Taxol Stabilization of Microtubules in Vitro: Dynamics of Tubulin Addition and Loss at Opposite Microtubule Ends†

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ABSTRACT: We have investigated the effects of taxol on steady-state tubulin flux and on the apparent molecular rate constants for tubulin addition and loss at the two ends of bovine brain microtubules in vitro. These microtubules, which consist of a mixture of 70% tubulin and 30% microtubule-associated proteins (MAPs), undergo a net addition of tubulin at one end of each microtubule (A end) and a precisely balanced net loss of tubulin at the opposite end (D end) at steady state in vitro. They do not exhibit to a detectable extent the "dynamic instability" behavior described recently for MAP-free microtubules, which would be evident as an increase in the mean microtubule length and a decrease in the number of microtubules in the suspensions [Mitchison, T., & Kirschner, M. (1984) *Nature (London) 312*, 237–242]. We used a double-label procedure in which microtubules were labeled with tritium and carbon-14 at A ends and carbon-14 at D ends to distinguish the two ends, combined with a microtubule collection procedure that permitted rapid and accurate analysis of retention of the two labels in the microtubules. We found that taxol slowed the flux of tubulin in a concentration-dependent manner, with 50% inhibition occurring between 5 and 7 μM drug. The effects of taxol on the apparent molecular rate constants for tubulin addition and loss at the two microtubule ends were determined by dilution analysis at an intermediate taxol concentration. The results indicated that taxol decreased the magnitudes of the dissociation rate constants at the two ends to similar extents, while exerting little effect on the association rate constants. The data indicate that taxol binding to the microtubule surface stabilizes microtubules by strengthening intertubulin bonding uniformly throughout the polymer.

Taxol, a macrocyclic alkaloid from the western yew *Taxus brevifolia*, is an unusual antimitotic agent of potential antineoplastic utility (Wani et al., 1971; Fuchs & Johnson, 1978). Other antimitotic drugs such as colchicine and vinblastine block formation of microtubules and also cause depolymerization of microtubules in cells [see Dustin (1978)]. In contrast, taxol appears to stabilize microtubules both in cells (Schiff & Horwitz, 1980; deBrabander et al., 1981; Heidemann & Gallis, 1980) and in vitro (see below). It also causes microtubules to aggregate laterally (called "bundling") both in vivo and in vitro (Albertini et al., 1984; Turner & Margolis, 1984), and in cells causes them to aggregate laterally with other cellular components (Antin et al., 1981). These unique properties have stimulated considerable interest in the action of the drug.

In vitro, taxol stabilizes microtubules against the depolymerizing action of dilution, cold, calcium ions, and a number of microtubule-disrupting drugs (Schiff et al., 1979; Parness & Horwitz, 1981; Kumar, 1981). Initiation of microtubule polymerization is enhanced by taxol, and the critical subunit concentration is reduced (Schiff et al., 1979). Taxol can even induce microtubule polymerization at 0 °C (Thompson et al., 1981). Recent studies of Kumar (1981) in which the rate of tubulin exchange was reduced approximately 5-fold at the ends of microtubules assembled from pure tubulin in the presence of taxol as compared with microtubules assembled from tubulin and microtubule-associated proteins (MAPs)[1] without taxol support the idea that taxol inhibits steady-state tubulin flux. However, the results are inconclusive, because assembly of tubulin induced by taxol was compared to assembly induced by MAPs, and no length measurements were made to determine the concentrations of microtubule ends (number concentration) in the two preparations or to determine whether the microtubule length distributions remained constant during the experiment. On the basis of a recent study on the effects of taxol on the incorporation of [^{3}H]guanine nucleotide labeled tubulin into microtubules formed in the absence of microtubule-associated proteins, Caplow & Zeeberg (1982) have suggested that the effect of taxol on microtubule polymerization might result from the ability of the drug to reduce the molecular rate constants for the loss of tubulin subunits at both microtubule ends to equivalent extents without affecting the association rate constants and without affecting directionality (flux efficiency).

In the present study, we have investigated the effects of taxol on the exchange of tubulin at the opposite ends of bovine brain microtubules at steady state, and on the apparent off rate constants at the two microtubule ends by dilution-induced disassembly. We have employed a MAP-containing microtubule protein preparation which exhibits tubulin flux in vitro, i.e., net tubulin addition at one end of each microtubule (assembly or A end) and a balanced net loss of tubulin at the opposite end (disassembly or D end). These microtubule

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[1] Abbreviations: MAPs, microtubule-associated proteins; Me_2SO, dimethyl sulfoxide; GTP, guanosine 5′-triphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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populations do not exhibit "dynamic instability" behavior to a detectable extent at steady state as reported recently for certain MAP-free microtubule preparations (Mitchison & Kirschner, 1984a,b). We utilized a taxol treatment protocol that did not appreciably change the concentration of microtubule ends as compared with untreated microtubules and employed a double-label protocol combined with a rapid filter assay to measure the effects of taxol on steady-state net tubulin addition and loss rates and on the apparent rate constants for tubulin addition and loss at opposite microtubule ends. The results indicate that taxol inhibits steady-state tubulin flux and causes an increase in microtubule stability by reducing the rate constants for tubulin loss at both microtubule ends to equivalent extents as suggested by Caplow & Zeeberg (1982), with little or perhaps no effect on the rate constants for tubulin gain at either end.

Materials and Methods

Preparation of Bovine Brain Microtubule Protein. Bovine brain microtubule protein consisting of approximately 70% tubulin and 30% microtubule-associated proteins (MAPs) was isolated without glycerol by modification [see Farrell & Wilson (1984)] of the procedure of Asnes & Wilson (1979) and stored frozen as microtubule pellets at −80 °C until used. For reassembly experiments, microtubule pellets were resuspended by Dounce homogenization in 100 mM MES, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.8 (MEM reassembly buffer), and after 15-min incubation at 0 °C, the solutions were centrifuged at 39500*g* for 20 min at 4 °C (SS-34 rotor, Sorvall). The resulting supernatants were used in all experiments and contained a GTP-regenerating system consisting of 10 mM acetyl phosphate and 0.1 IU/mL acetate kinase (MacNeal et al., 1977). All experiments were carried out at 30 °C. After reassembly to steady state, the microtubule polymer mass and the mean microtubule lengths remained constant during the course of the experiments (3 h) (see Discussion).

