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In Vitro Reconstitution of Calf Brain Microtubules: Effects of Macromolecules†

James C. Lee,‡ Neil Tweedy, and Serge N. Timasheff*

ABSTRACT: A comparative study has been carried out of the ability to reconstitute into microtubules of tubulins prepared by the Weisenberg and the cycle procedures. It was found that further purification of cycle tubulin by phosphocellulose chromatography made its ability to polymerize identical with that of Weisenberg tubulin. By adding to either tubulin the isolated proteins which copurify with tubulin in the cycle procedure, it is possible to reduce their critical concentrations of microtubule formation to a value identical with that of cycle tubulin. It was demonstrated quantitatively that the effect of these nontubulin proteins could be mimicked by a variety of polycationic molecules, the most effective one being poly(L-lysine). A possible mechanism is described by which growing microtubules could be stabilized by subsequent addition of the nontubulin proteins. The conclusion is drawn that, at present, it is not known whether the proteins which copurify with tubulin are specific components of the microtubule system, or simply artefactually coprecipitated impurities.

Since the initial report by Weisenberg (1972) of his discovery of conditions which lead to the in vitro self-assembly of brain tubulin into microtubules, a vast literature devoted to this subject has appeared. The aims of the various studies have been the elucidation of the mechanisms of self-assembly and of the regulation of the assembly process by metabolic effectors and drugs. The material used in most of these studies was either partially purified protein or crude homogenate of brain protein (Borisy & Olmsted, 1972; Olmsted & Borisy, 1973, 1975; Shelanski et al., 1973; Gaskin et al., 1974; Kirschner et al., 1974; Weingarten et al., 1975; Erickson, 1974; Dentler et al., 1975). The polymerization-depolymerization cycle procedure (Shelanski et al., 1973) has been the method of choice for preparing tubulin. This method in its various versions yields preparations which are 75-85% pure by the criterion of sodium dodecyl sulfate gel electrophoresis (Kirschner et al., 1974; Weingarten et al., 1975; Erickson, 1974; Borisy et al, 1974; Murphy & Borisy, 1975; Murphy et al., 1977), the remainder being a mixture of proteins, the molecular weights and amounts of which are a function of the exact conditions of preparation employed by the investigators. The observation that these nontubulin protein components coprecipitate in a constant ratio with tubulin through repeated cycles of assembly-disassembly has been used as a basis for the conclusion that these components are proteins specifically associated with microtubules rather than contaminants (Borisy et al., 1974, 195; Murphy & Borisy, 1975; Weingarten et al., 1975; Sloboda et al., 1975, 1976). Murphy & Borisy (1975) have reported that microtuble formation is promoted by heavy molecular weight components, while Weingarten et al. (1974, 1975) have concluded that dimeric tubulin is totally devoid of the ability to form microtubules and that the in vitro assembly of microtubules required the presence in stoichiometric amounts of nontubulin proteins with molecular weights considerably lower than those described by Murphy & Borisy (1975), which are found in their preparations and which they named "τ factor". In fact, tubulin dimers (5.8 S), purified by the cycle method followed by gel column chromatography, have been described repeatedly as being incapable of polymerization into microtubules by themselves (Kuriyama, 1975; Kirschner & Williams, 1974; Keates & Hall, 1975). Sandoval & Cuatrecasas (1976) compared by gel filtration chromatography tubulins prepared by various procedures (Weisenberg

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‡ Present address: E. A. Doisy, Department of Biochemistry, St. Louis University, St. Louis, Missouri 63104.

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et al., 1968; Shelanski et al., 1973; Hinman et al., 1973). All of these preparations were resolved into two major fractions. The protein in the excluded volume was equated to the protein obtained in similar fractions described in the literature and was assumed to be capable of reconstitution into microtubules. The protein eluted later consisted mainly of tubulin dimers and was assumed to be incapable of self-assembly into microtubules. Examination by sodium dodecyl sulfate gel electrophoresis (Sandoval & Cuatrecasas, 1976) revealed the presence in all preparations of three proteins with molecular weights of 32 000, 60 000, and 100 000, distributed asymmetrically between tubulin dimers and polymers, leading these authors to conclude that these proteins, either by themselves or as a group, may control the formation of protofilaments and microtubules. A similar conclusion was reached by Keates & Hall (1975).

Contrary to these reports, Lee & Timasheff (1975; (Lee et al., 1975) have shown that totally dissociated (5.8 S), purified tubulin prepared by the complete, modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973; Frigon & Timasheff, 1975a) can be reconstituted into microtubules. This protein appears to be devoid of the heavy molecular weight components as shown by sodium dodecyl sulfate gel electrophoresis at a loading of 350 μg of protein (Lee & Timasheff, 1975). The reconstitution of this tubulin into microtubules does not require the presence either of other proteins or of preexisting nucleation centers, at least within the limits of resolution of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, sedimentation velocity, and sedimentation equilibrium analysis. This observation, that microtubules can be reconstructed from totally dissociated tubulin dimers, has been confirmed by Erickson & Voter (1976), Himes et al. (1976), Weber and co-workers (Wehland et al, 1977; Herzog & Weber, 1977), and most recently by Borisy and co-workers (Murphy et al., 1977). It appears, therefore, that all the information necessary for microtubule growth is present in tubulin. The reaction requires the presence of GTP; it is enhanced by magnesium ions and the sequestration of calcium ions (Lee et al., 1975; Lee & Timasheff, 1975, 1977; Timasheff et al., 1976a). Glycerol at high concentrations strengthens the microtubule growth reaction, although microtubule formation can proceed in its absence (Lee & Timasheff, 1977; Herzog & Weber, 1977; Himes et al., 1977).

