Tubulin–Colchicine Complexes Differentially Poison Opposite Microtubule Ends†

Kevin W. Farrell* and Leslie Wilson

ABSTRACT: The kinetics of radiolabeled guanosine 5′-triphosphate–tubulin dimer addition to preformed microtubule copolymers, containing large numbers of tubulin–colchicine complexes (TCs), were examined at apparent equilibrium. The results indicated that radiolabeled dimer addition to copolymers occurs predominantly by a "treadmilling" reaction, analogous to that described for unpoisoned microtubules, and that some labeled dimer uptake also occurs by equilibrium exchange. The data further showed that TCs decrease the steady-state treadmilling reaction in a concentration-dependent manner. Since microtubule copolymers exhibited a treadmilling reaction, it was possible to differentially radiolabel opposite copolymer ends with [^{3}H]- and [^{14}C]guanine nucleotides and thus to measure the effects of TCs on dimer loss from opposite copolymer ends upon copolymer dilution. Dimer loss from both copolymer ends was inhibited in a concentration-dependent manner, but dimer loss from copolymer net assembly (A) ends (defined under steady-state conditions) was inhibited to a far greater extent than that from the opposite, net disassembly (D) copolymer ends. TCs therefore exhibited a graded, polar poisoning action, with copolymer A-end association and dissociation rate constants being far more susceptible to TC inhibition than those at the opposite copolymer D ends. The potential significance of this TC effect for regulating microtubule spatial orientation in vivo is discussed.

There has been considerable interest in the mechanism of action of the plant alkaloid colchicine since it was initially discovered to be a potent inhibitor of mitosis some 45 years ago [reviewed by Dustin (1978)]. It is well established that the action of colchicine on the mitotic spindle and, similarly, many of the other actions of colchicine on cellular processes are due to the disruption of microtubules. A large number of investigators have demonstrated that colchicine can bind with high affinity to tubulin, the dimeric protein subunit of microtubules [see Wilson & Bryan (1974) and Luduena (1979) for reviews] and that tubulin–colchicine complexes (TCs)[1] inhibit microtubule polymerization by adding to the microtubule ends (Margolis & Wilson, 1977). However, despite considerable effort, the mechanism underlying the ability of colchicine to inhibit microtubule polymerization remains controversial. The mechanism may be important to understand in some detail, because of the possibility that cells might produce natural regulatory molecules that work in a manner similar to colchicine to control microtubule assembly (Lockwood, 1979; Sherline et al., 1979).

The early observation of Olmsted & Borisy (1973), that inhibition of microtubule assembly in crude brain extracts occurred at colchicine concentrations well below the concentration of soluble tubulin in the extract, suggested that the drug was not stoichiometrically inactivating the tubulin. This initial observation was confirmed by Margolis & Wilson (1977), who further showed that the basis of the substoichiometric poisoning phenomenon was the formation of TCs which add to microtubule ends and inhibit subsequent growth. Differences of opinion arose, however, regarding the mechanism by which TCs prevented tubulin addition to microtubule ends. Following the addition of [^{3}H]colchicine to steady-state microtubules in vitro, Margolis et al. (1980) found that a stoichiometry of 0.5 TC bound per microtubule assembly end was sufficient to slow the rate of net dimer addition to this end by 50% and that even at saturating colchicine concentrations only eight to nine TCs were bound per microtubule. These investigators also found that the rate of tubulin loss from the assembly end was greatly retarded, in addition to the rate of tubulin gain. These results, together with the earlier observations of Margolis & Wilson (1977), were interpreted in terms of a "capping" phenomenon, in which very small numbers of TCs added to microtubule assembly ends effectively irreversibly and, at sufficiently high drug concentration, essentially prevented further tubulin addition and loss (an end-blocking or end-depleting mechanism).

The validity of a simple capping mechanism became doubtful, however, in light of the results of Lambeir & Engelborghs (1980) and Keates & Mason (1981), who, using preformed TCs, found that the binding of TCs to microtubule ends was readily reversible under conditions approximating no net growth. Similar results were obtained by Deery & Weisenberg (1981), who further found that both ends of steady-state microtubules can be in equilibrium with TCs. These results, which conflict with those of Margolis et al. (1980), are difficult to reconcile with a simple capping and end-blocking mechanism. In further disagreement with a simple capping mechanism, Sternlicht & Ringel (1979) found that preformed TCs could coassemble with tubulin dimers to form microtubule copolymers containing large numbers of TCs. These authors proposed that colchicine inhibits microtubule assembly by a copolymerization mechanism, in which copolymers retained assembly-competent ends, but with reduced affinity for tubulin and TCs (an end-conserving or copolymerization mechanism).

Farrell & Wilson (1980) examined the mechanism by which preformed TCs inhibit tubulin addition to microtubules reassembled to steady state from sea urchin sperm tail tubulin. In agreement with the results of Sternlicht & Ringel (1979),

† From the Department of Biological Sciences, University of California, Santa Barbara, California 93106. *Received August 29, 1983.* This work was supported by National Institutes of Health Grants GM26732 (K.W.F.) and NS13560 (L.W.).

[1] Abbreviations: ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; Me_2SO, dimethyl sulfoxide; MES, 2-(*N*-morpholino)ethanesulfonic acid; microtubule protein, tubulin plus all microtubule-associated proteins that copurify with tubulin through cycles of in vitro assembly and disassembly; MAPs, microtubule-associated proteins; TCs, complexes of tubulin and colchicine; GDPase, guanosine diphosphatase; Tu, tubulin; MT, microtubule.

0006-2960/84/0423-3741$01.50/0

their data supported a poisoning mechanism by which tubulin and TCs copolymerize at microtubule ends. Furthermore, at high tubulin:TC ratios, an end-conserving mechanism appeared to operate, and the results were interpreted in terms of repeated cycles of transient assembly–blockage followed by repair. However, at low tubulin:TC ratios, the poisoning mechanism seemed to change, and below a critical tubulin:TC ratio, the poisoning effects of TCs apparently became cooperative, with the result that inhibition of assembly appeared to become complete.