Preparation of Microtubules Differentially Labeled with [^{3}H]- and [^{14}C]Guanine Nucleotides: Determination of Steady-State Flux Rates. To determine simultaneously the rate of tubulin gain at A ends and loss at D ends of taxol-treated microtubules at steady state, we prepared microtubules differentially labeled with [^{3}H]- and [^{14}C]guanine nucleotide (Farrell & Jordan, 1982; Jordan & Farrell, 1983). The procedure makes use of the observation that labeled GTP binds exchangeably to soluble tubulin and the labeled guanine nucleotide becomes nonexchangeable upon incorporation of the tubulin into microtubules (Weisenberg et al., 1976; Margolis & Wilson, 1978). At steady state in vitro in the presence of GTP, net tubulin addition occurs at one microtubule end, operationally defined as the net assembly or A end, and net loss of tubulin occurs at the opposite end, operationally defined as the net disassembly or D end (Wilson & Margolis, 1978; Jordan & Farrell, 1982; Farrell & Wilson, 1984). When microtubule protein is polymerized to steady state in the presence of [^{14}C]GTP, the microtubules become uniformly labeled with [^{14}C]guanine nucleotide. Upon pulsing of the ^{14}C-labeled steady-state microtubules with [^{3}H]GTP, the [^{3}H]guanine nucleotide becomes incorporated at A ends and serves as the marker for A-end net tubulin addition, while the [^{14}C]guanine continues to be added at A ends and lost from D ends in an unaltered fashion. The rates of A-end ^{3}H-nucleotide and D-end [^{14}C]guanine nucleotide loss are then determined by addition of a 10.5-fold unlabeled GTP chase, after which the microtubules are collected on glass fiber filters (Wilson et al., 1982) for analysis of label incorporation, protein content, and microtubule number concentration (Margolis & Wilson, 1978; Jordan & Farrell, 1983; Farrell & Wilson, 1984).

Microtubules were initially labeled with [^{14}C]guanine nucleotide by polymerizing them to steady state in the presence of [^{14}C]GTP (final concentration of GTP 200 μM, 2–4 μCi/mL; 3–8 Ci/mol of GTP final specific activity). After drug addition (see below), a trace quantity of [^{3}H]GTP was added to the ^{14}C-labeled microtubules to initiate the [^{3}H]-guanine nucleotide pulse for A-end uptake (20–60 μCi/mL, 200–234 Ci/mol of GTP final specific activity; the final GTP concentration was negligibly altered) (see Discussion for an analysis of the contribution of equilibrium exchange to labeled dimer incorporation). A 10.5-fold excess of unlabeled GTP was added approximately 40 min after initiation of the [^{3}H]GTP pulse to serve as a chase for both isotopes.

Steady-state rates of tubulin incorporation at A ends were calculated from linear regression lines of the rates of [^{3}H]GTP uptake into steady-state and taxol-treated microtubules from 5 to 45 min after label addition and were corrected for microtubule number concentration. Data are corrected throughout the ^{3}H-nucleotide pulse for the presence of short microtubules which, if present, would become labeled at both ends during the [^{3}H]GTP pulse (any microtubules shorter than 1.04 μm in length under the conditions used in this study). Similarly, steady-state rates of tubulin loss at microtubule D ends were calculated from linear regression analyses of the rates of [^{14}C]GTP loss from steady-state and taxol-treated microtubules after being chased with excess unlabeled GTP (corrected for number concentration). Loss rate data were also corrected for the presence of any short microtubules, which would have lost all [^{14}C]guanine nucleotide during the GTP chase.

Method for Addition of Taxol to Steady-State Microtubules. It was not possible to construct double-labeled microtubules prior to adding taxol, because the additional assembly produced by taxol could occur at both microtubule ends and result in addition of [^{3}H]guanine nucleotide at microtubule D ends. Microtubules also could not be assembled from solutions of microtubule protein in the presence of taxol, because the drug would cause a large increase in the formation of short microtubules, thereby significantly increasing the microtubule number concentration as compared with control microtubules. Thus, it was necessary to assemble microtubules in the presence of [^{14}C]GTP first, then add taxol to the steady-state microtubules, permit the microtubules to relax to a new plateau, and then add the [^{3}H]GTP pulse. For this protocol to work, the microtubules must be treadmilling in the presence of the drug (see Results and Discussion). It was also necessary to use high total microtubule protein concentrations so that a high proportion of the microtubule protein would already be assembled prior to taxol addition. This procedure minimized taxol-induced changes in the quantity of polymer mass and in microtubule number concentration.

The stoichiometry of guanine nucleotide incorporation varied from microtubule preparation to preparation between 0.4 and 0.8 mol of guanine nucleotide per mole of tubulin in microtubules and was always constant within the same microtubule preparation. The stoichiometry of labeled guanine nucleotide incorporation was unaffected by treatment of microtubules with taxol. For example, in one control experiment, aliquots of a bovine brain microtubule protein preparation at 4.0 mg/mL were assembled to steady state in the absence or presence of 5 μM taxol at 30 °C with [^{3}H]GTP. After 60 min of incubation, the two samples were layered onto cushions of 50% sucrose in 4 mL of MEM assembly buffer and centrifuged

at 30 °C for 1 h at 50000 rpm (Beckman SW 50.1 rotor) (Margolis & Wilson, 1978). Analysis of label incorporation revealed that microtubules assembled without taxol contained 0.67 mol of labeled guanine nucleotide per mole of tubulin in the microtubules, while the microtubules assembled in the presence of taxol contained 0.71 mol of guanine nucleotide per mole of tubulin in microtubules. Addition of taxol to microtubules already containing labeled nucleotide similarly had no effect on the stoichiometry of the labeled nucleotide.

Estimation of Apparent Dissociation Rate Constants. The method and assumptions for estimating apparent dissociation rate constants at opposite microtubule ends by dilution analysis after differentially labeling with ^{3}H- and ^{14}C-nucleotides have been discussed in detail previously (Farrell & Jordan, 1982; Jordan & Farrell, 1983). Apparent rate constants for tubulin dissociation from both ends of microtubules were calculated from the initial rates of loss of labeled nucleotides following a 100-fold dilution of polymers from steady state. Aliquots (0.3 mL) of steady-state polymers were added to 29.7 mL of warm reassembly buffer containing 0.2 mM unlabeled GTP plus the GTP-regenerating system and rapidly mixed. Samples (3 mL) were then removed at 15-s intervals during the next 90 s, stabilized in 17 mL of stabilizing buffer (see below) at 30 °C, and processed by glass fiber filter assay. Dimer loss from microtubule A ends was calculated from the ^{3}H-nucleotide remaining in microtubules during depolymerization. Loss from both polymer ends was similarly determined from the ^{14}C-nucleotide data from which D-end loss was obtained by subtraction of A-end loss. Estimates of the association rate constants at both microtubule ends of control- and taxol-treated microtubules were calculated by using the measured apparent dissociation rate constants of control microtubules and microtubules treated with 5 μM taxol, flux rates measured in the presence and absence of 5 μM taxol, and critical subunit concentrations measured in the presence and absence of 5 μM taxol, according to the equation:

$$k_+ = (k_- + \phi)/C_c$$

where k_- is the dissociation rate constant, k_+ is the association rate constant, ϕ is the steady-state flux rate, and C_c is the critical subunit concentration. Because the initial rate of microtubule disassembly induced by dilution is not a linear function of the initial concentration of unassembled tubulin (Farrell et al., 1983; Carlier et al., 1984), the dissociation constants reported should be regarded only as apparent constants. They do not necessarily indicate the values of the rate constants for tubulin loss at microtubule ends under steady-state conditions. The calculated apparent association rate constants are to be viewed similarly. The values reported are intended solely to illustrate the relative effects of taxol on microtubule dynamics at the two ends.

