

MICROTUBULE PROTEIN PREPARATIONS FROM C₆ GLIAL CELLS AND THEIR SPONTANEOUS POLYMER FORMATION

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ABSTRACT

C₆ cell tubulin is indistinguishable from hog brain tubulin with respect to its molecular weight, amino acid composition, and colchicine-binding activity. Moreover, microtubule assembly systems from both sources form the same structures: rings, ribbons, tubules, and drug-induced polymers. There is, nevertheless, a difference between the cultured cell and brain systems which lies in the nature of their microtubule-associated accessory proteins. C₆ microtubule preparations exhibit few rings at 0°C, have low polymerization yield, and have a low content of accessory proteins. The addition of brain accessory proteins enhances the numbers of rings, and the yield of microtubules, to levels comparable with those of brain preparations. The polymerizing ability of C₆ microtubule protein decays much faster than that of brain, but it can be restored by the addition of brain accessory protein. The results suggest that C₆ accessory proteins are more labile than their brain counterparts.

KEY WORDS cultured cell tubulin ·
microtubule-associated accessory proteins ·
quantitative electron microscopy

Dynamic assembly and disassembly of cytoplasmic microtubules seem important to cellular functions such as cell division and the generation and maintenance of cell shape. Therefore, much recent research aims to understand the control of microtubule assembly and disassembly *in vivo*, at the molecular level. Studies of microtubule formation from component protein subunits were advanced by procedures for reversible *in vitro* assembly of mammalian brain microtubules (33). Recently, it was shown that spontaneous assembly reactions observed in brain homogenates and purified microtubule protein are not true tubulin self-assembly processes because, under the conditions ordi-

narily used, there is a requirement for certain copurifying microtubule-associated accessory proteins (16, 22, 27, 29, 31). Although this requirement is not absolute—it can be circumvented, at sufficiently high protein concentrations, by the presence of dimethylsulfoxide (15), Mg⁺⁺ (12, 14, 19), or various polycations (10)—microtubule-associated accessory proteins are attractive candidates for regulators of *in vivo* tubulin polymerization. If they play a role in regulation, they might well differ between cells in different physiological states.

Most studies of cytoplasmic microtubule assembly *in vitro* have used microtubule protein isolated from mammalian brain, because for some time such protein provided the only known system for reversible polymerization. However, for many purposes cultured cells would be a preferred

source of microtubule protein. Such systems would allow studies of tubulin of homogeneous cell types, and furthermore, in vitro experiments could be correlated with *in vivo* phenomena such as cell cycle events or cell morphology changes. For these reasons, we developed (35) a spontaneous microtubule assembly system from cultured rat glial cells (clone C₆). In this report, we further characterize the components of this assembly system.

MATERIALS AND METHODS

Cells

Rat glial cells, clone C₆, were grown in suspension (36), and mouse fibroblast cells (Balb/c-3T3) in roller bottles (37). Pelleted cells were resuspended in equal volumes of assembly buffer (100 mM sodium 2(*N*-morpholino)ethane sulfonate (MES), pH 6.4, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetate (EGTA), 0.5 mM MgCl₂, 1 mM GTP; [33]) and sonicated (35). The mouse fibroblast cells were grown for 3–4 d in medium containing 1 μCi/ml of a mixture of ³H-amino acids (New England Nuclear, Boston, Mass.) to label protein.

Microtubule Protein

Microtubule protein was purified by reversible polymerization-depolymerization cycles (28). Centrifugations were done in a Beckman 40 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) at 40,000 rpm for 1 h. Cell extracts were prepared from sonicated cell suspensions by centrifugation at 4°C (35). The extracts were diluted 1:1 with assembly buffer containing 8 M glycerol and incubated at 37°C for 30 min before centrifugation at 25°C. The pellets (referred to as pellet 1) contained 7–10% of the total protein originally in the extract; 20–25% of this precipitated protein was tubulin. The pellets were resuspended in assembly buffer (about one-tenth the initial extract volume), depolymerized on ice for 1 h, and centrifuged at 4°C, yielding supernates consisting of 70–80% pure tubulin. This one-cycle purified material was, for some experiments, repolymerized with glycerol, centrifuged, depolymerized, and centrifuged again, as above, resulting in two-cycle purified tubulin of ~90–95% purity. The yield after two cycles was ~1.5% of the total extract protein or 0.45 mg tubulin/g cells, wet weight. Cultured cell microtubule protein was always used immediately after preparation. Two-cycle purified hog brain microtubule protein was prepared in a similar fashion (28, 32), but was stored in 8 M glycerol at -20°C. It was purified further without further use of glycerol (17).

Microtubule-Associated Accessory Proteins

Accessory proteins were isolated from C₆ or hog brain

microtubule protein preparations by phosphocellulose chromatography (31). Pelleted microtubules were resuspended in chromatography buffer (25 mM MES, pH 6.4, 0.5 mM MgCl₂, 0.1 mM EDTA), chilled 1 h at 0°C, and centrifuged. The supernates containing 5–15 mg (in 0.7 ml) depolymerized microtubule protein, were loaded onto 2.5-ml phosphocellulose columns at 4°C, that had been equilibrated with chromatography buffer. The columns were washed with 15 ml of chromatography buffer, and 1-ml fractions were collected at a flow rate of 6 ml/h. The fractions containing tubulin were used immediately. The accessory proteins were eluted with chromatography buffer made 0.8 M in NaCl and desalting on 10-ml Biogel P-6 columns (Bio-Rad Laboratories, Richmond, Calif.) (equilibrated with 50 mM ammonium acetate, pH 6.4, 10 mM 2-mercaptoethanol). The protein was lyophilized and resuspended in small volumes of the same buffer. The resultant 5–10 mg/ml concentrated solutions of accessory proteins were stored frozen at -20°C.