Thus, the mechanism of action of colchicine is clearly more complicated than originally envisaged. It is likely that the widely different results obtained in previously published reports are reflecting at least in part the complexity of the poisoning mechanism.

In the present study, we have examined the effects of TCs at opposite ends of microtubule copolymers of tubulin and TCs, by using a radiolabeling protocol which allows opposite polymer ends to be distinguished. The data indicate that the mechanism of action of colchicine involves a differential kinetic stabilization of opposite microtubule ends, with both the association and dissociation rate constants being decreased at each end, but with the effect exerted far more strongly at the microtubule net assembly ends than at the net disassembly ends. This mechanism is consistent with previous results obtained in this laboratory and can account for a least some of the variation in results that has been recently reported.

Materials and Methods

Reagents. Colchicine, MES, EGTA, GTP (type I), acetyl phosphate, and acetate kinase were obtained from Sigma Chemical Co. [Ring C, *methoxy*-^{3}H]colchicine (13–20 Ci/mmol), [^{3}H]GTP (10 Ci/mmol), and [^{14}C]GTP (8–10 Ci/mmol) were purchased from New England Nuclear Corp., Inc. Bio-Gel P-10 was obtained from Bio-Rad Laboratories.

Preparation of Bovine Brain Microtubule Protein.[1] Bovine brain microtubule protein, consisting of approximately 70% tubulin and 30% MAPs, was isolated without glycerol by a modification of the procedure of Asnes & Wilson (1979). Initial homogenization of brains was carried out in 20 mM sodium phosphate, 100 mM sodium glutamate, 1 mM EGTA, 1 mM dithiothreitol, and 0.2% sodium azide at pH 6.90. The use of pH 6.90 rather than pH 6.75 in the initial homogenization (Murphy & Hiebsch, 1979) and the inclusion of dithiothreitol resulted in higher final yields as compared with the original procedure (Asnes & Wilson, 1979). Polymerization during the second and third cycles was carried out at pH 6.75. Three cycles of assembly and disassembly were used to purify the microtubule protein, with the warm incubations carried out for 30, 10, and 30 min at 30 °C for the first, second, and third assembly cycles, respectively. All centrifugation steps were the same as those described previously, except that centrifugation of the solubilized microtubule protein at 4 °C after the second cold depolymerization was carried out at 150000*g* for 40 min. (50Ti rotor; Beckman Model L5-50 preparative ultracentrifuge). This step served to remove a GDPase activity that interferes at steady state with maintenance of constant levels of GTP. Microtubules, after the third polymerization step, were centrifuged through 50% sucrose cushions as a final purification step (Margolis & Wilson, 1978) and stored as pellets at −75 °C until used. The modifications of the original procedure of Asnes & Wilson (1979) yielded a microtubule protein preparation which, when assembled to steady state, produced microtubules that exhibited considerably larger rate constants than those obtained by using microtubule protein prepared exactly according to the method of Asnes & Wilson [e.g., compare rate constants from Farrell & Jordan (1982) with the data in Figure 5 of this paper].

For reassembly experiments, the microtubule pellets were resuspended in 100 mM MES, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.8 (Mes reassembly buffer), depolymerized on ice for 15 min, and centrifuged at 48000*g* for 20 min at 4 °C (SS-34 rotor; Sorval). 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.

Preparation of Tubulin–Colchicine Complexes. The method of preparing TCs was similar to that used previously for *Strongylocentrotus purpuratus* outer doublet tubulin (Farrell & Wilson, 1980). Briefly, purified microtubule protein (3–5 mg/mL) in Mes reassembly buffer (no GTP) was incubated with 0.1 mM colchicine for 10 min at 30 °C, followed by chilling on ice for 15 min. The chilling step was necessary to dissociate polymers formed before significant TC complex formation occurred. Subsequently, the microtubule protein solution was incubated for a further 1 h at 30 °C. TCs (actually a mixture of TCs and MAPs) were separated from unbound colchicine by gel filtration using 1 cm × 18 cm columns of Bio-Gel P-10. To prepare tubulin–[^{3}H]colchicine complexes, the above procedure was followed, except that [^{3}H]colchicine was included in the incubation mixture at a final specific activity of 0.1–0.2 Ci/mmol. When the tritium content of the TC and MAP mixture was analyzed to determine the stoichiometry of complex formation, a value of approximately 0.7–0.8 mol of colchicine bound per mol of tubulin was obtained.

Preparation of Tubulin–Colchicine Complex/Tubulin Copolymers. Microtubule protein (3–6 mg/mL) was incubated with different concentrations of TCs prepared with either unlabeled or ^{3}H-labeled colchicine, as appropriate. The TC concentrations were chosen to give TC mole fractions[2] in solution of 0–0.2. The total microtubule protein concentration (including colchicine-bound tubulin) was adjusted to the same final value for all samples within a single experiment. Assembly was initiated by adding 0.1 mM [^{14}C]GTP (2–4 μCi/mL, 3–8 Ci/mol final specific activity) or unlabeled GTP, as appropriate. Attainment of steady state was determined by light scattering at 350 nm.

To determine the stoichiometry of TCs in TC/tubulin copolymers, microtubule protein and tubulin–[^{3}H]colchicine complexes were coassembled to steady state in the presence of 0.1 mM [^{14}C]GTP. This procedure labels the microtubules throughout their lengths with [^{14}C]guanine nucleotide, as well as with tritium due to the coassembly of the labeled TCs and ^{14}C-nucleotide-labeled tubulin dimers. Aliquots (100 μL) were then removed and stabilized in 4 mL of Mes reassembly buffer at 30 °C, containing 25% (v/v) glycerol, 10% (v/v) Me_2SO, and 2.5 mM ATP (stabilizing buffer). The molar concentrations of colchicine and guanine nucleotide in the copolymers were determined by collecting the copolymers on glass fiber filters (Whatman GF/F, 2.5 cm in diameter), extracting the radiolabeled ligands by incubation at room temperature with 1.5 mL of 0.1 N NaOH for 2 h, and scintillation counting (Wilson et al., 1982).