Filtration Assay for Analysis of Net Tubulin Addition to Microtubule A Ends, Net Loss from D Ends, and Steady-State Flux. The filtration assay for determination of labeled GTP incorporation into and loss from microtubules during assembly and steady-state treadmilling was modeled after the technique developed originally by Maccioni & Seeds (1978) and has been described in detail by Wilson et al. (1982). Briefly, aliquots (40 μL) of the radiolabeled microtubule suspension were diluted into 4 mL of microtubule stabilizing buffer at 30 °C, consisting of 30% glycerol (v/v), 10% Me_2SO (v/v), and 5.6 mM ATP in 100 mM MES, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.75, 30 °C. This step served to stop all tubulin uptake and loss. ATP was included in the stabilizing buffer to minimize nonspecific nucleotide adherence to the filter. Diluted suspensions of stabilized microtubules were then mixed by gentle vortexing and filtered immediately through Whatman GF/F 2.4-cm glass fiber filters on a Millipore 25-mm glass microanalysis support equipped with a stainless-steel screen filter support. The filters, which retained the microtubules quantitatively, were subsequently washed 3 times with 4-mL volumes of stabilizing buffer at 30 °C. Finally, each filter was transferred to a scintillation vial to which 10 mL of Fluorosol (National Diagnostics, Inc.) was added for determination of radioactivity.

Microtubule Length Determination. At desired times, samples were diluted 40-fold with 0.2% glutaraldehyde at 30 °C, and the microtubules were allowed to adhere to collodion films on copper electron microscope grids for 30 s. The grids were treated with a 0.1% solution of cytochrome *c* for 15 s, rinsed with water, and negatively stained with 1% uranyl acetate. Photographic prints were prepared at a final magnification of 6000×. A MOP-3 image processor was utilized to accumulate and process the length data, and the number of microtubules counted for each sample ranged from approximately 300 to 400.

Protein Assay. Protein was determined by the method of Lowry et al. (1951) with serum albumin as a standard.

Reagents. Taxol was obtained from Dr. Matthew Suffness of the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. Taxol was initially dissolved in 100% Me_2SO and stored frozen as a 5 mM stock solution in 12.5% MEM reassembly buffer (see below) and a final Me_2SO concentration of 87.5% (v/v). MES, EGTA, GTP (types I and II), acetate kinase (EC 2.7.2.1), and acetyl phosphate were obtained from Sigma Chemical Co., St. Louis, MO. [^{3}H]GTP was obtained from ICN, Irvine, CA, and [^{14}C]GTP was obtained from Research Products International, Mt. Prospect, IL. All other chemicals were reagent grade.

Results

Concentration Dependence for the Ability of Taxol To Inhibit Tubulin Addition to Microtubule A Ends at Steady State. We wanted to analyze the effects of taxol on net tubulin addition and loss at the ends of steady-state microtubules and on the kinetics of dilution-induced tubulin loss from microtubule ends at taxol concentrations that affected the rates of tubulin addition and loss by approximately 50%. Thus, initially we measured tubulin uptake at A ends of steady-state microtubules over a broad taxol concentration range using a single-label protocol to establish the appropriate taxol concentration for study. Fifty percent inhibition of the net tubulin incorporation rate occurred at approximately 7 μM taxol in the experiment shown in Figure 1. In several similar experiments, the taxol concentration that inhibited net tubulin uptake at the A ends of steady-state microtubules half-maximally varied between 5 and 7 μM drug.

Effect of Taxol Addition to Steady-State Microtubules. Addition of 6.5 μM taxol to suspensions of steady-state bovine brain microtubules produced an immediate increase in the mass of assembled polymer as determined by light-scattering measurements (Figure 2). Polymer mass relaxed to an apparently stable plateau 7–9% higher than the steady-state plateau of the control sample within 30 min after drug addition. No further change in the polymer mass occurred in the presence of taxol for at least an additional 140 min. Microtubule length determinations on the control and taxol-treated microtubule suspensions used in the experiment shown in Figure 2 are shown in Figure 3. The mean microtubule length in the control microtubule suspension was 12.1 μm, while the mean length of the taxol-treated microtubule suspension was 13.3 μm, a 9% increase. Addition of taxol to the

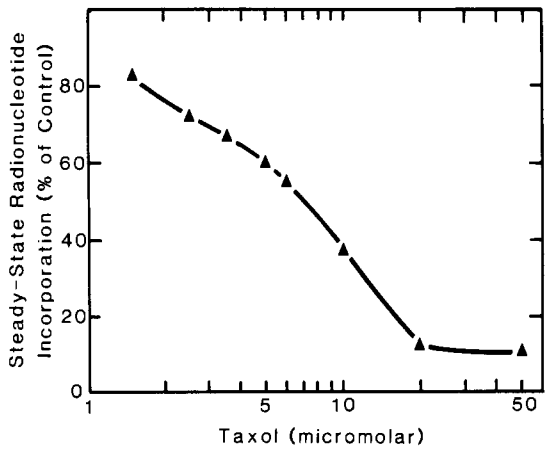

FIGURE 1: Concentration dependence of the ability of taxol to inhibit the steady-state incorporation of tubulin into microtubules. Bovine brain microtubule protein (5.25 mg/mL total protein) was polymerized to steady state at 30 °C as described under Materials and Methods in the absence of [^{3}H]GTP. At 45 min (approximately 30 min after reaching steady state), desired concentrations of taxol were added to aliquots of the microtubule suspension, and incubation was continued. Forty minutes after taxol addition, [^{3}H]GTP (50 μCi/mL, final specific activity 5.0 mCi/mmol) was added both to a non-taxol-treated and to the taxol-treated microtubule suspensions, and after an additional 45 min of incubation, 40-μL aliquots of each suspension were collected on glass fiber filters and analyzed for labeled nucleotide incorporation. Data in the presence of taxol are reported as the percentage of radioactive guanine nucleotide uptake of control (untreated) samples. Control microtubules incorporated 32 230 cpm/40 μL of microtubule suspension.

