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Polymerization of the Tubulin–Colchicine Complex: Relation to Microtubule Assembly†

José Manuel Andreu,‡ Terence Wagenknecht, and Serge N. Timasheff*

ABSTRACT: The polymerization of purified tubulin–colchicine complex, which results in polymers different from microtubules under microtubule-promoting conditions, has been characterized. It proceeds as a nucleated condensation polymerization, requires Mg^{2+}, and is inhibited by small concentrations of Ca^{2+}. Polymerization requires GTP binding, but GDP is inhibitory. The GTPase activity proceeds, but it is unlinked to polymerization. The thermodynamic characteristics of the growth reaction, namely, the apparent changes of free energy, enthalpy, entropy, heat capacity, and preferential interaction with H^+ and Mg^{2+}, are very similar to those of microtubule assembly. It is proposed that the interactions responsible for the two types of polymerization are very similar and that the molecular mechanism of microtubule inhibition by colchicine may consist in a drug-induced distortion of the normal protomer bonding geometry.

Colchicine has been used for many years as an inhibitor of mitosis (Dustin, 1978). In 1968 Weisenberg et al. (1968) purified the protein tubulin, which is the major constituent of microtubules and the main cellular receptor of colchicine, and to which the alkaloid binds tightly (Wilson & Bryan, 1974). It has been well established that colchicine inhibits substoichiometrically mitosis (Taylor, 1963) and the assembly of microtubules in vitro (Olmsted & Borisy, 1973). Microtubule assembly in vitro conforms to a nucleated helical condensation polymerization mechanism (Gaskin et al., 1974; Lee & Timasheff, 1975, 1977), just like the assembly of filamentous actin (Oosawa & Asakura, 1975). Margolis & Wilson (1977) have shown in their study of steady-state microtubules assembled in vitro that addition of tubulin promoters occurs preferentially at one end of the polymer and release preferentially at the other. This process, which is accompanied by the hydrolysis of bound GTP at the growing end (Carlier & Pantaloni, 1981), results in a net flow of promoters through the constant size polymer. The flow through, or treadmilling, is poisoned by colchicine which acts at the ends (Margolis & Wilson, 1977, 1978; Margolis et al., 1980), leading to the proposal that the tubulin–colchicine complex binds tightly to the growing end of microtubules and inhibits kinetically the addition of further promoters (Margolis & Wilson, 1977). On the other hand, a study of the effects of the tubulin–colchicine complex on the non-steady-state seeded assembly of microtubules has led to the conclusion that tubulin bound to colchicine copolymerizes with unliganded tubulin, reducing the affinity of the polymer for new protomers (Sternlicht & Ringel, 1979).

In a kinetic study of the effects of the tubulin–colchicine complex on small amplitude growth of microtubules, Lambeir & Engelborghs (1980) have found that the binding of tubulin–colchicine to microtubules inhibits growth, is rapid and reversible, and proceeds with an estimated association constant of the same order of magnitude as that of unliganded tubulin. The particular mechanisms of the inhibition of assembly proposed in each of the above studies are likely the consequence of the different experimental conditions used and of the approaches applied to a complex problem and need not necessarily be contradictory.

It has been recognized that the precise mechanism by which tubulin–colchicine terminates growth is not known (Margolis & Wilson, 1981; Lambeir & Engelborghs, 1980). Which are then the basic molecular phenomena that result in the inhibition of polymerization? It is clear that, for inhibition of the assembly of the normal polymer, colchicine binding has to cause changes in the normal protein–protein interactions between protomers. Knowledge of the nature of intermolecular contacts formed and of the detailed geometries of the normal and drug-altered tubulin additions to a growing microtubule should most likely lead to an understanding of the basic mo-

† From the Graduate Department of Biochemistry (J.M.A. and S.N.T.) and the Rosenstiel Basic Medical Sciences Research Center (T.W.), Brandeis University, Waltham, Massachusetts 02254. *Received June 4, 1982; revised manuscript received December 15, 1982.* This is Publication No. 1440 of the Graduate Department of Biochemistry, Brandeis University. Supported by Grants CA 16707 and GM 14603 from the National Institutes of Health and Fogarty International Fellowship TW02983 (J.M.A.).

‡ Present address: Instituto de Immunologia y Biología Microbiana C.S.I.C., Velázquez 144, Madrid 6, Spain.

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lecular mechanism of microtubule inhibition by the antimitotic drug, in addition to being relevant to microtubule assembly. In an approach to this question, we have characterized the various aspects of the interaction of purified calf brain tubulin with colchicine. First, the contributions of the different parts of the colchicine molecule to its binding to tubulin were established (Andreu & Timasheff, 1981a, 1982a). Then we examined the induction of the GTPase activity in dimeric tubulin by colchicine binding (Andreu & Timasheff, 1981b) and have shown that the tubulin–colchicine complex possesses a conformation different from that of the drug-unliganded protein (Andreu & Timasheff, 1982b). Very recently it has been reported that the tubulin–colchicine complex undergoes cooperative polymerization under the same in vitro conditions that promote microtubule assembly, the resulting polymer having a geometry different from those of microtubules (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982c). The preliminary characteristics of the drug-induced polymerization reaction indicate to us interactions very similar to those of normal microtubule assembly. However, since the polymers formed were not microtubules, we proposed that the basic mode of action of the antimitotic drug may be the generation of an incorrect bonding geometry between tubulin molecules (Andreu & Timasheff, 1982c). In this paper, we report the detailed characterization of tubulin–colchicine polymerization, comparing with normal microtubule assembly the effects of temperature, H^+, Mg^{2+}, Ca^{2+}, nucleotides, and polycations.

Materials and Methods

Chemicals. Colchicine was from Aldrich (lot no. 01987). GTP was from Sigma, type II-S. [*methoxy*-^{3}H]Colchicine, 20 Ci/mmol, and [γ-^{32}P]GTP,[1] 20–40 Ci/mmol, were from New England Nuclear. Sephadex G-25 was from Pharmacia. Guanidine hydrochloride was from Heico. Glycerol was from Fisher. Polylysine was from Mann (M_r 100000–200000) and lysozyme from Worthington. Glutaraldehyde, electron microscopy grade, and uranyl acetate were from Polysciences.

Tubulin and Tubulin–Colchicine Complex. Soluble purified calf brain tubulin was prepared as described before (Lee et al., 1973; Andreu & Timasheff, 1982a). Its concentration was measured spectrophotometrically by using extinction coefficients of E_{276} = 1.16 L g^{-1} cm^{-1} in neutral aqueous buffer and E_{275} = 1.09 L g^{-1} cm^{-1} in 6 M guanidine hydrochloride (Andreu & Timasheff, 1982b). Stable tubulin–colchicine complex (E_{276} = 1.23 L g^{-1} cm^{-1} in neutral buffer and E_{275} = 1.16 L g^{-1} cm^{-1} in 6 M guanidine hydrochloride) was prepared as described before (Andreu & Timasheff, 1982b) by incubation of millimolar concentrations of protein and ligand for 10 min at 25 °C, followed by removal of excess colchicine and equilibration in desired buffer by fast Sephadex G-25 chromatography. It contained 0.97 ± 0.05 mol of [^{3}H]-colchicine/10^5 g of tubulin and could be dissociated by photolysis of the ligand or exposure to 6 M guanidine hydrochloride (Andreu & Timasheff, 1982b). Cycle microtubule protein was prepared by polymerization and depolymerization in the absence of glycerol, following the procedure of Asnes & Wilson (1979). Cycle tubulin–colchicine complex was prepared by the same procedure as employed with the purified protein and contained approximately 0.8 mol of colchicine/10^5 g of protein. GTP hydrolysis by the tubulin–colchicine complex was measured by the release of [^{32}P]phosphate as described before (Andreu & Timasheff, 1981b).

Sedimentation Velocity. Protein solutions were centrifuged in double sector cells with regular and wedge shaphire windows in an AnD rotor, using a Beckman Model E analytical ultracentrifuge equipped with electronic speed control and RTIC unit.