Since [^{3}H]colchicine bound to tubulin with a stoichiometry of approximately 0.7–0.8 mol of colchicine per mol of tubulin

[2] TC mole fraction is the fractional molar ratio of TCs to total tubulin (both colchicine free and colchicine bound) in solution prior to initiation of assembly.

under the conditions used and the stoichiometry of labeled guanine nucleotide in the microtubules or copolymers was also the same, the stoichiometry of TCs in the copolymers could be determined from the molar ratio of colchicine to nucleotide in the copolymers. The average number of TCs per copolymer was obtained from the product of the colchicine stoichiometry and the number of tubulin molecules in an average-length copolymer (Farrell & Jordan, 1982).

Determination of Steady-State Flux Rates. To determine the rate of incorporation of tubulin dimers (including colchicine-bound dimers) into steady-state microtubules and copolymers, we exploited the fact that GTP exchangeably binds to the E site of unassembled tubulin dimers and becomes nonexchangeable upon dimer incorporation 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, while an equivalent net loss of tubulin occurs from the opposite end, defined as the net disassembly or D end (Margolis & Wilson, 1978; Farrell & Jordan, 1982). Thus, when added to a suspension of microtubules at steady state, radiolabeled GTP binds to soluble tubulin and becomes incorporated at the A end (see Results and Discussion sections for an analysis of the contribution of equilibrium exchange to labeled dimer incorporation). The rate of incorporation, which is a measure of the rate of tubulin flux from one end of the microtubule to the other, can then be determined by collection of the microtubules (or copolymers) and analysis of label incorporation, protein content, and microtubule number concentration (Margolis & Wilson, 1978; Farrell & Jordan, 1982; Jordan & Farrell, 1983).

Microtubules, or copolymers of tubulin and TCs, at steady state (determined by light scattering at 350 nm) were added to dried [^{3}H]GTP (120–130 Ci/mol of GTP final specific activity) and gently mixed. Aliquots (100 μL) were removed immediately after mixing and at 10-min intervals for a period of 1 h, and the microtubules or copolymers were immediately diluted into 4 mL of stabilizing buffer at 30 °C. Label incorporation was then determined by the glass fiber filter assay (Wilson et al., 1982). Steady-state rates of tubulin incorporation (flux rates) were calculated from linear regression analysis of the rate of [^{3}H]GTP uptake into steady-state polymers from 10 to 60 min following label addition (e.g., Figure 2a) and corrected for polymer number concentration (Farrell & Jordan, 1982).

Determination of Dissociation Rate Constants. The method for determining apparent dissociation rate constants at opposite microtubule ends after differentially labeling with ^{3}H- and ^{14}C-nucleotides has been described in detail elsewhere (Farrell & Jordan, 1982; Jordan & Farrell, 1983). This method has been adhered to herein, for both microtubules and copolymers. Microtubule protein solutions containing 0.1 mM [^{14}C]GTP (2–4 μCi/mL; 3–8 Ci/mol of GTP final specific activity) were incubated at 30 °C in the presence of different concentrations of unlabeled TCs. The total microtubule protein concentration was the same for all samples within a single experiment. This procedure resulted in the formation of copolymers with different tubulin:TC ratios and which were labeled throughout their lengths with [^{14}C]guanine nucleotide. At steady state (determined by light scattering at 350 nm), the ^{14}C-labeled polymers were pulsed with [^{3}H]GTP (20–60 μCi/mL; 200–234 Ci/mol of GTP final specific activity) for 60–120 min. Thus, the microtubules or copolymers became labeled with both ^{3}H- and ^{14}C-nucleotides at the A ends and remained labeled with ^{14}C-nucleotide alone at the D ends.

Apparent rate constants for tubulin dissociation from both ends of microtubules or copolymers were calculated from the initial rates of loss of labeled nucleotides following a 30-fold dilution of the polymers from steady state. Aliquots (0.5 mL) of steady-state polymers were added to 14.5 mL of warm reassembly buffer containing 0.1 mM unlabeled GTP and rapidly mixed. Samples (2.0 mL) were then removed at 10–15-s intervals during the next 2–3 min, stabilized in 3.0 mL of warm 70% sucrose (w/v) in Mes reassembly buffer, and processed by the glass fiber filter assay. Dimer loss (including colchicine-bound dimers) from copolymer A ends was calculated from the ^{3}H-nucleotide remaining in microtubules or copolymers during depolymerization. Loss from both polymer ends was similarly determined from the ^{14}C-nucleotide data from which D-end loss could be obtained by subtraction of A-end loss.

Because of the time required to process experimental samples, the first 10–15 s of the depolymerization reaction was not measured. The possibility existed, therefore, that an early and very rapid disassembly reaction was overlooked. This appeared unlikely, however, since theoretical curves fitted to the disassembly data by nonlinear regression analysis, when extrapolated to time zero, yielded values for the ^{3}H- and ^{14}C-nucleotide in polymers in close agreement with those measured in steady-state polymers immediately prior to dilution.

The methods and assumptions used in calculating the dissociation rate constants from the ^{3}H- and ^{14}C-nucleotide loss rates have been described in detail elsewhere (Farrell & Jordan, 1982; Jordan & Farrell, 1983). We have recently observed that the initial rate of microtubule disassembly, induced by microtubule dilution, is not a linear function of the initial concentration of unassembled tubulin (Farrell et al., 1983), as current theory for simple protomer–polymer systems predicts [e.g., see Oosawa & Asakura (1975)]. This suggests further levels of complexity for microtubule disassembly, and hence, the dissociation rate constants should be regarded as apparent constants and do not necessarily indicate the values of the rate constants under steady-state conditions. The values reported for the dissociation rate constants are intended solely to illustrate quantitatively the differential effects of TCs at opposite microtubule copolymer ends. Because of the nonlinearity of the rate plots, it was also not possible to quantify the effects of TCs on the association rate constants from the available data.

Results

We have recently described a method by which assembly and disassembly reactions at opposite microtubule ends may be measured (Farrell & Jordan, 1982; Jordan & Farrell, 1983). This method is predicated on the observation that microtubules attain a steady state in which net tubulin dimer addition occurs at one microtubule end, while an equivalent net loss of dimers occurs from the opposite microtubule end, resulting in a unidirectional flux or "treadmilling" of dimers through steady-state microtubules (Margolis & Wilson, 1978; Farrell et al., 1979). This microtubule property has made it possible to label opposite microtubule ends differentially with guanine radionucleotides, and hence to measure the assembly–disassembly reactions at each microtubule end [see Materials and Methods; see also Jordan & Farrell (1983)].

