Kinetic and Steady-State Analysis of Microtubules in the Presence of Colchicine†

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ABSTRACT: The effects of colchicine on bovine brain microtubules under steady-state conditions have been studied by combined kinetic and equilibrium analysis. Colchicine induces an initially rapid rate of depolymerization when added to microtubules which are at steady state. The initial rate of disassembly follows the kinetics of colchicine binding to free tubulin. However, disassembly is incomplete, and a new steady-state concentration of microtubules is established provided that a sufficient concentration of colchicine–tubulin is present. When steady state is attained from the disassembly direction, colchicine decreases the fraction of tubulin which is participating in the assembly reaction, without measurably changing the apparent critical concentration for polymerization. The extent of depolymerization of microtubules by colchicine is greater the lower the content of microtubule-associated proteins (MAPs). Microtubules at steady state in the presence of either colchicine or GDP do not exhibit subunit flow which occurs in microtubules at steady state in GTP. Colchicine–tubulin will stabilize microtubules in the presence of MAPs but will not support microtubule elongation. Microtubules at steady state in the presence of colchicine depolymerize upon dilution at about the same rate as untreated microtubules, and, in either case, disassembly appears to occur from both ends of the microtubule. These observations appear to be inconsistent with simple reversible assembly mechanisms but may be explained by a model based upon the cooperative interactions of MAP–tubulin oligomers.

The antimitotic drug colchicine is an inhibitor of microtubule assembly both in vivo and in vitro (Taylor, 1965; Inoue & Sato, 1967; Weisenberg, 1972). The microtubule subunit protein tubulin binds colchicine at a high affinity site with a stoichiometry of 1 mol of colchicine per mol of tubulin (Weisenberg et al., 1968), although complete inhibition of microtubule assembly can occur when only a small fraction of tubulin is complexed with colchicine (Olmsted & Borisy, 1973; Wilson & Bryan, 1974; Margolis & Wilson, 1977; Sternlicht & Ringel, 1979). In spite of the extreme sensitivity of tubulin polymerization to colchicine, only limited disassembly of microtubules is generally observed when saturating concentrations of colchicine are added to microtubules in vitro (Haga & Kurokawa, 1975; Herzog and Weber, 1977; Deery et al., 1978; Farrel et al., 1979; Wallin & Larsson, 1979). In a similar fashion, some microtubules in vivo have been found to be stable in the presence of colchicine, even though colchicine is able to prevent the formation of such microtubules (Hokfelt & Dahlstrom 1971; Mayor et al., 1972; Schnepf & Deichgraber, 1976). No consensus exists concerning the cause for either the extreme sensitivity of microtubule asembly to colchicine or the failure of colchicine to depolymerize some microtubules.

Two models have been presented to explain the effects of colchicine on microtubule assembly. The first model was proposed by Wilson and co-workers and is based upon their observation of subunit flow through microtubules (Margolis & Wilson, 1978). They proposed that colchicine–tubulin adds to the net assembly end of the microtubule and forms an irreversible "cap" which blocks further subunit addition to that end (Margolis & Wilson, 1977). Depolymerization is assumed to occur only through the slow loss of tubulin from the opposite, net disassembly end. The depolymerization rate in colchicine will therefore be equal to the subunit flow rate and to the intrinsic subunit dissociation rate from the disassembly end of the microtubule. The reported flow rates ranged from about 7%/h for microtubules in GTP to about 30%/h for microtubules incubated in ATP (Margolis & Wilson, 1979). These rates were said to be similar to depolymerization rates obtained in the presence of podophyllotoxin (a colchicine analogue), although faster rates have been reported for the initial rate of depolymerization observed after addition of the drug (Farell et al., 1979; Karr & Purich, 1979).

In contrast, the model proposed by Sternlicht & Ringel (1979) is based upon the observation that low concentrations of colchicine–tubulin can copolymerize with drug-free tubulin. Their data indicate that colchicine inhibits asembly by reducing tubulin–tubulin binding affinities. This results in an increased critical concentration, and a decreased equilibrium constant, for microtubule assembly. They proposed that colchicine–tubulin incorporated into the microtubule inhibits assembly through a cooperative interaction with reduces the affinity of tubulin for the assembly sites on the microtubule. The extent of inhibition depends upon the fraction of colchicine incorporated into the microtubule, while the ability of colchicine–tubulin to incorporate into the microtubule depends upon the concentration of MAPs.

In the experiments reported here, the kinetic and steady-state properties of microtubules in the presence of colchicine have been studied. These results demonstrate that at steady state both ends of the microtubule can be in reversible equilibrium with colchicine–tubulin and that this equilibrium depends upon the presence of MAPs. Both colchicine and GDP are found to prevent subunit flow through the microtubule. These results can be interpreted in terms of a proposed cooperative, oligomer-addition model for microtubule assembly (Weisenberg, 1980).

Materials and Methods

Tubulin was isolated from fresh beef brain by a modification of the method of Shelanski et al. (1973), as described previously (Zackroff et al., 1980). The buffer used for purification contained 0.1 M Mes[1] adjusted to pH 6.6 with NaOH, 1 mM

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EGTA, 0.5 mM $MgCl_2$, and 25% glycerol. Tubulin was purified by two cycles of assembly and disassembly and was stored at −70 °C until needed. Prior to experimentation, a third cycle of assembly and disassembly was performed. Just before use, the protein solution was passed through at least 10 volumes of packed Sephadex G-25 (Neal & Florini, 1973) to remove most remaining traces of glycerol and nucleotides. The protein was then clarified by centrifugation at approximately 100000*g* for 15 min. Except for polymerization steps, where the temperature was generally 33–35 °C, the temperature was maintained at 0–4 °C.

Total protein concentrations were determined by the method of Hartree (1972) by using bovine albumin as a standard. The proportions of tubulin and MAPs were determined by Na-$DodSO_4$ gel electrophoresis after the method of Laemmli (1970). Coomassie blue stained gels were scanned at 650 nm, and the weight of the cutout protein peaks were obtained for relative protein quantitation. Tubulin polymerized by three cycles of asembly and disassembly contained approximately 70% tubulin and 30% MAPs and other proteins.

