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Tubulin bound to colchicine forms polymers different from microtubules

(protein self-assembly/antimitotic drugs)

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ABSTRACT **The purified tubulin–colchicine complex undergoes *in vitro* polymerization under the same conditions that promote the assembly of microtubules from purified tubulin. The need for a critical concentration, the apparent free energy change of the reaction, and the effects of divalent cations and nucleotide binding indicate interactions similar to those involved in microtubule formation. The large polymers formed are not microtubules, suggesting that the mode of action of the antimitotic drug may be the production of an incorrect bonding geometry between tubulin molecules.**

Colchicine is an effective inhibitor of mitosis (1) and microtubule assembly *in vitro* (2). This drug binds to tubulin to form a tight complex (3) and induces a conformational change in the protein (4–7). The tubulin–colchicine complex inhibits microtubule growth substoichiometrically (8, 9). Various mechanisms involving "capping" (8) or copolymerization (10) have been proposed to explain this phenomenon. Recently, evidence has been presented indicating that the binding of the tubulin–colchicine complex to microtubules is rapid and reversible (11). The estimated association constant is of the same order of magnitude as the association constant for unliganded tubulin binding to microtubules (11, 12).

These results suggest that the overall characteristics and strength of the protein–protein interactions between tubulin protomers in microtubules are not substantially different from those of the binding of drug-liganded tubulin to the microtubule. Nevertheless, the consequences of the two reactions are strikingly different because binding of colchicine-liganded tubulin results in inhibition of the growth of the normal polymer. On the other hand, the soluble tubulin–colchicine complex exhibits a GTPase activity similar to the GTP hydrolysis that occurs during microtubule assembly (5, 13), suggesting a conformational resemblance between colchicine-liganded tubulin and tubulin within microtubules (5, 14).

In view of these facts, it is apparent that a detailed description of the structure and molecular interactions of tubulin and colchicine-liganded tubulin should result in a better understanding of the mechanism of microtubule assembly and its inhibition by the drug. We report here the assembly of the purified tubulin–colchicine complex into polymers that share several of the properties of microtubules, although they are morphologically different from them.

MATERIALS AND METHODS

Purified calf brain tubulin was prepared as described (15, 16). The tubulin–colchicine complex was prepared by 10-min incubation of 0.4–0.6 mM tubulin with 1 mM colchicine (Aldrich) at 25°C (7). Attainment of equilibrium under these conditions is consistent with a recent kinetic study (6). Excess ligand was removed by fast gel chromatography (7). This procedure afforded an essentially stoichiometric complex that contained 0.9 mol of colchicine per mol of tubulin as determined with [^{3}H]colchicine (New England Nuclear). No significant dissociation was detected during 2 hr at room temperature. The complex was quantitatively dissociated by 6 M guanidine hydrochloride and more than 90% dissociated by selective photoconversion of bound ligand to lumicolchicine (7).

Tubulin self-association was examined by sedimentation velocity using double sector cells in An-D rotors and a Beckman model E analytical ultracentrifuge equipped with electronic speed control, RTIC temperature control, and photoelectric scanner. Microtubule assembly *in vitro* was performed in assembly buffer (10 mM sodium phosphate/0.1 mM GTP/1 mM EGTA/16 mM $MgCl_2$/3.4 M glycerol, pH 7.0) at 37°C and the reaction was followed turbidimetrically (17). Polymerization of tubulin–colchicine complexes was performed under the same conditions or in the same buffer without glycerol and EGTA. Polymerized samples were treated with 0.5% glutaraldehyde (Polysciences, Warrington, PA), adsorbed to carbon-coated Formvar grids, stained with 1% uranyl acetate, and observed in the electron microscope (Philips EM 3001).