We proposed to use the differential radiolabeling protocol to examine the effects of TCs at opposite ends of microtubule copolymers. However, it was necessary, first, to characterize copolymer formation under our experimental conditions and, second, to determine whether the microtubule copolymers

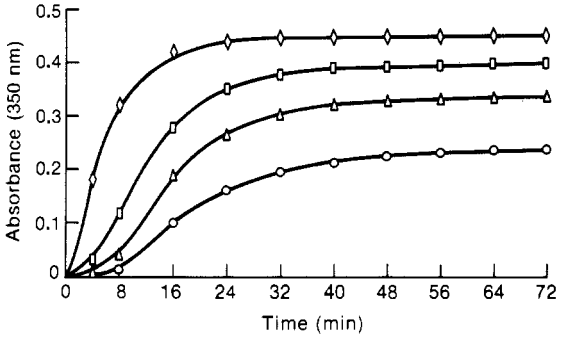

FIGURE 1: Inhibition of microtubule assembly by preformed TCs. Microtubule protein solutions containing 0.1 mM [^{14}C]GTP and a GTP-regenerating system were incubated with different concentrations of tubulin–[^{3}H]colchicine complexes at 30 °C, and assembly was followed by light scattering at 350 nm. The total tubulin concentration was the same for each sample. Both the final extent of assembly and the average polymer length decreased as a function of the initial TC mole fraction in solution. (◇) No TC control, L = 7.9 μm, N = 3.0 × 10^{-10} M; (□) 0.04 TC mole fraction, L = 5.3 μm, N = 3.7 × 10^{-10} M; (△) 0.08 TC mole fraction, L = 3.1 μm, N = 4.2 × 10^{-10} M; (○) 0.12 TC mole fraction, L = 1.99 μm, N = 4.6 × 10^{-10} M. L is the average polymer length, and N is the number concentration determined at apparent equilibrium. The average number of TCs per copolymer at apparent equilibrium was the following: (□) 179; (△) 157; (○) 168. Representative TC:tubulin molar ratios in the copolymers at apparent equilibrium are given in Table I.

exhibited a treadmilling reaction. This latter point was of paramount importance, since it would not have been possible to differentially radiolabel opposite copolymer ends in the absence of a unidirectional "flux" of tubulin subunits through the copolymers.

Characterization of Copolymer Formation. Increasing concentrations of preformed tubulin–[^{3}H]colchicine complexes were added to microtubule protein solutions containing 0.1 mM [^{14}C]GTP; the final tubulin concentration (drug free plus drug bound) in each sample was the same. Assembly was initiated by warming to 30 °C and was followed to plateau by light scattering at 350 nm. At plateau (ca. 60–70 min), aliquots were removed for processing by the filter disk assay, from which the polymer number concentration and the average number of TCs per microtubule were determined (Materials and Methods). Assembly inhibition was determined from light-scatter tracings as well as from the ^{14}C-nucleotide in microtubule copolymers, relative to that in untreated, control microtubules.

The results show that TCs inhibited both the initial rate and the final extent of assembly in a concentration-dependent manner (Figure 1). Furthermore, large numbers of TCs became incorporated into the microtubules. At plateau, significant TC:tubulin ratios within microtubule copolymers were observed (Table I), from which, together with the average copolymer length, it was possible to calculate the average number of TCs incorporated per copolymer. For example, with an initial TC mole fraction in solution of 0.12, as many as 168 TCs per microtubule copolymer were incorporated when sampled at plateau (see legend of Figure 1).

This result confirms the observations of Sternlicht & Ringel (1979) and strongly suggests that copolymer formation occurred under our experimental conditions. This conclusion is further supported by the observation that the initial stages of microtubule elongation, in both the presence and absence of TCs (Figure 1), approximated a first-order process (data not shown; Sternlicht & Ringel, 1979). A requirement for this condition is that the number of assembly-competent ends remains constant.

The foregoing results thus strongly support the notion that, under the experimental conditions employed in this study, TCs coassemble with tubulin dimers to form microtubule copolymers which contain large numbers of TCs and which possess assembly-competent ends.

Table I: Inhibition of Polymer Formation by Preformed TCs Relative to Untreated, Control Microtubules[a]

TC mol fraction in solution	TC:Tu ratio in copolymers	% inhibition		
		initial rate	final extent	steady-state flux
0.02	0.01	17	6	30
0.04	0.02	35	11	71
0.08	0.03	54	26	92
0.12	0.05	72	48	97

[a] Microtubule protein solutions containing 0.1 mM GTP were incubated with increasing TC mole fractions at 30 °C, and assembly was followed by light scattering at 350 nm. The reported TC mole fractions in solution are those present immediately prior to initiation of assembly. TC:tubulin (Tu) molar ratios in the copolymers were measured after the copolymers had attained apparent equilibrium, by using tubulin–[^{3}H]colchicine and [^{14}C]GTP (see Materials and Methods). Initial rates of polymer formation were determined by linear regression analysis of the linear portions of assembly curves, such as those shown in Figure 1. Final extents of assembly, taken from the same assembly curves as used for initial rate determinations, were measured after the absorbance had remained constant for 15 min. Steady-state flux rates were determined from duplicate samples assembled to steady state and pulsed for 60 min with [^{3}H]GTP (see Materials and Methods). Flux rates were corrected for polymer number concentration prior to calculation of the extents of inhibition.

Analysis of Labeled Dimer Incorporation and Loss: Evidence That Microtubules and Microtubule Copolymers Treadmill. Two general mechanisms have been described by which net incorporation of radiolabeled tubulin dimers into unlabeled steady-state microtubules may occur in vitro. The treadmilling mechanism (Margolis & Wilson, 1978), which leads to a unidirectional flux of tubulin dimers through steady-state microtubules, has been described above. The second mechanism proposes that labeled dimers can become incorporated into microtubules at equilibrium, or into microtubules at steady state, owing to the random distribution of the microscopic addition and loss reactions at microtubule ends around the macroscopic equilibrium (equilibrium exchange or "diffusion" mechanism; Zeeberg et al., 1980). These two mechanisms are not mutually exclusive and, in principle, may occur simultaneously in the same microtubule system.