For experiments in which the effects of MAPs were being studied, microtubule protein was further purified by either 33–50% ammonium sulfate precipitation (Arai & Kuziro, 1976) or phosphocellulose chromatography as described by Weingarten et al. (1975).

Measurement and Analysis of Microtubule Assembly and Disassembly. The kinetics of microtubule assembly and disassembly were monitored by turbidity measurements at 340 nm (Gaskin et al., 1974). Polymerization was performed in a 1-cm path-length water-jacketed cuvette at a temperature of 33 °C. The tubulin solutions contained 0.1 mM GTP and a GTP regenerating system of 20 mM acetyl phosphate and 0.05 IU/mL acetate kinase (MacNeal et al., 1977). Steady state, defined by no detectable change in turbidity, was attained within 100 min. Steady-state conditions were confirmed by a constant protein content of sedimented microtubule pellets for up to 3 h after the attainment of a constant turbidity.

The microtubule-dependent turbidity was determined by the difference in the plateau turbidity and the turbidity remaining following depolymerization at 0 °C. The relationship between the turbidity at 340 nm and the microtubule protein concentration was determined by the method of Johnson & Borisy (1977). A nearly linear dependence was observed between turbidity of microtubule samples and the microtubule protein concentration determined by centrifugation (Zackroff et al., 1980). A slope of 0.3 $A\ mg^{-1}\ mL^{-1}$ was obtained, and the turbidity was assumed to be directly proportional to the microtubule concentration under the conditions used.

Microtubule formation was verified by electron microscopy of samples negatively stained with 0.5% urany acetate. For quantitation, fields of microtubles were photographed at a magnification of 4600×, and microtubule lengths and numbers were determined directly from the photographic negatives.

Colchicine Binding Assay. The kinetics of colchicine binding to tubulin were determined essentially by the method of Sherline et al. (1974). After colchicine was added to tubulin solutions, 0.8-mL samples were removed at various times and mixed with 6–7 mg of activated charcoal at 0 °C for 10 min. The charcoal was then pelleted at 2200 rpm and the protein supernatant filtered to remove trace charcoal. The colchicine concentration was determined by the OD of the solution at 350 nm. The tubulin content was determined by determining total protein content and correcting for the fraction of nontubulin proteins obtained from analysis of electrophoretic gels.

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

Table I: Map-Dependent Stability[a]

	% microtubule depolymerization	
	2 mM GDP	0.05 mM colchicine
unfractionated	<5	<5
ammonium sulfate fractionated	14	25
phosphocellulose fractionated	52	65

[a] Microtubules were polymerized in 25% glycerol, 0.025 M Mes, 5×10^{-5} M GTP, 5 mM Mg, and 1 mM EGTA. Protein concentration was 2.8 mg/mL. Final equilibrium level after addition of depolymerizing agents was determined when no further decrease in turbidity occurred within 30 min; 100% depolymerization was taken to be the OD of the sample after 15 min at 0 °C.

Labeling of Microtubules with [^3H]GTP. Microtubules were uniformly labeled by polymerizing third cycle tubulin with 0.1 mM [^{3}H]GTP (11 Ci/mmol; 0.5 μCi/mL tubulin) until a steady state was attained essentially as described by Margolis & Wilson (1978). For prevention of further labeling, microtubules were chased with a 20-fold excess of unlabeled GTP (2 mM) or 2 mM GDP for GDP steady state.

The net assembly end of the microtubules was labeled by the addition of [^{3}H]GTP (10 μL/mL tubulin of 0.05 mCi/mL in H_2O) to solutions of 0.1 mM GTP steady-state microtubules for 45 min, folowed by a 2 mM GTP chase to stop labeling.

Analysis of Labeled Microtubules. Labeled microtubules were separated from free [^{3}H]GTP by layering 0.7–1 mL of the microtubule sample on 4 mL of a 50% glycerol, 0.1 M Mes, and Mg^{2+}EGTA cushion and centrifuging at 30 °C for 70 min at 43000 rpm (Beckman SW50.1 rotor) to pellet the microtubules. The supernatants were carefully removed, and each tube was washed 3 times with warm 60% glycerol–Mes buffer without disturbing the pellets which eliminated contaminating radioactivity. The washed microtubule pellets were resuspended in 0.5 mL of 0 °C 0.1 MES buffer, and aliquots were taken for protein assay and scintillation counting.

Results

MAP-Dependent Microtubule Stability. Microtubules purified by cycles of assembly and disassembly contain a number of microtubule-associated proteins (MAPs). MAPs promote microtuble assembly (Weingarten et al., 1975; Sloboda et al., 1976; Cleveland et al., 1977; Berkowitz et al., 1977) but are not an absolute requirement since glycerol and high concentrations of Mg will promote the polymerization of MAP-free tubulin (Lee & Timasheff, 1977). MAPs apparently act to decrease the rate of subunit disassembly from microtubules (Murphy et al., 1977; Sloboda & Rosenbaum, 1979) and have been observed to increase the stability of microtubules to depolymerization of GDP (Zackroff et al., 1980) and colchicine (Haga & Kurokawa, 1975; Herzog & Weber 1977; Deery et al., 1978).

For studies on the effect of MAPs on microtubule depolymerization in the presence of colchicine, third cycle microtubule protein was further purified by either ammonium sulfate precipitation (Arai & Kuziro, 1976) or phosphocellulose chromatography (Weingarten et al., 1975) to remove some or all of the MAPs. The fractionated or unfractionated third cycle microtubule protein was polymerized at 33 °C in reassembly buffer plus 25% glycerol and 5 mM $MgCl_2$, and the new steady-state extent of polymerization was determined following the addition of 5×10^{-5} M colchicine (Table I). Microtubules which contain all of the original MAPs are only

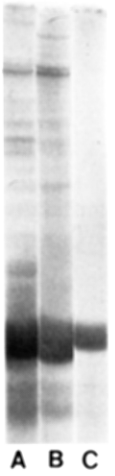

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of microtubules which remain after treatment with 0.5 mM colchicine and 2 mM GDP. The microtubules were assembled in reassembly buffer plus 25% glycerol and 5 mM $MgCl_2$ to allow assembly of MAP-free tubulin. Electrophoresis was performed with 5% acrylamide gels after the method of Laemmli (1970). (A) The microtubules were prepared from protein purified by three cycles of assembly and disassembly. (B) The protein was prepared as in (A), followed by ammonium sulfate fractionation. (C) The protein was prepared as in (A), followed by fractionation on phosphocellulose.