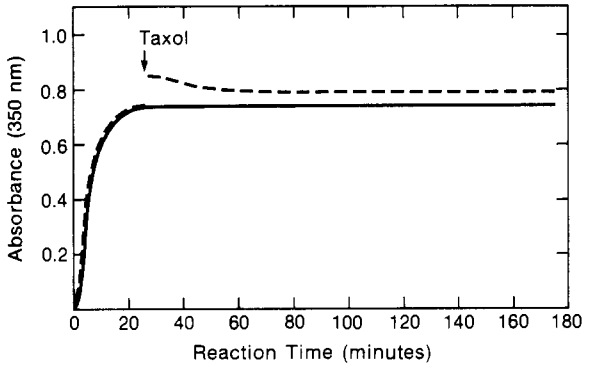

FIGURE 2: Influence of taxol addition to steady-state microtubules: analysis by turbidimetry. Two identical portions of a bovine brain microtubule protein solution, 5.37 mg/mL total protein, were assembled to steady state in reassembly buffer at 30 °C and monitored by turbidimetry at 350 nm. Taxol, 6.5 μM, was added to one portion at 26 min of incubation (arrow), and incubation of the untreated (solid line) and taxol-treated (dashed-line) microtubule suspensions was continued for approximately 150 additional minutes.

steady-state microtubule suspension did not result in the formation of a detectable number of new short microtubules (Figure 3B). Because the small increase in microtubule mass correlated well with the increase in mean microtubule length in taxol-treated suspensions, and because there was no measurable formation of new short microtubules after taxol addition, we can conclude that the microtubule number concentration was not changed by the protocol used.

The effect of 5 μM taxol on the critical subunit concentration was determined both by sedimentation analysis and by filter assay, with similar results. Results by sedimentation analysis are shown in Figure 4. Control microtubules exhibited a critical microtubule protein concentration (tubulin plus MAPs) of 0.31 mg/mL, while addition of 5 μM taxol reduced the critical concentration approximately 3-fold to 0.09 mg/mL. By filter assay, control samples of microtubules

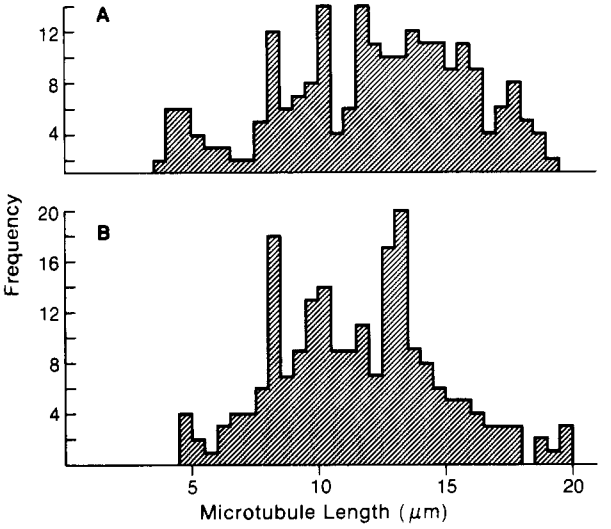

FIGURE 3: Microtubule length distributions in the presence and absence of 6.5 μM taxol. Microtubule length distributions were determined for the control and taxol-treated microtubule suspensions used in the experiment of Figure 2, at 42 min after initiation of assembly (15 min after addition of taxol in the taxol-treated sample). The mean microtubule length of the control microtubules was 12.1 μm (panel A), and the mean microtubule length of the taxol-treated microtubules was 13.3 μm (panel B). Under the labeling conditions used, control microtubules were labeled with tritium for a distance of 1.04 μm at their A ends, and taxol-treated microtubules were labeled for a distance of 0.50 μm. No microtubules were fully labeled with tritium. Approximately 200 microtubules were measured for each analysis.

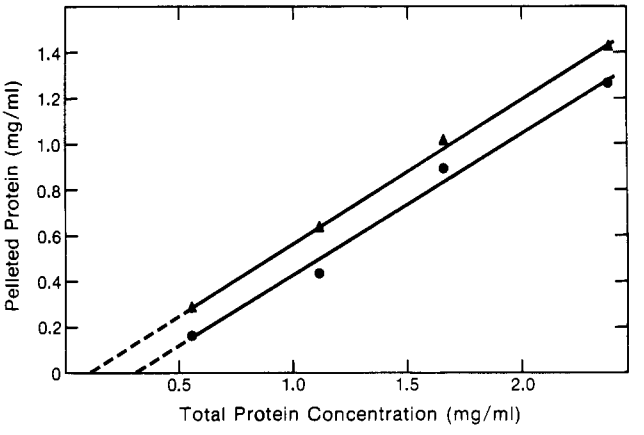

FIGURE 4: Estimation of the critical subunit concentration in the presence and absence of 5 μM taxol. Bovine brain microtubule protein solutions at concentrations indicated on the abscissa of the figure were polymerized to steady state at 30 °C in the presence of 0.2 mM GTP and a GTP-regenerating system as described under Materials and Methods. After 45 min of incubation, 5 μM taxol was added to one aliquot of each microtubule protein concentration, and incubation was continued. After a further 40 min of incubation, 0.45-mL aliquots of taxol-treated and untreated (control) microtubule suspensions were added to 0.75 mL of 70% sucrose in assembly buffer at 30 °C to stabilize the microtubules (Margolis & Wilson, 1978), and the samples were layered on 4 mL of 50% sucrose in assembly buffer and centrifuged at 30 °C for 60 min at 50 000 rpm (Beckman SW 50.1 rotor, L5-50 preparative ultracentrifuge). Any differences at each microtubule protein concentration in the ratio of free taxol to total microtubule surface (presently undeterminable) have not been taken into consideration in this analysis. Control microtubules (circles); 5 μM taxol (triangles). The mean microtubule length at 2.4 mg/mL control microtubule suspension was 8.6 μm and 9.8 μm in the taxol-treated suspensions. The mean microtubule length at 0.55 mg/mL was 9.8 μm in the control microtubule suspension and 11.1 μm in the taxol-treated suspension.

exhibited a critical concentration of 0.35 mg/mL (tubulin plus MAPs), while microtubules treated with 5 μM taxol exhibited a critical concentration of 0.14 mg/mL. These results are

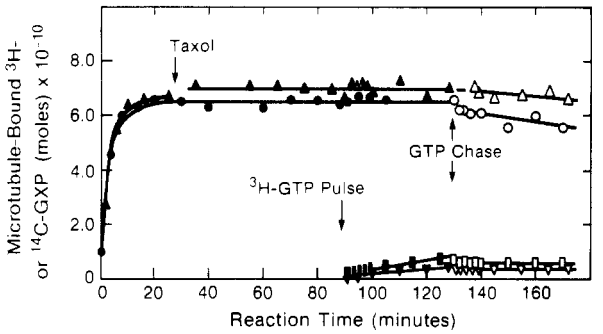

FIGURE 5: Effect of taxol on the incorporation of tubulin at A ends and loss of tubulin at D ends of steady-state microtubules: double-label protocol. Two identical portions of a bovine brain microtubule protein solution, 5.37 mg/mL total protein, were polymerized to steady state at 30 °C in reassembly buffer containing [^{14}C]GTP to label the microtubules uniformly with [^{14}C]guanine nucleotide (the same microtubule protein solution used in the experiments described in Figures 2 and 3). At 28 min (arrow), 6.5 μM taxol was added to one portion, and incubation of the untreated and taxol-treated microtubule suspensions was continued [[^{14}C]guanine nucleotide data: (closed circles) control microtubules, (closed triangles) taxol-treated microtubules]. A [^{3}H]GTP pulse was added to both the control and taxol-treated samples at 89 min of incubation [[^{3}H]guanine nucleotide data: (closed rectangles) control microtubules, (closed inverted triangles) taxol-treated microtubules]. A 2.1 mM unlabeled GTP chase was initiated at 129 min (arrow) and continued for an additional 45 min (open symbols denote chase conditions).