The equations of Zeeberg et al. (1980) lead to diagnostic predictions regarding the dynamics of subunit–polymer exchange in microtubule systems, depending on the type of mechanism operating. For a system in which an equilibrium exchange or diffusion mechanism were solely operative, radiolabeled dimer incorporation into preformed microtubules should increase linearly as a function of the square root of the time of incubation in the label. In contrast, label uptake should occur linearly with time for a steady-state microtubule system [see also Kristofferson & Purich (1981) and Jordan & Farrell (1983)]. If both mechanisms were simultaneously operative, labeled dimer incorporation into preformed microtubules should initially be rapid, since both mechanisms would contribute to dimer uptake. The contribution of the equilibrium exchange component to labeled dimer uptake would, however, rapidly attain a maximal value and saturate. As a result, continuing dimer uptake would decrease in rate and become a linear function of time, since only the steady-state treadmilling reaction would make a net contribution to further dimer incorporation [e.g., see Jordan & Farrell (1983)].

To determine which of the above models best describes our microtubules and microtubule copolymers, microtubule protein

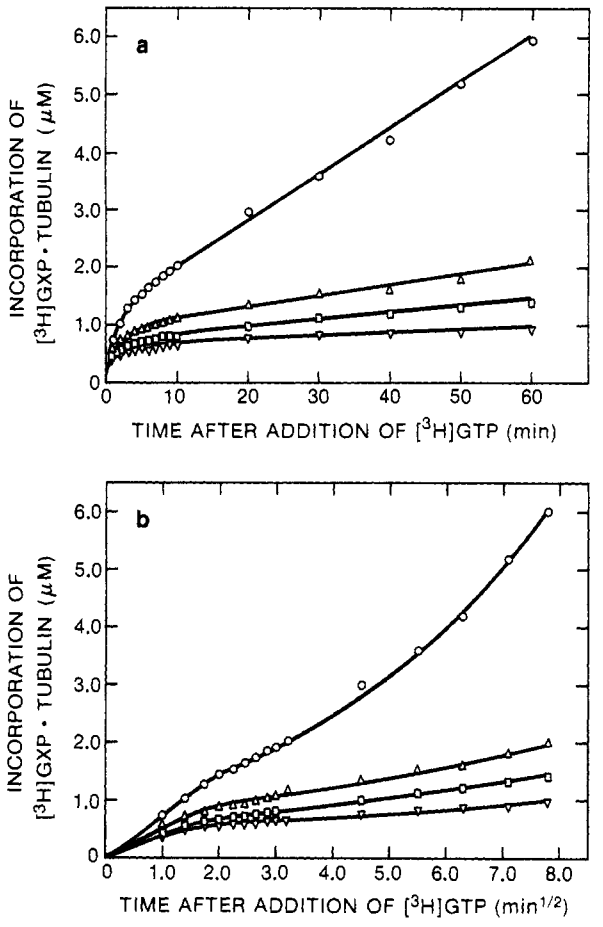

FIGURE 2: Incorporation of [^{3}H]GTP into microtubules and copolymers at apparent equilibrium. Microtubules or microtubule copolymers of tubulin and TCs were assembled to plateau, as determined by light scattering at 350 nm. At plateau, trace amounts of [^{3}H]GTP were added to the microtubules and copolymers, and radiolabel incorporation was assayed by removing polymer aliquots for processing by the filter disk method. Routinely, the ^{3}H-nucleotide labeled 5–15% of an average-length microtubule or copolymer at the end of a 60-min pulse with [^{3}H]GTP. (a) Radiolabel incorporation as a function of time after addition of [^{3}H]GTP. (b) The same data as in (a) plotted as a function of the square root of time after radiolabel addition. The initial TC mole fraction in solution prior to initiation of assembly was (○) 0 (control microtubules), (△) 0.02, (□) 0.04, and (▽) 0.08.

solutions or microtubule protein solutions containing TCs were assembled to plateau, as determined by light scattering at 350 nm. At plateau, the polymers were pulse labeled with 0.1 mM [^{3}H]GTP and aliquots removed for processing by the filter disk assay (see Materials and Methods).

The results (Figure 2a) clearly show an initial, rapid burst of label incorporation into both microtubules and copolymers over approximately the first 2–4 min after addition of labeled GTP, followed by a slower, linear rate of labeled dimer uptake for the remainder of the experiment (60 min). The presence of TCs in the microtubule copolymers decreased, in a concentration-dependent manner, the extent and rate of both the initial "burst" of labeled dimer incorporation and also the slower linear phase of incorporation. Throughout the entire period of label uptake, polymer mass, as determined by light scattering at 350 nm, remained constant.

The labeled dimer uptake data in Figure 2a were replotted as a function of the square root of time in [^{3}H]GTP (Figure 2b). Labeled dimer incorporation into both microtubules and copolymers increased linearly over the period corresponding to the rapid burst phase in Figure 2a but was nonlinear for the remainder of the experiment. This is particularly evident in the case of the unpoisoned, control microtubules, but less so for TC copolymers, since TCs dramatically reduce copolymer rate constants.

These results are consistent with both an equilibrium exchange and a steady-state treadmilling mechanism simultaneously contributing to labeled dimer incorporation into microtubules and microtubule copolymers. Although the upward curvature of the $t^{1/2}$ plots for copolymers may be slight, nevertheless the data do indicate that copolymers treadmill, a conclusion underscored by the results of the pulse–chase experiments described below. We cannot, however, exclude the possibility that the initial, rapid burst of label uptake may also partly be due, for example, to exchange of labeled nucleotide in solution with unlabeled nucleotide exchangeably bound to tubulin dimers over a short region at the very ends of microtubules [e.g., see Carlier & Pantaloni (1981)].

