Polymorphism of Tubulin Oligomers in the Presence of Microtubule-Associated Proteins. Implications in Microtubule Assembly†

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ABSTRACT: The interaction between tubulin and microtubule-associated proteins (MAPs) in solutions of cycled microtubule protein has been studied by using radioactively labeled MAPs. Kinetic data of MAP association to microtubules in the polymerization process indicate that an oligomer P of tubulin and MAPs is the polymerizing species. Analysis of MAP binding to microtubules formed from solutions in which the ratio MAPs/tubulin was varied shows evidence for a polymorphism of tubulin–MAP oligomers. When the ratio MAPs/tubulin is decreased by addition of dimeric tubulin to 3 times cycled microtubule protein, an oligomer P′ less rich in MAPs than P and unable to incorporate in microtubules is formed. The data further show that while τ, MAP_1, and MAP_2 can bind to oligomer P, only MAP_1 and MAP_2 can bind to oligomer P′. Therefore, the interactions of τ factor and of MAP_1 and MAP_2 with tubulin follow different patterns.

Microtubule protein isolated from pig brain by conventional methods of polymerization–depolymerization cycles (Shelanski et al., 1973; Weingarten et al., 1974; Borisy et al., 1975) is known to consist mainly of tubulin and of roughly 15% accessory proteins, called microtubule-associated proteins (MAPs), which bind tightly to polymerized tubulin and copurify with it. These MAPs are distributed in two classes of proteins unrelated to each other: high molecular weight MAP_1 and MAP_2 (M_r 300K and 280K, respectively) and the τ factor proteins (M_r 65K–55K). Both types are present in all microtubule protein preparations, in relative amounts differing according to the purification method used (Scheele & Borisy, 1976). Both MAP_1 and MAP_2 (Murphy & Borisy, 1975; Slobada et al., 1976) and τ (Weingarten et al., 1975) can promote microtubule assembly in vitro, and their role in microtubule nucleation and elongation has been demonstrated (Witman et al., 1976; Murphy et al., 1977). At low temperature, MAPs appear essentially bound to ring-shaped tubulin oligomers (Erickson, 1974; Murphy & Borisy, 1975). These rings, however, do not appear to be direct intermediates in microtubule assembly: Time-resolved synchrotron X-ray scattering studies of microtubule protein solutions undergoing assembly (Mandelkow et al., 1980; Bordas et al., 1983) revealed that rings broke down into fragments and eventually tubulin dimers before microtubule formation; however, the association of such fragments was involved in nucleation. The same conclusions were derived independently from biochemical studies (Pantaloni et al., 1981). However, since rings disappear during the polymerization process and only dimeric tubulin is found in equilibrium with microtubules (Johnson & Borisy, 1977), the kinetic mechanism through which microtubules with bound MAPs grow from a whole microtubule protein solution has not been elucidated yet.

At steady state, the equilibrium binding of MAPs to microtubules addresses the general problem of binding of large ligands to a homogeneous lattice of potential binding sites, as is the case for proteins binding to DNA (McGhee & von Hippel, 1974) or tropomyosin binding to actin (Wegner, 1979). The theoretical treatment of microtubule stabilization by MAP binding within such a model has been developed (Hill & Kirschner, 1983). Indeed our previous work (Pantaloni et al., 1981; Carlier & Pantaloni, 1982) showed that the relative amount of MAPs bound to microtubules per polymerized tubulin could be varied within a large range with varying experimental conditions and that the resulting stability of microtubules was correlated to the ratio MAPs/tubulin in the microtubule. However, no further experimental analysis of the equilibrium between MAPs and microtubules and of its implication in the mechanism of microtubule assembly has been reported. Indeed, such an approach is made difficult by the fact that in experiments of microtubule reconstitution from the separated components tubulin and MAPs, a low yield has generally been observed by several groups, showing that at least one of the components in the reconstituted system was not in its original state. Further studies showed evidence for the presence of several enzymes in microtubule protein preparations such as protein kinases (Sloboda et al., 1975; Vallee, 1980; Burns & Islam, 1981) and phosphatases (Jameson et al., 1980; Sheterline, 1977). Evidence has been shown for the involvement of the phosphorylated states of MAPs in the regulation of microtubule assembly in vitro (Jameson & Caplow, 1981) and in vivo (Pallas & Solomon, 1982; Bershadsky & Gelfand, 1981). Reconstitution experiments of purified MAPs and tubulin may fail to reproduce the original functional status of whole microtubule protein obtained through cycles of polymerization.

In order to clarify the interaction between tubulin and MAPs in microtubule assembly, it was thought more appropriate, in a first step, to trace MAPs present in a cycled microtubule protein preparation with radioactively labeled MAPs able to coassemble with microtubules. The results presented here show that this technique proved valuable in studying the kinetics of MAP binding to microtubules during the polymerization process and permitted an approach to the variety of possible interactions between tubulin and the different MAP species.

Materials and Methods

Chemicals. 2-(*N*-Morpholino)ethanesulfonic acid (MES) was from Calbiochem. Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), acetyl phosphate, and acetate kinase were from Sigma. Guanosine 5′-triphosphate was purchased from Boehringer. Sodium dodecyl sulfate (SDS) and other gel electrophoresis reagents were from

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Eastman Kodak Co. All other chemicals were analytical grade.

Iodination experiments were carried out by using either the iodination kit of New England Nuclear consisting of immobilized lactoperoxidase and glucose oxidase, working with carrier-free $Na^{125}I$, or, alternatively, monoiodinated ^{125}I-labeled Bolton and Hunter reagent (2000 Ci/mmol, New England Nuclear).

Taxol was purified from *Taxus baccata* at the Institut de Chimie des Substances Naturelles and was a kind gift from Drs. P. Potier and D. Guénard.

Tubulin Purification. Whole microtubule protein was prepared through two cycles of polymerization–depolymerization according to Shelanski et al. (1973) and stored at −20 °C in 8 M glycerol buffer. Before each experiment, a third cycle of polymerization was performed in the presence of 4 M glycerol. The microtubule pellet was resuspended in 0.1 M MES buffer, pH 6.8, containing 1 mM EGTA and 0.5 mM $Mg(CH_3CO_2)_2$. The solution was then chromatographied through Sephadex G-25 (PD10 prepacked columns from Pharmacia) to eliminate glycerol and free nucleotides and clarified by centrifugation at 50000*g* at 4 °C for 30 min. Pure tubulin dimer was obtained by phosphocellulose chromatography of 3 times cycled microtubule protein according to Weingarten et al. (1975). The column was run with 25 mM MES buffer, pH 6.8. After concentration by ultrafiltration in an Amicon cell equipped with a Diaflo PM30 membrane (Pharmacia), the solution was adjusted to 0.05 M MES buffer, 0.5 mM EGTA, 0.25 mM $Mg(CH_2CO_2)_2$, and 4 M glycerol and stored at −80 °C. Before use, dimeric tubulin was chromatographed through a Sephadex G-25 column under the same conditions as microtubule protein. Tubulin concentration was then determined spectrophotometrically by using an extinction coefficient $\epsilon_{0.1\%}^{278nm} = 1.2$ mg^{-1} cm^2 (Detrich & Williams, 1978).