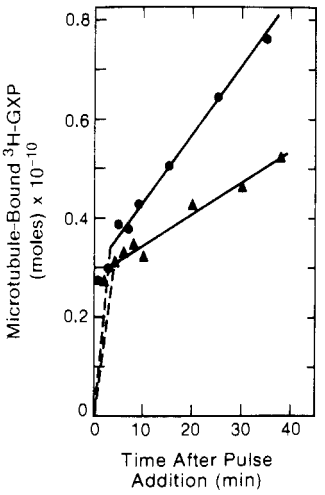

FIGURE 6: Incorporation of [^{3}H]guanine nucleotide into untreated and taxol-treated microtubules at apparent equilibrium. Data represent the pulse portion of the experiment shown in Figure 5. (Circles) Control microtubules; (triangles) taxol-treated microtubules.

consistent with those obtained previously by Schiff et al. (1979) and are in reasonable agreement with the increase in microtubule mass determined by light-scattering measurement (Figure 2) and the increase in mean microtubule lengths determined by negative-stain electron microscopy after addition of taxol (Figure 3).

Effect on Net Tubulin Uptake at the A Ends and Net Loss at the D Ends after Addition of Taxol to Steady-State Microtubules. Results of a typical double-label experiment designed to measure net tubulin addition at A ends and net tubulin loss at D ends at apparent steady state in the absence and presence of 6.5 μM taxol are shown in Figure 5 in their entirety and in Figures 6 and 7 to illustrate critical portions of the experiment in expanded form.

Similar to results obtained by light scattering, analysis of [^{3}H]guanine nucleotide incorporation revealed that microtubule polymerization reached a steady-state plateau within 20–25 min of incubation. Addition of 6.5 μM taxol to one

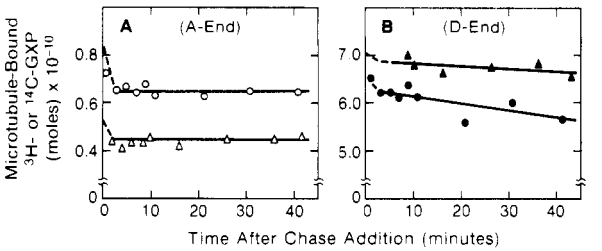

FIGURE 7: Loss of radiolabeled tubulin from A and D ends of control and taxol-treated microtubules. Data from the chase portion of the experiment are shown in Figure 5. (A) ^{3}H-Labeled nucleotide data; A-end label loss: (circles) control microtubules; (triangles) taxol-treated microtubules. (B) ^{14}C-Labeled nucleotide data; D-end label loss: (circles) control microtubules; (triangles) taxol-treated microtubules. The intercept positions on the ordinate for each data set represent the radiolabel incorporation immediately prior to addition of the chase.

portion of the suspension at 28 min after initiation of polymerization rapidly increased the polymer mass to a stable plateau approximately 7.7% greater than that attained in the control sample. Addition of a [^{3}H]GTP pulse at 90 min of incubation resulted in a 2–4-min burst of incorporation in both samples, followed by a linear incorporation of label (Figure 6). The rate of net tubulin addition at microtubule A ends in the control microtubule suspension was determined by using the linear region of the data beginning after the initial burst at 5 min and was calculated to be approximately 44 molecules of tubulin per minute per microtubule (tubulin addition rate of 0.73/s). Taxol at 6.5 μM had little effect on the initial rapid burst of incorporation but inhibited the linear incorporation of label by 48% to approximately 21 molecules of tubulin per minute per microtubule (tubulin addition rate of 0.35/s).

Addition of excess unlabeled GTP as a chase for both isotopes was performed 40 min after initiation of the [^{3}H]GTP pulse. At A ends (^{3}H label) of control microtubules, there was an initial loss of label which amounted to 21% of the tritium pulse followed in less than 3 min by a stable plateau lasting at least 45 min during which no additional label loss occurred (Figure 7A). These data are inconsistent with a purely equilibrium exchange mode of label incorporation during the [^{3}H]GTP pulse and indicate that labeled dimer incorporation into preformed microtubules occurred predominantly by a steady-state flux reaction (see Discussion). At A ends (^{3}H label) of taxol-treated microtubules, there was an initial loss of label that amounted to 18% of the tritium pulse followed within 2 min by a stable plateau during which no additional label loss occurred. These results are similar to those obtained with control microtubules and indicate that label incorporation into taxol-treated microtubules also occurred predominantly by a flux reaction (see Discussion).

At D ends of control microtubules (^{14}C label, Figure 7B), there was a small initial burst of label loss followed within 1 or 2 min by a linear rate of label loss that continued throughout the remainder of the experiment (45 min). The rate of net tubulin loss at D ends was determined by using the linear region of the data beginning after the initial burst of label loss and was 45.6 molecules of tubulin per minute per microtubule (tubulin loss rate of 0.76/s). This value is indistinguishable from that obtained with the tritium label for the rate of net tubulin addition at A ends of the microtubules and demonstrates that control microtubules were indeed at steady state with net A-end tubulin gain balanced precisely by the net rate of D-end tubulin loss.

Taxol at 6.5 μM inhibited the net rate of tubulin loss at D ends by 47% to approximately 22 molecules of tubulin per minute per microtubule (tubulin loss rate of 0.36/s). The