The above data suggest that both microtubules and microtubule copolymers attain a steady state under our experimental conditions. This conclusion is further supported by the following pulse–chase experiment. First, microtubule solutions, with and without TCs, were assembled to plateau in the presence of 0.1 mM [^{14}C]GTP. The microtubules and copolymers thus became labeled throughout their lengths, and hence at both microtubule ends, with ^{14}C-nucleotide. At steady state, the microtubules and copolymers were pulsed for 1 h with trace amounts of [^{3}H]GTP. After 1 h, aliquots were removed from the microtubules and microtubule copolymer suspensions and processed by the filter disk assay to determine the extents of labeled dimer uptake. An excess chase of unlabeled GTP (2.5 mM) was then immediately added to the remaining microtubules and copolymers, and aliquots were removed for processing at timed intervals for the following hour.

The results show that greater than 92% of the ^{3}H-nucleotide incorporated into both microtubules and copolymers during the 1-h pulse was retained after the 1-h chase with unlabeled GTP (Figure 3). This result further supports the argument that microtubules and microtubule copolymers exhibit a steady-state treadmilling reaction: greater than 95% of labeled dimers are expected to be retained in microtubules after a chase period of equal duration to the pulse period, even for steady-state systems with highly inefficient treadmilling reactions (Zeeberg et al., 1980). In contrast, if an equilibrium exchange mechanism were solely responsible for labeled dimer incorporation, only 41.4% of the label incorporated during the pulse should have been retained following a chase period of equal duration.

The kinetics of ^{14}C-nucleotide loss during the chase period with unlabeled GTP similarly support the idea that microtubules and copolymers attain a steady state. Following an initial, rapid loss of ^{14}C-nucleotide during the first 5 min of the chase period, ^{14}C label loss occurred linearly with time (Figure 3), as expected on the basis of a steady-state treadmilling reaction. The initial, rapid loss of ^{14}C label amounted to less than 5% of the ^{14}C-nucleotide present in the microtubules or copolymers prior to addition of the unlabeled GTP chase. This may represent labeled dimer loss by equilibrium exchange, the converse of the rapid burst of label incorporation during the [^{3}H]GTP pulse period (Figure 2a).

The essentially complete retention of the ^{3}H-nucleotide pulse during the chase period with unlabeled GTP, yet simultaneous and linear loss of ^{14}C-nucleotide (Figure 3), is entirely consistent with microtubules and copolymers achieving a steady state.

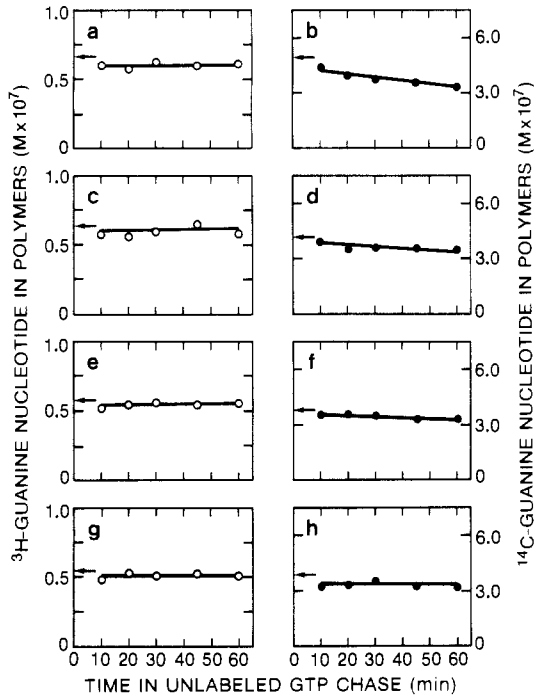

FIGURE 3: Loss of [^{3}H]- and [^{14}C]guanine nucleotides from double-labeled microtubules and microtubule copolymers at apparent equilibrium. Microtubules and microtubule copolymers were assembled to plateau in the presence of 0.1 mM [^{14}C]GTP. At plateau, the microtubules and copolymers were pulse labeled with [^{3}H]GTP for 1–2.5 h, following which an excess "chase" of unlabeled GTP was added. Since the rate of labeled dimer uptake into copolymers at plateau was slower than that into control MTs, it was necessary to pulse label the copolymers with [^{3}H]GTP for longer periods (up to 2.5 h) than with control MTs to obtain similar extents of label uptake. Loss of ^{3}H-nucleotide (open symbols) and ^{14}C-nucleotide (closed symbols) during the chase period was monitored by the filter disk assay (see Materials and Methods). The initial TC mole fraction in solution prior to initiation of assembly was (a, b) 0, (c, d) 0.02, (e, f) 0.04, and (g, h) 0.08. Arrows indicate the concentrations of the appropriate nucleotides in the microtubules or copolymers immediately prior to addition of the unlabeled GTP chase.