Polymerization. Microtubule assembly in vitro was performed in PG[1]–1 mM EGTA–16 mM $MgCl_2$–3.4 M glycerol buffer, pH 7.0. The reaction was started by warming the solution contained in a jacketed cuvette from 10 to 37 °C (half-warming time was approximately 20 s); it was followed turbidimetrically (Gaskin et al., 1974; Lee & Timasheff, 1977) at 450 nm. The polymerization of tubulin bound to colchicine was carried out under the same conditions, or in PG–16 mM $MgCl_2$ buffer, pH 7.0, in the absence of glycerol. The reaction was also followed turbidimetrically. Once the plateau was reached, the polymer was sedimented by centrifugation at 3000*g* for 10 min at the polymerization temperature ±2 °C. The amounts of protein sedimented and remaining in the supernatant were measured both by spectrophotometry and by the radioactivity of bound ligand. [^{3}H]Colchicine remained bound to tubulin after a cycle of polymerization and depolymerization. The turbidity of tubulin–colchicine polymer suspensions was stabilized against cold depolymerization by a 2-min fixation with 0.5% glutaraldehyde, after which the samples were adsorbed onto carbon-coated formvar grids, negatively stained with 1% uranyl acetate, and examined in a Phillips EM 301 electron microscope.

Results

Solution Properties of the Tubulin–Colchicine Complex and Inhibition of Microtubule Assembly. Tubulin liganded to colchicine is characterized by a quenched intrinsic fluorescence, small perturbations in its circular dichroism, and a slow GTPase activity (Andreu & Timasheff, 1982b). The binding of colchicine does not induce any association or change in the sedimentation coefficient of tubulin in PG buffer, pH 7.0 (Andreu & Timasheff, 1982c); i.e., the protein remains as a single 5.8S sedimenting species (Frigon & Timasheff, 1975a). In PG–16 mM $MgCl_2$ buffer, pH 7.0, tubulin–colchicine sedimented at 20 °C with a bimodal schlieren profile (Andreu & Timasheff, 1982c) characteristic of the Mg^{2+}-induced self-association with the formation of 42S double rings known to occur in the unliganded protein (Frigon & Timasheff, 1975a). Since it had been reported that colchicine liganding to tubulin increased the self-association affinity of tubulin (Weisenberg & Timasheff, 1970), this reaction was examined quantitatively. Tubulin solutions of equal concentrations, one of them containing 3 × 10^{-4} M colchicine and the other not, were run simultaneously in double sector cells,[2] with typical results depicted in Figure 1. A small, but consistently reproducible decrease in the area of the slow peak (by 5–10%) with a corresponding increase in the fast peak was observed in the colchicine-containing protein solution (upper schlieren profile of Figure 1). According to the criteria of the Gilbert theory (Gilbert, 1955), this indicates an enhancement of the self-association reaction, corresponding to a small increase in the standard free energy change of the self-association, by less than −0.2 kcal mol^{-1}, in the liganded protein (Frigon & Timasheff, 1975b).

[1] Abbreviations: PG, 10 mM sodium phosphate–0.1 mM GTP; Mes, 4-morpholineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GTP, guanosine triphosphate; GDP, guanosine diphosphate; MAPs, microtubule-associated proteins.

[2] Under the conditions employed the binding reaction is known to be more than 90% complete in 30 min (the approximate time needed to start the sedimentation velocity run) as shown in fluorescence experiments (Andreu & Timasheff, 1982b).

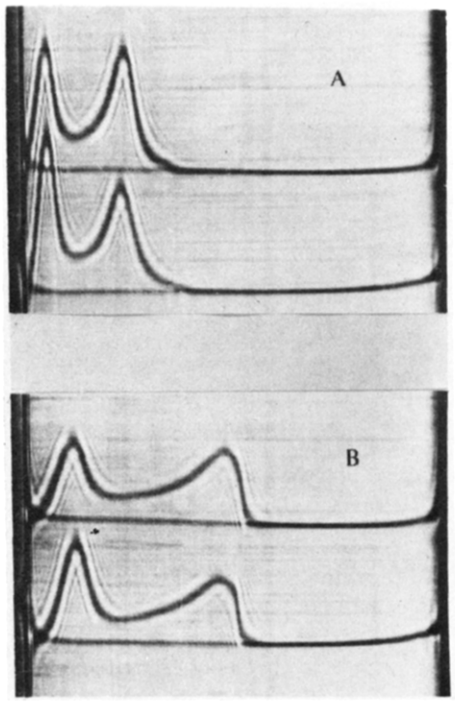

FIGURE 1: Effect of colchicine on the sedimentation velocity pattern of 9.1 mg mL^{-1} tubulin in PG–16 mM $MgCl_2$ buffer, pH 7.0, 20 °C. (A) Twelve minutes after reaching the speed of 48 000 rpm (bar angle was 65°). (B) Same run, 20 min after reaching speed. The upper profile of each photograph corresponds to the protein solution aliquot containing 3×10^{-4} M colchicine (in a cell with wedge window), while the lower profile corresponds to the control without the drug.

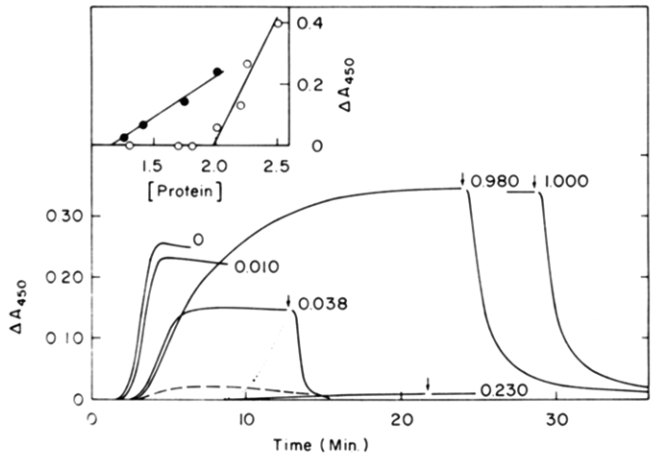

FIGURE 2: Inhibition of microtubule assembly in vitro by tubulin–colchicine complex and polymerization of tubulin–colchicine. The turbidimetric assembly assay in buffer containing 3.4 M glycerol (see Materials and Methods) was employed to follow the polymerization of mixtures of tubulin and tubulin–colchicine complex at a constant total protein concentration of 2.35 mg mL^{-1}. The numbers by each tracing are the mole ratio of liganded to total protein. The reaction was started by heating to 37 °C; the arrows indicate recooling to 10 °C. The dashed line was the result of adding 5×10^{-5} M colchicine to unliganded protein at the beginning of the assembly reaction, while the dotted line was obtained with 10^{-4} M colchicine. The inset shows the effects of total protein concentration on the plateau turbidity of unliganded (open circles) and fully liganded (filled circles) protein.

The microtubule assembly inhibitory activity of the tubulin–colchicine complex was tested by mixing unliganded and liganded tubulins, maintaining constant the total protein concentration, in microtubule assembly buffer, and following the assembly reaction turbidimetrically. As shown in Figure 2, where the numbers on each tracing are the mole ratio [tubulin–colchicine]/[total tubulin], the liganded protein was a potent inhibitor of microtubule assembly. A 50% inhibition of the plateau turbidity was obtained with ca. 5% of liganded molecules in the population, in semiquantitative agreement with the results of Margolis & Wilson (1977), who used cycle microtubule protein and found half inhibition with ca. 2% of bound molecules.

Table I: Effects of Buffer Composition on the Polymerization of the Tubulin–Colchicine Complex[a]

buffer (pH 7.0)	C_r (mg mL^{-1})
PG–16 mM $MgCl_2$	1.13 ± 0.09
PG–16 mM $MgCl_2$ (sedimentation of protein)	1.18 ± 0.05
PG–16 mM $MgCl_2$ (sedimentation of ^3H-labeled ligand)	1.21 ± 0.14
PG–16 mM $MgCl_2$–3.4 M glycerol	1.9 ± 0.2
Mes G–16 mM $MgCl_2$	0.4 ± 0.1
Mes G–16 mM $MgCl_2$–30 mM NaCl	1.6 ± 0.2

[a] Polymerization was performed at 37 °C. The critical concentration, C_r, was determined from turbidity measurements except where indicated. No polymerization was observed in the absence of Mg^{2+} or GTP.