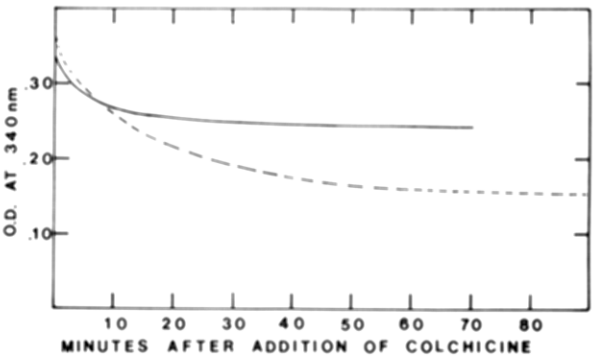

FIGURE 2: Microtubule depolymerization and attainment of a new steady state following the addition of colchicine. Microtubule protein was prepared by three cycles of assembly and disassembly (solid line) or by phosphocellulose chromatography to remove MAPs (dashed line). The protein concentration in each case was 2.6 mg/mL. The MAP-free protein was assembled in the presence of reassembly buffer plus 25% glycerol.

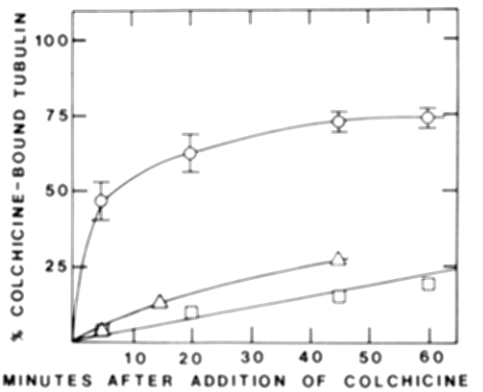

FIGURE 3: Binding of colchicine in the presence and absence of microtubules. Third cycle microtubule protein at 2.5 mg/mL was incubated with 5×10^{-5} M colchicine, and, at the indicated times, the amount of bound colchicine was determined as described under Materials and Methods. (□) Colchicine was added to tubulin at 0 °C, and incubation was carried out at 0 °C. (○) Colchicine was added to protein at 0 °C, and incubation was carried out 33 °C. No microtubule assembly occurred in this case. (△) Colchicine was added to assembled microtubules at 33 °C. The fraction of MAPs and other nontubulin proteins was determined by gel electrophoresis and was corrected for in the percent of tubulin with bound colchicine.

slightly affected by colchicine and depolymerize by only about 5%. Microtubules formed from the ammonium sulfate fractionated protein lack most of the 58 000–65 000-dalton MAPs (Figure 1B). These microtubules were depolymerized by about 25% by colchicine. Microtubules formed from phosphocellulose-purified protein contain no detectable MAPs (Figure 1C). These microtubules are the most labile and depolymerize by about 65%.

The extremely small amount of depolymerization induced by colchicine in the unfractionated protein and the failure of even the MAP free tubulin to completely depolymerize are probably due to the stabilizing effects of glycerol and Mg on microtubules. These results, nevertheless, demonostrate that MAPs decrease the sensitivity of microtubules to colchicine. It is interesting to note that the extent of GDP-induced depolymerization is similar to that obtained with colchicine. This suggests that similar mechanisms may be determining the extent of depolymerization in both cases.

Kinetics of Colchicine-Induced Microtuble Disassembly. The rate of colchicine-induced disassembly was determined by the change in turbidity after the addition of 5×10^{-5} M colchicine to either MAP-containing microtubules in the glycerol-free reassembly buffer or MAP-free microtubules in the presence of 25% glycerol and 5 mM Mg. In both cases, there is an initial, relatively rapid disassembly (Figure 2). In the experiment shown, the rates were 0.018 A/min for MAP-containing microtubules and 0.025 A/min for MAP-free microtubules. These rates are equivalent to 6%/min total tubulin in the microtubules lost and 7%/min for MAP and MAP-free microtubules, respectively, although rates as high as 20%/min were commonly observed. The observed disassembly rates depend upon the number concentration of microtubules, which was not determined in these experiments. However, these rates are comparable to those obtained by dilution of microtubules (Karr & Purich, 1979) but are much greater than the rates reported by Margolis & Wilson (1978) and Farrell et al. (1979) for subunit flow and depolymerization rates in podophyllotoxin.

Although the concentration of colchicine used in these experiments is several times greater than that needed to saturate all the tubulin, complete depolymerization is not obtained with MAP-containing microtubules, even in the absence of glycerol. Note that the failure to obtain complete depolymerization cannot be explained by a slow rate of depolymerization since a new plateau is reached within 60 min after addition of colchicine.

Kinetics of Colchicine Binding. It has been suggested (Inoue & Sato, 1967; Garland, 1978; Borisy et al., 1975) that colchicine induces microtubule disassembly by binding to free subunits, thereby preventing their participation in microtubule equilibrium. If this is the case, then the rate and extent of binding of colchicine to tubulin in equilibrium with microtubules should be related to the kinetics of microtubule disassembly. This will be true, however, only if the rate of colchicine binding is relatively fast compared to the intrinsic microtubule subunit dissociation rate.

The rate of colchicine binding is temperature dependent and is very slow at 0 °C, while at polymerization temperatures, it is nearly complete after 20 min at 0.05 mM colchicine (Figure 3). When unpolymerized protein is incubated with colchicine, 75–80% of the tubulin will bind colchicine. The failure of 100% of the tubulin to bind colchicine is unexplained but apparently is due to denaturation of some tubulin during

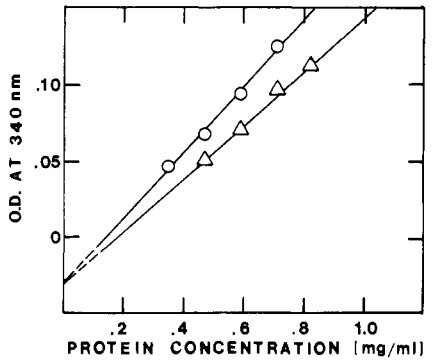

FIGURE 4: Analysis of microtubule steady state in the presence of colchicine. Microtubules at steady state in GTP and in the presence (Δ) or absence of colchicine (O) were diluted, and the absorbance at 340 nm was determined after a new steady state was established. The concentration of colchicine was 0.05 mM, and the concentration of GTP was maintained at 0.1 mM with a GTP regenerating system. The slope of each graph is proportional to the fraction of protein participating in polymerization. The extrapolated intercept on the absorbance axis is proportional to the critical concentration.