Analytical Procedures

We measured colchicine binding by the method of Frigon and Lee (11), and protein by the method of Lowry et al. (21). Proteins separated by electrophoresis on polyacrylamide slabs using a discontinuous, sodium dodecyl sulfate (SDS)-containing system (18), were stained with Coomassie Brilliant Blue R-250, and quantitated by densitometry at 550 nm.

Electron Microscopy

Pellets of polymerized C₆ tubulin were resuspended in 1.0 mM EGTA, 0.5 mM MgCl₂, 1.0 mM GTP, buffered at pH 6.4 with either 50 mM ammonium acetate or 25 mM MES. Specimens were spray-deposited with 5% uranyl acetate stain onto carbon-coated collodion grids using a dual nebulizer (17). For quantitative work, tomato bushy stunt virus was added to samples before spraying (17).

RESULTS

A critical concentration (C_c) of cell extract protein was necessary for effective polymerization of tubulin (24). The data in Fig. 1 can be extrapolated to a C_c of ~2 mg/ml total protein. Because we estimate the tubulin concentrations of these extracts at ~9%, the C_c for tubulin is ~0.2 mg/ml. Protein A, which pelleted with polymerized tubulin, and coelectrophoresed with actin (35), showed a C_c of ~0.5 mg/ml extract. After the first polymerization, both the putative actin and tubulin depolymerized at 0°C and reformed polymers at 37°C. Because the C_c for actin was lower than that of tubulin, repeated cycles of polymerization-depolymerization at low protein concentration could enrich for this protein rather than tubulin.

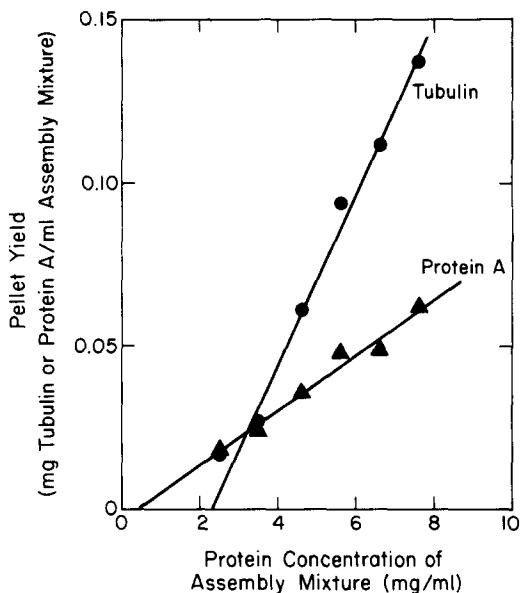


FIGURE 1 Effect of protein concentration on pellet yields of tubulin and protein A from cell extracts. To produce the dilutions shown, aliquots of cell extract were diluted to 9 ml by the addition of assembly buffer, and glycerol to 4 M. The mixtures were incubated at 37°C for 30 min and then centrifuged. Portions of the resuspended pellets were electrophoresed on 10% polyacrylamide SDS gels which were subsequently scanned densitometrically. The proportions of tubulin and protein A were determined by automatic integration.

For purified C₆ tubulin, data (Fig. 2) can be extrapolated to a C_c of 0.2–0.3 mg/ml. This figure is similar to that of Gaskin et al. (13) for hog brain tubulin, but the higher concentration of glycerol in the present experiments precludes exact comparison (6).

Although the tubulin that spontaneously polymerized from cell extracts represented ~2% of the extract total protein, the total amount of tubulin in the extracts was much larger, as estimated from extrapolation of the colchicine-binding assays (Fig. 3). The colchicine-binding decay rates of one-cycle (Fig. 3B) and two-cycle (Fig. 3A) purified C₆ microtubule protein were similar, and extrapolation of the curves to zero time showed 0.2 mol of colchicine bound per 110,000 mol wt tubulin dimer. If these apparent binding stoichiometries of purified tubulin, 2.1 nmol of colchicine-mg tubulin, can be applied to crude extract, then the zero-time binding of 0.21 nmol/mg crude protein (Fig. 3C) implies a tubulin concentration of ~10% in the cell extract. Although this concentration must be regarded as a crude approximation

because the true zero time for binding decay is some undetermined time before the start of the assay, and the calculations assume purity of the tubulin preparations, it does agree with a figure of 9% obtained by vinblastine precipitation and gel electrophoresis.

Comparison of C₆ Tubulin with Other Tubulins

The colchicine-binding decay rates of microtubule protein preparations of different purities were all similar to that of brain tubulin, within experimental error. Crude C₆ cell extracts had a decay half-life of 173 ± 21 min (SD); one-cycle purified protein, 230 ± 37 min; and two-cycle purified protein, 181 ± 78 min. Purified hog brain microtubule protein (three cycles) had a half-life of 293 ± 20 min, in agreement with published data (38). Like brain tubulin (38), C₆ tubulin decays slower at 0°C, with a half-life of 388 ± 44 min when assayed at 37°C after incubation on ice.