Polymerization of Tubulin–Colchicine. When in the same experiment the mole ratio of liganded protein was increased to near unity, there was again a development of turbidity, which was reversible by cooling but had a longer lag time and slower kinetics than microtubule polymerization at the same protein concentration (see tracings 0.980 and 1.000 of Figure 2). Addition of 10^{-4} M colchicine to the plateau aggregate had no effect on the turbidity, whereas in the case of microtubules it caused a slow depolymerization (not shown). The concentration dependence of the plateau turbidity of pure tubulin–colchicine complex followed a straight line (Figure 2, inset), which extrapolated to a critical concentration, C_r, of approximately 2 mg mL^{-1}. No turbidity developed below this concentration, and above it the turbidity followed a linear dependence on concentration with a slope markedly steeper than that of microtubules. The wavelength dependence of the turbidity (Andreu & Timasheff, 1982c), $\Delta A \propto \lambda^{-2.1}$ instead of $\Delta A \propto \lambda^{-3.0}$ characteristic of long thin rods such as microtubules (Gaskin et al., 1974), indicated that the aggregates were most probably not microtubules, in spite of the fact that they were formed reversibly under the same solvent conditions, and with a critical concentration, a characteristic of highly cooperative polymerization (Oosawa & Asakura, 1975; Timasheff, 1981). Tubulin–colchicine also polymerized readily in the absence of glycerol (PG–16 mM $MgCl_2$ buffer, pH 7.0), permitting to pellet it by sedimentation at 30000*g* for 10 min. Determination of the critical concentration, C_r, simultaneously by turbidity, sedimentation of the protein, and sedimentation of the labeled bound ligand yielded the same value within experimental error, as shown in Table I. Above the critical concentration, essentially all of the protein added was incorporated into the polymer (Andreu & Timasheff, 1982c), indicating that it was all active. No polymerization was observed in the absence of Mg^{2+} or GTP. Substitution of Mes for phosphate resulted in a lower C_r, and an increase in ionic strength by addition of NaCl resulted in higher C_r (Table I), identically to the effects observed in microtubule assembly (Lee & Timasheff, 1977).

The tubulin–colchicine aggregates could be seen in a phase contrast microscope as globular particles or clusters of such particles, indicating a fairly large size for the polymers or secondary aggregation products (the smallest particles were of ca. 0.2 μm). The appearance of these particles correlated with the turbidity, i.e., there were none in unheated samples, or below C_r, and their formation was cold reversible. Mild

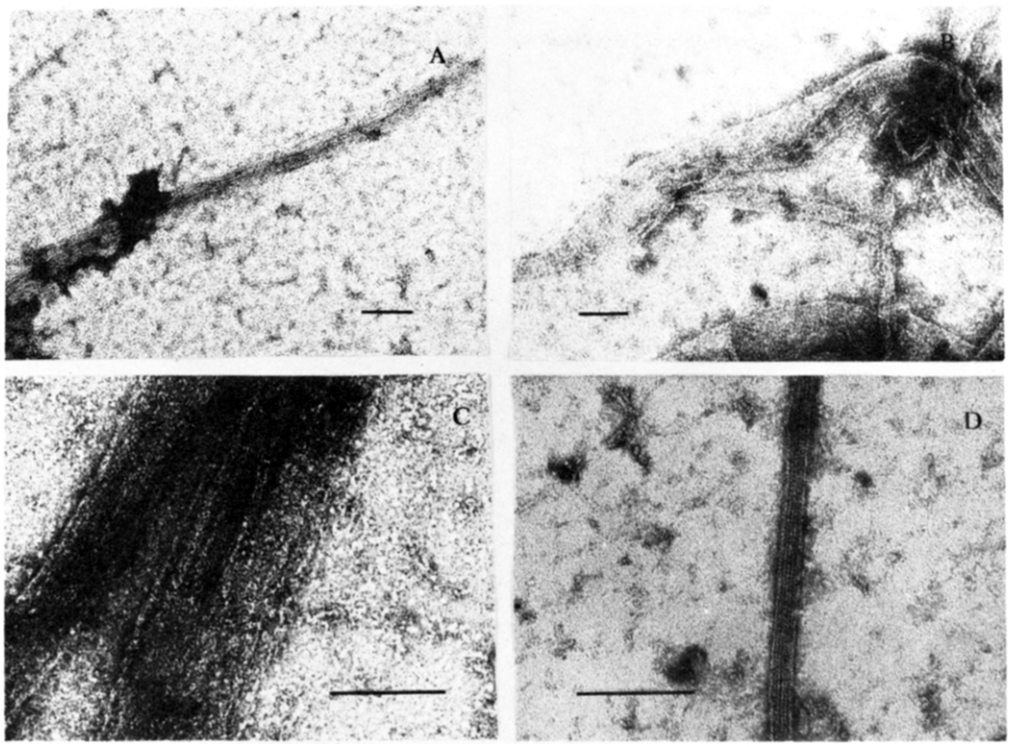

FIGURE 3: Electron micrographs of the tubulin–colchicine aggregate (A–C) and microtubules (D). Polymerization was performed in assembly buffer containing 3.4 M glycerol, and the aggregates were fixed with glutaraldehyde (see Materials and Methods). Initial protein concentrations were 2.6 (A–C) and 2.0 (D) mg mL^{-1}. The bars indicate 100 nm.

fixation with glutaraldehyde (see Materials and Methods) stabilized against cold depolymerization both the turbidity and the particles seen by phase contrast. Electron microscopic observation of unfixed, negatively stained specimens did not reveal any large organized structures. Examination of fixed samples showed amorphous structures as well as large aggregates, which appeared to be fibrous (Figure 3A) or sheetlike (Figure 3B). When some fine structure could be detected (Figure 3C), globular particles ca. 3 nm in diameter were distinguishable in the associated filaments. Microtubule controls, polymerized from unliganded tubulin and treated under identical conditions, had the familiar appearance, consisting of particles 3–4 nm in diameter arranged in protofilaments organized in hollow cylinders, with a diameter of 25 ± 2 nm (Figure 3D).

Effects of Ca^{2+}, GTP, GDP, and Polycations. In view of the observed similarities between tubulin–colchicine polymerization and microtubule assembly, we proceeded to characterize the effects of Ca^{2+}, GTP and GDP binding, polycations, and cycle microtubule protein on this reaction, as well as its relation to the rate of GTP hydrolysis. As shown in Figure 4, addition of 10^{-4} M $CaCl_2$ to a tubulin–colchicine solution, 1.6×10^{-2} M in $MgCl_2$, produced a partial inhibition of the polymerization (tracing c) with respect to the control without Ca^{2+} (tracing b). Addition of 10^{-4} M $CaCl_2$ to polymerized tubulin–colchicine (tracing b) resulted in a turbidity decrease to a new plateau close to the sample inhibited from the start of the reaction. Removal of Ca^{2+} by EGTA led to a nearly complete reversal of the inhibition (tracings b and c). The polymer could also be depolymerized by cooling to 10 °C. It is evident, therefore, that the Ca^{2+} at the assayed concentration was partially inhibitory under our conditions and that the effect was reversible.

Soluble tubulin–colchicine is known to exhibit a weak GTPase activity (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981b). Under the present conditions, soluble tubulin–colchicine hydrolyzed 0.016 ± 0.004 mol of [γ-^{32}P]GTP (10^5 g of protein)$^{-1}$ min^{-1} in 10 mM sodium phosphate–16 mM $MgCl_2$–10^{-4} M GTP, pH 7.0 at 37 °C. Addition of 3.4 M glycerol to the buffer raised the hydrolysis rate 4-fold to 0.065 ± 0.005 min^{-1} under identical conditions. These rates were not modified by polymerization. As shown in Figure 5, the time course of [^{32}P]phosphate release (filled circles) was linear and proceeded at the same rate as in samples below the critical concentration (open circles).

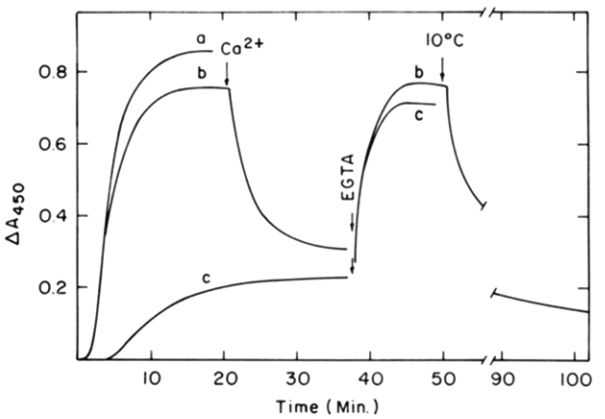

FIGURE 4: Effects of Ca^{2+} on the polymerization of 2.9 mg mL^{-1} tubulin–colchicine in PG–16 mM $MgCl_2$–3.4 M glycerol buffer, pH 7.0 at 37 °C. Sample a contained 10^{-4} M Ca^{2+} and 10^{-3} M EGTA; the polymerization of (b) was started without additions, and 10^{-4} M Ca^{2+} and 10^{-3} M EGTA were added at the points indicated; the polymerization of (c) was started in the presence of 10^{-4} M Ca^{2+}, and EGTA was added where indicated by the arrow.