Another apparent difference in the self-assembly of tubulins prepared by the polymerization-depolymerization and complete Weisenberg procedures is found in the apparent standard free energy of addition of a subunit to a growing microtubule, $\Delta G°_{app}$, when the data are analyzed in terms of the polymerization theory of Oosawa & Kasai (1971). For tubulin prepared by the polymerization-depolymerization procedure, $\Delta G°_{app}$ is more negative (Gaskin et al., 1974) than that for the highly purified protein (Lee & Timasheff, 1975). It must be noted, however, that these values were obtained in different media and, therefore, refer to nonidentical standard states.

A further difference between the two types of tubulin is found in their sedimentation velocity properties. The highly purified protein can enter into rapidly reversible equilibrium with 42S double ring structures in the presence of magnesium ions, all molecules of the protein being thermodynamically identical with respect to this reaction (Frigon & Timasheff, 1975a,b); Weingarten et al. (1974, 1975), on the other hand, have reported that cycle prepared tubulin consists of two ultracentrifugal components not in equilibrium with each other, one being 5.8S tubulin dimers, the other being manifested by a highly skewed, hypersharp peak, with a reported sedimentation coefficient, $s°_{20,w}$, of 36 S.

It is evident that tubulins purified by different procedures, which yield products of different degrees of homogeneity, may exhibit highly different physical properties. As a first step toward a better understanding of these differences, it seemed reasonable to ask the question whether the observed differences are reflections of the intrinsic properties of the protein or are effects induced by exogenous factors, and, if so, whether the last are biologically significant components of the system, or are artefacts of the method of protein purification. An effort was initiated, therefore, to test these possibilities. To this effect, a comparison was carried out of the solution behavior of tubulin purified by the two types of procedures. It is hoped that the results presented in this paper will help to start sorting out the apparent contradictions found in the literature.

Materials and Methods

Ribonuclease A (lot 55C-8250), spermine (lot 95C-0143), and spermidine (lot 25C-0086) were purchased from Sigma Chemical Co. Poly(L-lysine) (lot T-1762) of molecular weight 100 000–200 000 and lysozyme (lot LYSF 647-8) were obtained from Mann Research Laboratories, Inc., and Worthington Biochemical Corp., respectively. Calf thymus histone f-1 was a generous gift of Dr. G. D. Fasman and A. Fulmer of Brandeis University. Fibrous cellulose phosphate P11 and ACS certified grade glycerol were purchased from Whatman Biochemical Ltd. and Fisher Scientific Co., respectively. All reagents were used without further purifications.

Calf brain tubulin was prepared by two procedures, namely, a modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973; Frigon & Timasheff, 1975a) and the polymerization-depolymerization method of Shelanski et al. (1973), as modified by Weingarten et al. (1974). Tubulin concentrations were determined spectrophotometrically in 6 M guanidine hydrochloride using an absorptivity of 1.15 L/(g·cm) at 274 nm (Lee et al., 1973). The purity of the protein was monitored by sodium dodecyl sulfate gel electrophoresis employing the procedure of Weber et al. (1972).

The formation of microtubules was followed by the turbidity method, first introduced by Gaskin et al. (1974). Two assembly buffers were used. The first consisted of 10^{-2} M sodium phosphate, 10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$, and 3.4 M glycerol at pH 7.0 and will be referred to as buffer A. The second consisted of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes),[1] 10^{-3} M ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 10^{-3} M GTP, and 5×10^{-4} M $MgCl_2$ at pH 6.6 and will be referred to as buffer B. Turbidity was measured at 350 nm on a Cary 118 recording spectrophotometer. The protein solutions were initially incubated at 10 °C in a water-jacketed cuvette which was thermostatically regulated by a Neslab circulating bath. Reconstitution of microtubules was initiated by circulating through the cuvette water at 37 °C, regulated by a Haake circulator. The equilibrium constant for the microtubule propagation reaction, K_p^{app}, was obtained from the turbidity data as the reciprocal of the critical concentration, assuming the validity for this system of the polymerization theory of Oosawa & Kasai (1971) as demonstrated by Gaskin et al. (1974). Electron microscopy was performed as described previously (Lee & Timasheff, 1975).

Cycle tubulin was further purified by phosphocellulose chromatography according to the method of Weingarten et

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

al. (1975). Cycle tubulin was dissolved in 2.5×10^{-2} M Mes, 5×10^{-4} M $MgCl_2$, 1×10^{-3} M β-mercaptoethanol, 1×10^{-4} M EDTA, pH 6.4, to a concentration of about 10–15 mg/mL. The protein solution was applied to a 4-mL column of phosphocellulose equilibrated with the same buffer at 4 °C. Fractions of 0.5–1 mL were collected. After elution of all the nonretained protein, the column was eluted with 1.0 M NaCl in the same buffer. The protein not retained was shown to be tubulin and designated as PC-tubulin. The protein eluted with 1.0 M NaCl was designated as microtubule-associated protein, or τ fraction.