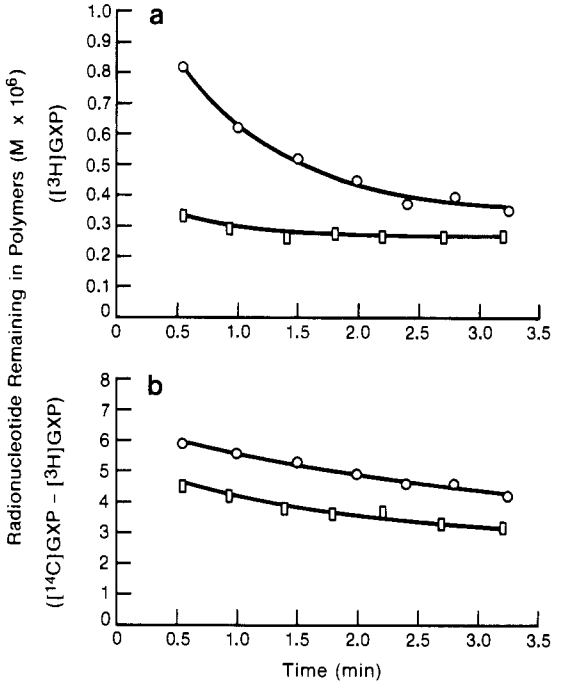

FIGURE 4: Effect of TCs on disassembly rates from (a) polymer A ends and (b) polymer D ends. Microtubule protein solutions were assembled to steady state at 30 °C in the presence of 0.1 mM [^{14}C]GTP (○) or [^{14}C]GTP and TCs (□, initial TC mole fraction in solution = 0.04). At steady state, microtubules or microtubule copolymers were incubated with trace amounts of [^{3}H]GTP for 1.5 h to label A ends. To induce polymer disassembly, 0.5-mL aliquots were added to 14.5 mL of warm (30 °C) reassembly buffer containing 0.1 mM GTP and samples removed over a 3.5-min time course for processing by the filter disk assay. Loss rates from opposite polymer ends were calculated from the ^{3}H and ^{14}C label remaining in the polymers (Materials and Methods). It took at least 15 s to process experimental samples for analysis; thus, it was not possible to obtain zero-time data points. Solid lines represent theoretical plots derived from fitting first-order decay curves to the data by least-squares nonlinear regression analysis.

In summary, analysis of the above labeled nucleotide incorporation and pulse–chase experiments, using the equations of Zeeberg et al. (1980), indicates that labeled dimer incorporation into preformed microtubules and copolymers occurs by a steady-state treadmilling reaction and, if our interpretation of the initial, rapid burst of label uptake is correct, also simultaneously by equilibrium exchange. However, even with the assumption that the initial burst of labeled dimer uptake can be ascribed entirely to equilibrium exchange (undoubtedly a gross overestimate, since it ignores the contribution from the treadmilling reaction, as well as possible nucleotide exchange at polymer ends), the equilibrium exchange contribution to total labeled dimer incorporation after a 1-h pulse is the minor of the two.

On the basis of the above considerations, it is reasonable to conclude that a 1-h steady-state pulse with [^{3}H]GTP would predominantly, if not overwhelmingly, label the net assembly (A) ends of microtubules, while leaving the opposite, net disassembly (D) ends labeled essentially with ^{14}C-nucleotide alone (see Materials and Methods). The distribution of the ^{3}H label in TC copolymers may be less polarized than in microtubules, since TCs severely decrease the A-end kinetics. Nevertheless, the above results indicated that ^{3}H labeling of copolymer A ends is still heavily favored.

Effect of TCs on Dimer Loss from Opposite Ends of Microtubule Copolymers. To examine the influence of TCs at opposite ends of microtubule copolymers, microtubule protein solutions containing increasing mole fractions of TCs were assembled to steady state in the presence of 0.1 mM [^{14}C]GTP. At steady state (determined by light scattering), net assembly A ends of microtubules and microtubule copolymers were additionally labeled with ^{3}H-nucleotide by incubation with [^{3}H]GTP for 1 h (Materials and Methods). The numbers of TCs incorporated into steady-state copolymers were determined from duplicate microtubule suspensions which had been reassembled to steady state in the presence of 0.1 mM [^{14}C]GTP and [^{3}H]TCs (Materials and Methods).

The double-labeled, steady-state microtubules and copolymers were then diluted and the rates of loss of ^{3}H- and ^{14}C-nucleotides determined, from which dimer loss rates from opposite microtubule and microtubule copolymer ends were calculated.

Typical copolymer disassembly curves are shown in Figure 4a,b. The depolymerization kinetics for control microtubule A ends closely approximated a first-order loss reaction (correlation coefficients = −0.92 to −0.98), reflecting an exponential loss of microtubule A ends (Karr et al., 1980; Kristofferson et al., 1980). In contrast, the presence of TCs in the microtubule lattice kinetically stabilized copolymer A ends to such an extent that extensive loss of these ends did not occur; for example, only a 21% loss of ^{3}H label occurred from copolymer A ends over a 3.5-min period, compared with a 57% loss from control microtubule A ends during the same interval (Figure 4a). The disassembly kinetics of copolymer A ends consequently appeared more linear than exponential. Simi-

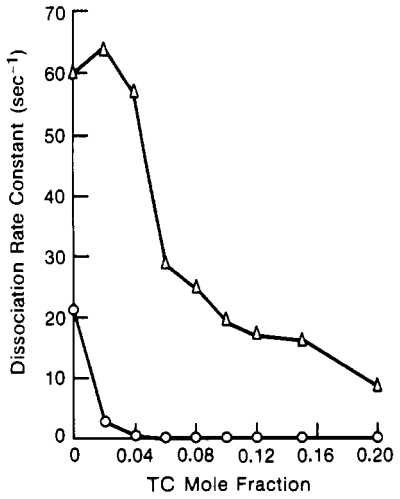

FIGURE 5: Apparent dissociation rate constants for polymer A ends (O) or D ends (Δ) as a function of the initial TC mole fraction in solution. Microtubule solutions containing increasing TC concentrations were assembled to steady state (e.g., see Figure 1). To induce microtubule disassembly, aliquots (0.5 mL) were diluted 30-fold into 14.5 mL of warm reassembly buffer containing 0.1 mM GTP. Dissociation rate constants were derived from microtubule or microtubule copolymer disassembly curves (e.g., see Figure 4) by nonlinear regression analysis and corrected for polymer number concentration.

larly, D-end loss rates appeared more linear for both microtubules and copolymers since extensive loss of ^{14}C label did not occur for either polymer (less than ca. 30%; e.g., see Figure 4b).

The presence of TCs in the microtubule lattice inhibited disassembly rates at both copolymer ends, relative to control microtubules. For any given TC concentration, however, copolymer A ends showed a far greater sensitivity to TC poisoning than did D ends. This differential sensitivity was particularly clear from the behavior of the apparent dissociation rate constants (Figure 5), derived from plots such as those shown in Figure 4. For example, at an initial TC mole fraction in solution of 0.04, the A-end rate constant was reduced by 97%, compared with that for control microtubules, whereas that for the D end was reduced by only 5%.

These results may represent an underestimation of the differential poisoning effects of TCs. During the steady-state pulse with [^{3}H]GTP, designed to label polymer A ends by the treadmilling reaction (Materials and Methods), some ^3H-nucleotide might also become incorporated at the D ends of the ^{14}C-labeled microtubules (or copolymers) by an equilibrium exchange mechanism. This would lead to an overestimation of the magnitude of A-end dissociation rate constants and an underestimation of D-end rate constants (see Determination of Dissociation Rate Constants under Materials and Methods). This would be true, in particular, at the highest TC concentrations, where D-end dissociation rate constants are proportionally much greater than the corresponding A-end constants.