Since GTP is required for polymerization, the effects of GTP and GDP binding were compared under conditions where GTP hydrolysis is minimal (absence of glycerol). The results are shown in Figure 6. Tracing a was obtained with 1.7 mg mL^{-1} tubulin–colchicine and 10^{-4} M GTP. Tracing b shows the effect of decreasing the GTP concentration to 10^{-5} M. No

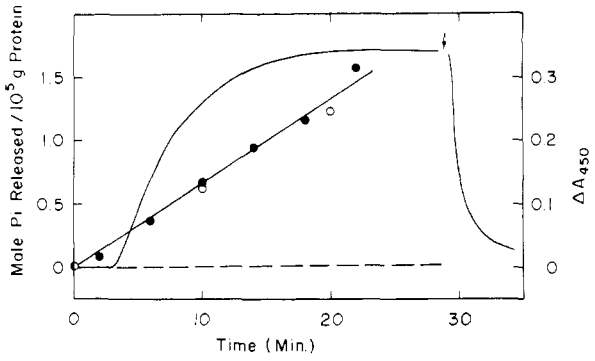

FIGURE 5: Hydrolysis of GTP during the polymerization of tubulin–colchicine. The filled circles represent the specific [^{32}P]phosphate release, and the solid line shows the turbidity generated by 2.4 mg mL^{-1} tubulin–colchicine in PG–16 mM $MgCl_2$–1 mM EGTA–3.4 M glycerol buffer, pH 7.0 at 37 °C. The open circles and the dashed line correspond to 1.2 mg mL^{-1} protein (C_r = 2 mg mL^{-1}) under identical conditions.

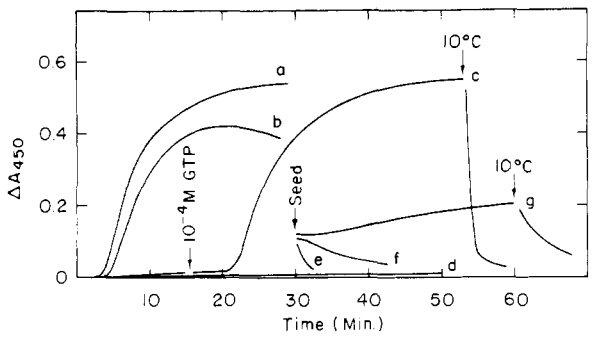

FIGURE 6: Effects of GTP and GDP on the polymerization of tubulin–colchicine in 10 mM sodium phosphate–16 mM $MgCl_2$ buffer, pH 7.0 at 37 °C. (a) 1.7 mg mL^{-1} protein and 10^{-4} M GTP. (b) 1.7 mg mL^{-1} protein and 10^{-5} M GTP. (c) 1.7 mg mL^{-1} protein, 10^{-4} M GTP added at the point indicated by the arrow. (d) 2 mg mL^{-1} protein and 10^{-4} M GDP. (e, f, and g) Seeded assembly in the presence of 10^{-4} M GDP. Protein (2.0 mg mL^{-1}) was polymerized with 10^{-4} M GTP during 30 min [giving a residual concentration of (9.04 ± 0.24) × 10^{-5} M GTP and (9.6 ± 2.4) × 10^{-6} M GDP] and diluted to a seed concentration of 0.18 mg mL^{-1} into 10^{-4} M GDP–tubulin–colchicine solutions to give final total protein concentrations of 0.18 (e), 1.72 (f), and 2.00 (g) mg mL^{-1} in buffer containing (8.2 ± 0.2) × 10^{-6} M GTP and (9.2 ± 0.1) × 10^{-5} M GDP.

exogenous GTP was added to sample c in which polymerization occurred only after the addition of 10^{-4} M GTP (position marked by the arrow). Sample d contained 2.0 mg mL^{-1} tubulin–colchicine and 10^{-4} M GDP. The lack of turbidity indicates that GDP was not promoting assembly. Tracings e, f, and g were obtained after 11-fold dilution by volume of plateau polymer samples into GDP-inhibited samples to give in each case a seed concentration of 0.18 mg mL^{-1} and final total protein concentrations of 0.18 (e), 1.72 (f), and 2.00 (g) mg mL^{-1} in a buffer which contained ca. 10^{-5} M GTP and 10^{-4} M GDP. These results are qualitatively consistent with the conclusions that (i) GTP binding is required for polymerization, (ii) GDP is incapable of inducing polymerization, and (iii) polymer growth may still occur in the presence of an excess of GDP over GTP, but with a critical concentration higher than that of GTP protein.

The effects of polycations are shown in Figure 7A; 5 μg mL^{-1} polylysine (tracing b) or 100 μg mL^{-1} lysozyme (tracing c) had no significant effects on the plateau turbidity of 1.6 mg mL^{-1} tubulin–colchicine (tracing a). Kinetic effects, however, were clearly evident in the diminution of the lag time, faster polymerization, and slower depolymerization. The possible effects of microtubule-associated proteins (MAPs)

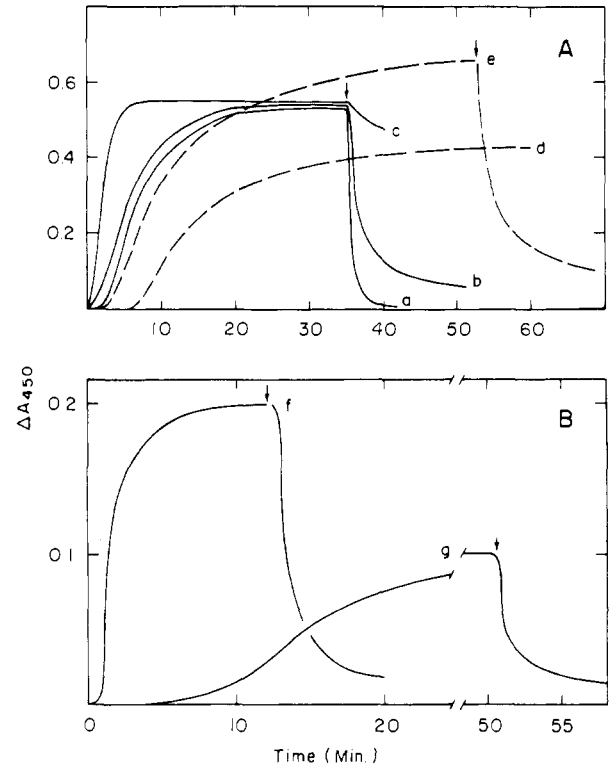

FIGURE 7: (A) Effects of polycations on the polymerization of 1.6 mg mL^{-1} tubulin–colchicine in PG–16 mM $MgCl_2$ buffer, pH 7.0 at 37 °C. (a) No addition; (b) 5 μg mL^{-1} polylysine; (c) 100 μg mL^{-1} lysozyme; (d) 175 μg mL^{-1} cycle tubulin; (e) 175 μg mL^{-1} cycle tubulin–colchicine complex. (B) Polymerization of cycle tubulin. (f) 2.6 mg mL^{-1} cycle tubulin in 20 mM sodium phosphate–100 mM sodium glutamate–0.5 mM $MgCl_2$–1 mM EGTA–1 mM GTP buffer, pH 6.75 at 37 °C; (g) 2.6 mg mL^{-1} cycle tubulin–colchicine complex (see Materials and Methods) in 10 mM sodium phosphate–16 mM $MgCl_2$–1 mM GTP buffer, pH 7.0 at 37 °C. Arrows indicate cooling of samples to 10 °C.

were checked by adding 10% by weight of cycle tubulin containing MAPs to purified tubulin–colchicine [final concentration of MAPs estimated to be ~40 μg mL^{-1}, according to the composition of the cycle preparation of Asnes & Wilson (1979)]. The observed retardation in the polymerization and slight decrease in the plateau turbidity (tracing d) can be attributed best to GTP depletion by the GTPase known to exist in the cycle tubulin preparations (David-Pfeuty et al., 1977). On the other hand, liganding of the cycle tubulin to colchicine increased the turbidity appreciably (tracing e), which is consistent with the participation of the cycle tubulin–colchicine complex in the polymerization reaction. These results indicate that MAPs have no major effects on the polymerization of tubulin–colchicine under the conditions assayed. The same cycle tubulin was able to polymerize under microtubule assembly conditions (Figure 7B, tracing f), and, once liganded to colchicine, generated cold-reversible turbidity under the conditions for the assembly of the purified tubulin–colchicine complex (tracing g).