In order to check whether there were any differences in behavior between tubulin prepared by the original Weisenberg et al. (1968) procedure and the fully purified tubulin (Weisenberg & Timasheff, 1970; Lee et al., 1973, Frigon & Timasheff, 1975a) as expected from the conclusions of Sandoval & Cuatrecasas (1976), calf brain tubulin purified by the original Weisenberg et al. (1968) procedure was fractionated on a 2 × 30 cm column of Sephadex G-150 equilibrated with buffer A at 4 °C. The protein was resolved into two peaks. One was found in the void volume, constituting 30–40% of the total protein. The other peak was retained on the column.

Results

A quantitative comparison was carried out of the self-assembly properties of tubulins prepared by the modified Weisenberg and cycle procedures, as well as of dimeric tubulin purified from the cycle preparation by phosphocellulose ion-exchange chromatography. These will be referred to as W-tubulin, c-tubulin, and PC-tubulin, respetively. These proteins were found to vary in homogeneity by sodium dodecyl sulfate gel electrophoresis. W-tubulin, purified by precipitation with magnesium chloride, displays 98–99% of the optical density under the tubulin band, the rest being present principally under a heavy molecular weight band. Prior to magnesium precipitation, only 91% of the total stained material migrated under the tubulin peak. Cycle tubulin, on the other hand, showed about 35% of nontubulin proteins, spread over a large number of bands. Passage through a phosphocellulose ion-exchange column removed most of these components, with tubulin accounting for approximately 95% of the total stain, the rest, consisting of a high molecular weight protein and several other faint bands.

The ability of these tubulin preparations to self-assemble was examined in both assembly buffers A and B, in which each of these proteins had been studied individually. Examination in assembly buffer A of tubulin purified by the Weisenberg et al. (1968) procedure, followed by fractionation on Sephadex G-150 (Sandoval & Cuatrecasas, 1976), resulted in an apparent microtubule propagation equilibrium constant, K_p^{app}, of 1.0×10^5 L/mol, as shown in Table I, A. The protein unfractionated on Sephadex G-150 displayed a value of $K_p^{app} = 0.9 \times 10^5$ L/mol. Both values of K_p^{app} are essentially identical with that of pure dimeric tubulin (Lee & Timasheff, 1975, 1977), indicating that the nontubulin proteins found in the Weisenberg et al. (1968) preparation have no significant effect on microtubule elongation.

In order to compare the self-assembling abilities of c- and W-tubulins, c-tubulin, prepared by the procedure of Weingarten et al. (1975), was examined in assembly buffers A and B at 37 °C. The results of turbidity measurements, presented in Table I, B, show that, in both media, self-assembly occurs with an identical critical concentration, C_r, of 0.2 mg/mL, which corresponds to an apparent equilibrium constant of 5.5×10^5 L/mol and an apparent standard free energy change for the addition of a subunit to a growing microtubule, $\Delta G°_{app} = -8.2$ kcal/mol. These results are in reasonable agreement with the published value of C_r of 0.4 mg/mL for reconstitution in buffer B (Gaskin et al., 1974). Similar experiments with W-tubulin in buffer B did not show any reversible turbidity even at a protein concentration of 18 mg/mL. In buffer A it reconstitutes into microtubules with a critical concentration of 0.9 mg/mL, i.e., $K_p^{app} = 1.2 \times 10^5$ L/mol, or $\Delta G°_{app} = -7.2$ kcal/mol. Therefore, in identical reassembly medium, buffer A, the ability to self-assemble into microtubules of c-tubulin is stronger than that of W-tubulin by −1 kcal/mol of 5.8 S dimer, or 15%. In electron microscopy, the two systems showed similar networks of filamentous structure, typical of microtubule reconstitution, indicating that the method of purification is not a significant source of uncertainty in the analysis of the turbidimetric data.

TABLE I: Dependence of K_p^{app} on Tubulin Preparation.

	C_r (mg/mL)	K_p^{app} (L/mol)
A. W-Tubulin		
i. Before gel permeation[a]	1.2 ± 0.1	0.9×10^5
ii. After gel permeation[a]	1.1 ± 0.1	1.0×10^5
iii. + C-tubulin in Mes buffer[b]	1.3 ± 0.1	0.9×10^5
iv. + C-tubulin in phosphate buffer[c]	0.8 ± 0.1	1.4×10^5
B. C-Tubulin		
i. In Mes buffer[b]	0.2 ± 0.1	5.5×10^5
ii. In phosphate buffer[c]	0.2 ± 0.1	5.5×10^5

[a] Assembly buffer is 10^{-2} M sodium phosphate, 10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$, 10^{-3} M EGTA, and 2.4 M glycerol at pH 7.0. [b] Assembly buffer is 0.1 M Mes, 10^{-3} M EGTA, 10^{-3} M GTP, and 5×10^{-4} M $MgCl_2$ at pH 6.6. [c] Assembly buffer is the same as in footnote *a* without EGTA.