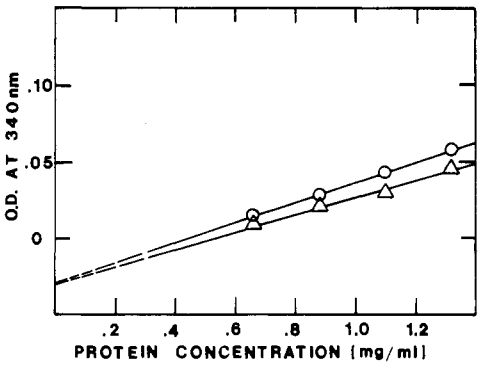

FIGURE 5: Analysis of microtubule equilibrium in the presence of colchicine and GDP. Microtubules were assembled in polymerization buffer with 0.1 mM GTP and then allowed to reach equilibrium in 2 mM GDP or GDP plus 0.05 mM colchicine. The protein was then diluted in buffer containing GDP (O) or in buffer containing GDP and colchicine (Δ), respectively. The absorbance was determined when a new equilibrium had been reached, as indicated by no further change in absorbance over 20 min.

preparation. When colchicine is added to assembled microtubules, the rate and extent of binding are greatly reduced (Figure 3).

The reduced binding of colchicine in polymerized tubulin solutions is most likely due to the inability of colchicine to bind to microtubules (Wilson & Mesa, 1973). The observed binding in this case must therefore be due to the binding of colchicine to free tubulin. Note that the fraction of tubulin which binds colchicine after 45 min is 27%, which is nearly identical with the 30% loss of tubulin from microtubules (Figure 2) obtained after the same time in this experiment.

Effect of Colchicine on Microtubule Equilibrium and Steady State. The concentration of free tubulin which is required to maintain microtubules at steady state is defined as the critical concentration. For a simple reversible, helical condensation mechanism such as described by Oosawa & Kasai (1962) for actin polymerization and later applied to microtubule assembly (Johnson & Borisy, 1977), the reaction is described by rate eq 1 where P, C_1, and M represent the

$$dP/dt = k_+C_1M - k_-M \quad (1)$$

molar concentrations of tubulin within the microtubule, free tubulin, and microtubule ends, respectively, while k_+ and k_- are the apparent association and dissociation rate constants. They are apparent rate constants only because they may be a function of the mechanism of polymerization-coupled GTP hydrolysis (see Discussion).

At steady state, $dP/dt = 0$ and the unpolymerized tubulin fraction is equal to the critical concentration and is given by the relationship

$$C_c = k_-/k_+ = 1/K_a \quad (2)$$

where K_a is the apparent polymerization equilibrium constant.

Note that the above expressions can be considered approximations only for microtubules which are at steady state in the presence of GTP. True equilibrium is not reached in the presence of GTP, and the apparent equilibrium constant can be shown to be related to the hydrolysis rate (Weisenberg, 1980).

For determination of the effects of colchicine on the microtubule equilibrium and steady state, serial dilutions were made of microtubules at steady state in either GTP or GDP, and the extent of polymerization was determined when the diluted samples had reequilibrated. A graph of polymer concentration (as measured by turbidity) vs. total protein concentration yields a straight line in both the absence or presence of colchicine (Figures 4 and 5).

The extrapolated y intercept represents the apparent critical concentration and may be expressed as

$$cA_{(MT)} = mC_{total} - C_c \quad (3)$$

where c is a constant of proportionality relating optical density units to the concentration of polymerized subunits and the slope m is proportional to the fraction of protein participating in the microtubule assembly reaction.

The slope of the line obtained when colchicine is present is significantly less than that obtained in its absence, while both lines extrapolate to approximately the same absorbance at zero protein concentration (-0.033 ± 0.004 A). This intercept is proportional to the critical concentration (eq 3) and is independent of the fraction of protein participating in polymerization. These results clearly indicate that colchicine induces microtubule disassembly by decreasing the fraction of tubulin participating in the microtubule steady-state reaction without significantly changing the apparent critical concentration and thus the equilibrium constant (eq 2).

Subunit Flow at Steady State in the Presence of Colchicine and GDP. It has been reported by Margolis & Wilson (1978) and Farrell et al. (1979) that, at steady state, net assembly occurs at one end of the microtubule (the net assembly end) while net disassembly occurs at the other end (the net disassembly end). At steady state, there would occur an elongation of the microtubule at the net assembly end which is balanced by an equivalent shortening at the opposite end. This requires that there be a difference in the critical concentrations at opposite ends of the microtubule (Bergen and Borisy, 1980; Kirschner, 1980). This can only occur if there is an energy source present (Wegner, 1976), and, in the case of microtubule assembly, the energy source is presumably GTP hydrolysis (Weisenberg, 1980). Thus, subunit flow should not occur in the presence of GDP while microtubules at steady state in the presence of colchicine may or may not exhibit subunit flow, depending upon the mechanism of action of colchicine.