RESULTS AND DISCUSSION

The results of the sedimentation velocity analysis of the purified tubulin–colchicine complex are shown in Fig. 1. In the absence of divalent cations, this complex sedimented as a simple symmetrical peak, as shown in Fig. 1*a*, where the dotted line is the 350 nm absorption profile due to the ligand distribution. This ligand boundary cosedimented with the protein, whose sedimentation velocity was found to be within experimental error the same as that of the unliganded tubulin $\alpha\beta$ heterodimer (18), namely 5.8S. It must be noted that the preparation of the stoichiometric tubulin–colchicine complex was based on the use of high ligand concentrations and, thus, involved very mild conditions (see *Materials and Methods*), instead of those commonly used (1 hr at 37°C) which result in partial denaturation of the protein (3). The tubulin–colchicine complex prepared under the present conditions was found to be active, inhibiting in substoichiometric amounts the assembly of unliganded tubulin into microtubules (19).

In 10 mM sodium phosphate/0.1 mM GTP/16 mM $MgCl_2$, pH 7.0, at 20°C, tubulin–colchicine sedimented with the bimodal profile (Fig. 1*b*) characteristic of the Mg^{2+}-induced self-association of tubulin into 42S double rings (18). This association was only slightly enhanced in the colchicine-liganded protein (7). When the same experiment was performed under identical conditions and protein concentration but at a higher temperature, 30°C, a portion of the material was found to sediment during the first stages of rotor acceleration and a marked de-

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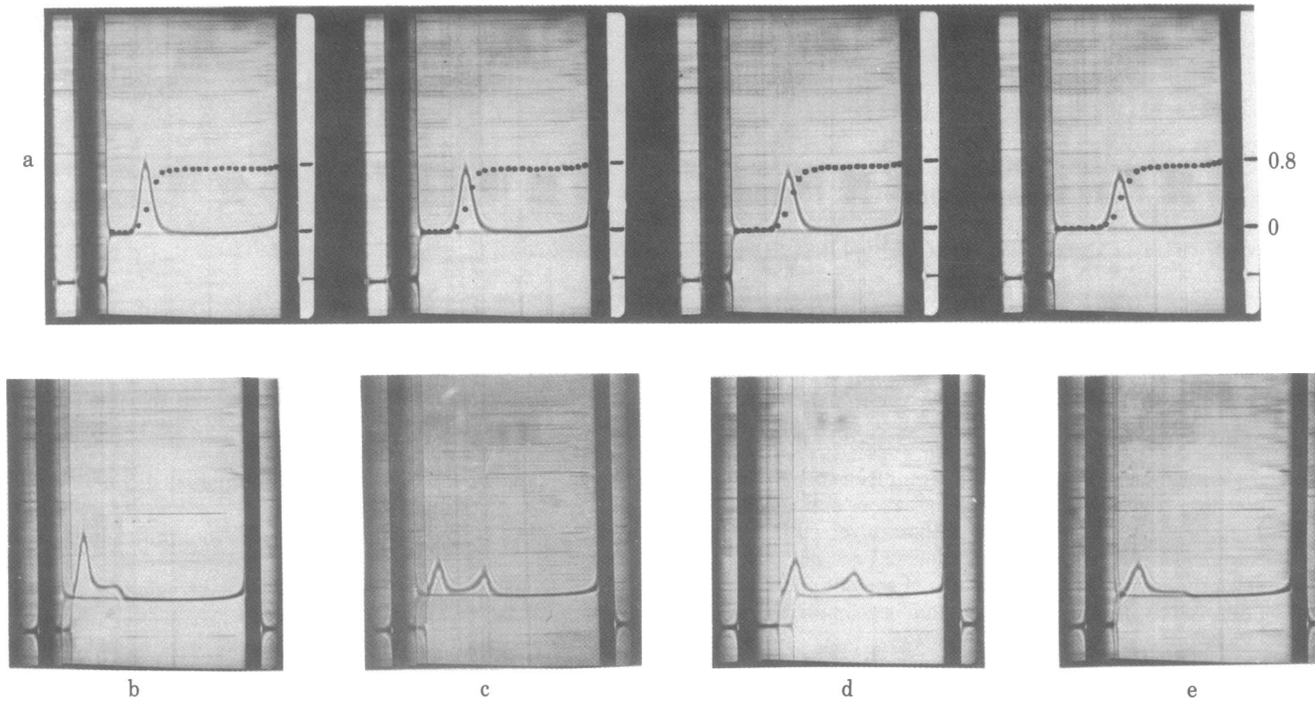