Radioactive Labeling of MAPs. MAPs were prepared from 3 times cycled microtubule protein according to Vallee (1982). Microtubule protein (2–3 mg/mL) was polymerized at 37 °C for 10 min, and then 100–120 μM Taxol was added. After 5 min, 0.23 M NaCl was added, and the solution was centifuged at 100000*g* for 20 min at room temperature. The supernatant containing the MAPs was dialyzed against 0.1 M MES buffer, pH 7.2, and concentrated about 5-fold in the dialysis bag against dry Sephadex G-200 for 2 h in the cold room before labeling.

Essentially two procedures were used for ^{125}I-labeling: tyrosine iodination by lactoperoxidase and lysine reaction with iodinated Bolton and Hunter reagent. It was checked that the same results were obtained with both methods. However, the efficiency of labeling was at least 1 order of magnitude higher with Bolton and Hunter reagent with which 10–30% incorporation of radiolabel was obtained. For this reason, this reagent was found more convenient.

In both cases an aliquot (25 μL) of the concentrated MAP fraction was reacted either with $Na^{125}I$ onto the immobilized lactoperoxidase–glucose oxidase enzyme system supplemented with glucose or with the Bolton and Hunter reagent following evaporation of the benzene solvent. The reaction was carried out at 0 °C for 1 h and stopped by addition of an excess of sodium bisulfide (for the lactoperoxidase method) or glycine (for the Bolton–Hunter method). The material was then chromatographed on Sephadex G-25 fine (0.5 × 10 cm) equilibrated in 0.1 M MES buffer, pH 6.8. The yield of ^{125}I-label incorporation in the MAPs was calculated from the elution profile of the column. The eluted labeled MAPs fraction was supplemented with 60% glycerol and stored at −80 °C.

In order to radioactively trace the MAPs in a given microtubule protein solution, an aliquot of the [^{125}I]-MAP stock solution was added to the solution of microtubule protein (two cycles of polymerization). A third cycle of polymerization was then performed as usual. This procedure permitted to separate, from the bulk of radioactively labeled MAPs, only the labeled MAPs maintained in their "native" state, i.e., able to bind to assembled microtubules in the same way as unlabeled MAPs. Routinely 50–60% of the initial radioactivity was found associated to the microtubule protein fraction after the third cycle of polymerization.

Polymerization Studies. Microtubule assembly was followed at 37 or 25 °C both by a turbidimetric method at 350 nm, as previously described (Carlier & Pantaloni, 1982), and by sedimentation at 160000*g* for 3.5 min in the airfuge. Polymerization buffer consisted of 0.1 M MES buffer, pH 6.8, containing 0.1 mM GTP, 1 mM EGTA, 2 mM magnesium acetate, 20 mM acetyl phosphate, and 0.5 unit/mL acetate kinase as a GTP-regenerating system.

Protein concentration was measured in the supernatant and initial solution by the Lowry method (Lowry et al., 1951). The amount of MAPs present in the same solutions was measured by counting an aliquot (25 μL) in Aquasol on a Packard Model 3310 liquid scintillation spectrometer.

Gel Electrophoresis and Autoradiography. Slab polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laëmmli (1970) except the running gel was a gradient from 11% to 4% acrylamide [the ratio bis(acrylamide)/acrylamide was 1/37.5]. Protein samples were denatured by boiling for 3 min in the presence of 2% SDS and 0.1% 2-mercaptoethanol. Electrophoresis was carried out either at room temperature overnight (60 V) or for 3 h in the cold room at a constant power of 7 W.

Gels were fixed in 10% acetic acid/5% trichloroacetic acid for 10 min, stained with 0.01% Coomassie blue in methanol/acetic acid/water/glycerol (43/10/43/4), and destained in the same solvent. The gels were dried over a gel backing paper (Bio-Rad) under vacuum and with overheating. Autoradiography of the dried gel when necessary was done with a X-Omat S X-ray film (Kodak) placed between two intensifying screens (Du Pont Cronex) inside an autoradiography cassette. The cassette was wrapped in an aluminum sheet and kept at −80 °C until revelation.

Electron Microscopy. Samples were negatively stained with 2% uranyl acetate following rapid fixation with 1% glutaraldehyde. Observation was done with an Hitachi HU IIB electron microscope.

Analytical Ultracentrifugation. The composition of microtubule protein solutions was analyzed by velocity sedimentation in a Spinco Model E analytical ultracentrifuge. Samples were run at 10 °C and 56000 rpm. Schlieren patterns were enlarged, and their areas, proportional to the protein concentration after correction for radial dilution, were measured by using a planimeter.

Results

Radiolabeling of Microtubule-Associated Proteins. Figure 1 shows that MAPs prepared according to Vallee (1982) as described under Materials and Methods consisted in high molecular weight MAP_1 and MAP_2 polypeptides accompanied by some proteolysis products (~200K; Sloboda et al., 1976), The four bands of τ protein, and a few lower molecular weight proteins which have been shown to be the assembly promoting 35K fragment of MAP_2 (Vallee, 1980) and the light chains

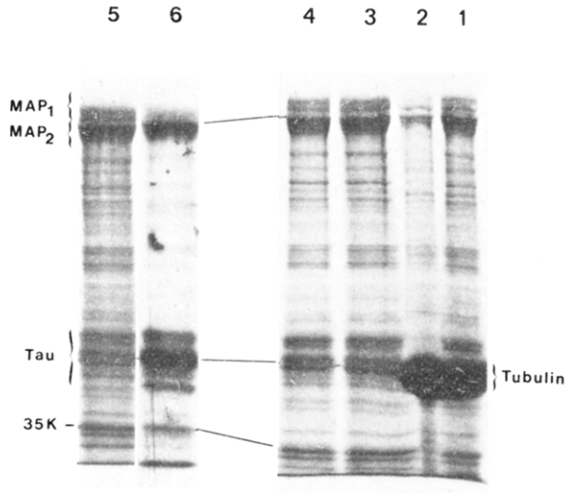


FIGURE 1: Preparation and radiolabeling of MAPs from whole microtubule protein. SDS gel electrophoresis of the different fractions in the preparation of MAPs: (1) Whole microtubule protein obtained through three cycles of polymerization. (2) Pellet of microtubules obtained by polymerizing fraction 1 in the presence of 100 μM Taxol and 0.23 M NaCl. A large depletion in MAPs is observed. (3) Supernatant of microtubules in fraction 2, containing MAPs. (4) Fraction 3 following chromatography through DEAE-Sephadex equilibrated in 0.1 M MES buffer containing 0.35 M NaCl. (5) MAPs obtained after labeling by Bolton and Hunter reagent of the same fraction as 3, but in a separate experiment. (6) Autoradiography of fraction 5.

of MAP_1 protein (Vallee & Davis, 1983).