The functional similarity of C₆ and a heterologous tubulin was established by copolymerizing C₆ tubulin with tubulin from mouse fibroblast cells.

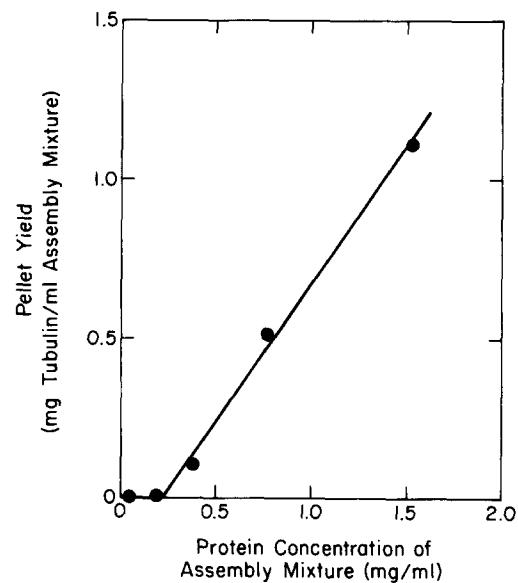


FIGURE 2 Effect of protein concentration on polymerization of one-cycle purified microtubule protein. Aliquots (0.8 ml) containing 2.44 mg of one-cycle purified C₆ microtubule protein were diluted to the concentrations shown by the addition of assembly buffer, and glycerol to 4 M. Tubulin polymerization and the analysis of pellets were performed as described in the legend of Fig. 1, except that gel scans were quantitatively evaluated by cutting out peaks and weighing.

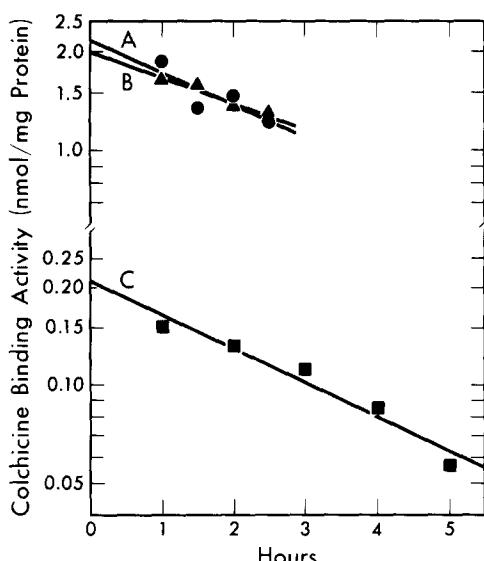


FIGURE 3 Decay of colchicine-binding activity. Cell extracts and depolymerized microtubule protein purified by one and two cycles of polymerization were prepared as described in Materials and Methods. Immediately after preparation, aliquots were taken from each fraction and preincubated at 37°C for various lengths of time. All colchicine-binding assays were run in triplicate; the data points shown represent the average values. The curves were constructed by linear least-squares fit to these values. The times shown include preincubation plus incubation with [³H]colchicine. The decay of two-cycle purified microtubule protein, at 0.044 mg/ml is shown in curve A (●), one-cycle purified microtubule protein at 0.057 mg/ml in curve B (▲), and crude cell extract at 1.51 mg/ml protein in curve C (■).

Two dilution series of microtubule assembly mixtures were prepared, one consisting of C₆ cell extract alone and the other of various combinations of C₆ and 3T3 cell extracts. Total protein concentrations were kept between 3 and 6 mg/ml. The amounts of tubulin polymerized after 30 min at 37°C, were determined by pelleting and gel electrophoresis. At equivalent total protein concentrations, the same amount of tubulin polymer was formed whether C₆ extract was used alone or in combination with 3T3 extract. That is, C₆ and 3T3 tubulins copolymerized additively. Moreover, when polymerization was performed with a mixture that contained radioactive 3T3 extract (0.7 mg/ml) below the critical concentration and unlabeled C₆ extract at a high concentration (4.5 mg/ml), the specific radioactivity was essentially the same after one cycle (77 cpm/μg) and two cycles (69 cpm/μg) of polymerization-depolymerization.

The specific radioactivities were also within 15% of each other after electrophoretic purification of the tubulin. This showed that tubulin molecules from both sources were incorporated into the same polymers.

The pathway of C₆ tubulin assembly was evaluated by electron microscopy (Figs. 4–6) and found to be qualitatively similar to that of brain tubulin assembly. Polymeric structures formed with hog brain tubulin under identical conditions resembled the ones in Figs. 4–6 in every respect, implying similar mechanisms of polymer formation for hog brain and C₆ cell tubulin. (For some of the quantitative differences, see the numbers in Tables I and II, and Discussion). In addition to the normal intermediates (9, 17, 26), polymers (Fig. 7) formed in the presence of 0.8 mM colchicine and 0.9 mM vinblastine were the same for C₆ and brain tubulin.