For examination of subunit flow, microtubules were labeled by assembly to steady state in the presence of 0.1 mM [^{3}H]GTP (Margolis & Wilson, 1978). This was followed by a chase with either 2 mM GTP or 2 mM GDP to remove the exchangeable label from the free subunits. Following the attainment of a new steady state, aliquots were centrifugated at various intervals through a 50% glycerol cushion, and the

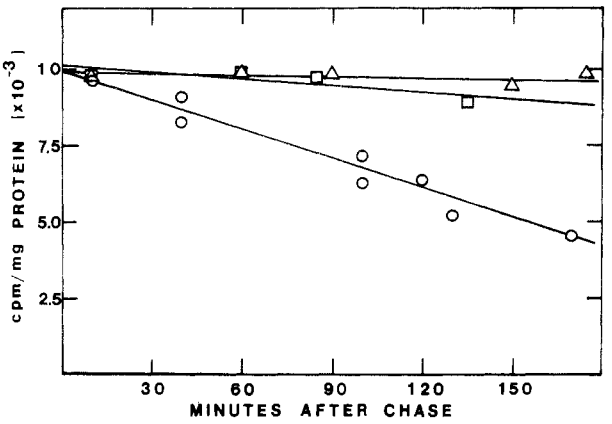

FIGURE 6: Effect of colchicine and GDP on the steady-state exchange of microtubule subunits labeled with [^{3}H]GTP. Third cycle microtubule protein at 2–3 mg/mL was polymerized at 33 °C to steady state in the presence of 0.1 mM [^{3}H]GTP (3.7×10^{12} cpm/mol). One sample (○) was then chased with 2 mM GTP. A second sample (□) was chased with 2 mM GDP. The third sample (△) was chased with 2 mM GTP in the presence of 5×10^{-5} M colchicine. At the indicated times, aliquots were removed, and the specific radioactivity of the microtubules was determined as described under Materials and Methods.

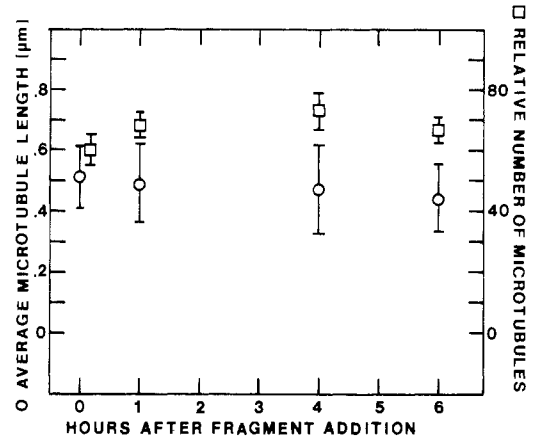

FIGURE 7: Stability of microtubule fragments in the presence of colchicine–tubulin. Third cycle tubulin was polymerized at 2.5 mg/mL, brought to a new steady state in colchicine, and fragmented sonication. To 50 μL of the fragment preparation was added 1 mL of 2.5 mg/mL microtubule protein preincubated in 0.5 mM colchicine and 5 mM GTP at 33 °C. At the indicated times, samples were negatively stained with 0.5% uranyl acetate and microtubule length and number were determined by electron microscopy. The bars indicate the standard error.

specific radioactivity of the pelleted microtubules was determined.

As expected, a loss of label occurs from microtubules at steady state in GTP. Over a 3-h incubation, the loss of label is roughly linear and occurs at a rate of about 20%/h (Figure 6). In the presence of GDP, however, no loss of label is observed. This result is consistent with the interpretation that the loss of label is due to subunit flow and can be considered as an essential control experiment.

In the presence of colchicine, no significant loss of label is observed (Figure 6), and it can be concluded that colchicine also inhibits subunit flow through the microtubule.

Effect of Colchicine–Tubulin on Microtubule Stability. The data presented thus far indicate that colchicine-induced disassembly of microtubules is incomplete and a new steady state can be established in the presence of colchicine. It may be argued, however, that the rate of colchicine-induced disassembly is simply too slow to be observed in these experiments. In order to increase the rate of disassembly, we have sonicated microtubules to increase the concentration of microtubule ends.

Microtubules at steady state in presence of GTP and 0.5 mM colchicine were sonicated to an average length of 0.5 μm. These microtubules were then diluted into 2.5 mg/mL third cycle purified microtubule protein which had been preincubated in 0.5 mM colchicine at 33 °C for 1 h. Samples were taken at intervals for 6 h and examined after negative staining with uranyl acetate. No significant change in either microtubule length or number was observed over this period (Figure 7), thus indicating that the colchicine–tubulin did not induce disassembly but was not capable (at 8 mg/mL) of elongating the fragments.

The ability of colchicine–tubulin to stabilize microtubules depends upon the presence of MAPs. When microtubule fragments were diluted into MAP-free tubulin (prepared by phosphocellulose chromatography) at 3 mg/mL and preincubated with 0.5 mM colchicine, complete depolymerization was observed within 5 min. Rapid disassembly also occurs when microbutules are diluted into buffer containing colchicine. Therefore these microtubules can be considered to be in a normal rapid equilibrium. The presence of MAPs in the dilution buffer does not prevent depolymerization, as was also

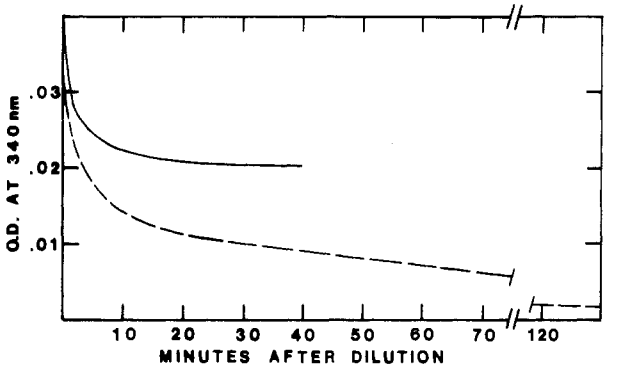

FIGURE 8: Rate of microtubule depolymerization following dilution in the presence or absence of colchicine. Microtubule protein at 2.6 mg/mL was reassembled to steady state at 33 °C in the presence of 0.1 mM GTP and a GTP regenerating system. To one aliquot, 5×10^{-5} M colchicine was added, a new steady state was established. The other aliquot remained free of colchicine. Microtubules in GTP only were diluted 10-fold in assembly buffer (solid line). Microtubules at steady state in colchicine were diluted in assembly buffer containing 5×10^{-5} M colchicine (dashed line).

observed with microtubules in GDP (Zackroff et al., 1980).

Kinetics of Dilution-Induced Disassembly of Microtubules in the Presence of Colchicine. The rapid depolymerization of microtubules in colchicine buffer mentioned above was more closely studied by determining the rates of disassembly by turbidity. Microtubules were polymerized to steady state in GTP and were then allowed to reach a new steady state in the presence of 0.05 mM colchicine. The microtubule solution was then rapidly diluted 10-fold while constant solvent conditions were maintained. This dilution decreases the free subunit concentration 10-fold below its steady-state value and thereby allows the disassembly reactions to be examined (eq 1).