An extreme case of the above situation, in which incorporation (by either treadmilling or equilibrium exchange) of [^{3}H]GTP dimers at copolymer A ends was essentially completely inhibited by TCs and, instead, labeled dimers added entirely to the "wrong" copolymer D ends by equilibrium exchange, did not occur. If this had occurred, upon microtubule dilution, the rates of loss of ^3H- and ^{14}C-nucleotides should have been identical. In fact, ^{14}C-nucleotide loss rates were always at least twice those of ^3H-nucleotides.

Thus, although equilibrium exchange labeling of microtubule copolymer D ends by ^3H-nucleotide, if it occurred to a significant degree, could lead to an underestimation of the differential poisoning effects of TCs, it is unlikely that this mechanism led to a "switch" in the copolymer ends labeled by the [^{3}H]GTP pulse.

Discussion

The foregoing results clearly show that TCs decrease the dissociation rate constants at opposite microtubule copolymer ends in a concentration-dependent and graded manner. Furthermore, the inhibitory effects of TCs are polar, with copolymer A ends being far more sensitive to TC action than D ends. Since TCs also inhibit the extent of polymer formation (Figure 1), as well as the steady-state flux rate (Figure 2a, Table I), it is likely that A- and D-end association rate constants are similarly affected.

The differential, or polar, poisoning effects of TCs at opposite copolymer ends, observed by using the double-labeling protocol, are consistent with previous observations from this laboratory using independent methods. When colchicine (Margolis et al., 1980) or TCs (Jordan & Farrell, 1983) were added to steady-state microtubules, pulse incorporation of labeled dimers (at the microtubule A ends) could be completely inhibited, yet dimer loss (from the opposite D ends), as measured by a decrease in polymer mass, continued. Other observations, also independent of the double-labeling protocol, are likewise consistent with the results obtained by using the double-labeling method. For a given TC mole fraction in solution, the initial rate of dimer addition to steady-state copolymers (at the A ends) was inhibited to a far greater extent than the initial rate of copolymer assembly from microtubule protein solutions (dimer addition at both A and D copolymer ends) (Table I). In spite of the differential poisoning effects of TCs, however, colchicine, and possibly competitive inhibitors of colchicine such as podophyllotoxin, cannot be used as a specific A-end inhibitor, although with a judicious choice of TC concentration a close approximation can be attained (e.g., for Figures 3 and 5, TC mole fraction = 0.04).

The polar poisoning effects of TCs may also partly explain the difference in the number of TCs required to inhibit microtubule assembly reported by Margolis & Wilson (1980) and Farrell & Wilson (1980) and by Sternlicht & Ringel (1979). In the first two studies, colchicine or TCs were added to steady-state microtubules and hence would measure TC poisoning of the more sensitive microtubule A ends. A small number of incorporated TCs (<10 per microtubule) was thus sufficient to poison dimer addition at the A ends completely. In contrast, Sternlicht & Ringel (1979) added TCs to microtubule protein solutions prior to initiation of assembly. The higher initial tubulin concentrations could lead to bidirectional microtubule assembly (at least initially), with concomitant incorporation of large numbers of TCs (ca. 100 per microtubule) at the relatively refractile D ends.

The results obtained above with the homologous microtubule system differ significantly from those recently reported by Bergen & Borisy (1983) using an axoneme-directed assembly system. These workers found that TC poisoning was nonpolar (i.e., opposite microtubule ends were affected to the same degree) and that only association rate constants were decreased; dissociation rate constants were unaffected. The reason for this discrepancy is unclear but is hardly surprising as the kinetic properties of homologous and heterologous "axoneme-seeded" microtubule systems differ significantly in the absence of colchicine [cf. Bergen & Borisy (1980) and Farrell & Jordan (1982)].

The polar poisoning effects of TCs lead to a dramatic enhancement of kinetic differences between opposite microtubule

ends. For example, with unpoisoned, control microtubules, dissociation rate constants for opposite microtubule ends differed by as little as 3-fold. In contrast, this difference increased to nearly 60-fold for copolymers assembled from tubulin solutions containing a TC mole fraction of 0.04 (Figure 5). Microtubules containing the "correct" TC composition, in effect, become copolymers capable only of unidirectional growth, rather than the bidirectional growth more easily possible with microtubules lacking TCs.

The role of microtubule organizing centers notwithstanding, copolymer formation may thus represent a mechanism for controlling microtubule spatial orientation in vivo. It has already been suggested that equilibrium differences at opposite microtubule ends [e.g., see Bergen & Borisy (1980) and Farrell & Jordan (1982)] may be employed in vivo to regulate microtubule spatial orientation (Kirschner, 1980): if the level of the unpolymerized tubulin pool were maintained above that required for net growth at one microtubule end, but below that for the opposite microtubule end, growth would only be possible at one end. Consequently, microtubules would have a parallel structural orientation, such as in each mitotic half-spindle (Euteneuer & McIntosh, 1981; Telzer & Haimo, 1981).

However, the equilibrium dissociation constants for opposite microtubule ends differ only slightly in vitro. If this were the case in vivo, it would require that the tubulin pool be regulated within very narrow limits to ensure correct microtubule organization. With a copolymer of tubulin and an endogenous colchicine-like molecule, however, control of microtubule spatial orientation could be shifted to a kinetic basis. Copolymer growth could be kinetically so slow at one microtubule end that elongation would essentially be unidirectional, thereby ensuring a parallel structural orientation. Furthermore, the constraint of maintaining the tubulin pool to within narrow limits would be relaxed; "correct" microtubule orientation could still be achieved even if the level of the tubulin pool were to vary significantly.

Acknowledgments

We thank Drs. M. A. Jordan and G. Taborsky for their helpful discussions and criticism. We also thank Herb Miller for preparing the large quantities of microtubule protein used in this study.

Registry No. GTP, 86-01-1.

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