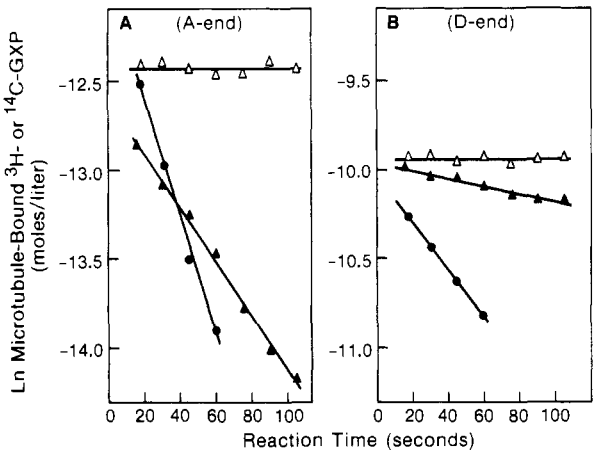

FIGURE 8: Effect of taxol on the dilution-induced disassembly from (A) microtubule A ends and (B) microtubule D ends (uncorrected for A-end carbon-14 loss). Double-labeled control and taxol-treated microtubules were constructed as described for the experiment of Figure 5, except that the microtubules were sheared by passage twice through a 25 gauge needle (4.98 mg/mL total microtubule protein). Mean length of control microtubules, 4.68 μm; mean length of taxol-treated microtubules, 4.82 μm (see length distribution in Figure 9). Under the labeling conditions used, control microtubules were labeled at their A ends with tritium for a distance of 0.43 μm, and taxol-treated microtubules were labeled with tritium for a distance of 0.21 μm. No microtubules were fully labeled with tritium. Both control and taxol-treated microtubules were diluted 100-fold into reassembly buffer (300 μL diluted to 30 mL) containing no taxol (closed symbols) or reassembly buffer containing 6.5 μM taxol (open symbols) 60 min after initiation of the [^{3}H]GTP pulse. Samples (3 mL) were stabilized in stabilization buffer at 30 °C at 15-s intervals, collected on glass fiber filters, and assayed for retained radiolabel (Materials and Methods). It took approximately 15 s to process experimental samples for analysis; thus, it was not possible to obtain data points prior to 15 s. Approximately 35% of the microtubule polymer mass had depolymerized in the control samples during the initial 15 s after dilution (40% of the tritium label remained at 15 s, and 65% of the carbon-14 label). (Circles) Control (untreated) microtubules; (triangles) taxol-treated microtubules. Solid lines represent theoretical plots derived from fitting first-order decay curves to the data by least-squares nonlinear analysis. Off rate constants were calculated by using linear regression lines of the data.

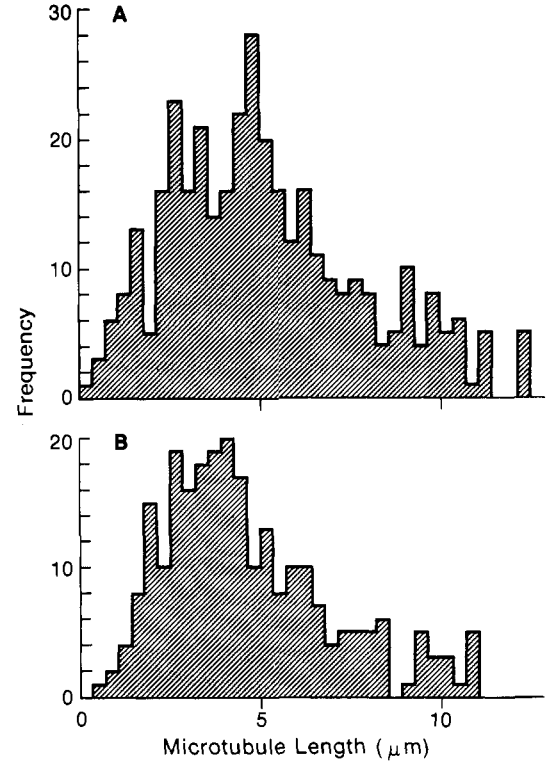

FIGURE 9: Length distributions for the sheared microtubules used in the experiment described in Figure 8. (A) Untreated microtubules; (B) taxol-treated microtubules.

degree of inhibition of D-end tubulin loss (47% inhibition, from 0.76/s to 0.36/s) was similar to the degree of inhibition of A-end tubulin gain (48% inhibition, from 0.73/s to 0.35/s) in the taxol-treated microtubule suspension, indicating that the taxol-treated microtubules were at steady state and were treadmilling, but at a reduced rate as compared to control microtubules.

Estimate of Molecular Rate Constants at Microtubule Ends of Taxol-Treated Microtubules. Apparent molecular rate constants at the A and D ends of microtubules can be estimated by dilution-induced disassembly measurements of double-labeled microtubules. However, the values obtained do not necessarily represent the true steady-state rate constants because the initial rate of tubulin loss is not a linear function of the initial free tubulin concentration (Farrell et al., 1983; Carlier et al., 1984). The faster than expected rate of tubulin loss when microtubule suspensions are diluted below the steady-state critical subunit concentration may be due to the loss of GTP–tubulin caps at microtubule ends and exposure of unstable GDP–tubulin cores (Carlier et al., 1984). Although the reactions giving rise to the measured initial rates of tubulin loss are not understood, apparent rate constants obtained, which probably reflect the rate-limiting reactions under dilution conditions, are still useful. Such apparent rate constants measured in the presence of taxol can be used to examine the effects of the drug at each microtubule end in a quantitative manner. Because the degree of deviation from linearity is similar for the initial loss rates from both the A and D ends (Farrell et al., 1983), the relative effects of taxol on the apparent rate constants for the two ends should not be artifactually altered by this methodology.

Results of a typical dilution experiment carried out with control and taxol-treated sheared and double-labeled microtubules appear in Figure 8. The length distributions of the microtubules prior to dilution are shown in Figure 9. This experiment was carried out similarly to the experiment described in Figure 5, with the control microtubules being diluted 60 min after the [^{3}H]GTP pulse rather than being chased undiluted with unlabeled GTP. In this experiment, dilution of the taxol-treated microtubules was carried out into taxol-free buffer 70 min after addition of the [^{3}H]GTP pulse. The depolymerization kinetics at A ends both of control and of taxol-treated microtubules (Figure 8A) closely approximated first-order loss reactions (correlation coefficients = 0.9975 and 0.9973) from which the initial rate of tubulin loss could be calculated. It has been proposed that the first-order approximation of such data reflects an exponential loss of microtubule ends (Karr et al., 1980; Kristofferson et al., 1980). The kinetics are complicated, and regardless of the basis for the loss, the approximate first-order nature is evident and permits a reasonable estimate of the initial rate of tubulin loss. Similar results were obtained upon analysis of label loss of D ends of both control and taxol-treated microtubules (Figure 8B).

The apparent molecular rate constants for control and taxol-treated microtubules appear in Table I. The A ends of control microtubules exhibited kinetics that were between 2 and 3 times slower than the D ends of the microtubules, as was previously reported (Farrell & Wilson, 1984). Taxol substantially slowed the apparent dissociation rate constants at both microtubule ends, with the extent of inhibition being similar at the two ends. Taxol also inhibited the apparent association rate constants at both ends; however, inhibition

Table I: Steady-State Parameters and Apparent Rate Constants of Untreated and Taxol-Treated Microtubules[a]

	A ends		D ends			
microtubules	k_- (s^{-1})	k_+ (M^{-1} s^{-1} × 10^{-7})	k_- (s^{-1})	k_+ (M^{-1} s^{-1} × 10^{-7})	flux rate (s^{-1})	critical subunit concn[b] (mg/mL)
untreated	39.8	1.8	105	4.8	0.73	0.31
taxol treated	9.5	1.4	22	3.3	0.35	0.09

[a] The rate constants are considered to be apparent rate constants for the reasons stated in the text. [b] Tubulin plus MAPs.

of the dissociation rate constants was considerably greater than inhibition of the association rate constants. Dilution of taxol-treated microtubules was also carried out into dilution buffer containing 6.5 μM taxol. Surprisingly, no loss of tubulin occurred at either the A or the D ends during the 100-s period of time the microtubules were monitored after dilution. Further, in a similar experiment, no loss of tubulin occurred from either microtubule end upon 30-fold dilution into 6.5 μM taxol for at least 60 min after dilution.