An initial rapid disassembly rate is observed following dilution (Figure 8). The observed rate depends upon the microtubule number concentration (which was not determined), but, for the same preparation of microtubules, the rates were nearly identical when dilution was performed on microtubules at steady state in the presence or absence of colchicine. Note that when the initial rates are corrected for the dilution of the microtubule number concentration the rate observed (0.007

A/min) is similar in magnitude to that obtained upon addition of colchicine to microtubules (Figure 2).

In addition to the initial rapid rate, a second 60-fold slower rate of depolymerization is observed when microtubules are diluted in the presence of colchicine. Microtubules diluted in the absence of colchicine do not exhibit the slow rate and appear to decay by a single first-order rate process.

Loss of Subunits from Microtubules during Dilution or Colchicine-Induced Depolymerization. When [^{3}H]GTP is added to microtubules at steady state, it is possible to preferentially label the net assembly end of the microtubule (Margolis & Wilson, 1978). It is then possible to determine the end from which subunit disassembly occurs by the change in specific radioactivity of the microtubules. If the labeled net assembly end is the site of preferential subunit disassembly, a decrease in the specific activity of the microtubules should be observed, while loss of subunits from the opposite end will result in an increase in specific activity. Loss of label from both ends will result in little or no change in specific radioactivity, depending upon the fraction of the microtubule which is labeled.

The polarity of disassembly was studied by pulse-labeling microtubules at steady state in the presence of 0.1 mM GTP for 45 min with [^{3}H]GTP. The microtubule solution was then chased with 2 mM GTP and divided into three aliquots. One sample was not depolymerized, another was partially depolymerized by a 50% dilution, and the third sample was partially depolymerized by the addition of 0.05 mM colchicine.

The diluted microtubules were depolymerized by 22% and the colchicine-treated microtubules by 27%. The specific radioactivity was 3800 cpm/mg before depolymerization and was essentially unchanged (3600 cpm/mg) following partial depolymerization by dilution or colchicine treatment. In addition, the disassembly under both conditions results in a similar decrease in the total radioactive counts in the microtubule pellets. No detectable protein or radioactivity was pelleted when samples were completely depolymerized at 0 °C, which indicates that radioactivity in the experimental samples is due to the presence of microtubules.

Discussion

Although colchicine has been known for many years for its ability to interfere with microtubule assembly, it is only recently that a detailed study of its mechanism of action has been undertaken. One of the reasons for this renewed interest is the realization that the action of colchicine is closely linked to the mechanism of microtubule assembly and that this mechanism is more complex than was originally believed. In the work reported here, we have examined particular aspects of the response of microtubules to colchicine which appear to yield valuable insights into the process of microtubule assembly.

The major conclusion reached from our experiments is that microtubules can be stable indefinitely in the presence of even high concentrations of colchicine. The stability of microtubules in the presence of colchicine is demonostrated most clearly by the experiment of Figure 7, in which microtubule fragments were exposed to 0.5 mM colchicine for several hours. This is about 20 times more colchicine than the amount of tubulin present, yet the microtubule fragments do not shorten, or elongate, detectably after 6 h of incubation. Stability of microtubules in vitro to colchicine treatment is not a fixed property but depends upon the content of microtubule-associated proteins (MAPs) that were present when the microtubules were assembled. Stability of microtubules in vitro in the presence of colchicine has been reported on several occasions (Haga & Kurokawa, 1975; Herzog & Weber, 1977; Wallin & Larsson, 1979), but an analysis of this phenomenon has not been previously performed.

There are several potential explanations for the failure of colchicine to induce complete depolymerization of microtubules assembled in the presence of MAPs. Before discussion of what we feel to be the most likely explanation, we would like to indicate those explanations that do not appear to be valid.

(*1*) *The concentration of colchicine used is inadequate to completely inactivate the tubulin present.* This is unlikely to be true since we use concentrations of colchicine that are several times greater than the concentration of tubulin, and direct measurements of colchicine binding (Figure 3) indicate that nearly stoichiometric binding is obtained to free tubulin.

(*2*) *The disassembly rate of microtubules in the presence of colchicine is extremely slow, and what appears to be a stable solution is actually undergoing slow depolymerization.* We see no evidence for a slow rate of depolymerization even when the microtubules are fragmented to increase the concentration of ends (Figure 7). We observed only a relatively fast, but limited, depolymerization upon addition of colchicine to microtubules (Figure 2), which is consistent with both the rate of colchicine binding (Figure 3) and the intrinsic rate of microtubule disassembly as determined by dilution (Figure 8). We do observe a slow rate of disassembly when microtubules at steady state in the presence of colchicine are subsequently diluted (Figure 8). But even in this case a new plateau can eventually be estabilished provided a sufficient concentration of colchicine–tubulin is present. A slow, continuous rate of depolymerization has been reported for microtubules exposed to podophyllotoxin, a colchicine analogue (Margolis & Wilson, 1978; Karr & Purich, 1979), but it is not clear from their data whether a new plateau is eventually reached.

(*3*) *Colchicine "caps" the end(s) of the microtubule and prevents subunit assembly or disassembly from the capped end.* Capping of microtubule ends has been proposed by others (Margolis & Wilson, 1977, 1978; Farrell et al., 1979; Karr & Purich, 1979) to explain the inhibition of microtubule assembly by colchicine. We see no evidence for such a phenomenon. The initial rate of depolymerization of microtubules following dilution is the same in the presence or absence of colchicine (Figure 8). Nor do we observe any effect of colchicine on the loss of labeled subunits from microtubule ends. If colchicine caps only one end of the microtubule, we are left with the problem of explaining why normal steady-state reactions appear to continue at the other end, but microtubule elongation does not occur even at high colchicine–tubulin concentrations.

In order to explain the action of colchicine, it is clearly necessary to understand the mechanism of microtubule assembly itself. We believe that the most important consideration for the present discussion is the existence of distinct reactions that determine net microtubule elongation, or shortening, and the reactions occuring at steady state. To make this point clear, we will briefly discuss the nature of microtubule assembly and steady-state reactions as they have been proposed to occur in the presence of GTP and MAPs (Weisenberg, 1980), conditions most often used for studies of microtubules in vitro.