The sources of the difference in microtubule propagation standard free energy between c- and W-tubulins were probed by fractionation and mixing experiments. First, to see whether c-tubulin contains a factor capable of enhancing the self-assembly of dimeric W-tubulin, aliquots of c-tubulin were added to W-tubulin at concentrations at which neither protein preparation develops turbidity on exposure to 37 °C. It was found that when 0.2–0.25 mg/mL of c-tubulin was added to a 1.5 mg/mL W-tubulin solution in buffer B, heating to 37 °C generated turbidity, which was reversible by cooling to 10 °C. The critical concentration of this mixture was 1.3 mg/mL, based on total protein. A similar result was obtained in buffer A. Thus, the addition of small amounts of c-tubulin to W-tubulin increases the value of K_p^{app} but the enhancement is insufficient to approach the value characteristic of c-tubulin.

These results pose the question: Is the difference in ability to form microtubules an intrinsic property of the tubulins or is it due to the presence, in cycle preparations, of copurified nontubulin proteins? To probe this, both W- and c-tubulins were fractionated on phosphocellulose ion-exchange columns at 4 °C, according to the method of Weingarten et al. (1975). In both preparations, the fraction not retained by the column, which by definition is tubulin, was equilibrated with reconstitution buffer A and heated to 37 °C, and the generated turbidity was recorded as a function of protein concentration. The results, shown in Table II, clearly indicate that passage through the phosphocellulose column did not affect the ability of W-tubulin to form microtubules, since the critical concentration remained 0.9 mg/mL both before and after ion-exchange chromatography. A totally different situation prevails with c-tubulin. With this protein, phosphocellulose column fractionation resulted in a dramatic change in its ability to

TABLE II: Dependence of K_{app} on Treatment of Tubulin.[a]

	C_r (mg/mL)	K_{app} (L/mol)
A. W-Tubulin		
i. Before phosphocellulose ion-exchange chromatography	0.9	1.2×10^5
ii. After ion-exchange chromatography	0.9	1.2×10^5
iii. Addition of 8% by wt lysozyme	0.6	1.8×10^5
iv. Addition of 0.5% by wt polylysine	0.1	11.0×10^5
v. Addition of 4% by wt "τ" factor	0.2	5.5×10^5
B. C-Tubulin		
i. Before phosphocellulose ion-exchange chromatography	0.2	5.5×10^5
ii. After ion-exchange chromatography	0.8	1.4×10^5
iii. Addition of τ factor to ii	0.3	3.7×10^5

[a] Assembly buffer is 10^{-2} M sodium phosphate, 10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$, and 3.4 M glycerol at pH 7.0.

self-assemble. The phosphocellulose-purified cycle prepared tubulin (PC-tubulin) reassembled in buffer A into microtubules with a critical concentration of 0.8 mg/mL, which is identical within experimntal error with the value obtained for W-tubulin. Unfractionated c-tubulin has a C_r value of 0.2 mg/mL. Therefore, purification of c-tubulin by phosphocellulose ion-exchange chromatography leads to a decrease in microtubule propagation free energy of almost 1 kcal/mol, from −8.1 to −7.3 kcal/mol, i.e., to the value characteristic of dimeric W-tubulin.

The fraction retained on phosphocellulose when c-tubulin is used as starting material was eluted with 1 M NaCl in the buffer with which the column had been equilibrated (see Materials and Methods). This material, according to Weingarten et al. (1975), is the "τ factor" fraction. In our hands, this fraction gave turbidity when heated in the reconstitution buffer and was found to contain tubulin, an observation similar to that of Sloboda et al. (1976). Since it has been reported (Weingarten et al., 1975) that "τ factor" proteins are stable to boiling, this fraction was further purified by heating in boiling water for 15 min and removing precipitated denatured proteins by centrifugation. The heat-treated "τ factor" did not generate any turbidity when heated to 37 °C in a reconstitution buffer. Addition of the heat-treated material to either W-tubulin or PC-tubulin resulted in a decrease of the tubulin concentration at which turbidity occurred on heating to 37 °C in buffer A. This turbidity, however, was not fully reversible by cooling to 10 °C. On the other hand, if this material was dialyzed and the dialyzate was used in its place, no turbidity was generated in purified tubulin, indicating that the active material was macromolecular in nature.

A quantitative evaluation of the enhancement of tubulin self-assembly by the heat-treated material was obtained from critical concentration measurements. As is shown in Table II, the addition of 4% by weight of the heat-treated "τ factor" to W-tubulin lowered the critical concentration to 0.2 mg/mL, a value identical with that obtained with whole c-tubulin. Similar addition of the heat treated material to PC-tubulin restored its critical concentration to that of unfractionated c-tubulin. Therefore, both PC- and W-tubulins are affected equally by this material, suggesting again that they are identical in their ability to form microtubules. The difference between c-tubulin and W-tubulin, when reconstitution is used as the criterion, appears, therefore, to lie simply in the presence in the former of copurified nontubulin proteins, the so-called microtubule-associated proteins or "τ factor" proteins. These proteins are evidently capable of enhancing the self-association

