the experiment with an ATP incubation period of 10 min (Fig. 3*a*) showed that under these conditions, ATP relaxed microtubule bundles at lower concentrations. In these experiments, the true ATP concentration undoubtedly differed from the added concentrations, due to contributions from cytosolic ATP and due to enzymatic hydrolysis. Perhaps the difference observed with the two incubation times may be attributed to cytosolic enzymes present in the crude extract that reverse the ATP effect at later times. To verify that higher concentrations of ATP were required to relax the microtubule bundles, we present a complete sedimentation profile of the taxol bundled microtubules incubated with or without 2.5 mM ATP for 30 min (Fig. 3*b*). It is apparent that taxol-bundled microtubules incubated with 2.5 mM ATP have returned to a sedimentation profile similar to that for microtubules that have not been exposed to taxol (compare Fig. 3*b*, open circles, and Fig. 1, *top*, closed circles). To assure that the ATP effect is not due to chelation of Mg^{++} from the buffer at high ATP concentrations, we also performed the indicated experiments in MME buffer supplemented with 5 mM $MgCl_2$, with no difference in result.

Taxol-bundled microtubules, isolated as pellets through sucrose cushions from rat brain crude extracts, when gently resuspended in a fully bundled state, resedimented through a second sucrose cushion as rapidly as through the first. In such resuspensions, the ATP effect on taxol-bundled microtubules could be tested without interference from cytosolic enzymes. Resuspended bundled microtubules were therefore exposed to increasing concentrations of ATP and repelleted. The ratio of sedimentation times (Fig. 3*c*) clearly indicates that ATP-dependent relaxation occurred in this more purified system, and confirms that relatively high concentrations of ATP were required to effect a relaxation. In careful time course experiments (data not shown) we found that relaxation was substantially completed by 30 min at 2.5 mM ATP, but that an additional slow component continued beyond this time.

Extraction of the Bundling Factor from Rat Brain Crude Extracts

We have shown (Fig. 3*c*) that taxol-bundled microtubules remain bundled on a second sedimentation run, and that resuspended pellets of bundled microtubules respond to ATP by relaxing. Although the bundling factor does not recycle quantitatively with microtubule protein, it is apparently retained on taxol-stabilized microtubules. Taxol-stabilized microtubules can be treated with high salt to specifically extract the microtubule-associated proteins from salt-resistant polymers (42). We therefore exposed taxol-bundled microtubules to increasing salt concentrations and tested for retained bundling activity by sedimentation assay. As shown in Fig. 4, *top*, NaCl concentrations >0.4 M increasingly release microtubule bundling; at concentrations >1 M, the sedimentation rate was reduced to that of microtubules not exposed to taxol. When 2 M NaCl was used, most of the associated proteins were released from the taxol-stabilized microtubules (Fig. 4, *bottom*). Proteins (or factors) that are responsible for bundling would not necessarily release from microtubules when their bundling activity is lost. Nonetheless, it is clear that little bundling activity was lost after exposure to 0.4 M NaCl even though the bulk of the associated proteins was displaced from