Discussion

We have used a double-label procedure in which microtubule A and D ends were differentially labeled with tritium and carbon-14 to investigate the effects of taxol on steady-state net tubulin addition at A ends and loss at D ends and to assess the effects of taxol on the apparent dissociation and association rate constants for tubulin addition and loss at each end. A drug addition strategy in which taxol was added to suspensions of ^{14}C-nucleotide-labeled microtubules assembled at high microtubule protein concentrations (5 mg/mL) prior to addition of the tritium label ensured minimal change in the concentration of microtubule ends and obviated the problem of tritium labeling of D ends that would have occurred if the taxol were added to the microtubules in the presence of both radioisotopes.

Properties of the Bovine Brain Microtubule Protein System and Double-Labeled Microtubules. In order to use the double-label protocol to analyze the effects of taxol addition on the rates of tubulin addition and loss at opposite microtubule ends, it is necessary to establish (1) that tubulin addition and loss occurred at the opposite ends of individual microtubules rather than at the ends of two different microtubule populations within the same suspension and (2) that the uptake of tritium-labeled tubulin and loss of carbon-14-labeled tubulin in the taxol-treated microtubules occurred predominantly by a flux mechanism rather than by simple equilibrium exchange [discussed previously for control microtubules by Farrell & Jordan (1982), Jordan & Farrell (1983) and Farrell & Wilson (1984)].

Recently, Mitchison & Kirschner (1984a,b) have observed that certain MAP-depleted preparations of brain tubulin undergo a behavior that they have called "dynamic instability". They found that both growing and shortening microtubule populations can coexist in vitro in the same microtubule suspension and suggested that the populations interconverted infrequently. Mitchison and Kirschner suggest further that the possible basis for the behavior is that the population of growing microtubules normally contains a GTP–tubulin cap at the microtubule ends and when the cap is lost, exposing a GDP tubulin core, the microtubules become extremely unstable and depolymerize rapidly [based upon the model of Carlier et al. (1984)]. These investigators have suggested that the interpretation that steady-state isotope exchange data of the kind used in this study revealing the net addition of tubulin at one end of a microtubule and the balanced net loss of tubulin at the opposite end of the same microtubule (flux, or treadmilling) may be incorrect. For example, if the majority of the microtubules were growing by equilibrium exchange at both their ends, and if some of the microtubules suddenly were to undergo a phase change and become extremely unstable, then the net loss of isotope would be occurring from those few rapidly disassembling microtubules, and the net addition would be occurring at both ends of the majority of the microtubules in suspension. The mass of total polymer would remain constant, but the number concentration of microtubules would decline, while the mean length of the remaining microtubule population would increase.

We have analyzed the behavior of the bovine brain microtubules used in this and preceding studies and have determined that dynamic instability behavior does not occur to a detectable extent under the conditions used [discussed in detail in Wilson & Farrell (1985)]. For example, if dynamic instability occurred in the microtubule system used in the present study, the mean length of the microtubule population should have increased, and the microtubule number concentration should have decreased. The increase in mean microtubule length in the microtubule population would have been identical with the rate of net tubulin addition to the microtubule population at apparent steady state as measured by the rate of incorporation of the tritium pulse. For example, this would have amounted to an easily detectable 3.9-μm increase in mean length from 12.1 to 16 μm in the control microtubule suspensions studied in the experiments of Figures 5–7 (from 30 to 180 min). No such increase occurred. The mean microtubule lengths in all control experiments have remained constant for the duration of the experiments [see also Wilson & Farrell (1985)]. Further, in previously published pulse–chase experiments, labeled nucleotide was retained in the microtubules for the time required to transit from one microtubule end to the other and then was lost [see Margolis & Wilson (1981)]. These results are incompatible with the possibility that dynamic instability contributed in a significant way to the isotope exchange. We conclude that the isotope exchange data we have obtained in the present study reflect the steady-state addition of tubulin at one microtubule end and loss from the opposite end and that the data cannot be attributed to length redistributions of the kind recently described in a MAP-depleted system by Mitchison & Kirschner (1984a,b).

Evidence that ^{3}H-labeled tubulin added predominantly at A ends and ^{14}C-labeled tubulin was lost predominantly from D ends by a flux mechanism rather than by equilibrium exchange derives from analysis of pulse–chase data (see Figures 5–7). Zeeberg et al. (1980) determined that if equilibrium exchange were solely responsible for tritium incorporation during a [^{3}H]GTP pulse, only 41.4% of the tritium-labeled dimers incorporated during a pulse would be retained in the microtubules during a chase of equal duration. If one includes the quantity of label incorporation represented by the intial 2–4-min burst of incorporation during a pulse and the initial 2–4-min burst of label loss during a chase in the data shown in Figure 6 (the weakest case for treadmilling incorporation of label), 80% of the label incorporated during the 40-min pulse was retained in the microtubules 40 min after initiation of the unlabeled GTP chase. This value is far from that which one would have observed if the labeled tubulin were being incorporated into the microtubules purely by equilibrium exchange.

If one assumes that the initial burst of ^{3}H-nucleotide uptake during a pulse and the burst of label loss during a chase predominantly represented equilibrium exchange [see Farrell & Wilson (1984)] and excludes the initial 2–4 min of the pulse and chase data in the calculation, then greater than 95% of the label incorporated during the linear phase of a pulse was retained during the linear phase of the chase. These data strongly support the conclusion that the tritium label incorporation represented by the linear phase of the data predominantly represents the flux incorporation of tubulin at microtubule A ends and the carbon-14 label loss represented by the linear phase of the data represents the flux loss of tubulin at microtubule D ends.

In addition, it is clear that during the chase period the rates of carbon-14 loss and tritium loss were very different (Figure 7); they would have been similar if the labeling had occurred predominantly by an equilibrium exchange mechanism. Furthermore, an upward deviation from linearity was apparent when the data shown in Figure 6 were plotted as a function of the square root of time (data not shown). This indicates that flux incorporation of label was occurring during the pulse (Zeeburg & Caplow, 1980). Finally, in experiments in which double-labeled microtubules were diluted from steady state to obtain apparent off rate constants, the ^{14}C-nucleotide-labeled tubulin was lost between 2 and 3 times faster than the ^{3}H-nucleotide-labeled tubulin, which is consistent only with a flux mechanism as being the predominant mechanism for labeled tubulin incorporation during construction of the double-labeled microtubules (Figure 8).