Accessory Proteins

A number of accessory proteins copurify with brain tubulin during repeated cycles in *in vitro* polymerization, and facilitate the *in vitro* formation of microtubules (16, 22, 27, 29, 31). C₆ microtubule protein preparations do not yield active accessory proteins when submitted to the phosphocellulose chromatography used to isolate them from hog brain preparations (31). In the chromatography of C₆ microtubule protein, the tubulin eluted entirely in the first peak (Fig. 9a). The second peak contained many proteins, but none comigrated in electrophoresis (Fig. 9b) with tubulin, nor did any bands correspond to the high molecular weight (~300,000 daltons) components of brain preparations (16, 22, 27, 29). A trace of a high molecular weight protein has been observed (35) in preparations of C₆ microtubule protein, but it does not match the high molecular weight proteins reported for brain, in amount, size, or multiplicity. Furthermore, it did not elute with either major fraction from phosphocellulose. A lack of high molecular weight components has been reported previously in other cell systems (7, 23). (In the material from peak II, there was a band of molecular weight slightly lower than tubulin, which might have been the 10-nm filament protein (39), or the 49,000-dalton component of Nagle et al. [23]).

Phosphocellulose-treated C₆ tubulin preparations did not form rings, ribbons, or microtubules when incubated at 37°C, alone or in the presence of 4 M glycerol, at maximum protein concentra-

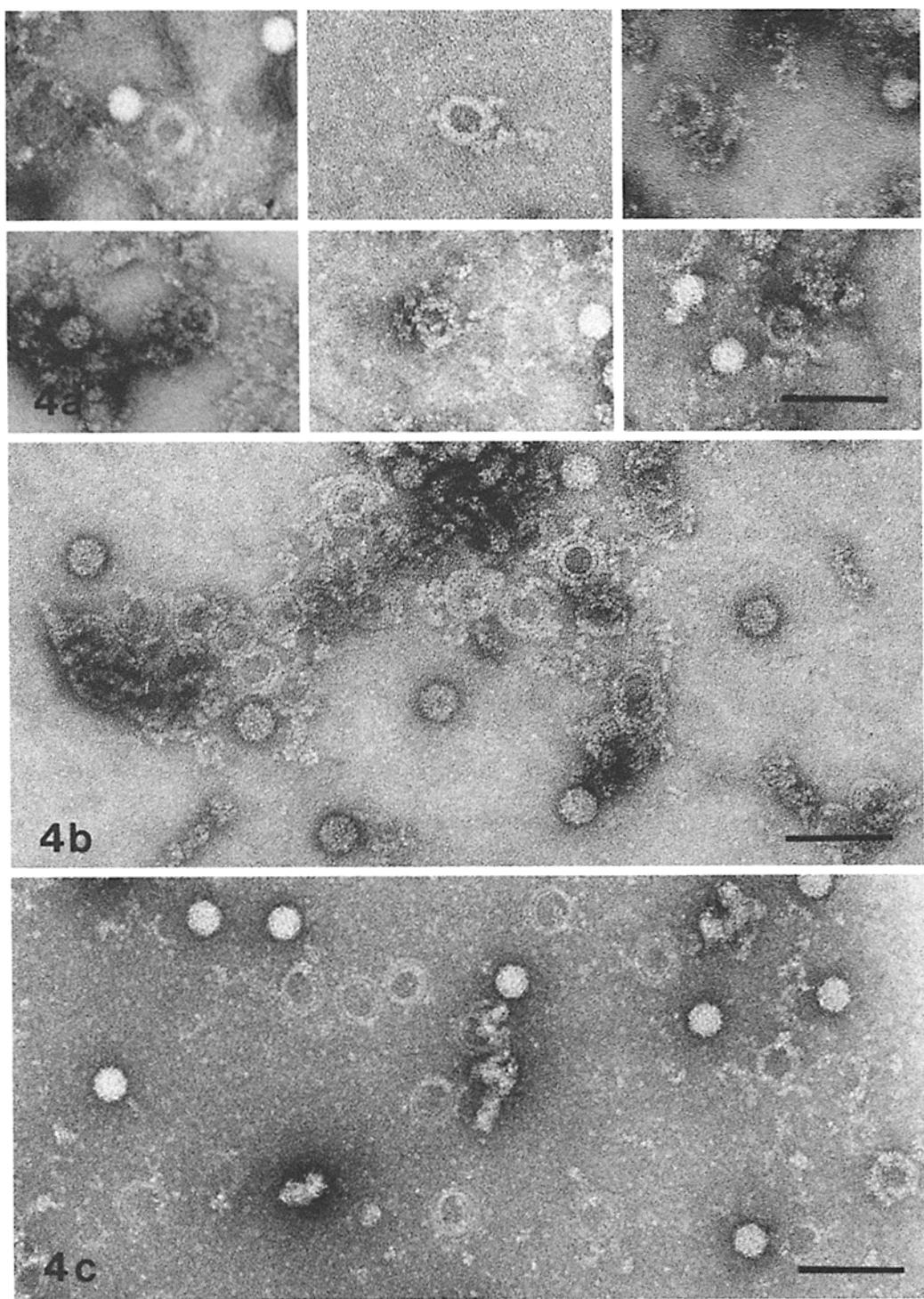


FIGURE 4 Rings in C₆ microtubule protein preparations at 0°C. (a) Freshly depolymerized one-cycle material, (b) with 1.8 mM CaCl₂ present, and (c) in the presence of added hog brain accessory proteins. Bars, 0.1 μm. × 158,000.