It is now generally accepted that microtubules reach a steady state, rather than a true equilibrium, because the turnover of subunits at microtubule ends is coupled to the hydrolysis of GTP (David-Pfeuty et al. 1978). However, the effect of GTP hydrolysis on the properties of the steady state has not been

previously discussed. An equation describing the influence of GTP hydrolysis on the kinetics of microtubule assembly has recently been proposed (Weisenberg, 1980). This equation is based upon the nucleation–condensation model of Oosawa & Kasai (1962) but takes into account the fact that the assembly species is GTP–tubulin while the disassembly species is probably GDP–tubulin. The rate of polymerization can then be approximated by

$$dP/dt = k_+MC_1 - k_-M/(1 + k_+C_1/v)$$

where k_+ is the association rate constant for GTP–tubulin, k_- is the dissociation rate constant for GDP–tubulin, M is the molar concentration of assembly sites, C_1 is the free subunit concentration, and v is a rate constant for GTP hydrolysis that applies only after subunit addition to the microtubule. At steady state, $dP/dt = 0$, and the above equation can be solved for C_1:

$$C_c = \frac{(1 + 4k_-/v)^{1/2} - 1}{2k_+/v}$$

Here C_c is the free subunit concentration at steady state, which is generally referred to as the "critical concentration".

The important conclusion from this analysis is that even when GTP hydrolysis is taken into account the critical concentration depends only upon a set of reaction rate constants. Thus, it cannot be readily distinguished from a true critical concentration which is equal to k_-/k_+, the inverse of the polymerization equilibrium constant.

If the rate of GTP hydrolysis, v, differs at opposite ends of the microtubule, then the critical concentrations will also differ. Under these conditions, subunit flow through the microtubule can occur. However, the critical concentration for the steady-state system will remain a function of a set of rate constants only, and an equilibrium type analysis can be performed, such as used in Figures 4 and 5.

Although it is possible to determine a critical concentration for the steady-state reaction of microtubules in GTP, the critical concentration is not simply related to the net assembly and disassembly rates. Although measurements of initial assembly and disassembly rates have been used to calculate the critical concentrations (Bergen & Borisy, 1980), such an analysis is probably not valid. This is because the measured net assembly rate is that of GTP–tubulin, while the net disassembly rate is that of GDP–tubulin. But these measurements do not take into account the conversion of GTP to GDP which is part of the steady-state reaction and which contributes to the actual critical concentration (see the above equation).

For purposes of understanding the mechanism of action of colchicine, the most important consequence of the above analysis is that net microtubule elongation, or shortening, and steady state represent distinct reactions. This distinction can exist not only because of the involvement of GTP hydrolysis in microtubule assembly but also because of the specific role of MAPs in microtubule elongation and steady-state reactions. It has been proposed (Weisenberg, 1980) that microtubule assembly proceeds in "rounds" of elongation steps. Each round starts with the addition of a MAP–tubulin oligomer to the end of the microtubule in a reaction that depends only upon tubulin–tubulin interactions between the end of the microtubule and the end of the oligomer. The next 12 oligomers (assuming a 13-sided microtubule) add adjacent to the previously added oligomers, and these reactions are stabilized cooperatively by MAP–tubulin interactions. Addition of the 13th oligomer recreates a "flat-ended" microtubule and completes a round of assembly.

To make this mechanism more specific, we can write a set of equations for the 13 reactions of a round of microtubule elongation. In these equations, T refers to free tubulin, which

$$M_N^* + T = M_N^1$$
$$M_N^1 + T = M_N^2$$
$$M_N^2 + T = M_N^3$$
$$\vdots$$
$$M_N^{12} + T = M_{N+1}^*$$

is assumed to be present in MAP—tubulin oligomers, M is the concentration of microtubule ends, the subscript N, $N + 1$, ... indicates the number of rounds of microtubule assembly completed, and the superscript indicates the number of projecting oligomers on the end of the microtubule. When the microtubule contains an integral number of oligomers, the end of the microtubule is flat, and it has no projecting oligomers to provide for a cooperative side-to-side interaction with another oligomer. This situation is indicated by an asterisk.

Because it is required for the start of each round of elongation, reaction 1 limits the rate of microtubule assembly. However, it can be shown (Weisenberg, 1980) that the reactions 2nd–12th dominate at steady state and determine the critical concentration. Thus we are again led to the conclusion that microtubule elongation and steady state reflect different reactions.

The conclusion that microtubule elongation and steady-state reactions can differ is, we believe, essential in understanding the process of microtubule assembly and the mechanism of action of colchicine. Becuase they differ, it is possible to specifically interfere with the elongation reaction while leaving the steady-state reaction essentially unaffected. If microtubule elongation is effectively prevented over laboratory times, then a "pseudo steady state" will be obtained. In a pseudo steady state, normal subunit steady-state turnover reactions still occur, but net microtubule elongation does not take place even if the subunit concentration is in excess of the critical concentration. The subunit that is present in excess of the critical concentration will appear to be "inactive" even though it may be participating in the steady-state reactions.

We believe that our results can be best explained by the ability of colchicine to block the net elongation reaction of microtubules while it leaves the shortening and steady-state reactions essentially unaffected. Colchicine can thus induce a microtubule pseudo steady state.

The conclusion that colchicine blocks microtubule elongation is based upon the failure of microtubules to undergo detectable elongation in concentrations of colchicine–tubulin up to 50 times greater than the estimated critical concentration. In addition, the ability of colchicine to inhibit subunit flow through the microtubule can be explained by its ability to block net elongation at the "assembly end" of the microtubule.

The conclusion that colchicine does not affect the net disassembly reactions of microtubules is based upon a direct comparison of the rate of depolymerization of normal microtubules and microtubules preincubated with colchicine (Figure 8). This conclusion is also supported by the disassembly of end-labeled microtubules, which indicates that colchicine has no effect on the loss of labeled and unlabeled subunits from either end of the microtubule.

The existence of normal steady-state reactions in the presence of colchicine is indicated by the results of the dilution experiments of Figures 4 and 5. The observation that a nonzero critical concentration is obtained for microtubules in the presence of colchicine indicates that subunit turnover re-

actions must be occurring. The similarity of the critical concentrations measured in the presence and absence of colchicine indicates, in addition, that colchicine has little effect on the microtubule steady-state reactions.