Thermodynamics of Polymerization: Effects of Temperature, Protons, and Magnesium Ions on the Critical Concentration. The presence of a critical concentration for polymerization (see above) indicates a highly cooperative behavior, characteristic of the nucleated condensation polymerization of proteins (Oosawa & Asakura, 1975). The reciprocal of the critical concentration, C_r^{-1}, is, to a close approximation, equal to the apparent binding equilibrium constant for the addition of a protomer to the polymer, K_{app} (Oosawa & Asakura, 1975; Lee & Timasheff, 1977; Ti-

Table II: Effects of Temperature on the Polymerization of the Tubulin–Colchicine Complex[a]

T (°C)	C_r (mg mL^{-1})	ΔG°_{app} (kcal mol^{-1})	ΔH°_{app} (kcal mol^{-1})	ΔS°_{app} (eu)
30.0	2.52 ± 0.11	−6.38 ± 0.03	28.3	114
32.8	1.64 ± 0.18	−6.70 ± 0.06	24.9	103
34.8	1.31 ± 0.20	−6.88 ± 0.09	22.5	95
36.5	1.06 ± 0.07	−7.05 ± 0.04	20.5	89
39.3	0.85 ± 0.14	−7.25 ± 0.14	17.1	78
41.1	0.68 ± 0.09	−7.43 ± 0.07	15.0	72
43.0	0.59 ± 0.14	−7.56 ± 0.15	12.7	64

[a] PG–16 mM $MgCl_2$ buffer, pH 7.0. ΔG°_{app} values were calculated directly from C_r (mg mL^{-1}) as $\Delta G^\circ_{app} = -RT \ln K_{app} = RT \ln (C_r/10^5)$ cal mol^{-1}. ΔH°_{app} and ΔS°_{app} are the result of a truncated van't Hoff fit with $\Delta C_p = -1200$ cal deg^{-1} mol^{-1} and ΔH°_{app} (36.5 °C) = 20.5 kcal mol^{-1} (see the text and Figure 8).

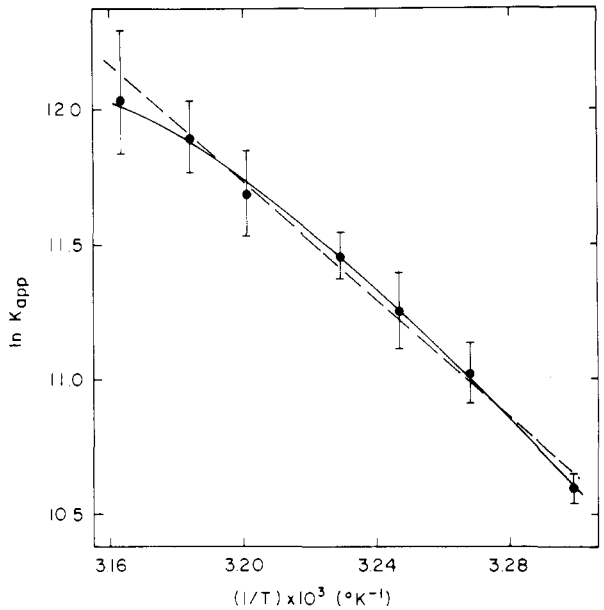

FIGURE 8: van't Hoff plot of tubulin–colchicine polymerization in PG–16 mM $MgCl_2$ buffer, pH 7.0. Bars indicate experimental errors. A least-squares linear fit of the data (dashed line) gives ΔH°_{app} = 21.7 kcal mol^{-1} and ΔS°_{app} = 93 eu, with the sum of the squares of deviations ammounting 0.039, whereas the least-squares nonlinear van't Hoff integral (see the text), shown by the solid line, gave the heat capacity, enthalpy, and entropy values listed in Table II and a sum of the squares of deviations of 0.001, indicating a better fit.

masheff, 1981; Erickson & Pantaloni, 1981). This allows a rigorous quantitative examination of the effects of solution variables on the growth reaction, provided there is a convenient method to measure C_r (Timasheff, 1981). In our case, the turbidity, although not necessarily a correct measurement of the amount of polymer formed, constitutes a convenient method for measuring the critical concentration, since it gives the same results as centrifugation (Table I).

The effect of temperature on the polymerization of tubulin–colchicine is summarized in Table II and the van't Hoff plot of Figure 8. The growth reaction was found to be endothermic in the range of temperatures studied (30–43 °C), and the data were fitted best by a nonlinear integrated van't Hoff equation (Atkins, 1978):

$$\ln K_{app}(T) - \ln K_{app}(T_1) = \frac{-\Delta H^\circ_{app}(T_1)}{R}\left(\frac{1}{T} - \frac{1}{T_1}\right) + \frac{\Delta C_p}{R}\left(\frac{T_1 - T}{T} + \ln T - \ln T_1\right) \quad (1)$$

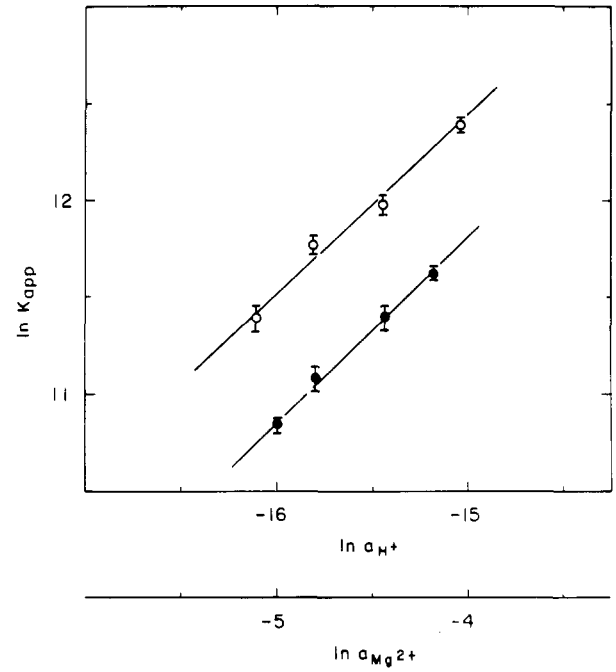

FIGURE 9: Wyman plot of tubulin–colchicine polymerization as a function of the activity of magnesium and hydrogen ions. (Filled circles) As a function of the calculated mean electrolyte activity of $MgCl_2$ (conditions, PG buffer pH 7.0, 37 °C); the concentration of free Mg^{2+} was obtained from the total concentration, correcting for $MgHPO_4$ formation (Tabor & Hastings, 1943; Greenwald et al., 1940) and Mg^{2+} binding to tubulin (Frigon & Timasheff, 1975b). (Open circles) As a function of H^+ activity, measured with a glass electrode (conditions, PG–16 mM $MgCl_2$ buffer, 37 °C); data were corrected for the variation of Mg^{2+} activity with pH.

where T is the experimental kelvin temperature and T_1 = 309.6 K. This analysis resulted in a constant apparent heat capacity change, ΔC_p = −1200 cal deg^{-1} mol^{-1}, and the positive apparent enthalpy and entropy changes listed in Table II. These characteristics are very similar to those previously reported for microtubule assembly and suggest the loss of ordered water during polymerization (Lee & Timasheff, 1977).