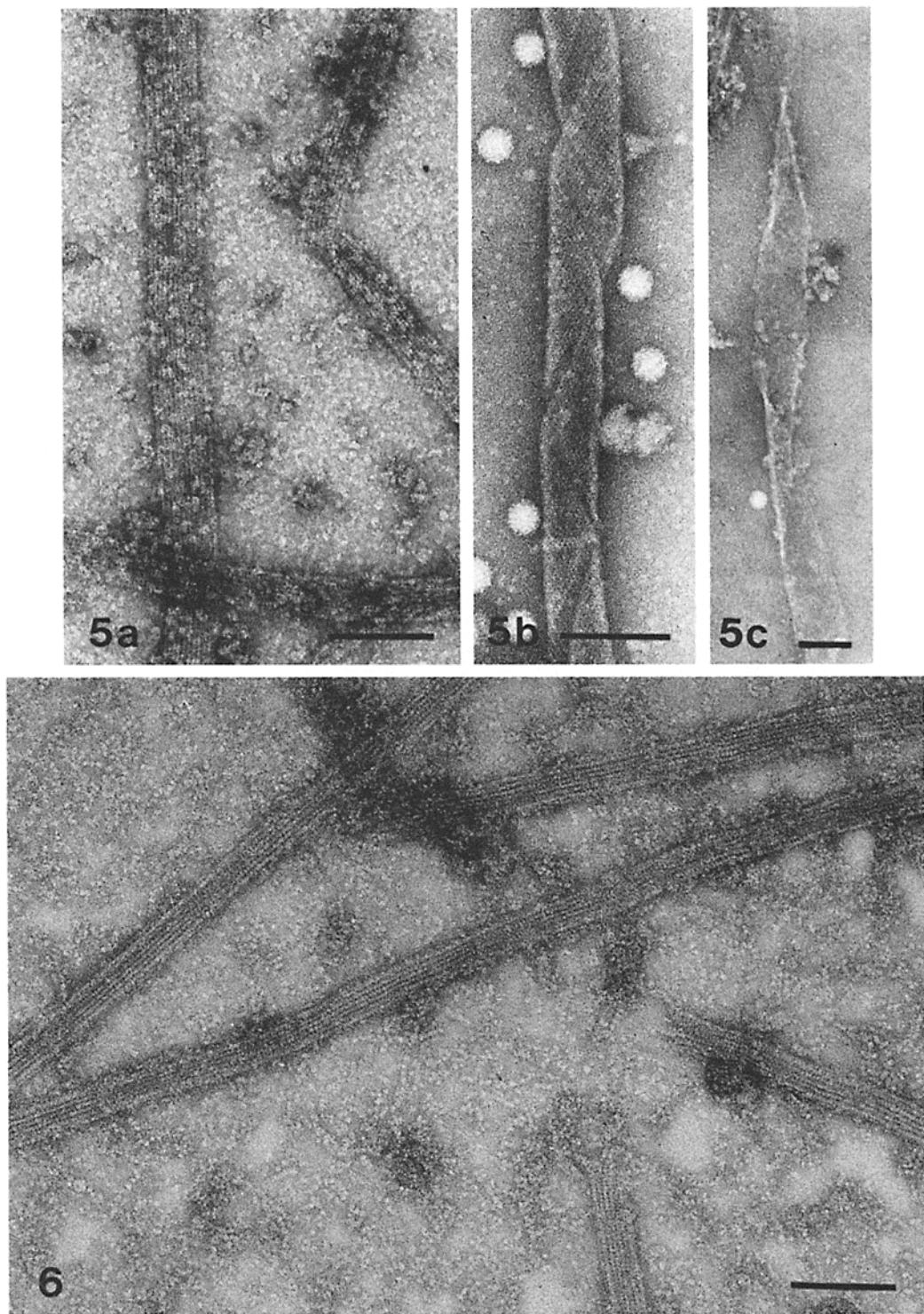


FIGURE 5 Planar and twisted ribbons formed during C_6 assembly. Found in one-cycle purified material polymerized ~3 min at 37°C. Bars, 0.1 μm . (a) $\times 157,000$, (b) $\times 161,000$, and (c) $\times 79,000$.

FIGURE 6 C_6 microtubules. Two-cycle purified material polymerized for 8 min at 37°C. Bar, 0.1 μm . $\times 157,000$.

TABLE I
Influence of Hog Brain Accessory Proteins on C₆ Microtubule Assembly

Exp	Microtubule protein concn	Brain accessory protein concn	% Microtubule protein as tubules
	mg/ml	mg/ml	
Two-cycle purified microtubule protein	3.63	0.00	1.6
	3.63	0.25	4.5
	2.27	0.64	25.8
One-cycle purified microtubule protein	1.00	0.00	0.0
	1.00	0.45	19.4
Phosphocellulose-purified tubulin	1.01	0.00	0.0
	1.02	1.24	20.6

C₆ microtubule protein was incubated for 10 min at 37°C with hog brain accessory proteins. The percentage of the microtubule protein present as tubules was determined by quantitative electron microscopy (17); the lower detection limit was 0.01%. Less than 10% of the polymers were ribbons in the above experiments, so, for simplicity, ribbons were scored as tubules. This could cause at most a 5% error, because the mass/length of ribbons is about half that of tubules. In exp 2, the one-cycle purified protein had aged 3 h.