This pattern did not change upon chromatographing the material over a small DEAE-Sephadex column in MES buffer containing 0.3 M NaCl, conditions under which tubulin remains bound to the resin. This ensured that no tubulin was present in this material. After labeling, all the bands stained by Coomassie blue on the gel appeared radioactively labeled, from the autoradiogram (Figure 1). Again it was checked that no labeled tubulin was present in the following way: radioactively labeled MAPs were copolymerized with 2 times cycled microtubule protein, microtubules were resuspended in buffer, and the solution was clarified by centrifugation at 4 °C and chromatographed on an analytical phosphocellulose column (0.5 × 10 cm). Autoradiography of the gels of the eluted fractions revealed (data not shown) that no radioactive material was associated to the tubulin fraction, and it was all eluted with the MAPs in the 0.8 M NaCl wash of the column.

Time Course of the Binding of Labeled MAPs to Microtubules in the Microtubule Assembly Process. Microtubule protein was obtained through a third cycle of polymerization carried out in the presence of radioactively labeled MAPs.

Polymerization was followed by a turbidimetric method and by sedimentation at short intervals of time. The amount of MAPs bound to microtubules at each time was derived from the measurements of radioactivity present in the bulk solution before polymerization and in the supernatant of sedimented microtubules. Polymerization was performed at 25 and 37 °C. Figure 2 shows that at both temperatures, the evolution with time of the amounts of sedimented protein and labeled MAPs followed roughly the same process. However, at 25 °C we repeatedly observed that the material sedimented at the early times ($t < 1$ min) has a slightly higher ratio MAPs/tubulin than later on in the polymerization process. Such an observation was not made at 37 °C because polymerization was faster at this temperature. It should be noticed that the curves of sedimented polymer (160000*g* for 3 min) and recorded turbidity could be superimposed only in the last 70% of the polymerization process. At the early times of

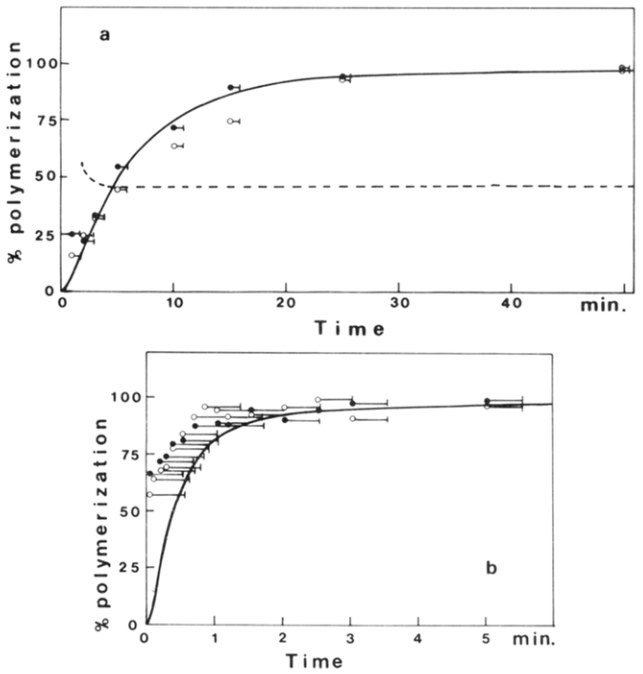


FIGURE 2: Time course of tubulin and MAP incorporation in microtubules upon polymerization of microtubule protein. Three times cycled microtubule protein containing radioactively labeled MAPs was prepared as described under Materials and Methods. Polymerization was followed at 25 (a) and 37 °C (b) both by turbidity (solid curve) and by sedimentation upon centrifugation in the airfuge at time intervals. (●) Weight amount of sedimented protein; (○) amount of sedimented MAPs, from radioactivity measurement. The symbols are located at the exact time at which centrifugation was started. A bar of 30 s indicates the reasonable minimum delay to be added for a polymer of 1000 S to be sedimented at 160000*g*. Tubulin was 2.12 mg/mL in (a) and 2.72 mg/mL in (b). The dashed line indicates the time course of the ratio MAPs/tubulin on sedimented polymer.

microtubule assembly, a significant amount of polymerized tubulin, carrying a high proportion of MAPs, could be sedimented in the airfuge at 160000*g* for 3 min. At the same time the turbidity developed in the cell had reached only 5–10% of the final value. Negative-staining electron microscopy showed that this polymerized material observed after 45 s of assembly at 25 °C did not consist of microtubules but of small aggregates of curly filaments packed together (data not shown). Similar observations have been made in the initial stages of the polymerization process by Bordas et al. (1983). This experiment does not indicate, however, whether these polymers are the precursors of microtubules or short-life dead-end adducts. At later times, only microtubules and no more aggregates could be observed. At 37 °C, the polymerization process was too fast to allow the same observations to be made conveniently. The main conclusion of these experiments is that not all MAPs are bound to the first formed microtubules, as would be expected within a scheme in which MAPs would have a high preferential affinity for microtubules and free MAPs be in equilibrium with microtubule-bound MAPs. In contrast, MAPs and tubulin seem to assemble into microtubules at the same rate, at least in the major part of the elongation process. Although these data do not permit it to be established unambiguously, they give support to the polymerization scheme initially postulated by Weisenberg (1980).

Binding of MAPs to Microtubules at Steady State. Stock solutions of microtubule protein containing different proportions of MAPs relative to tubulin were made up by mixing known amounts of 3 times cycled microtubule protein and pure tubulin dimer as described under Material and Methods.