The effects of H^+ and Mg^{2+} were examined in terms of the linked functions theory of Wyman (1964). Under the assumption of a two-state reaction, the relationship between the ligand activity, a_x, and the polymer growth association constant, expressed in moles of unliganded protomer, K_p, is (Lee & Timasheff, 1977; Timasheff, 1981)

$$\left(\frac{\partial \ln K_p}{\partial \ln a_x}\right)_{a_{i\neq x}} = \left(\frac{\partial m_x}{\partial m_p}\right)^{\text{polymer}}_{\mu_x, m_{i\neq p}} - \left(\frac{\partial m_x}{\partial m_p}\right)^{\text{protomer}}_{\mu_x, m_{i\neq p}} \quad (2)$$

where m_x and m_p are the molal concentrations of ligand and protein (expressed as the α–β hetero dimer). The right-hand side of this equation expresses the difference in the preferential interaction of the ligand with the protein between the two end states of the reaction. If the observed association constant, K_{app}, is a good approximation of K_p, and the ligand concentration is small enough to neglect the effects of the displacement of bound water, the measured value of $(\partial \ln K_{app}/\partial \ln a_x)$ becomes, to a close approximation, the difference between the numbers of ligand molecules bound to the protein molecule in the polymer and protomer states, $\Delta\bar{\nu}_x$ (Lee & Timasheff, 1977). This analysis was applied to the polymerization of tubulin–colchicine in PG buffer, 37 °C, and the results, plotted as $\ln K_{app}$ vs. $\ln a_{H^+}$ and $\ln a_{Mg^{2+}}$, are presented in Figure 9. Polymerization was clearly favored by increasing ionic activities in both cases, the slopes being 0.94 ± 0.05 and 0.96 ±

Table III: Properties of the Polymerization of the Tubulin–Colchicine Complex Compared with Microtubule Assembly in Vitro[a]

property	tubulin–colchicine polymerization	microtubule polymerization
type of polymerization	nucleated condensation	nucleated condensation,[d] treadmill mechanism[e]
morphology of polymer	associated filaments (tentative)	helical tube
thermodynamics of the growth reaction		
ΔG°_{app} (kcal mol^{-1}; 37 °C)	−7.1	−7.0 (−5.9)[b,d]
ΔH°_{app} (kcal mol^{-1}; 37 °C)	19.4	2.15[d]
ΔS°_{app} (eu; 37 °C)	85	30[d]
$\Delta C^\circ_{p,app}$ (cal deg^{-1} mol^{-1})	−1200	−1500[d,f]
$\Delta\bar{\nu}_{H^+}$	0.94	0.86[d]
$\Delta\bar{\nu}_{Mg^{2+}}$	0.96	0.78[d]
qualitative effects		
Ca^{2+}	inhibition	inhibition[d]
ionic strength	inhibition	inhibition[d]
polycations	no enhancement; lag suppression	enhancement[g]
role of nucleotides		
GTP binding	required	required[d]
GTP hydrolysis rate (min^{-1}) by soluble protein	0.02 (0.07)[c]	not detected[h,i]
GTP hydrolysis rate (min^{-1}) by protein in polymer	0.02 (0.07),[c] not coupled to polymerization	0.13,[i] induced by polymerization; proceeding for one cycle at an intrinsic rate of 0.25[h]
GDP effects	inhibition	inhibition[j]

[a] Polymerization of tubulin–colchicine was in PG-16 mM $MgCl_2$ buffer, pH 7.0, except where indicated. Microtubule assembly was performed in the same buffer, containing 3.4 M glycerol and 1 mM EGTA, except where indicated and in studies from other laboratories. [b] Without glycerol. [c] With 3.4 M glycerol. [d] Lee & Timasheff (1977). [e] Margolis & Wilson (1977). [f] Hinz et al. (1979). [g] Lee et al. (1978). [h] Carlier & Pantaloni (1981). [i] Andreu & Timasheff (1981b). [j] Carlier & Pantaloni (1978).

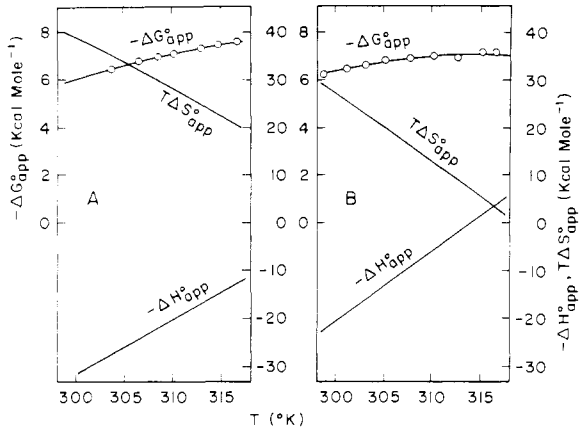

FIGURE 10: (A) Enthalpy and entropy contributions to the apparent standard free energy change of polymerization of tubulin–colchicine. The points are the experimental results and the solid lines the parameters resulting from the truncated van't Hoff analysis (see Figure 8 and Table II). The solvent was PG–16 mM $MgCl_2$ buffer, pH 7.0. (B) Enthalpy and entropy contributions to the apparent standard free energy change of polymerization of tubulin during microtubule assembly [data of Lee & Timasheff (1977) obtained in PG–16 mM $MgCl_2$–1 mM EGTA–3.4 M glycerol buffer, pH 7.0].

0.05 for H^+ and Mg^{2+}, respectively. Both values are close to the previously reported ones for microtubule assembly (Lee & Timasheff, 1977) and, as in that case, may be accounted for best by assuming that one additional proton and one additional magnesium ion are bound per tubulin–colchicine protomer incorporated into the polymer.

Discussion

Characteristics of the Polymerization of Tubulin Liganded by Colchicine: Comparison with Microtubule Assembly. The binding of colchicine to tubulin induces modifications in the properties of the soluble protein, which are consistent with a structural change [see Results and Andreu & Timasheff (1982b)] and are not accompanied by aggregation. The stable tubulin–colchicine complex inhibits substoichiometrically microtubule assembly in vitro. In the presence of 1.6×10^{-2} M Mg^{2+} and low to room temperatures, colchicine binding results in a small enhancement of the normal Mg^{2+}-induced tubulin self-association into double ring structures. Warming of the solution, however, leads to the formation of large aggregates (Andreu & Timasheff, 1982c). This polymerization, which proceeds under the conditions of microtubule assembly, is characterized by a lag time, cold reversibility, presence of a critical concentration, and a high degree of cooperativity consistent with a nucleated condensation polymerization mechanism (Oosawa & Asakura, 1975). The polymers formed are not microtubules, as testified by the wavelength dependence of their turbidity and their appearance in the electron microscope. The observation under our conditions of filamentous, twisted, and planar shapes, seen in the fixed specimens, suggests a structure of associated filaments. It is quite possible that our observation procedures are not adequate for the determination of the exact structure or that the polymers formed are ordered only over a few protomers but devoid of any easily recognizable long-range order. The significant points, however, are that the polymers formed are not microtubules[3] and that the characteristics of the polymerization of the tubulin–colchicine complex conform to an association pattern different from that of nonspecific amorphous protein precipitation.

The in vitro assembly of tubulin exhibits a high degree of polymorphism, the formed structures being highly dependent on pH, divalent cations, and other solvent conditions (Matsumura & Hayashi, 1976; Burton & Himes, 1978; Scheele & Borisy, 1979). Colchicine has been reported to cause the formation of filamentous protein aggregates at acid pH (Matsumura & Hayashi, 1976) or in the presence of the GTP

[3] In their examination of the same system, Saltarelli & Pantaloni (1982) describe the tubulin–colchicine polymers as "curly", or coiled structures. The difference between the morphologies of the aggregates seen by electron microscopy could arise from several sources: (i) different preparations of tubulin; (ii) different buffer compositions (the morphology of tubulin aggregates is known to be strongly affected by small changes in the ionic composition of the environment); (iii) different methods of sample preparation for electron microscopy (Saltarelli and Pantaloni worked with unfixed samples, while our samples were fixed in solution with 0.5% glutaraldehyde). The important point, however, is that both studies have shown that the polymers formed from tubulin–colchicine are not microtubules.

analogue guanyl-5′-yl methylenediphosphonate (Sandoval & Weber, 1979). Vinblastine, a drug that binds to sites on the tubulin molecule different from the colchicine binding site, induces an isodesmic tubulin self-association (Lee et al., 1975; Na & Timasheff, 1980) and, in the presence of Mg^{2+}, the formation of tubulin spirals and paracrystals that have been observed in vivo (Bensch & Malawista, 1968) and in vitro (Haskins et al., 1981; Na & Timasheff, 1982). In the present study, the colchicine-induced polymerization is related to microtubule assembly not only by proceeding under identidcal solvent conditions but also by its thermodynamic and qualitative characteristics, which are summarized in Table III. The apparent thermodynamic parameters of the growth reaction derived from the van't Hoff analysis of the critical concentration (see Results) appear in Figure 10 where they are compared with the corresponding parameters for microtubule assembly [data of Lee & Timasheff (1977)]. The last were obtained in the presence of 3.4 M glycerol, i.e., of a nonspecific thermodynamic booster of the reaction which facilitates the study of the temperature dependence (Lee & Timasheff, 1977). The polymerization of the tubulin–colchicine complex, on the other hand, was analyzed in the absence of glycerol because this cosolvent enhances the GTPase activity of the liganded protein, leading to the appearance of GDP in the system and a possible inhibition of the polymerization (see Results). Keeping this limitation in mind, there is a striking similarity between the two temperature diagrams of Figure 10. Both reactions proceed with a negative heat capacity change (of equal value within experimental error); they are endothermic over most of the temperature range examined, and they are driven by the positive entropy change in the system. Actually, the $\Delta H°_{app}$ and $\Delta S°_{app}$ values of microtubule polymerization are very close to those of tubulin–colchicine polymerization if they are shifted by 12–15 °C. Thus, for microtubule assembly, $\Delta H°_{app} \simeq 0$ at 38.5 °C, whereas the prediction for tubulin–colchicine polymerization is $\Delta H°_{app} \simeq 0$ at 53.5 °C, i.e., at a temperature not accessible to study due to the thermal denaturation of the protein.