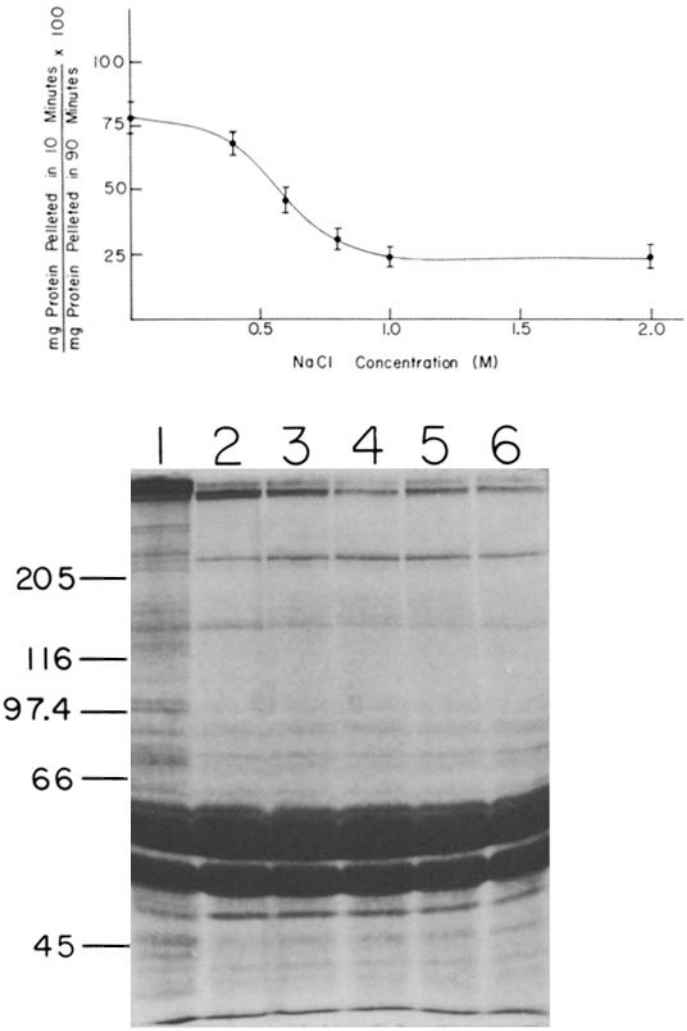

FIGURE 4 Effect of NaCl on taxol-bundled rat brain crude extract microtubules. Rat brain crude extract microtubules were prepared as described in Materials and Methods and bundled with 35 μM taxol as in Fig. 1. (*top*) The taxol-bundled microtubules were incubated in NaCl at the concentrations indicated for 30 min at 30°C. The extent of bundling that remained after the salt incubation was determined as described in Fig. 3*a*. Results represent $\bar{\chi} \pm$ SEM for four experiments. Similar results were obtained with bundled microtubules extracted with $CaCl_2$ in the millimolar range (see text). (*bottom*) PAGE was performed on the NaCl-treated samples (*top*) after they were pelleted through sucrose (lane *1*, 0.0 M NaCl; lane *2*, 0.4 M; lane *3*, 0.6 M; lane *4*, 0.8 M; lane *5*, 1.0 M; lane *6*, 2.0 M). 250 μg protein was loaded on each lane to increase the intensity of the bands between the high molecular weight microtubule-associated proteins and α- and β-tubulin. The scale at the left indicates approximate molecular weights $\times 10^{-3}$.

the polymers at this salt concentration. We also note that the protein doublet of ~100,000 daltons, which is unique to taxol-bundled microtubules (Fig. 1*c*), was almost quantitatively eluted at 0.4 M NaCl, whereas bundling was almost quantitatively retained. This result does not rule out the 100,000-dalton protein doublet as the active species, however, since a small residual population can still display substantial bundling activity. In both the salt treatment and ATP experiments, the loss of bundling, as determined by the sedimentation assay, was confirmed by electron microscopy analysis (not shown). Similar experiments performed with increasing $CaCl_2$ concen-

trations showed that Ca^{++} in the millimolar range relaxes microtubule bundles and releases substantial amounts of associated proteins from the taxol-bundled microtubules (data not shown).

To test if the bundling factor, pelleted with taxol-bundled microtubules, could be salt eluted in active form, an add-back experiment was performed. We assembled microtubules composed of phosphocellulose-purified tubulin and proteins that were salt released from sucrose cushion–isolated microtubule bundles, and these microtubules were tested for their capacity to bundle with taxol. We found that the protein fraction isolated with NaCl from taxol-bundled microtubules retained the capacity to induce microtubule bundles (Fig. 5) and that this bundling activity was still strictly dependent on the presence of taxol.

DISCUSSION

Taxol has two major effects in cells: it stabilizes microtubules (7, 32) and it induces microtubule bundling (7, 25, 32). Microtubule stabilization can readily be reproduced in vitro (31), even with pure tubulin (14, 26). However, extensive microtubule bundling induced by taxol has not previously been reported in vitro. (A recent report by Albertini et al. [1] indicates a small extent of bundling of recycled microtubules by taxol.) The reason is now clear; taxol-induced bundling requires a factor(s) present in the crude extract of brain tissue which is lost during recycling of microtubule protein. Taxol binds to tubulin (17, 26) and is not known to bind to microtubule-associated proteins. Therefore, it is possible that taxol induces a conformational change in tubulin that alters the binding stability of an associated linker protein.

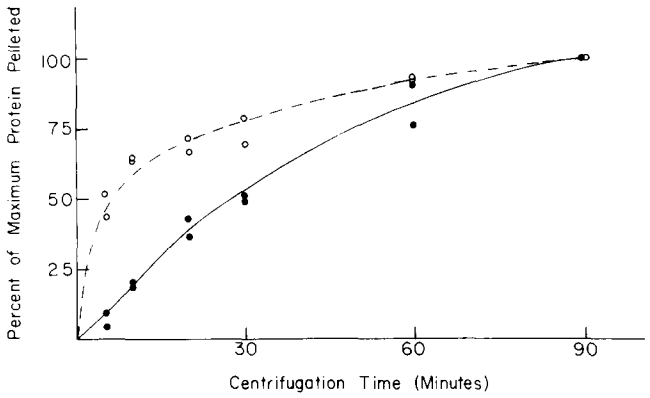

FIGURE 5 Effect of taxol on microtubules assembled from a mixture of phosphocellulose-purified tubulin and a salt extract from taxol-bundled microtubules. Rat brain crude extract microtubules prepared as described in Materials and Methods were bundled with 35 μM taxol as in Fig. 1 and isolated through 35% sucrose at 150,000 *g* for 60 min at 25°C in a Beckman SW50.1 rotor. The isolated bundles were washed with MME containing 35 μM taxol and collected by centrifugation at 41,000 *g* for 30 min at 4°C in a Sorvall SS34 rotor. The pellet was resuspended with MME containing 2 M NaCl and 35 μM taxol and incubated for 30 min at 30°C. All the proteins released by the salt were separated from the taxol-stabilized microtubules by centrifugation at 41,000 *g* for 30 min at 4°C in a Sorvall SS34 rotor. After the salt was removed from the supernatant with a G-25 Sephadex column, the protein eluate was used to resuspend pelleted phosphocellulose-purified tubulin prepared as described in Materials and Methods. After homogenization with a loose-fitting pestle, incubation on ice for 20 min, and centrifugation at 41,000 *g*, 4°C for 10 min, the supernatant, adjusted to a tubulin/microtubule-associated protein ratio of approximately 3:1, was assembled with 1 mM GTP for 30 min at 30°C. The assembled microtubules were then tested for the capacity to bundle with taxol as described in Fig. 1. (●) control; (○) taxol-treated microtubules. Two experiments were performed as shown.