FIG. 1. Sedimentation profiles of tubulin–colchicine. Sedimentation was from left to right. (*a*) Tubulin–colchicine at 4.4 mg/ml in 10 mM sodium phosphate/0.1 mM GTP, pH 7.0, at 20°C for 34, 42, 50, and 58 min (left to right) after attainment of speed (60,000 rpm; bar angle, 55°). The dotted line is a superimposed absorption profile obtained with the photoelectric scanner. Performing the experiment at 30°C gave also a single symmetrical peak. (*b*) Tubulin–colchicine at 4.5 mg/ml in 10 mM sodium phosphate/0.1 mM GTP/16 mM $MgCl_2$, pH 7.0, at 20°C for 10 min after attainment of speed (48,000 rpm; bar angle, 55°). (*c*) Same as *b*, except at 30°C for 8 min. (*d*) At 7.2 mg/ml under the same conditions as *c*, 10 min. (*e*) At 2.6 mg/ml under the same conditions as *c*, 12 min.

crease in the area under the slow peak was noticed without a corresponding increase in the rapid peak, indicating the formation of very large aggregates (Fig. 1*c*). This was confirmed by observation of the protein concentration dependence of the sedimentation profile at 30°C (Fig. 1 *c*, *d*, and *e*). The total area under the boundary—i.e., the amount of observable sedi-