TABLE II
Influence of Hog Brain Accessory Proteins on C₆ Ring Formation

Exp	Microtubule pro-tein concn	Brain accessory protein concn	% Microtubule protein as rings
	mg/ml	mg/ml	
A	2.48	0.00	0.13
	2.48	0.69	7.80
B	6.34	0.11	1.43
	6.23	0.21	3.15
C	5.93	0.51	6.90
	5.47	1.01	12.85

One-cycle purified C₆ microtubule protein was incubated for 5 min at 4°C with hog brain accessory proteins. The percentage of the microtubule protein present as rings was determined by quantitative electron microscopy (17).

tions of 3.0 and 1.3 mg/ml, respectively. The readdition of peak II material repeatedly failed to induce microtubule assembly in these phosphocellulose-treated preparations. The presence of protease inhibitors, soy bean trypsin inhibitor (250 µg/ml) or phenylmethylsulfonyl fluoride (50 µg/ml), from cell disruption through the entire isolations

tion of peak II material did not change the results.

Phosphocellulose-treated C₆ tubulin did form microtubules when peak II material from hog brain was added to it. The hog brain accessory proteins increased polymer formation of ordinary C₆ microtubule protein preparations (Table I, exp 1) and restored the polymerizing ability of phosphocellulose-treated C₆ tubulin as well (Table I, exp 3). Polymer formation from aged C₆ microtubule protein was observed, when supplemented with brain accessory proteins even when the polymerization of the C₆ preparation by itself had decayed to an undetectable low level (Table I, exp 2 and reference 36). As the level of brain accessory protein was raised, there was an increase in the number of rings observed at 4°C (Table II and Fig. 4c) that appeared to correspond to an increase in microtubule assembly at 37°C (Table I). The effectiveness of brain accessory proteins in promoting polymerization was equal for phosphocellulose-treated tubulin from hog brain and C₆ cells.

Addition of hog brain microtubule-associated accessory proteins to C₆ microtubule protein preparations offset the decay of the latter's polymerizing ability. The polymerizability of one-cycle purified C₆ tubulin mixed with brain accessory proteins (Fig. 10) decayed at 0°C, with a half-life of 880 ± 490 min, in contrast to a half-life of only 65 ± 15 min in the absence of the brain proteins.

The form of tubulin polymer shown in Fig. 8 has not been described before, as far as we know. This polymer, consisting of two or more tubules laterally aggregated, was observed only in experiments in which accessory proteins were in high ratio to microtubule protein. For example, in exp 2 of Table I, at an especially high weight ratio, 1.8:1 (not shown) of accessory protein to microtubule protein, 70% of the polymeric mass consisted of doublet or multiplet tubules.

DISCUSSION

The experiments described show that C₆ tubulin is chemically and functionally similar to other tubulins. It has the same apparent molecular weight and amino acid composition (35) as other tubulins (5, 8, 25, 34), and copolymerizes with heterologous tubulin. The colchicine-binding capacity and its decay rate were about the same for C₆ tubulin as for hog brain tubulin, purified by the same procedures. The extrapolated binding capacity of 0.2 mol of colchicine per mol dimer is reasonably close to those of 0.5–1.0 previously reported for