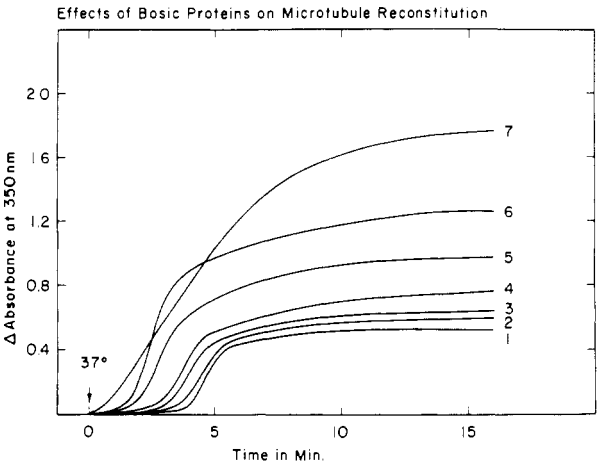

FIGURE 1: Turbidimetric measurements of W-tubulin in the presence of basic proteins and polycations. The conditions are: 10^{-2} M sodium phosphate, 10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$, and 3.4 M glycerol, pH 7.0 and 37 °C. Tubulin concentration in all cases is 1.94 mg/mL. The identity and final concentration of additive are: (1) none; (2) 0.16 mg/mL spermidine; (3) 0.27 mg/mL RNase A; (4) 0.02 mg/mL spermine; (5) 0.20 mg/mL histone f-1; (6) 0.14 mg/mL lysozyme; and (7) 0.13 mg/mL poly(L-lysine).

reaction whether tubulin is prepared by the modified complete Weisenberg procedure or by the Shelanski method of polymerization–depolymerization cycles followed by ion-exchange chromatography on a phosphocellulose column. Their presence, however, does not appear to be essential for in vitro microtubule growth. What is the role, then, of the tubulin copurified proteins?

Erickson & Voter (1976) have pointed out that the microtubule-associated proteins (MAP), or "τ factor" proteins which copurify with tubulin in the cycle preparations, behave as weak polycations on ion-exchange columns (Erickson & Voter, 1976). This is consistent with their interactions with a variety of polyanions (Bryan et al., 1975a,b) and suggests that they complex with tubulin oligomers by electrostatic forces. It seemed of interest, therefore, to test whether the effect of these proteins on the self-association of purified tubulin to microtubules is restricted exclusively to these proteins, as might be expected of highly specific essential factors, or if it could be a more general property of polycations. The enhancement of tubulin self-association to tubular structures by various polycations has been reported in the literature (Behnke, 1975; Jacobs et al., 1975; Erickson, 1976; Levi et al., 1975; Erickson & Voter, 1976). This effect, however, has never been tested with purified dimeric protein, W-tubulin; nor has it been examined quantitatively. Therefore, turbidimetric measurements were carried out in buffer A on W-tubulin to which various polycations (polylysine, spermine, spermidine) and basic proteins (lysozyme, ribonuclease A, and histone f-1) had been added. The results are presented in Figure 1. It is evident that all the cations increase the turbidity in the plateau region, with poly(L-lysine) being the most effective. The turbidity generated by heating could be reversed in all cases by cooling to 10 °C, although the reversibility was not complete in the presence of high concentrations of poly(L-lysine) and lysozyme.

The effect of polycation concentration on W-tubulin self-association was examined for lysozyme and poly(L-lysine). The results shown in Figures 2A and 2B indicate that the turbidity exceeding that of the control tubulin is essentially proportional to the amount of poly(lysine) or lysozyme added, in a manner similar to that found on addition of the microtubule associated proteins to tubulin purified by phosphocellulose chromatog-

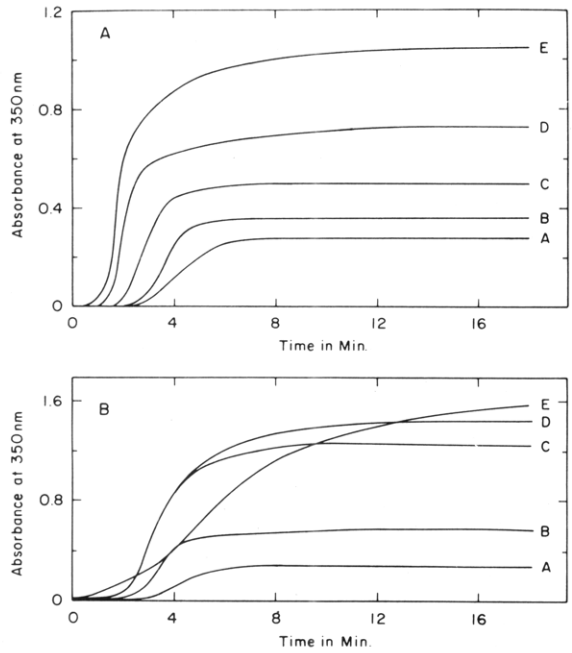

FIGURE 2: Turbidimetric measurements of W-tubulin at 37 °C in assembly buffer A as a function of additive concentrations. In all cases tubulin concentration is 1.5 mg/mL. (A) The additive is lysozyme and the final concentrations are: (A) none; (B) 28 μg/mL; (C) 56 μg/mL; (D) 84 μg/mL; and (E) 140 μg/mL. (B) The additive is poly(L-lysine) and the final concentrations are: (A) none; (B) 2.5 μg/mL; (C) 7.5 μg/mL; (D) 12.5 μg/mL; and (E) 25 μg/mL.