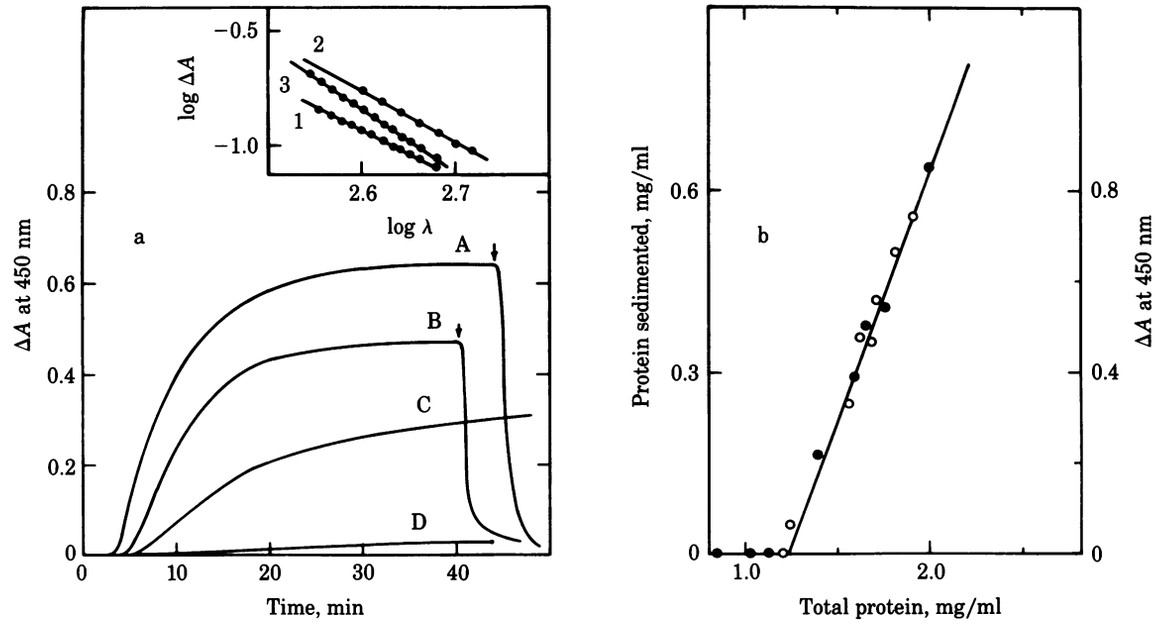


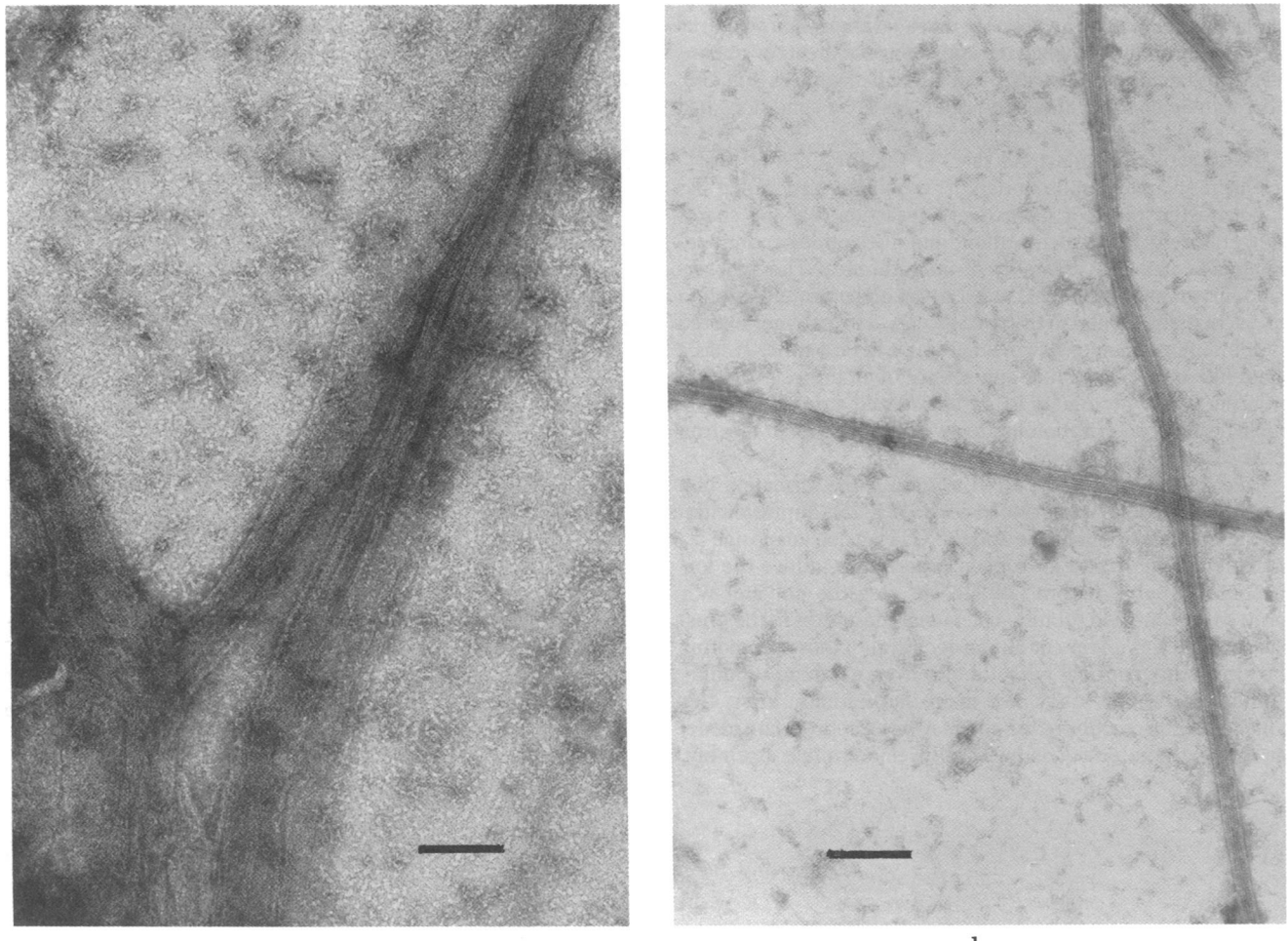
FIG. 2. (*a*) Time course of tubulin–colchicine turbidity development in 10 mM sodium phosphate/0.1 mM GTP/16 mM $MgCl_2$, pH 7.0. The reaction was initiated at zero time with a temperature jump from 10°C to 37°C; cooling is indicated by the arrows. Curves: A, 1.80 mg of protein per ml; B, 1.70 mg/ml; C, 1.55 mg/ml; D, 1.55 mg/ml in 1 mM $CaCl_2$. The tubulin–colchicine complex in the same buffer without Mg^{2+} or GTP gave no turbidity increase. (*Inset*) Wavelength dependence of the turbidity of the tubulin–colchicine complex (curve 1), the tubulin–colchicine complex in a buffer containing 3.4 M glycerol and 1 mM EGTA (curve 2), and a control of microtubules polymerized in a buffer with glycerol and EGTA (curve 3). (*b*) Dependence of polymer concentration on total protein concentration. The tubulin–colchicine solutions were heated at 37°C for 45 min and centrifuged at 30,000 × *g* at 37°C (± 1°C) for 15 min, and the amount of protein sedimented was measured (solid circles). In another set of samples the plateau turbidities were measured (open circles).