Changes in associated proteins are reproducibly seen in taxol cross-linked microtubules, but the role of these unique proteins will be determined only by purification. We have developed procedures for the solubilization and assay of cross-linking factors, and these methods will allow purification of the active moieties. One should note that a bifunctional cross-linking molecule needn't be abundant to form stable microtubule-microtubule associations. It may therefore be too small a fraction of the total associated proteins to be observable on gels. The unique protein doublet of ~100,000 daltons associated with taxol-stabilized crude extract microtubules (Fig. 1, *top*, arrow) is possibly associated with cross-linking although it is greatly reduced by salt extraction when cross-linking is still retained. In all other cases we have examined, cross-linking appears correlated with the presence of the 100,000-dalton doublet.

The in vitro bundling of brain microtubules has been reported to occur in three systems not using taxol. Clustering of three-times-cycled chick brain microtubules has been reported to be mediated by microtubule-associated proteins (36). We report here that microtubules formed from either beef brain or rat brain recycled microtubule protein do not bundle; however, we have not as yet assayed for bundling after the addition of cyclic AMP and ATP to these microtubules. Bundling of microtubules composed of porcine tubulin has been reported to occur with either a high molecular weight protein from squid axon (24) or with fodrin (13), a spectrin-like high molecular weight protein from mammalian brains which cross-links actin filaments in vitro (4). Bundling with these linkers has been reported to be severely attenuated when using microtubules containing associated proteins, but the taxol dependency of bundling induced by these factors has not been tested in that system.

In our study we have used a sedimentation assay that clearly distinguishes cross-linked microtubules as a rapidly sedimenting population, and can therefore be used to measure the extent of cross-linking. However, although sedimentation is used to gauge microtubule bundling, a linear relation between the extent of bundling and sedimentation time has not been demonstrated, nor has it been implied. The relation is quite likely to be highly complex since it depends on the relative packing density of the bundles formed. Nonetheless, it can be reproducibly affirmed that after taxol treatment microtubules both bundle extensively (Fig. 1, *lower left*) and sediment more rapidly than nontreated control microtubules (Fig. 1, *top*), which indicates that the assay is a valid measure of bundling. The studies just mentioned, which use high molecular weight proteins to bundle microtubules (13, 24), have used turbidity change as a measure of cross-linking. In those studies, it is not clear whether this change is due to a cross-linking rather than to changes in the assembly state. (Changes in the assembly state are not a factor in our experimental measurements since the assembly state of taxol-stabilized microtubules does not change [31, 42].) We have routinely observed, in the course of these experiments, extensive changes in sedimentation rate accompanied by minimal changes in turbidity. Therefore, we question the validity of turbidity measurements as a gauge of cross-linking. It remains to be determined if the taxol-induced cross-linking is due to any of the cross-linking

agents previously described or to a unique factor.

It is possible that part of the pathology of taxol poisoning is related to the stabilization of normally transitory bonds between microtubules. We are proceeding to isolate the responsible factors and to determine their in vivo functions. Although we have not yet identified the agent that mediates taxol-induced cross-linking, our observation that the taxol-stabilized bundling is ATP labile suggests a physiological regulatory pathway for cross-linking. This is no surprise, since most microtubule linkage systems thus far described have been ATP sensitive (8, 28, 29, 33, 34, 36). ATP lability may indicate either a phosphorylation-sensitive linkage or an ATPase dependent cycling linkage, as described for dynein-microtubule interactions in flagellar motility (6). We do not yet know by what mechanism taxol-induced microtubule bundling is regulated, but we do know that the time course of bundle relaxation by ATP is quite long (in excess of 30 min with 2.5 mM ATP, data not shown). If the linkage is phosphorylation sensitive, relaxation may require a long time because the linker protein in the innermost part of a bundle cannot be released by phosphorylation until the outermost sections of the bundle have been phosphorylated. It is interesting that we find high ATP concentrations are required to relax microtubule bundles. These concentrations are similar to those recently reported to be necessary to release cytoplasmic dynein bonds on microtubules (12). It is possible that taxol-stabilized cross-links involve cytoplasmic dynein which does not ordinarily cycle with microtubules in vitro. If relaxation of taxol-bundled microtubules occurs by a cycling ATPase, the long time course may be due to the parallel sliding of microtubules over long distances. The purification of the cross-linking factor(s) mediating taxol-induced microtubule bundling will help determine how the bundles form and are regulated by ATP.

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