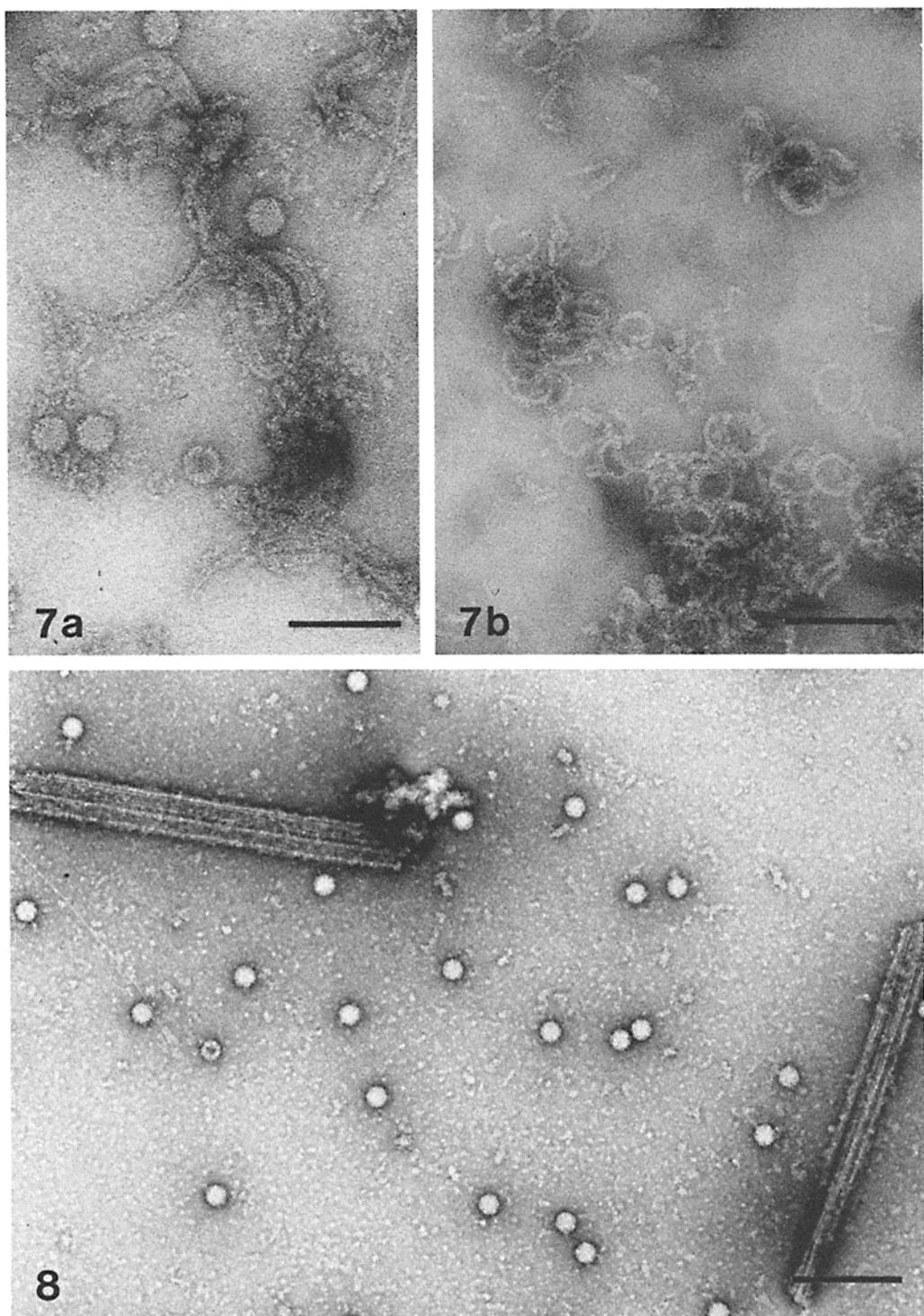


FIGURE 7 Effects of anti-microtubular drugs. (a) Multiply-whorled and partial "snake" structures observed upon incubation for 8 min at 37°C with 0.8 mM colchicine. (b) Coiled, single and double ring forms observed in the presence of 0.9 mM vinblastine after 20 min at 0°C followed by 5 min at 37°C. Bars, 0.1 μ m. $\times 164,000$.

FIGURE 8 Laterally aggregated tubules. C₆ microtubule protein at 1.0 mg/ml with added brain accessory proteins at 1.8 mg/ml, was incubated 10 min at 37°C. Bar, 0.1 μ m. $\times 161,000$.

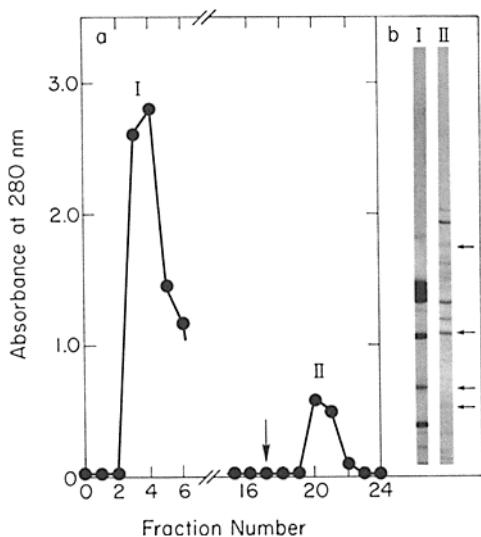


FIGURE 9 Fractionation of microtubule proteins. One-cycle purified microtubule protein was fractionated by phosphocellulose chromatography (31). Portions of the highest peak I fraction and the pooled peak II fractions were then analyzed on an 8% polyacrylamide SDS slab gel. (a) Column elution profile, absorbance at 280 nm. Chromatography buffer containing 0.8 M NaCl was applied at fraction 17 (arrow). (b) Electrophoresis of peak I (35 µg) and peak II (15 µg) fractions. The arrows at the right-hand side of the panel designate the position of protein markers, run in parallel, of the following molecular weights (from top to bottom): 67,000, 43,000, 36,000, and 33,000 daltons.

brain tubulin (11, 34, 38) in light of the factors complicating interpretation of this number as discussed above.

The assembly process of C₆ tubulin is like that of hog brain tubulin with respect to the forms of intermediate and secondary structures. Although infrequent, rings were observed in depolymerized C₆ preparations. Our results with cultured cell tubulin, like previous reports on brain tubulin, are consistent with the notion that rings are intermediates in assembly, but do not allow us to exclude other possibilities (3, 7, 20). Polymerizable C₆ tubulin preparations always had rings, and the presence of higher numbers of rings at 4°C always correlated with greater ability to polymerize at 37°C. Furthermore, because very few rings might be required for assembly, a paucity of rings does not prove that they are unnecessary. If an opened-ring segment of 58 tubulin monomers was sufficient to nucleate an average tubule of 2 µm (6,000 monomers), then polymerization of the observed ~5% of the tubulin would only require ~0.05% of the proteins to be rings.