Table I: Binding of Radioactively Labeled MAPs to Microtubules at Steady State[a]

sample	total protein concn (mg/mL)	total MAP concn (cpm)	microtubule amount (mg/mL)	amount of MAPs polymerized (cpm)	MAPs polymerized (%)	polymerized MAPs per polymerized tubulin (A.U.)
A	0.62	1630	0.38	860	53	2260
	0.81	2010	0.51	1050	52	2060
	1.01	3000	0.66	1840	61	2790
	1.34	3980	0.96	2670	67	2780
	2.02	6110	1.61	4270	70	2650
	2.70	7910	2.23	5840	74	2620
B	0.95	690	0.27	150	22	560
	1.33	1120	0.39	320	28	820
	1.71	1500	0.43	440	29	1020
	2.09	1640	0.58	340	21	590
	2.66	2300	0.90	500	22	560
	3.33	2690	1.25	550	21	440

[a] Three times cycled microtubule protein in which MAPs are radioactively labeled was prepared. This solution was divided in two parts. One part, called A, was used as such. To the other part, pure phosphocellulose-tubulin was added in such amounts that the proportion of MAPs to tubulin was 30% of its value in A. This stock solution was called B. A and B solutions were polymerized to steady state and diluted at 37 °C to different final concentrations. The amounts of total and sedimented protein and radioactive MAPs were then determined.

These solutions, differing fron one another by the ratio MAP/tubulin, were polymerized to steady state at 37 °C. The time necessary to reach steady state was assessed turbidimetrically. The kinetics will be developed in a forthcoming paragraph. At steady state, each sample was subdivided, by dilution with warm buffer, into several samples thus containing polymerized tubulin at different known concentrations, the ratio MAP/tubulin being kept constant. Five minutes after dilution, the samples were centrifuged and processed for determination of the amount of microtubules. Figure 3 shows the plots of the amount of microtubules formed vs. total tubulin concentration for a series of microtubule protein solutions more or less rich in MAPs. Each curve corresponds to a given proportion of MAPs relative to tubulin. It can be observed that at a given tubulin concentration, less microtubules are formed when less MAPs are present. However, when the ratio MAPs/tubulin was decreased, the plots deviated from the linearity routinely observed for the 3 times cycled microtubule protein preparation and exhibited a positive curvature. In addition, all plots converged on the abscissa toward the same critical concentration of 0.15 ± 0.03 mg/mL. This latter observation has already been made in a preliminary report (Weisenberg et al., 1981). In view of the complexity of these results, the same experiment was made with labeled MAPs initially present in the stock solutions. The amount of MAPs bound to microtubules at steady state could be derived from the measurement of radioactivity present in the supernatant of sedimented microtubules. The data shown in Table I establish the comparison between 3 times cycled microtubule preparation, solution A (leading to a linear plot of microtubule amount vs. tubulin concentration), and a preparation containing 3-fold less MAPs per tubulin, solution B (leading to a curved plot). It appeared that microtubules formed from all dilutions of B solution had a lower amount of MAPs bound per polymerized tubulin than in the corresponding A series, at all tubulin concentrations. However, the data also indicated that while 60–70% of the MAPs present in the A solutions were bound to assembled microtubules, this proportion was only 20–25% for the B solutions. This last result is puzzling, since the addition of dimeric tubulin to microtubule protein was expected, within a simple scheme, to shift the equilibrium between free MAPs and microtubule-bound MAPs toward more associated species. In contrast, the data show that this simple scheme does not apply here since the interaction between MAPs and microtubules rather seems weakened by the addition of dimeric tubulin to microtubule protein.

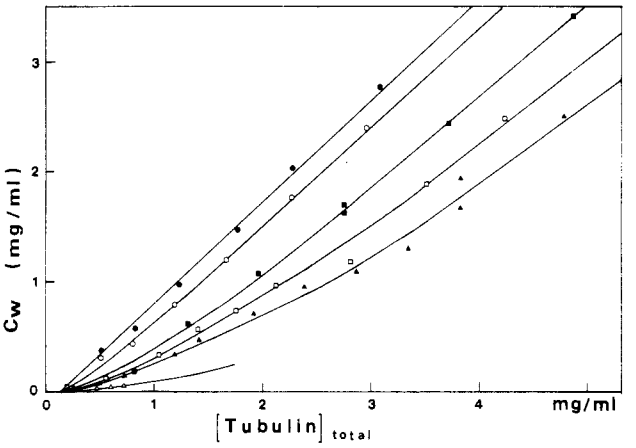


FIGURE 3: Plots of the amount of microtubules formed vs. total tubulin concentration, for solutions of microtubule protein more or less rich in MAPs. Solutions containing different relative amounts of tubulin and MAPs were prepared by adding different known amounts of dimeric tubulin to 3 times cycled microtubule protein. The amount of MAPs per tubulin was the following when 100% was used as a reference for the ratio MAP/tubulin in 3 times cycled microtubule protein: (●) 100; (○) 90; (■) 70; (□) 64; (▲) 57; (△) 23.

In agreement with this last result, when the time courses of MAP binding to microtubules during the assembly process were compared in two solutions containing the same amount of MAPs but different amounts of tubulin, they were found to be homothetic curves and about 3-fold less MAPs were again found bound to microtubules formed from the solution containing the lower proportion of MAPs.

That less MAPs bound to microtubules upon addition of pure tubulin dimer was further assessed by gel electrophoresis of the supernatants and pellets of microtubules. Figure 4 shows that while very few MAPs were observed in the supernatant of microtubules formed from unmodified microtubule protein, the major part of high molecular weight MAP_1 and MAP_2 polypeptides appeared in the supernatant of microtubules formed from the tubulin-enriched material. Correlatively microtubules then were largely depleted in these polypeptides. Analytical ultracentrifugation of the solution detected, apart from the major 6S tubulin peak, a very small proportion of several discrete fast migrating peaks which might correspond to tubulin polymers carrying MAP_1 and MAP_2. Such polymers were not observed in the supernatant of microtubules formed from standard whole microtubule protein. In contrast with the high molecular weight MAPs it is worthwhile noting

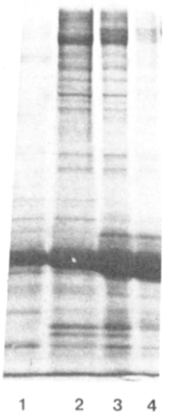


FIGURE 4: Dissociation of MAP_1 and MAP_2 from microtubules upon tubulin dimer addition. Three times cycled microtubule protein at 4.45 mg/mL was polymerized at 37 °C either alone or in the presence of 1.75 mg/mL dimeric tubulin. Supernatants SA and SB were collected. Microtubules were sedimented at 110000*g* for 30 min and resuspended in the same volume of buffer at 0 °C, and the solution was clarified by centrifugation at 0 °C at 70000*g* for 20 min, yielding microtubule protein solutions MA and MB. Equal volumes of fractions SA and SB and fractions MA and MB, respectively, were denatured and processed for SDS–polyacrylamide gel electrophoresis. (1) SA (44 μg); (2) SB (93 μg); (3) MA (110 μg); (4) MB (100 μg).