A further thermodynamic similarity was found in the linkage of H^+ and Mg^{2+} binding to polymerization, which showed that one additional ion of each species became bound to the protein during its incorporation into the polymer, just as in the case of microtubules. Microtubule growth is also enhanced by glycerol, with the apparent binding of one additional glycerol molecule per protomer polymerized which, in fact, reflects a decrease in preferential exclusion of glycerol from the protein domain (Lee & Timasheff, 1977), since glycerol binds negatively to tubulin (Na & Timasheff, 1981). In the present experiments, glycerol did not enhance the polymerization of tubulin–colchicine. The observed increase in the critical concentration in the presence of this cosolvent, however, is most probably a secondary consequence of the enhancement of GTPase activity by glycerol. Therefore, at present, no rigorous conclusion can be made on the intrinsic effect of glycerol on the polymerization.

The qualitative effects of 10^{-4} M Ca^{2+} were, just as in microtubule assembly, inhibition of tubulin–colchicine polymerization, although in the latter case the inhibition was not complete, suggesting only an increase in the critical concentration instead of the total effect of Ca^{2+} on microtubule polymerization (Lee & Timasheff, 1977). Further qualitative similarities are found in the effects of Mes buffer and ionic strength (see Results and Table I).

GTP or nonhydrolyzable analogues are required for microtubule assembly (Weisenberg et al., 1976); GDP is inhibitory, although tubulin–GDP can still be active in supporting polymer growth (Carlier & Pantaloni, 1978). These characteristics can be interpreted in terms of structural differences between tubulin–GTP and tubulin–GDP. Microtubule assembly is accompanied by nucleotide hydrolysis, with a consequence that the growth rates at the two ends of the polymers are different and protomers flow through the assembled structure (Wegner, 1976; Margolis & Wilson, 1977; Hill, 1980, 1981; Engelborghs & Van Houtte, 1981). Carlier & Pantaloni (1981) have shown recently that this GTPase activity is induced by the polymerization process and proceeds through one cycle only with an intrinsic rate of 0.25 min^{-1} under their experimental conditions. This indicates that the conformations of the monodisperse protein and the protein in the polymer are different, a property known to be widespread in protein assembly systems. It has been called autostery (Caspar, 1980). GTP is also required for the polymerization of the tubulin–colchicine complex, and GDP is inhibitory. In this system, however, both the unassociated protein and the protein in the polymer hydrolyze GTP at the same constant rate of 0.07 min^{-1} (in the presence of 3.4 M glycerol), which is comparable to typical initial rates of hydrolysis during microtubule assembly under the same conditions [0.13 min^{-1} (Andreu & Timasheff, 1981b)]. These observations are consistent with the following notions: (i) there exist structural differences between GTP–tubulin–colchicine and GDP–tubulin–colchicine which make the former state more active in polymerization; (ii) GTP hydrolysis is completely uncoupled from polymerization; (iii) the GTP site is accessible in the polymer. Therefore, the tubulin–colchicine polymer can be predicted not to treadmill, and consequently, its formation should be close to a pure nucleated condensation polymerization, in both its thermodynamic and kinetic properties. If the activation of the GTPase were taken as a conformational parameter, it could be inferred that changes related to the ones linked to normal microtubule polymerization are already expressed in the soluble tubulin–colchicine complex (David-Pfeuty et al., 1979).

The effects of polycations on the polymerization of tubulin–colchicine present clear differences from microtubule assembly. Synthetic polycations, exogenous cationic proteins, and the microtubule-associated proteins enhance microtubule assembly, probably by binding tightly to the polymer as a result of cooperative interaction of each polycation molecule with a number of associated tubulin protomers (Lee et al., 1978). In the case of tubulin–colchicine, the present results indicate that, except for some kinetic effects, polylysine and lysozyme do not enhance polymerization under the conditions used and, preliminarily, that microtubule-associated proteins do not seem to have a significant effect. Thus, the polycations interact equally with both the unassociated protein and the polymer; i.e., the cooperative interaction with the polymer is lost, suggesting a different spatial arrangement of the groups on the tubulin which are responsible for this interaction. It is not known at present whether such a modification of the tubulin–polycation interactions by colchicine binding could play a role in the inhibition of microtubule assembly in vivo by colchicine (Deery & Weisenberg, 1981) or by postulated endogenous colchicine-like substances (Sherline et al., 1979; Lockwood, 1979). The differences found between tubulin–colchicine polymerization and microtubule assembly, namely, the uncoupling of nucleotide hydrolysis from polymerization, the accessibility in the polymer of the exchangeable nucleotide site, the different effects of polycations, and the different morphology, allow a very clear distinction between the two

modes of polymerization. The encountered qualitative similarities in the effects of GTP and GDP binding, Ca^{2+}, and ionic strength and, most important, the strong resemblance in the apparent thermodynamic characteristics of the two systems (Table III and Figure 10) indicate that a part of the mechanism of assembly must be common and that the two polymerizations are likely driven by similar protein–protein and protein–solvent interactions. The tubulin–colchicine system may, therefore, constitute a relatively simple model for a part of the interactions involved in microtubule assembly.

Mechanism of Microtubule Inhibition by Colchicine. The observation that colchicine-liganded tubulin polymerizes (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982c) and the characteristics of this self-assembly described in this paper do not in any way contradict the concept that colchicine inhibits microtubule assembly substoichiometrically. In fact, the tubulin–colchicine complex has this inhibitory property, as shown in Figure 2. The lack of previous reports of tubulin–colchicine polymerization in the presence of GTP may be ascribed to the requirement that the liganded protein must be at a concentration above the critical concentration normal for a given set of conditions, as well as to the extensive use of cycle microtubule protein preparations, which may render more difficult the observation of this phenomenon due to the GTPase contents of such preparations. Thus, the present results do not in any way invalidate any of the models proposed for the inhibition of microtubule assembly by colchicine (see the introduction), but to the contrary, they provide new insights into the basic molecular mechanism of the process.

The fact that the tubulin–colchicine polymers are different from microtubules is a clear indication that binding of the drug perturbs the normal protein–protein interactions which give rise to the natural polymer. How does this phenomenon occur? To answer this question, let us examine other modes of tubulin self-association, relevant to microtubule assembly. It has been proposed from structural observations that the Mg^{2+}-induced tubulin rings are actually coiled microtubule protofilaments (Erickson, 1974; Kirschner & Williams, 1974; Voter & Erickson, 1979; Mandelkow et al., 1982). Such a situation would be fortunate, since the apparent thermodynamics of the Mg^{2+}-induced self-association of purified tubulin into rings are known (Frigon & Timasheff, 1975b) for the solvent conditions employed in the present study. Colchicine binds both to soluble tubulin and to rings (Lambeir & Engelborghs, 1980), but not to microtubule walls (Margolis et al., 1980), and it strengthens somewhat ring formation (see Results). On the other hand, colchicine does not affect the vinblastine-induced polymerization (David-Pfeuty et al., 1979) which has been proposed to be mediated by enhanced longitudinal interactions (David-Pfeuty et al., 1979). It can be concluded, therefore, that colchicine does not affect significantly the longitudinal (protofilament forming) interactions between tubulin protomers (David-Pfeuty et al., 1979). At this point two types of models can be proposed.