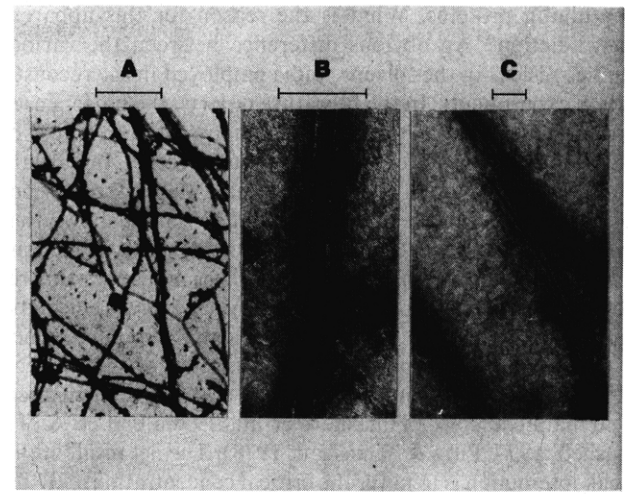

FIGURE 3: Electron micrographs of W-tubulin in assembly buffer A at 37 °C in the presence of (A) 0.5% poly-L-lysine at 15 333×; (B) 0.5% poly(L-lysine) at 103 333×; and (C) 8% lysozyme at 40 000×. The bar in A corresponds to 500 nm; in B and C, it corresponds to 100 nm.

raphy (Bryan et al., 1975a,b). This effect, which is the expected one when complexing is stoichiometric, is particularly evident in the case of lysozyme, while the poly(lysine) saturation may be approaching already at 12.5 μg/mg when the tubulin concentration is 1.5 mg/mL. Indeed, when poly(L-lysine) concentration is increased to 12.5 μg/mL, the shape of the turbidity increase curve changes, suggesting either a change in the mode of aggregation or the breakdown of conformity to the fundamental assumptions of the pertinent light-scattering theory (Berne, 1974); for example, passage to conditions at which the Rayleigh–Gans approximation is no longer valid or at which external interference due to strong repulsive forces and partial ordering in solution becomes significant (Fournet, 1951; Kirkwood & Mazur, 1952). For both polycationic additives, the turbidity was lowered when the temperature was decreased to 10 °C, but it did not return to its original value at high polycation concentrations. Above a given concentration of polycation, the magnitude of the residual turbidity was roughly proportional to the amount of macromolecular additive present in the system.

The aggregates, both at 37 and 10 °C, were examined by electron microscopy. The results at 37 °C in the presence of 0.5% (w/w) lysozyme are shown in Figure 3. The structures at 10 °C were essentially identical with those observed at 37 °C. At low magnification networks of filamentous structures are seen, as shown for poly(L-lysine) in Figure 3A. At higher magnification, the observed structures in the presence of poly(L-lysine) are similar to those normally identified as reconstituted microtubules (Kirschner & Williams, 1974; Erickson, 1974). Although there are indications of some irregularities, or decorations, on the surfaces of these tubules, in particular in the presence of lysozyme, more complicated structures, such as duplex microtubules (Jacobs et al., 1975) or double-walled microtubules (Erickson & Voter, 1976), were not observed.

The ability of these polycations to mimic microtubule copurified proteins in the reconstitutin reaction was tested quantitatively by examining the effects of lysozyme and poly(L-lysine) on the critical concentration and, therefore, the apparent microtubule growth constant, K_p^{app}, of W-tubulin, using macromolecular additive to tubulin ratios at which the turbidity is more than 95% reversible. The results, presented in Table II, show that both lysozyme and poly(L-lysine) can lower the critical concentration for the formation of microtubules from W-tubulin. In fact, poly(L-lysine) seems particularly effective, since its inclusion to the extent of 0.5% by weight was enough to lower the critical concentration to 0.1 mg/mL. This effectiveness of poly(L-lysine) in promoting microtubule formation cannot be due solely to the chemical nature of its monomeric residues, since in the presence of 10^{-2} M lysine, K_p^{app} has a value of 1.6×10^5 L/mol (Lee & Timasheff, 1977). Furthermore, 1,4-butanediamine at a level of 0.3 mg/mL does not enhance microtubule assembly, suggesting that polymeric cations are much more effective in enhancing microtubule formation than small basic molecules.

Discussion

The results reported in this paper clearly indicate that, when turbidimetric monitoring of microtubule assembly is used as a criterion, the difference between tubulins prepared by the modified Weisenberg procedure and the cycle method results from the presence in c-tubulin preparations of some nontubulin proteins which can be separated from tubulin by ion exchange chromatography. Under proper conditions, purified tubulin, whether prepared by the modified complete Weisenberg procedure, or isolated by phosphocellulose chromatography from cycle tubulin, can be reassembled into microtubules without the addition of any other proteins, with identical apparent standard free energies of polymer growth. Therefore, the basic information required for microtubule formation seems to be present in the tubulin molecule. Addition of protein copurified with tubulin in cycle preparations to 5.8 S tubulin, purified by either method, results in identical increases in the apparent standard free energy of polymer propagation and, essentially, causes both tubulins to reassemble in a manner similar to whole cycle tubulin. These observations are in contrast to reports in the literature (Kuriyama, 1975; Erickson, 1974; Kirschner & Williams, 1974) that purified dimeric tubulin does not reassemble into microtubules without the presence of the copurified