that τ protein was not released in the supernatant of microtubules upon addition of tubulin dimer. Unfortunately, the two lower apparent molecular weight components of τ comigrate with tubulin, and only the higher bands of τ can be seen on the gel in Figure 5 in the presence of tubulin. Therefore, in order to investigate this point more thoroughly, the same experiment was repeated with radioactively labeled MAPs. To a solution of microtubule protein containing radioactive MAPs were added increasing amounts of dimeric tubulin, the partial concentration of initial microtubule protein (and therefore of MAPs) being kept constant. After polymerization and centrifugation of the samples, the protein content of the supernatants and microtubule fractions was assayed by SDS gel electrophoresis followed by Coomassie blue staining. Autoradiography of the dried gel showed the distribution of MAPs (Figure 5). Again the increased dissociation of MAP_1 and MAP_2 (plus 35K peptide) from microtubules upon addition of increasing amounts of dimeric tubulin was noticed. The autoradiogram further shows that the two faster migrating bands of τ protein were at least partially present in the supernatant of microtubules, in a slightly decreasing amount upon increasing the amount of tubulin dimer added. It is noticed, by comparison with the autoradiogram of MAPs alone shown on Figure 1, that the electrophoretic migration of these bands of τ protein is artifactually accelerated by the presence of a relatively much larger amount of tubulin which migrates in the same region and introduces a local alteration of the electric field.

In the microtubule fraction, the four bands of τ protein were observed and obviously remained roughly at the same amount in all samples. Consequently, when microtubule protein is copolymerized with large enough amounts of dimeric tubulin, microtubules having exclusively τ bound should be obtained. Such an experiment was attempted and was done in the following way: microtubule protein (three cycles) was polymerized, at 1.51 mg/mL, either alone (sample A) or in the presence of 4 mg/mL pure phosphocellulose–tubulin (sample B). After centrifugation, the microtubule pellets were resuspended in buffer, and the solutions were clarified by centrifugation and processed for analytical ultracentrifugation and

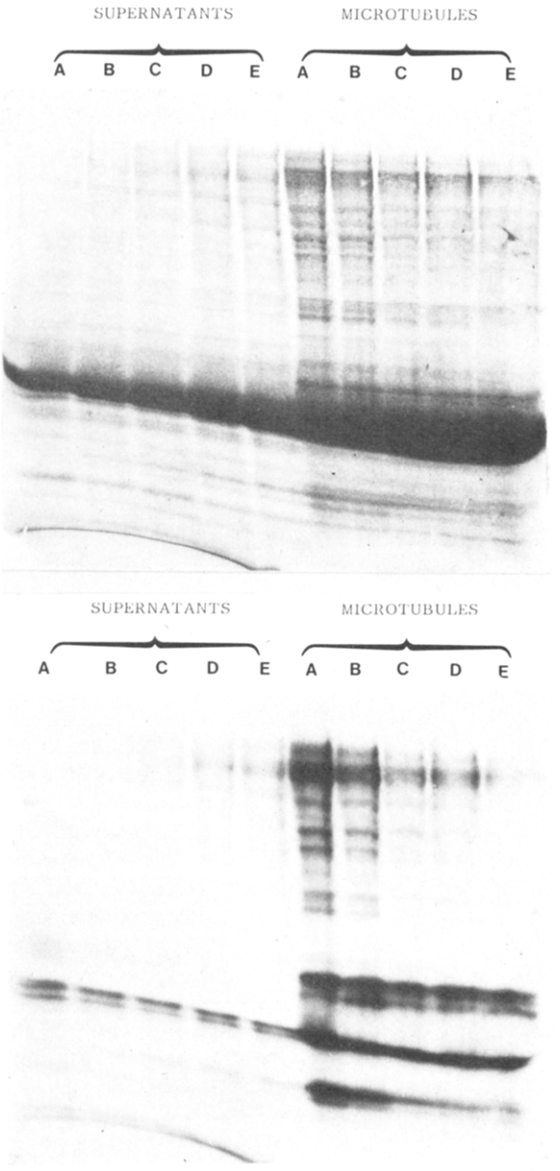


FIGURE 5: Increased dissociation of MAP_1 and MAP_2 and not of τ protein, from microtubules upon copolymerization of microtubule protein with increasing amounts of dimeric tubulin. Microtubule protein at 2.14 mg/mL (cycled with labeled MAPs) was polymerized with the following amounts of dimeric tubulin (in mg/mL): (A) 0; (B) 1.1; (C) 1.3; (D) 2.0; (E) 2.9. Supernatant and microtubule protein fractions were obtained as described in Figure 4 and electrophoresed. The top figure shows the Coomassie blue staining of the gel and the bottom figure the autoradiogram of the different fractions as indicated.

electron microscopy. Figure 6 shows that while double rings could be observed in the 4 times cycled A solution, they had disappeared in B and instead short linear polymers were observed. Analytical ultracentrifugation of A and B solutions at 2.7 and 3.6 mg/mL, respectively, confirmed the electron microscopy data: while solution A contained 49% rings ($s_{20w,c}$ = 34 S) and 51% dimeric 6S tubulin, in solution B, the polymeric species of tubulin represented no more than 13% of total protein, with 3.5% in the 34S and 9% in a new 21S polymeric species. Solution B thus obtained and containing a low amount of MAPs (essentially τ) per tubulin was nevertheless able to polymerize rapidly.

These experiments demonstrate that the two classes of MAPs, namely, the high molecular weight MAP_1 and MAP_2 polypeptides on one hand and τ protein on the other hand, do not exhibit the same behavior in their interactions with tubulin and microtubules.