(*1*) *Colchicine Blocks the Lateral Interactions between Tubulin Protomers.* This model is based on the decomposition of the polymer-forming interactions into lateral and longitudinal contributions, which is a simplified, although conceptually very useful representation of the thermodynamics of microtubule assembly. It was suggested by the colchicine-induced formation of tubulin ribbons consisting of three to four protofilaments in the presence of guanyl-5′-yl methylenediphosphonate (Sandoval & Weber, 1979), and it has been explored in detail by Saltarelli & Pantaloni (1982). It is consistent with the simplest interpretation of the experimental facts that both the colchicine site (Margolis et al., 1980) and the exchangeable nucleotide site (Weisenberg et al., 1976) are blocked in the microtubule wall, if both sites are assumed to be in the lateral interaction zones of the tubulin molecule. The basic tubulin–colchicine polymer could then be expected to be made of one protofilament or of two protofilaments in inverted lateral association (D. Pantaloni, B. McEwen, and S. Edelstein, unpublished results). Our experimental results, however, do not fit readily into this attractive model. First, the structural properties of the polymer, namely, light scattering, optical, and electron microscopic appearances, suggest dimensions large relative to a protofilament. Second, and most important, the presence of a critical concentration in the polymerization of tubulin–colchicine is at variance with a linear indefinite isodesmic association, but it points to a highly cooperative two- or three-dimensional nucleated condensation polymerization. These difficulties could possibly be overcome by postulating a two-phase polymerization, the first phase being a linear association along longitudinal bonds and the second a highly cooperative association of the formed polymers to give large aggregates by an as yet unknown mechanism.

A third problem arises, however, when the standard free energy changes for the addition of one tubulin molecule during microtubule growth and during tubulin–colchicine polymerization under the same solvent conditions (Table III) are decomposed into longitudinal bonding, lateral bonding, and entropic contributions, following the procedure of Erickson & Pantaloni (1981), and the ring formation (Frigon & Timasheff, 1975a,b) is assumed to include only the logitudinal bonding (Voter & Erickson, 1979) and an entropic contribution. The resulting parameters (J. M. Andreu and S. N. Timasheff, unpublished results) cannot be fitted easily into the simple concept that colchicine acts by blocking the lateral interactions.

(*2*) *Colchicine Induces a Distortion in the Normal Bonds between Protomers without Suppressing Any of Them.* An alternate model which we would like to propose both for the polymerization of the tubulin–colchicine complex and the inhibition of microtubule assembly is based on the distortion in the geometry of the normal bonds between protomers. This hypothesis is consistent with the great similarities found between the two modes of association, as well as with the difference in the final geometries of the two polymers. This hypothesis makes no assumptions as to which particular protein–protein bonds are altered. The alteration could involve simply a change in the contact angle between adjacent protomers and does not need to imply a perturbation of the contact free energy. How does this hypothesis explain the inhibition of microtubule assembly by colchicine? Considering the similarities between the microtubule assembly growth reaction (tubulin–tubulin interaction) and the tubulin–colchicine polymer growth reaction (liganded tubulin–liganded tubulin interaction), it seems reasonable that the addition of tubulin–colchicine to a microtubule (liganded tubulin–tubulin interaction) should be characterized by a free energy change similar to that of the other two reactions, i.e., between −6 and −7 kcal mol^{-1} (Table III). In fact Lambeir & Engelborghs (1980) estimated from their kinetic study, performed under conditions different from ours, that the association constants for the addition of tubulin and tubulin–colchicine to microtubules differ by only −1 kcal mol^{-1}, the reversible addition of the liganded protein being favored. So, in a hypothetical equilibrium situation, the binding of the tubulin–colchicine complex to a microtubule end would have a statistical advantage of approximately 5 times over that of the unliganded

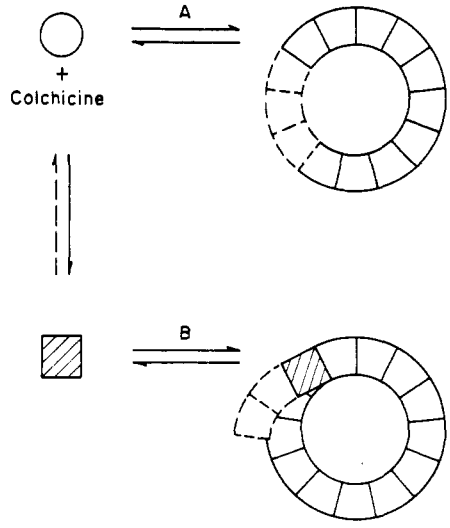

FIGURE 11: Conceptual schematic representation of microtubule inhibition by colchicine. The top view of a growing end is represented. Tubulin in solution (circle) is represented with a different conformation from tubulin in the microtubule (circular crown sectors) and tubulin liganded to colchicine (squares). The addition of unliganded tubulin to the polymer (reaction A) is compatible with further addition of protomers (indicated by the dashed lines). The addition of liganded tubulin (reaction B) is not compatible with the correct formation of a new layer of protomers since they fall out of correct contact with the previous layer (see the text).

protein. It is more likely, however, that the important factors are the actual rates of addition and release of the two species of protomers at the two ends of the polymer.

Let us take an actively growing end of either an elongating or a steady-state treadmilling microtubule. When a new protomer adds on, it may either remain linked or be removed before a new growth by one unit takes place. This depends on the interaction of a second bound protomer with the first one which increases the number of contacts of the first one and, therefore, decreases the probability of its dissociation. At some point, and due to its interaction with neighboring protein molecules, the newly incorporated protomer undergoes a structural change resulting in activation of its GTPase (Carlier & Pantaloni, 1981). When a drug-liganded protomer is added to the polymer, it would make interactions similar to those of the unliganded molecules, but with a slightly different geometry and possibly without a conformational change, since it is already an active GTPase. The distortion should not be large, since this would preclude the binding of any unliganded molecule in the vicinity of the liganded protein and require a relatively large number of drug-liganded molecules for an effective inhibition because of the reversibility of protomer addition. A small distortion would permit the further addition of a few unliganded protomers in the vicinity of the one bound to the drug, until the newly added ones fell out of proper register with the rest of the polymer, stopping growth. Since the drug-liganded protomer is secured in place by tubulin molecules added later, its dissociation would be improbable, constituting in this manner a statistical "cap" (Margolis & Wilson, 1977), stemming from a very limited degree of copolymerization (Sternlicht & Ringel, 1979). In fact a colchicine-mediated small alteration of the α–β subunit contacts can be inferred from the effect of this drug on the dissociation of the dimeric protein (Detrich et al., 1982). Going back to the simplified distinction between longitudinal and lateral interactions, we have described this effect schematically in Figure 11, in which the lateral interaction geometry has been hypothetically changed by ligand binding from the tube-forming one (angle of 152° between vicinal protomers) to a sheet-forming one (bond angle of 180° between protomers). It is evident that addition of the protomer at an incorrect angle rapidly makes microtubule growth impossible.

In general, a perturbation of the correct microtubule-bonding geometry by ligand binding may be due to (i) a simple steric hindrance by the ligand bound in the contact zone, (ii) a change in the shape of the tubulin molecule, and (iii) a change in the protein dynamics and flexibility. The fact that the colchicine site is not accessible in microtubules would favor the first possibility, while the colchicine-induced conformational change could be responsible for mechanisms of the second or third type. The third mechanism is conceptually attractive: it permits us to attribute a regulatory purpose to the polymerization-linked conformational change (Caspar, 1980). In fact, in the case of microtubules, a structural change is required for the hydrolysis of GTP (Carlier & Pantaloni, 1981), which imparts the in vitro flow-through property to the polymer (Hill, 1980, 1981). Although treadmilling has not been observed in vivo, it has been proposed to take part in chromosomal movement (Margolis & Wilson, 1981). It is conceivable that the binding of colchicine could confer to tubulin, or to a part of it, a different, perhaps tighter, dynamic structural state related to the state of polymerized tubulin. This would cause the protein to polymerize with an even slightly higher affinity, but the product would lack the flexibility necessary for the autosteric transition and the correct bonding characteristics of normal microtubule polymerization.

Acknowledgments

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Registry No. Mg, 7439-95-4; Ca, 7440-70-2; GTP, 86-01-1; GDP, 146-91-8; GTPase, 9059-32-9; hydrogen ion, 12408-02-5; polylysine, 25104-18-1; (*S*)-poly[imino[1-(4-aminobutyl)-2-oxo-1,2-ethanediyl]], 38000-06-5; lysozyme, 9001-63-2.

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