Similar results were obtained with taxol-treated microtubules. The degree of ^{3}H-nucleotide retention after a 40-min pulse and 40-min chase of the taxol-treated microtubules was almost identical with that of control microtubules [80% retention of the pulse if the initial 2–3-min burst incorporation is included in the calculation and >95% retention if the linear portions of the data are used (Figure 6)]. Further, during the unlabeled GTP chase, there was little loss of ^{3}H-nucleotide, while the ^{14}C-nucleotide was lost at a rate equal to the rate at which ^{3}H-nucleotide was incorported during a ^{3}H-nucleotide pulse. Finally, dilution of double-labeled taxol-treated microtubules resulted in a 3-fold difference in the rates of ^{3}H-nucleotide and ^{14}C-nucleotide loss rates, again indicating that the label uptake and loss occur predominantly by a flux mechanism and that the two isotopes differentially label the opposite microtubule ends.

Though the cellular function(s) of the reactions that produce the disparity between the two microtubule ends that result in microtubule flux in vitro at steady state is (are) not known, it seems reasonable that the flux reactions are important and that they could be utilized by cells in one or several ways to control microtubule growth or function. In the present study, the flux reactions have been utilized to prepare microtubules differentially labeled with tritium and carbon-14 at their two ends.

Effects of Taxol. Addition of taxol to steady-state microtubules produced a concentration-dependent decrease in the net uptake of tubulin at microtubule A ends, with half-maximal inhibition occurring between 5 and 7 μM drug. In this concentration range, the drug produced an approximately 3-fold decrease in the critical subunit concentration, a compensatory 7–9% increase in the mean microtubule length, and essentially no detectable alteration in the microtubule number concentration. After addition of 6.5 μM taxol to the steady-state microtubules, the incorporation rate as determined with the tritium label incorporation data was reduced 48%, and the loss rate as determined with the carbon-14 data was reduced 47%. These data indicate that the microtubules reestablish a new steady state after taxol addition, with a precisely balanced net addition of tubulin at A ends and loss of tubulin at D ends, but with the net addition and loss of tubulin reduced as compared to untreated microtubules equally at the two ends.

Estimates of the molecular dissociation rate constants at microtubule A and D ends were obtained by 100-fold dilution of untreated and taxol-treated double-labeled microtubules into assembly buffer containing no taxol (Figure 8). The apparent rate constants do not take into consideration any loss of taxol from the microtubule surfaces that might have occurred upon dilution, because the free taxol concentration under steady-state conditions in the presence of 6.5 μM added taxol is not presently determinable. Thus, the values for the apparent rate constants may be underestimates of the true steady-state effect of taxol. Nevertheless, the data are useful in that the relative effects of taxol at the two ends have been revealed. The data indicate that taxol slowed the rate of tubulin loss from the two microtubule ends to exactly the same extent, which indicates that the drug interacts with the surfaces of microtubules and stabilizes inter-tubulin bonding uniformly throughout the microtubule surface lattice. Estimates of the molecular association rate constants at both microtubule ends were made by using measured values for the apparent dissociation rate constants, flux rates, and critical concentration in the presence of taxol. The values obtained for the association rate constants, like those for the dissociation rate constants, are intended solely to illustrate quantitatively the effects of taxol at the microtubule ends. We interpret the results to indicate that taxol exerts little or no effect on the association rate constant at either microtubule end.

Interestingly, 100-fold dilution of taxol-treated microtubules into the same total concentration of taxol that was added initially to the steady-state microtubule suspension resulted in no loss of tubulin from either end. This effect is striking in view of the observation that under steady-state conditions, tubulin was lost at a measurable rate (flux rate = 0.35/s). This must mean that when the taxol-treated microtubules were diluted into more taxol, an extremely rapid binding of additional taxol to the microtubule surfaces must have occurred (considerably faster than the loss of tubulin) and also indicates that the microtubules treated with 6.5 μM taxol were far from saturated with the drug.

Consistent with this interpretation are the results of Parness & Horwitz (1981), who studied the binding of tritium-labeled taxol to microtubules assembled in vitro from calf brain microtubule protein. They found that taxol bound to the microtubules with a $K_{d,app}$ of 0.87 μM and that binding became saturated at a calculated ratio of approximately 0.6 mol of taxol per mole of tubulin in the microtubules. Our data indicate that half-maximal inhibition of the rate of tubulin flux under the conditions employed in this study occurred at 5–7 μM taxol. If we assume that taxol binding to tubulin in microtubules is a simple reversible bimolecular reaction with one taxol binding site per tubulin dimer, at equilibrium the binding reaction would be represented by the equation:

$$K_d/[Tu]_f = [Ta]_f/[Ta{\cdot}Tu]$$

where $[Ta]_f$ is the free taxol concentration, $[Tu]_f$ is the concentration of unbound tubulin in the microtubules, and [Ta·Tu] is the concentration of taxol–tubulin complex in the microtubules. Using the value of 0.87 μM for the K_d as determined for the binding of taxol to calf brain microtubules (Parness & Horwitz, 1981), we can estimate the ratio of $[Ta]_f/[Ta{\cdot}Tu]$ at 7 μM taxol, since at 7 μM taxol at least 80% of the tubulin

in the microtubules would not be bound by drug under the conditions used (for example, see Figure 5, 35 μM tubulin in microtubules). We calculate the ratio of $[Ta]_f/[Ta{\cdot}Tu]$ to be approximately 0.031 at 7 μM taxol. Thus, the quantity of free taxol can be considered negligible, and most of the added taxol was bound to the microtubules. At half-maximal inhibition of tubulin flux (i.e., 7 μM taxol), approximately one molecule of taxol was bound per five molecules of tubulin in the microtubules.

Two effects of taxol have been documented to occur in cells and in vitro: (1) microtubule stabilization and promotion of microtubule polymerization; (2) microtubule bundling. It seems reasonable that the ability of taxol to reduce the dissociation rate constants equally for tubulin at the two microtubule ends could account for the increase in microtubule mass and stability that has been observed. However, it is not so easy to visualize how such a mechanism could result in the phenomenon of microtubule bundling. It seems reasonable to assume that bridging of one microtubule to another or of microtubules to other cellular components occurs with some normal frequency in cells due to transient attachment and detachment of cross-bridge molecules at microtubule surfaces. Perhaps stabilization of intermolecular tubulin bonding by taxol results in a conformational change or stabilization of a specific tubulin conformation at the microtubule surface that results in an increased affinity of microtubule cross bridges with microtubule surfaces.

It is interesting that at the taxol concentration that inhibited steady-state tubulin flux by 50%, there was little or no effect on the initial burst of label incorporation which occurred during the first 2–3 min of a pulse and on the initial 2–3-min burst of label loss after initiation of a chase. One possible explanation is that the initial burst of incorporation and loss in this microtubule preparation represent exchange of guanine nucleotide with microtubule ends rather than exchange of tubulin–guanine nucleotide complexes. Another possibility is that the tubulin at the very end regions of the polymers does not interact with the drug. Finally, it is also interesting that taxol, a natural plant product, exerts such a profound and specific stabilization of microtubules in cells. Perhaps taxol is mimicking the action of a natural regulator of microtubule assembly. If so, a search for taxol-like microtubule assembly regulators would be a very fruitful area for further research.

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Registry No. Taxol, 33069-62-4.

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