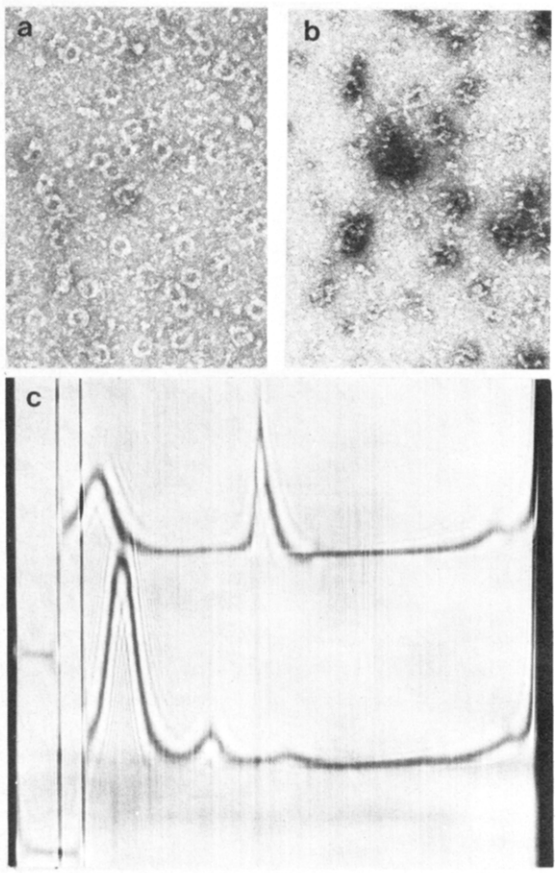


FIGURE 6: Electron microscopy and analytical ultracentrifugation analysis of the polymer content of microtubule protein obtained after a fourth cycle of polymerization of microtubule protein assembled at 1.51 mg/mL without (MA) or with addition of 4 mg/mL dimeric tubulin (MB). (a) Negative staining of MA solution showing numerous rings. (b) Negative staining of MB solution showing the almost complete disappearance of rings (one is left on the field) and appearance of small rod polymers. Bar = 0.25 μM. (c) Analytical ultracentrifugation schlieren picture taken 28 min after after starting centrifugation of MA and MB solutions at 2.7 and 3.6 mg/mL, respectively. Centrifugation was done at 10 °C, 56 000 rpm. (Upper trace) MA; (lower trace) MB. Gel electrophoresis showed that MB contained about 4–5-fold less MAP_1 and MAP_2 per tubulin than MA. In MA, the peak corresponding to double ring ($s_{20,wc}$ = 34.2 S) represented 49% of the protein. In MB, only 3.5% protein was present in this peak and 10% in a peak of $s_{20,wc}$ = 21 S.

Kinetic Approach of the Interactions between MAPs and Tubulin Involved in Microtubule Assembly. The effect of the proportion of MAPs present in microtubule protein solutions on the kinetics of microtubule assembly was studied by adding increasing amounts of tubulin dimer to a constant amount of microtubule protein (three cycles). The extent of reversible turbidity development, the pseudo-first-order rate constant of the growth process, and the initial rate of depolymerization upon cooling the solution to 4 °C were measured. This latter parameter provides a measurement of the microtubule number concentration (Johnson & Borisy, 1977; Karr et al., 1980; Carlier, 1983). Figures 7 and 8 show that while the amount of microtubules increases upon addition of dimeric tubulin to microtubule protein, the pseudo-first-order rate constant for the elongation process, k_{obsd}, either increases or decreases according to the relative amounts of tubulin and MAPs. At a high concentration of microtubule protein, the addition of dimeric tubulin resulted in an enhancement of the rate of polymerization, and k_{obsd} increased. The corresponding increase in the rate of microtubule depolymerization at 4 °C owing to an increase in the microtubule number concentration

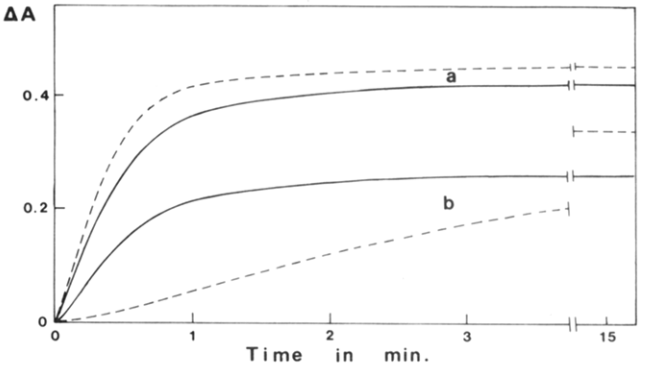


FIGURE 7: Kinetics of polymerization of microtubule protein in the absence (solid curves) or presence (dashed curves) of dimeric tubulin. The time course of turbidity development at 350 nm was recorded. Temperature was 37 °C. In (a), microtubule protein was 4.89 mg/mL (± 1.89 mg/mL dimeric tubulin). In (b), microtubule protein was 2.45 mg/mL (± 2.84 mg/mL dimeric tubulin).

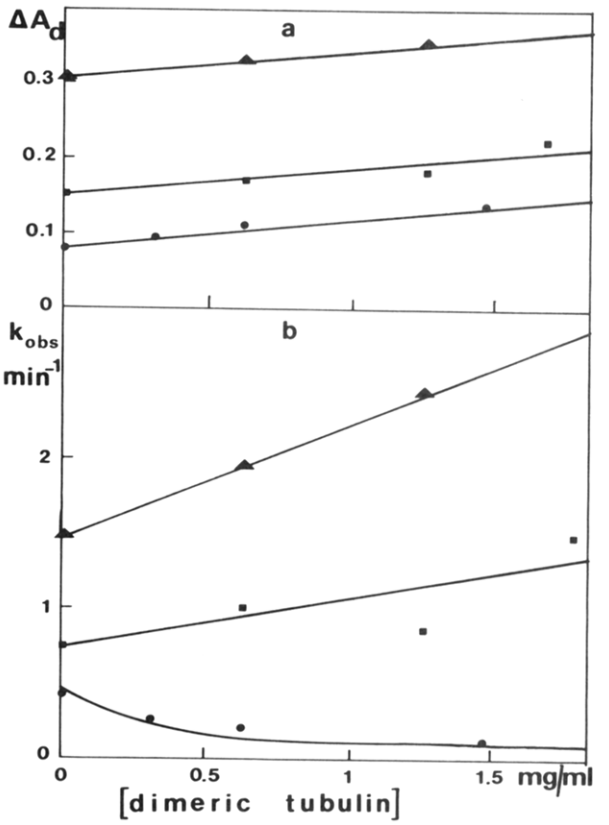


FIGURE 8: Kinetics of microtubule assembly upon addition of different amounts of dimeric tubulin to the following constant amounts of 3 times cycled microtubule protein: (●) 0.85 mg/mL; (■) 1.41 mg/mL; (▲) 2.82 mg/mL. (a) Increase in the amount of microtubules formed, from the extent of turbidity decrease ΔA_d upon cooling the polymerized solution to 4 °C; (b) change in the observed pseudo-first-order rate constant for the elongation process.

confirmed that the increase in k_{obsd} was due to an activation of the nucleation reaction. On the other hand, at low concentrations of microtubule protein, the addition of dimeric tubulin produced a marked inhibition of the rate of polymerization. The value of k_{obsd} decreased continuously, and almost total inhibition (lag time > 30 min) could be obtained. Initial rate of depolymerization at 4 °C decreased accordingly, indicating that the nucleation step, here too, was affected. Although this does not appear in the experiments presented in Figure 7 and 8, a strong inhibition of nucleation eventually yielded a lower amount of microtubules formed at steady state. This complex behavior, showing an alternate activation–in-

hibition of polymerization pattern, was qualitatively reproducible. It was, however, observed that the quantitative relative amounts of tubulin and MAPs necessary to observe either activation or inhibition of the nucleation varied from one experiment to the other. The reason for this scattering remains unclear and may lie in some uncontrolled critical property of MAPs varying from one preparation to the other.