The C₆ and hog brain systems differed in that only the former showed lateral aggregates of microtubules at high concentrations of added accessory proteins. The novel aggregation might be due to cross-bridging by accessory proteins extending (1, 22) from the microtubular surface, or it may be due to cationic cross-linking of anionic microtubules (e.g., as perhaps occurs in vinblastine paracrystals, although these do not, in general, have the microtubular structure, but see reference 30). Despite this difference in their behavior, it cannot be concluded reliably that C₆ and hog brain tubulins themselves differ, as long as the absolute purity of their preparations might be questioned. The similarity of all their other polymeric forms—rings, ribbons, and even colchicine “snakes” and vinblastine coils—argues against significant differences between the two kinds of tubulin.

Despite similarities in their tubulins, there are distinct differences in the behavior of the assembly systems from cultured glial C₆ cell and hog brain microtubules. First, C₆ tubulin polymerized with a low yield. Both colchicine-binding and vinblastine precipitation data indicated that only a fraction of the tubulin present in the extracts underwent a

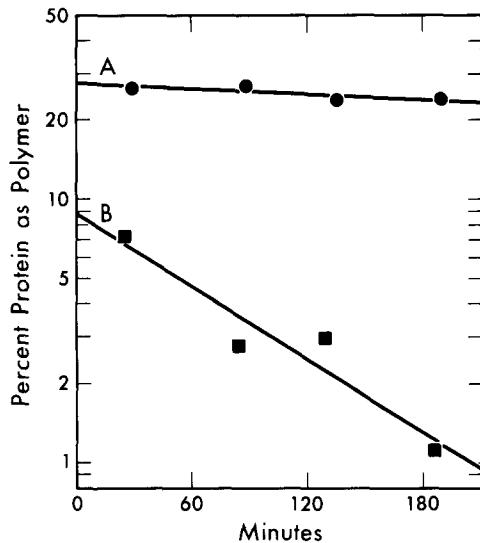


FIGURE 10 Effect of hog brain accessory proteins on the decay of C₆ microtubule protein polymerizing ability. One-cycle purified C₆ microtubule protein was diluted to 6.3 mg/ml by the addition of hog brain accessory proteins at 1.2 mg/ml (curve A, ●) or buffer (curve B, ■) and incubated at 0°C. At the times indicated, aliquots were incubated for 10 min at 37°C and the amount of polymer was determined by quantitative electron microscopy.

cycle of spontaneous reversible polymerization in the presence of glycerol, and even lower yields were obtained with purified microtubule protein preparations which contained only a low, residual concentration of glycerol. In these latter preparations, often only 5–10% of the protein would polymerize compared to yields near 50% from hog brain preparations. Second, C₆ rings were scarce, usually amounting to only 0.1–0.2% of the cycled protein compared to 20–50% observed in depolymerized brain preparations. Third, preparations of C₆ cell tubulin always contained fewer electrophoretically distinct components than did comparably isolated hog brain tubulin, especially in the high molecular weight and 55,000- to 75,000-dalton regions. These observations would be easily explained if C₆ microtubule preparations were deficient in microtubule-associated accessory proteins. The deficiency could be due either to a low endogenous concentration of accessory protein factors in C₆ cells or to a rapid decay of the accessory proteins which prevents their effective co-isolation with tubulin polymers.

The lability of the accessory proteins of C₆ preparations is supported by our recent report (36) that the microtubule-polymerizing ability decays much more rapidly than the colchicine-binding activity. Their instability during desalting and concentrating operations could explain our failure to isolate functional C₆ accessory proteins which could induce microtubule formation by phosphocellulose-purified tubulin. The inactivation of the accessory proteins does not appear to be simply the result of proteolysis. Even if proteases or other inhibitory catalytic factors were active at 0°C, and were responsible for the accessory protein decay, they would have to be specific for the glial factors since C₆ tubulin polymerizability decayed extremely slowly in the presence of hog brain accessory proteins (Fig. 10). Moreover, two protease inhibitors had no effect on our ability to isolate functional accessory proteins, nor was any proteolytic loss of minor components or appearance of new low molecular weight bands detected in electrophoresis of C₆ preparations left on ice for as long as 48 h. If the decay or deficiency of C₆ microtubule-associated accessory proteins is not due to proteases or other external agents, then the accessory proteins from the two sources must differ qualitatively, although accessory proteins from brain microtubules may substitute for C₆ factors.

Despite recent demonstrations that the require-

ment for accessory proteins may be circumvented, there is significant support for the view that accessory proteins are important to microtubule formation in vivo, including observations that a flagellar protein is capable of acting as a promoting factor (2), and that the immunolocalization pattern in mouse fibroblasts treated with antibody to accessory protein coincides with that of antitubulin and is colcemid-sensitive (4). We think that the fact that C₆ accessory proteins differ from hog brain factors, even though the latter heterologous factors will function in the C₆ system, gives further support to the notion that accessory proteins are important in vivo because this observation accords so well with biological expectations. The lability and possible low levels of accessory proteins would seem very appropriate for cells in which the tubulin/microtubule system is a dynamic one, while it is equally plausible that the more or less perpetually polymerized microtubules of the brain would use higher concentrations of comparatively stable accessory proteins.

Nagle et al. (23) have carried out experiments that relate to the present report. They showed that tubulin from another cultured cell, neuroblastoma, will also copolymerize with hog brain tubulin and that hog brain accessory proteins facilitate polymerization of neuroblastoma tubulin. Nagle et al. failed to detect rings in the neuroblastoma system, whereas rings were present in our C₆ microtubule preparations.

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