nontubulin proteins. What is the reason for this apparent contradiction? An obvious difference between the various studies resides in the solvent media employed in the reconstitution experiments. In the literature reports, the buffers used are either piperizine-*N*,*N*′-bis(2-ethanesulfonic acid) or morpholineethanesulfonic acid, without glycerol. In our laboratory, pH 7.0 sodium phosphate buffer is used in the presence of 3.4 M glycerol. Lee & Timasheff (1977) have shown that the identity of the buffer ions does not markedly affect the self-assembly reaction. Glycerol, however, enhances this reaction, most likely through a nonspecific general thermodynamic solvent effect on the chemical potential of tubulin, which is amenable to treatment by a combination of the Wyman (1964) linked function concepts and multicomponent thermodynamic theory (Timasheff et al., 1976a,b; Lee & Timasheff, 1977; Pittz & Timasheff, 1978). The net result of this weak interaction is to lower the critical concentration at 37 °C from ca. 10 mg/mL in dilute buffer to 1.0 mg/mL in 3.4 M glycerol (Lee & Timasheff, 1977), i.e., to make it amenable to easy measurement in the laboratory. Glycerol, however, need not enter into the actual tubulin–tubulin reaction and acts, most probably, through preferential exclusion from contact with the protein. Therefore, it is clear that the lack of formation of microtubules from purified tubulin reported from other laboratories simply reflects the fact that the total tubulin concentration used was below the required critical concentration for the given solvent system. In c-tubulin, the nontubulin proteins simply enhance the reaction to such an extent that a thermodynamic booster, such as glycerol, is no longer needed to lower the critical concentration to an experimentally easily manageable, or indeed detectable level. This enhancement, in fact, need not be very strong, since a change in ΔG°_{app} of only 1.4 kcal/mol is sufficient to change the critical concentration by a factor of ten. However, since c-tubulin stripped of the nontubulin proteins can still be reconstituted into microtubules, as shown by Himes et al. (1976), and Weber and co-workers (Wehland et al., 1977; Herzog & Weber, 1977), as well as in the present paper, it would seem unnecessary to assign to these proteins the role of factors "essential for the assembly of microtubule" (Weingarten et al., 1975), although some involvement in the in vivo microtubule system is certainly not excluded.

If these proteins are not factors essential for microtubule growth, are they tubule specific? It has been shown both in this paper and elsewhere (Levi et al., 1975; Erickson & Voter, 1976; Jacobs et al., 1975; Behnke, 1975; Murphy et al., 1977) that various basic proteins and polycations can promote the self-assembly process in a manner akin to that of the proteins copurified with tubulin. Tubulin is an acidic protein and the carboxyl termini of both chains are very rich in acidic side chains (Lu & Elzinga, 1976). The nontubulin proteins found in various cycle preparations of tubulin are either cationic or have regions rich in cationic character (Sloboda et al., 1976; Erickson & Voter, 1976). The possibility exists, therefore, that the copurification of these proteins with tubulin in a low ionic strength buffer reflects only trivial nonspecific electrostatic interactions between polyanionic (tubulin) and polycationic macromolecules present in the same tissue extract, under conditions at which Debye–Huckel screening of the charges by the ionic atmosphere is insufficient to reduce the electrostatic free energy of interaction (Timasheff, 1970). This could lead to the precipitation of a nonspecific complex coacervate (Veis, 1970). Great caution would seem in order, therefore, in assigning possible in vivo roles to the copurified nontubulin proteins on the basis of reconstitution and other solution experiments alone.

The mechanism through which the various proteins complexed with tubulin enhance the reconstitution process is not clear. As suggested above, a simple possibility could be electrostatic interactions with constellations of charged groups on the surface of formed tubulin aggregates, reducing the unfavorable electrostatic free energy of the assembled structure through the formation of additional intertubulin linkages in a macroligand-facilitated pathway. This would stabilize the structure against dissociation, for example, by colchicine (Haga & Kurokawa, 1975). Such interaction could be either specific or nonspecific, depending on whether specifically matching constellations of charges on the two macromolecules are required or not.

An argument advanced for specificity (Borisy et al., 1974, 1975) is based on the observations that the composition of the assembled material over a number of cycles of assembly–disassembly remains constant and that the interaction between tubulin and the microtubule-associated proteins appears to be stoichiometric (Borisy et al., 1974, 1975; Murphy & Borisy, 1975; Weingarten et al., 1975; Bryan et al., 1975a; Sloboda et al., 1976; Cleveland et al., 1977). Let us examine whether these criteria are sufficient to establish specificity of interaction.

Consider the following totally general model reaction. Suppose that the solution contains tubulin, T, which can associate into microtubules with an association constant K_2, for the addition of each subunit to the growing microtubule and a second protein, A, which can complex with assembled structures by some interaction which may be either specific or nonspecific and the nature of which need not be specified, one molecule of A adding for each n tubulin molecules incorporated into microtubules, with a binding constant, K_a. If M represents a growing microtubule at a given stage of elongation and $T^{(i)}$ are tubulin dimers consecutively adding to it, this model can be expressed through the set of reactions

$$\begin{aligned}
&M + T^{(1)} \rightleftharpoons MT^{(1)};\ K_2\\
&MT^{(1)} + T^{(2)} \rightleftharpoons MT^{(1)}T^{(2)};\ K_2\\
&\vdots \qquad\qquad (1)\\
&MT^{(1)} \ldots T^{(n-1)} + T^{(n)} \rightleftharpoons MT^{(1)} \ldots T^{(n)} = MT_n;\ K_2\\
&MT_n + A \rightleftharpoons MT_nA;\ K_a
\end{aligned}$$