The stability of microtubules in colchicine depends upon the presence of MAPs (Table I, Figures 1 and 2) and is presumably related to the role of MAP–tubulin interactions as discussed above. We propose that colchicine inhibits the rate-limiting reaction for microtubule elongation (reaction 1) while having little effect on the steady-state reactions. Colchicine affects reaction 1 specifically because it depends exclusively upon tubulin–tubulin interactions which are destabilized by colchicine. The steady-state reactions are stabilized by MAP–tubulin interactions, and this makes them much less sensitive to colchicine inhibition.

An additional comment must be made about the steady-state reactions. It is not necessary that the steady state involve the turnover of intact MAP–tubulin oligomers at the end of the microtubule. It appears more likely that free subunits are undergoing reversible interactions with sites at the ends of the microtubule. These sites may contain MAPs, in which case the argument above still holds, and this reaction is relatively insensitive to colchicine. There may also exist sites at the ends that do not incorporate MAPs. Subunits interacting at these sites would be inhibited by colchicine. We suspect that the extent of microtubule depolymerization observed after addition of colchicine reflects the relative proportion of MAP-free sites. This explains why the extent of depolymerization in colchicine depends upon the MAP content. Note that a very complicated analysis may be necessary to explain the details of the response to colchicine because the distribution of MAPs may not be uniform along the microtubule. We would predict, for example, an enrichment of MAPs at microtubule ends following partial depolymerization with colchicine.

The present report has concerned the response of microtubules to saturating concentrations of colchicine, but there has also been considerable interest in the effects of substoichiometric concentrations of colchicine. Microtubule elongation can be prevented by concentrations of colchicine that are below the concentrations of tubulin present, and there have been several explanations suggested for this phenomenon (Margolis & Wilson, 1978; Sternlicht & Ringel, 1978; Karr & Purich, 1979; Farrell et al., 1979). We wish to suggest another possible explanation. According to our model, the elongation of microtubules requires the addition of MAP–tubulin oligomers to the end of the microtubule. One can easily conceive that the binding of a single colchicine to the end subunit of a linear oligomer would result in complete inhibition of oligomer interaction with the microtubule. The oligomers may presumably contain subunits with bound colchicine at positions other than the end. The extent of inhibition may, in this case, depend upon the fraction of subunits in the oligomer that contain colchicine. Colchicine-containing subunits could inhibit the cooperative side-to-side interactions of oligomers. Such an explanation may explain the observations of Sternlicht & Ringel (1978), which indicate a cooperative inhibition of colchicine–tubulin incorporation into the microtubule.

There has been considerable interest in polarity of microtubules and how this affects the action of colchicine. There are two aspects of microtubule polarity that are important, although these have been frequently confused. The ends of the microtubule may differ in their critical concentrations or they may differ in the rate at which they approach equilibrium, or steady state. The difference in critical concentration is energy dependent (Wegner, 1976) and must involve GTP hydrolysis since this is the only source of energy for microtubule assembly. Thus our failure to observe subunit flow in the presence of GDP (Figure 6) can be viewed as an essential control experiment for the interpretation of results obtained in GTP.

The ability of colchicine to inhibit subunit flow could be the result of colchicine interfering with the normal mechanism by which GTP hydrolysis is coupled to polymerization. This can only be considered as an interesting possibility, however. Our results indicate that colchicine prevents net microtubule elongation, and this by itself will prevent subunit flow since it requires that net elongation occur at one end of the microtubule.

The net elongation end of microtubules that can be demonostrated to exist at steady state when subunit flow occurs has been frequently confused with the rapidly elongating end that is observed when microtubule assembly is induced. The rate of net assembly or disassembly is proportional to the activation energy for polymerization and must proportionally be the same for both the forward and reverse reactions. The relative rates of equilibration at opposte ends of the microtubule are fundamentally distinct from the relative critical concentrations, which are determined by net free energy changes. It has been reported that the fast equilibrating end of the microtubule is the same as the end with the lower critical concentration (Bergen & Borisy, 1979); however, as noted above, this analysis is theoretically flawed and experimentally it is consistent with other interpretations. The conclusion reached by Bergen and Borisy may, nevertheless, be valid. We have observed a slow rate of depolymerization following the initial fast rate when microtubules at pseudo steady state in colchicine were diluted. This observation may be a result of the differences in the critical concentrations and rates of equilibration at opposite ends of the microtubule. Normally for microtubules in GTP, the total rate of equilibration is determined only by the fast equilibrating end. However, if microtubule elongation reactions are blocked by colchicine, then each end of the microtubule should reach steady state at its own rate. If the end of the microtubule with the highest critical concentration is the end with the slowest rate of equilibration, then it will continue to depolymerize after the other end has reached its steady state. Thus our data appear consistent with the view that the fast equilibrating end of the microtubule is the end with the lower critical concentration.

Do our observations have any biological relevance? We can only speculate at this time, but the observation that microtubules may be stable in the presence of colchicine even though their elongation is prevented may have relevance when the possibile regulatory role of endogenous colchicine analogues is considered (Sherline et al., 1979; Lockwood, 1979). Our results suggest that natural colchicine analogues could be used to regulate microtubule length while they allow normal microtubule steady-state reactions to continue (Zackroff et al., 1980). The stability of microtubules depends upon the presence of MAPs, and this suggests a possible modulating role for MAPs in microtubule regulation by the above mechanism. The phenomenon of subunit flow through the microtubule has been confirmed, and we have shown, as expected, that it requires the presence of GTP as an energy source. Such an energy-dependent process could act as a basis for microtubule-dependent motility. It is interesting in this regard that we have shown that colchicine blocks subunit flow and that it has also been found that colchicine blocks axonal transport even in cases in which it does not disrupt microtubules (Fernandez et al., 1970; Norstrom, 1975).

Since submission of this paper a report has appeared (Lambeir & Engelborghs, 1980) which concludes that the affinity of tubulin and colchicine–tubulin for microtubule ends is of the same order of magnitude and that colchicine–tubulin interacts reversibly with the microtubule ends. These conclusions are consistent with ours.

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