menting components—did not increase significantly when the total protein concentration was increased above 4 mg/ml and the excess protein went to the bottom of the cell. Under these conditions, unliganded tubulin does not form aggregates larger than rings (18).

Heating of tubulin–colchicine solutions to 37°C in the same buffer resulted in the generation of turbidity that was reversible upon cooling, dependent on Mg^{2+} and GTP, and sensitive to Ca^{2+} (Fig. 2*a*). Polymerization was also obtained in a buffer containing 3.4 M glycerol. However, as shown in Fig. 2*a Inset*, the wavelength dependence of the turbidity of the tubulin–colchicine aggregate ($\Delta A \propto \lambda^{-2.1}$) was clearly different from that of a microtubule control polymerized in the presence of glycerol ($\Delta A \propto \lambda^{-2.8}$), indicating that the tubulin–colchicine polymer does not follow the Berne (20) turbidity criterion ($\Delta A \propto \lambda^{-3.0}$) for long thin rods such as microtubules. The aggregates could be easily sedimented at 30,000 × *g*. No polymerization was detected at concentrations below 1.2 mg/ml (Fig. 2*b*). Above this concentration, 90% of the excess protein was incorporated into the sedimenting polymer. Similar results were obtained in measurements of the concentrations of sedimented [^{3}H]colchicine bound to tubulin, showing that the drug remained associated with the protein throughout the procedure.

There being such a critical concentration is diagnostic of a highly cooperative behavior, and it is characteristic of the nucleated condensation polymerization of protein assemblies like actin filaments and microtubules (17, 21). It is a consequence of the formation of a bidimensional lattice of protein–protein bonds which thermodynamically favors large polymers over small linear assemblies formed during nucleation (22). In these systems the experimentally determined critical concentration, C_r, is close to equal to the reciprocal of the equilibrium binding constant for the addition of one protomer to the growing polymer and therefore is a convenient way to measure the apparent free energy change of the growth reaction because $\Delta G^\circ_{app} = RT\ln C_r$ (17, 21).

The turbidity of the tubulin–colchicine polymer at 450 nm showed a good correlation (Fig. 2*b*) with its mass concentration in the observed range ($\Delta A \simeq 1.3$ liters $g^{-1}cm^{-1}$), making it possible to use turbidity as a simple empirical method for estimating the critical concentrations. However, it is likely that in our case the turbidity values cannot be related rigorously to the mass of protein polymerized (23) as in the case for microtubules (20) which give lower turbidity values (approximately 0.2 liter $g^{-1}cm^{-1}$ at 450 nm). Using this approach, we found that, under the conditions of Fig. 2*b*, the growth of the tubulin–colchicine polymer was characterized by an apparent free energy change, ΔG°_{app}, of −7.0 kcal/mol (1 cal = 4.184 J) of protein, whereas microtubule assembly gives −5.9 and −7.1 kcal/mol of protein in the same buffer in the absence and presence of 3.4 M glycerol, respectively (17). A further similarity was revealed by the temperature dependence of C_r, which indicated



a b

FIG. 3. Electron micrographs of negatively stained specimens of the tubulin–colchicine complex (*a*) and unliganded tubulin (*b*), both polymerized in buffer containing 3.4 M glycerol. The bar indicates 100 nm.