Discussion

The obtention of radioactively labeled MAPs able to reassociate with microtubules has proven a useful tool to study the interaction between tubulin and MAPs involved in microtubule assembly in vitro.

Previous experiments using unrelated techniques (Mandelkow et al., 1980; Pantaloni et al., 1981) had suggested that fragments of the ring oligomers, before their complete dissociation into tubulin subunits, could associate laterally to form the nuclei of microtubules. However, little was known about the elongation process at the molecular level. Two extreme cases could be a priori distinguished: In a first model, MAPs could have an exclusive high affinity for microtubules. Elongation would then proceed through addition of dimeric tubulin subunits onto the nuclei. Due to the high affinity of MAPs for them, these initially formed microtubules would be largely saturated by the majority of MAPs present in solution. As polymerization of dimeric tubulin proceeds, the bound MAPs would then scatter along the growing microtubules. In this case, the amount of MAPs bound per polymerized tubulin would decrease in the time course of the polymerization process. Moreover, the kinetics of such a process would not be first order, since the time-dependent decrease in the saturation of microtubules by MAPs implies a corresponding time-dependent increase in the dissociation rate constant of tubulin from microtubules, according to Murphy et al. (1977). In another alternative model, MAPs would exist not only in the free and microtubule-bound states but also bound to an intermediate oligomer which would be the polymerizable species of tubulin. This small oligomer would be present in small amounts, as a kinetic intermediate, in the polymerizing microtubule protein solutions, in order to reconcile with the ultracentrifugation observations showing that essentially 6S tubulin coexists with growing microtubules. Such a model is well supported by our data showing that the ratio of MAPs/tubulin on microtubules is kept constant during the polymerization process, except at the early initiation step (visible at 25 °C), in agreement with our previous results (Pantaloni et al., 1981). This model also agrees with the observation of exponential kinetics of polymerization. While the theoretical possibility of oligomeric intermediate forms of tubulin in the polymerization process has been proposed by Weisenberg (1980), no experimental evidence for such a pathway has been shown until now. Recent data of time-resolved synchrotron X-ray scattering (Bordas et al., 1983) do not reject the possibility that small oligomers can participate in microtubule elongation, while larger transient intermediates like sheets are excluded. A closely related possibility also consistent with our data is that short oligomers having MAPs bound and dimeric tubulin assembled at comparable rates to those microtubules, so that a single first-order growth process is observed. It should, however, be noticed that under other buffer conditions, biphasic polymerization kinetics have been observed (Barton & Riazi, 1980). The role of pH in determining the nature of the kinetics has been emphasized recently (Bayley et al., 1983). In addition, we observed that single exponential kinetics were no more observed if the concentration of Mg^{2+} ions, in our polymerization buffer, was lowered below

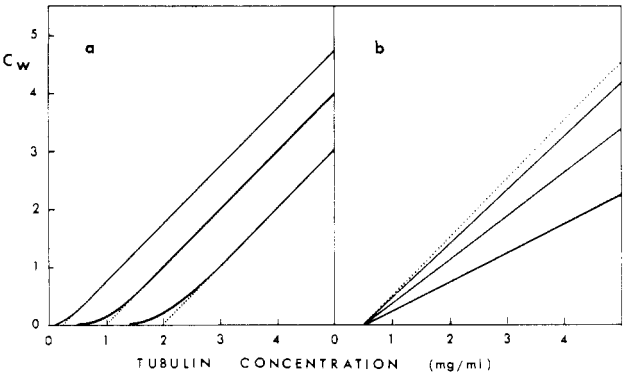


FIGURE 9: Two possible schemes qualitatively describing the interaction between MAPs and microtubules. (a) MAPs bind to microtubules exclusively (with no nearest-neighbor interactions) and decrease the critical concentration. (b) MAPs bind alternatively to microtubules and to a nonmicrotubular polymer. c_w is the weight amount of microtubules formed; the ratio MAPs/tubulin is kept constant in each curve and decreases, from left to right in (a) and from top to bottom in (b). The dotted line is the ideal curve with no formation of nonmicrotubular polymer (slope = 1).

0.5 mM. For the sake of simplicity, the interaction between MAPs and tubulin in microtubule assembly under this complex situation has not been explored further in the present work.

Models. With regard to the possible theoretical types of interactions between MAPs and microtubules at equilibrium, two different models can be considered.

(1) If MAPs have an exclusive affinity for microtubules and if nearest-neighbor interactions are neglected, the variation of the tubulin critical contentration C_1 with the amount M_0 of MAPs present in solution is described by the following equation (Engelborghs, 1983):

$$c_1 = c_{1,0}(1 + KM)^{-1/m} \quad (1)$$

where $c_{1,0}$ is the critical concentration in the absence of MAPs and M is the concentration of free MAPs. M is the apparent maximal stoichiometry of polymerized tubulin to microtubule-bound MAPs. Assuming that MAPs have a high affinity for microtubules, in a region of high tubulin concentrations as compared to the critical concentration, eq 1 can be written as

$$c_1 = c_{1,0}[1 - m(M_0/c_0)]^{1/m} \quad (2)$$

where M_0 and c_0 are the total MAP and tubulin concentrations. Equation 2 is identical with the one already proposed to fit previous data (Pantaloni et al., 1982) and shows that plots of the amount of microtubules vs. total tubulin obtained from solutions in which the ratio MAPs/tubulin (M_0/c_0) was kept constant should be parallel straight lines of slope equal to 1 and extrapolating to a critical concentration should be only dependent on the ratio M_0/c_0, according to 2. In the region of tubulin concentration close to the critical concentration, however, 2 is no longer valid, and therefore, the plot exhibits a positive curvature (Figure 9a); i.e., microtubules can exist below the extrapolated critical concentrations, since a higher density of MAPs on microtubules can be obtained.