For this scheme the apparent equilbrium constant for the addition of one tubulin dimer to a growing microtubule, $K_p^{app} \equiv C_r^{-1}$, determined from measurements of the critical concentration, will be (Lee & Timasheff, 1977)

$$K_r^{app} = K_2(1 + K_a[A])^{1/n} \qquad (2)$$

Equation 2 shows that binding of protein A to be assembled structure will give rise to an apparent increase in the microtubule growth equilibrium constant. This, in fact, is a necessary consequence of linked function theory (Wyman, 1964). Kinetically such a stabilizing effect should manifest itself mostly through a decrease in the rate of depolymerization, since the depolymerization process must be preceded by a dissociation of the macroligand from the microtubules. Let us make a sample calculation using the poly(L-lysine) data from Figure 2B. Saturation seems to occur at a mass ratio of tubulin to polylysine of close to 100 to 1. Since the molecular weights of tubulin dimer and this particular sample of poly(lysine) are similar, this corresponds to a molar ratio of ca. 100:1 at saturation, setting $n = 100$. Taking from Table II the experimentavalues of $K_2 = 1.2 \times 10^5$ and $K_p^{app} = 1.1 \times 10^6$ L/mol results in a value of K_a of about 10^{100}, which corre-

sponds to a standard free energy of binding of the polycation to a microtubule of ca. −140 kcal/mol of poly(L-lysine), or approximately −1.5 kcal/mol tubulin in the microtubule. The interaction with each tubulin dimer is weak. Yet a large stabilizing effect can be produced through the cooperation of the nonspecific weak interactions of a large number of charged groups of a polyelectrolyte with charges on the surface of the assembled structure, e.g., a microtubule, with an appropriate surface charge density. A net result of the strong total interaction is that all of the poly(lysine) will coprecipitate with microtubules each time that these are formed. On the other hand, the weak standard free energy of interaction between dissociated dimeric tubulin and the polycation could lead to the formation of only a negligibly small amount of complex when assembled structures are absent, and, in the dissociated (5.8 S) state, tubulin would not be liganded. Furthermore, monomeric L-lysine does not enhance the assembly reaction (Lee & Timasheff, 1977). This is due to the absence of cooperativity of interaction between the basic groups which is found in poly(L-lysine). The origin of the cooperativity in poly(L-lysine) resides in the covalent linkages between the lysine residues. These eliminate the loss of cratic entropy[2] each time that a lysyl group is bound to a microtubule, once the first contact is made, with a resulting gain in the standard free energy of binding per residue (Kauzmann, 1959; Aune et al., 1971; Frigon & Timasheff, 1975b; Timasheff et al., 1976a).

Similar calculations can show that, for an increase of K_p^{app} from 1.2×10^5 to 5.5×10^5 (the values for W- and c-tubulins), if $n = 5$ and for reasonable strengths of the interaction between tubulin and the additive, e.g., with $\Delta G°$ of binding = −3.5 kcal/mol of tubulin dimer within a microtubule, the amount of nontubulin polyelectrolyte remaining free in solution during each cycle of polymerization will be insignificant, not more than 10^{-9} M, or 0.1 μg/mL for a polymer with a molecular weight of 100 000, i.e., of similar size to that of the proteins copurified with tubulin in cycle preparations. As a consequence, the nontubulin protein will copurify with tubulin at a constant ratio through repeated cycles of assembly–disassembly, and the interaction will appear to be stoichiometric. Since this mechanism contains no assumptions about specificity, it is evident that the criteria advanced for specificity of interaction, while consistent with this concept, are not sufficient, and the conclusions concerning specificity reached on the basis of constancy of composition of assembled structures through repeated cycles, therefore, need not be valid.

The present study, combined with the previous reports (Lee & Timasheff, 1975, 1977), strongly indicates that the ability to form microtubules is an intrinsic property of tubulin, since purified tubulin, in the absence of microtubule-associated proteins, can self-assemble into microtubules with thermodynamics similar to those observed in vivo. A number of naturally coprecipitated proteins and of added polycations can enhance this reaction by complexing with the assembled structures, via interactions which may be totally nonspecific. In vitro, none of these are essential for microtubule growth, although their involvement in nucleation (where traces might be sufficient) cannot be excluded due to the limits of analytical procedures, such as sodium dodecyl sulfate gel electrophoresis. Whether they have any significance for the in vivo properties of microtubules is a question which cannot be answered by any of the reported observations based on reconstitution or other solution experiments. It seems premature, therefore, to equate these proteins with a factor which "operates in vivo as a significant regulatory element for microtubule assembly" (Weingarten et al., 1975).

It should be pointed out that some circumstantial evidence suggesting that involvement in the in vivo system of proteins copurified with tubulin has been presented. This consists of preliminary immunofluorescence experiments (Sherline & Schiavone, 1977; Connolly et al., 1977) and of reports that electron micrographs of natural microtubules and microtubules reconstituted in the presence of microtubule associated proteins exhibit similar decorations (Murphy & Borisy 1975; Sloboda et al., 1976; Amos, 1977). Yet the possible significance of these observations must await furhter investigation. Sloboda et al. (1976) have carefully analyzed the in vitro evidence; yet, quite perspicatiously, they caution against drawing conclusions at present on the significance of these proteins for the in vivo process of microtubule formation, a position which we fully support. It is also consistent with the recent report by Williams and co-workers (Berkowitz et al., 1977).

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