ΔC_p, ΔH°_{app}, and ΔS°_{app} values (19) not far from those of microtubule assembly (17). These results support the idea that the interactions involved in the two types of polymerization are similar.

A study of the effects of solution variables and macromolecules on the polymerization of the tubulin–colchicine complex (19) has shown that, similar to microtubules (17), the polymer formation is accompanied by the binding of one additional proton, is dependent on Mg^{2+}, and is inhibited by Ca^{2+}. Furthermore, it has indicated that: (*i*) the polymerization of tubulin–colchicine is more favored when GTP is bound to the protein rather than when GDP is bound, despite the fact that tubulin–colchicine hydrolyzes GTP (7, 13) in a manner not coupled to polymerization; (*ii*) polycations such as polylysine do not enhance polymerization; and (*iii*) tubulin prepared by cycles of polymerization and depolymerization in the absence of glycerol (24) and then associated with colchicine generated turbidity under conditions similar to those for the purified tubulin–colchicine complex.

What is the morphology of the tubulin–colchicine polymer? The characteristics of the polymerization process suggest the formation of at least a two-dimensional polymer probably larger than microtubules. Particles of glutaraldehyde-fixed tubulin–colchicine polymer were examined under the phase-contrast microscope. Their presence was well correlated with that of the turbidity (not in unheated samples or samples of concentration $<C_r$), indicating fairly large dimensions either of the polymer itself or of secondary aggregation products. An electron microscopic examination of the polymer revealed amorphous material and large structures that had the appearance of sheets or ribbons made of filaments on a background of small oligomers (Fig. 3*a*). Microtubule controls obtained under the same conditions had the usual long thin rod morphology (Fig. 3*b*).

These results demonstrate that, in the presence of Mg^{2+}, colchicine-bound tubulin undergoes a polymerization that has the characteristics of a self-assembly reaction (21) and shares temperature, pH, divalent cation, and nucleotide binding dependences with the assembly of microtubules. These observations strongly suggest that at least part of the protein–protein interactions are similar in the two systems. The polymer formed by tubulin–colchicine, however, is not a helical tube. It has a sheet-like or a ribbon-like appearance, but the regularity of ordering of the protomers is not known and a definitive morphological characterization has to await more detailed electron microscope studies.

Colchicine has been reported to induce the formation of a filamentous protein aggregate at acidic pH (25). Suprastoichiometric amounts of colchicine and the GTP analog guanosine 5′-methylenediphosphate have been reported to induce the formation of tubulin ribbons (26). In our case the polymer was formed from purified tubulin–colchicine complexes in the presence of GTP and under conditions identical to those promoting the formation of normal microtubules from unbound tubulin.

Our observations carry two main implications. First, this polymerization reaction of tubulin may be an appropriate model for part of the interactions involved in microtubule assembly, with the advantage that the colchicine complex is more stable than the unbound protein (7). Second, these results shed light on the mechanism of microtubule inhibition by antimitotic drugs. Regardless of the specific mechanism of substoichiometric microtubule inhibition by colchicine that is invoked (8–12), one of the most likely primary events leading to such an inhibition is the distortion of the correct bonding geometry between tubulin protomers as a result of drug binding. This can itself be the result of a steric effect of the bound ligand or a ligand-induced conformational change of the protein. The polymerization of colchicine-bound tubulin in a manner closely related to microtubule assembly, but resulting in a polymer with a different morphology, can be regarded as direct evidence for such a distortion of the protein–protein interactions by the antimitotic drug.

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