(2) If MAPs can bind to microtubules with an affinity K and a stoichiometry m and to another oligomeric nonmicrotubular species with an affinity K', and stoichiometry m', then the following equation should apply (Engelborghs, 1983):

$$c_1 = c_{1,0}\frac{(1 + K'M)^{1/m'}}{(1 + KM)^{1/m}} \quad (3)$$

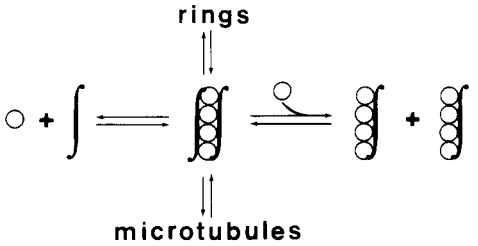
Although the ultimate expression of c_1 vs. M_0 and c_0 is not

easy in this case, it can be qualitatively proposed that if K' is not much smaller than K, a constant proportion of MAPs and tubulin, at a given M_0/c_0 ratio, is involved in a nonmicrotubular complex. If this complex forms with a stoichiometry m' higher than m, the slope of the plot of the amount of microtubules vs. tubulin concentration will decrease upon decreasing the ratio M_0/c_0 (Figure 9b).

The data presented here indicate that τ binding to microtubules seems to obey the first model, while high molecular weight MAP_1 and MAP_2 can bind alternatively to microtubules and to a nonmicrotubular polymer according to the second model. Qualitatively, the experimental plots shown on Figure 3 can be understood as a sum of the theoretical plots in Figure 9. In agreement with this view, it is currently observed that in cycled microtubule protein preparations, the slope of the plot of the amount of microtubules vs. total tubulin concentration is often smaller than 1. This result has, until now, been interpreted within the view that some part of the tubulin is denatured. However, it can be checked (unpublished data from our laboratory) that 100% of the tubulin present in the supernatant of these microtubules can bind colchicine and is able to polymerize into microtubules upon addition of 4 M glycerol and 5 mM magnesium ions (which are the conditions of pure tubulin polymerization). We thus favor the idea that tubulin and high molecular weight MAP_1 and MAP_2 could be distributed both in microtubules and in a nonmicrotubular tubulin oligomer, in proportions depending upon the concentrations of the respective constituents.

Analysis of the kinetics of microtubule assembly further indicates that the data cannot be accounted for by a simple single model within which MAPs would have an exclusive affinity for microtubules. The inhibition of polymerization by an excess of dimeric tubulin is inconsistent with it. The measurement of a lower amount of MAP_1 and MAP_2 bound to microtubules argues for the formation of another oligomeric species of tubulin carrying MAPs but unable to assemble in microtubules and provides a possible explanation for the complex kinetic pattern observed.

The following tentative scheme qualitatively describes the different interactions between tubulin (O) and MAPs ($\int$) at 37 °C:



This scheme emphasizes the poymorphism between two types of tubulin oligomers P and P′, the equilibrium between P and P′ depending on the ratio MAPs/tubulin in solution. In this scheme, oligomer P is the polymerizing species of tubulin, and P′ is an oligomer containing less MAPs per tubulin molecule than P. (For this reason, electrostatic repulsion forces would take place between P′ oligomers and prevent their incorporation in microtubules.) The introduction of P′ here represents a modification of the scheme proposed by Weisenberg (1980). It should be noted that this scheme is qualitative with respect to the relative number of tubulin subunits in P and P′: although the ratio of MAPs to tubulin is larger in P than in P′, the size of P′ could be smaller than that of P. The fact that P′ does not cosediment with microtubules, however, indicates it has a sedimentation coefficient under 200 S. Whether or not the pathway involving the participation of MAP_1 and MAP_2 in polymer P′ is of biological significance in the cell remains an open question. Nonetheless, some reports point to a non strictly microtubular location of high molecular weight MAPs in cells (Hill et al., 1981; Hill, 1983; Leterrier et al., 1982; Pytela & Wiche, 1980).

Within the above scheme, our results indicate that while MAP_1, MAP_2, and τ can participate in the P species, only MAP_1 and MAP_2 can form with tubulin the P′ oligomer. Indeed, in contrast to MAP_1 and MAP_2, τ protein was essentially bound to microtubules independently of the relative amount of MAPs present in the tubulin solutions. However, the four bands composing τ factor described by Cleveland et al. (1977) did not seem to associate to microtubules with the same affinity despite their close relationship: while the two higher apparent molecular weight τ polypeptides (60K–65K) exhibited a very high affinity for microtubules, the two lower apparent molecular weight components (57K–55K), which comigrate with tubulin on SDS–polyacrylamide gels, were partially found in the supernatant. It will ve valuable, in the future, to investigate whether phosphorylation of the 57K–55K components of τ protein regulates its binding to microtubules in vitro, as is the case in vivo for the τ-like 80K and 69K polypeptides in nonnervous cells (Pallas & Solomon, 1982).

Because of this complexity revealed by our experiments, it was not possible, at the present stage, to go much deeper in the analysis of microtubule stabilization upon MAP binding. A technical problem in the quantitative evaluation of our results also lies in the different specific radioactivities of MAP_1, MAP_2,and τ proteins, depending both on their different reactivities in the labeling reaction and on their variable relative amounts, from one preparation to the other, in the bulk microtubule protein solution. For these reasons, the work presented here only intends to be a first step. Its main objective is to show that, since all MAPs do not interact with tubulin in the same way, it is not possible to study the binding of whole MAPs to microtubules, in reconstitution experiments. Obviously it will be necessary, in the future, to deal with a homogeneous system of tubulin interacting with one defined MAP component, to study more thoroughly its thermodynamics. In this respect, the results reported here show that it is technically possible to eliminate MAP_1 and MAP_2 and obtain microtubules in equilibrium with tubulin and τ.

The different behavior of MAP_1, MAP_2, and τ protein observed in vitro may reflect different cellular functions of the microtubules having either MAP_1 and MAP_2 or τ bound. This view is supported by biochemical and immunofluorescence studies showing that τ (or τ-like protein) was a conserved and widespread MAP while high molecular weight MAPs were more specifically found associated with dendritic microtubules (Cleveland et al., 1979; Black & Lasek, 1980; Duerr et al., 1981; Matus et al., 1981; Hill et al., 1981). In the microtubule protein preparation, homogenization of brain tissue and subsequent cycles of polymerization yield mixed populations of microtubules to which high molecular weight proteins and τ bind in a competitive scheme depending on solution variables. It is hoped that a better understanding of the function of these proteins will allow the purification of one single microtubular system specifically.

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