Microtubule-Associated Proteins and the Stimulation of Tubulin Assembly in Vitro†

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ABSTRACT: Microtubules, purified by cycles of assembly and disassembly in vitro, are composed of tubulin and several microtubule-associated proteins (MAPs). When the MAPs were separated from the tubulin by phosphocellulose chromatography, the tubulin no longer assembled at 37 °C as measured by turbidity. If the MAPs and tubulin were recombined and warmed to 37 °C, microtubules assembled. MAPs stimulated tubulin assembly by affecting both the initiation and elongation processes. The effect on initiation was indicated by results showing an increase in initial rate and a decrease in average microtubule length as the MAP:tubulin ratio was increased. The effect on elongation was indicated by results showing that a greater mass of microtubules was assembled at the apparent equilibrium as the MAP:tubulin ratio was increased. The initiation and elongation activities of the MAPs could be separated by storing the MAPs at 4 °C during which time the initiating activity decreased while the ability to affect the total amount of assembly remained constant. The decrease in initiating ability was correlated with the loss of the two major components of the MAP fraction, MAPs 1 and 2.

Since the discovery of the conditions necessary for the in vitro polymerization of microtubules from brain homogenates (Weisenberg, 1972), it has become possible to study specific aspects of the in vitro assembly reaction. At least three proteins have been shown to maintain a constant ratio to tubulin during assembly and disassembly of microtubules in vitro, suggesting that they are integral components of the microtubule. These proteins have been called MAPs[1] for microtubule-associated proteins. The most prominent of these are two high-molecular-weight proteins called MAP 1 and MAP 2 (Sloboda et al., 1975), which have been shown to compose the filaments coating the surface of in vitro-assembled brain microtubules (Dentler et al., 1975; Murphy and Borisy, 1975). The third MAP known to be associated with microtubules at a constant ratio is a cyclic AMP-stimulated protein kinase that specifically phosphorylates MAP 2 (Sloboda et al., 1975). In addition to these proteins, several other proteins with molecular weights less than MAP 2 are present in preparations of in vitro-assembled brain microtubules (Borisy et al., 1975; Murphy and Borisy, 1975; Weingarten et al., 1975; Granett et al., 1975), but it is not known whether these proteins are present in a fixed ratio to tubulin.

When in vitro-assembled microtubules are depolymerized at 4 °C, molecular sieve chromatography can be used to resolve two structural forms: 46-nm diameter ring-shaped aggregates that have a sedimentation coefficient of 30 or 36 S tubulin dimers that have a sedimentation coefficient of 6 S (Kirschner and Williams, 1974; Kirschner et al., 1974; Olmsted et al., 1974; Rebhun et al., 1974; Weingarten et al., 1974; Weisenberg, 1974; Borisy et al., 1975; Scheele and Borisy, 1976). When these two fractions are analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the MAPs are detected only in the ring-containing fraction, not in the tubulin dimer fraction (Erickson, 1974; Dentler et al., 1975; Haga and Kurokawa, 1975; Keates and Hall, 1975; Kuriyama, 1975; Granett et al., 1975; Sloboda et al., 1976). The ring fraction, composed of the MAPs and tubulin, readily assembles into microtubules when warmed to 37 °C with a critical concentration for assembly lower than that observed in the unfractionated microtubule protein preparation (Granett et al., 1975; Sloboda et al., 1976). The purified tubulin dimers alone do not assemble to a significant extent but can be stimulated to assemble by the addition of the fraction containing rings and MAPs (Erickson, 1974; Haga and Kurokawa, 1975; Kuriyama, 1975; Granett et al., 1975; Sloboda et al., 1976).

Tubulin can be removed from the MAP and ring-containing fraction by ion-exchange chromatography using DEAE-Sephadex (Murphy and Borisy, 1975; Granett et al., 1975; Sloboda et al., 1976) or phosphocellulose (Weingarten et al., 1975). The tubulin fractions obtained by either of these methods no longer contain ring-shaped aggregates and do not assemble into microtubules when warmed to 37 °C. However, the tubulin dimers can be stimulated to assemble by the addition of the MAP fraction. This MAP-stimulated assembly appears to be preceded by the formation of the rings, since the MAP fraction can induce the formation of rings when combined with tubulin dimers at 4 °C (Murphy and Borisy, 1975; Weingarten et al., 1975; Granett et al., 1975), and, when the temperature is raised to 37 °C, the rings disappear within the first few minutes of the assembly reaction even though an apparent equilibrium is not reached for 15–20 min (Borisy et al., 1975; Dentler and Rosenbaum, 1975; Kirschner et al., 1975).

In this report it is shown that (a) for a constant concentration of tubulin, the addition of increasing concentrations of the MAP fraction, isolated by phosphocellulose chromatography, results in a linear increase in both the *initial rate* and the *total amount* of microtubule assembly, (b) the increase in initial rate appears to result from the formation of initiation centers, since

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[1] Abbreviations used are: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DEAE, diethylaminoethyl; MAP, microtubule-associated protein; HMM, heavy meromyosin; ATPase, adenosine triphosphatase; GTP, guanosine triphosphate; AMP, adenosine monophosphate.

the average microtubule length at the apparent equilibrium point of the reaction decreases with increasing MAP concentrations, (c) as the MAP fraction is stored at 4 °C for up to 8 days, the *total amount* of assembly for a given MAP fraction:tubulin ratio remains the same, although the *initial rate* of the assembly reaction decreases, and (d) during storage of the MAP fraction at 4 °C the prominent bands corresponding to MAP 1 and MAP 2 seen on sodium dodecyl sulfate–polyacrylamide gels break down into a heterogeneous population of polypeptides with molecular weights greater than that of tubulin but less than that of MAP 2. A preliminary report of this work has appeared (Sloboda and Rosenbaum, 1975).

Materials and Methods

Purification of Tubulin and Microtubule-Associated Proteins. Microtubule proteins were purified from calf brain by cycles of assembly and disassembly in vitro, using a modification of the method of Shelanski et al. (1973). Brains were homogenized at 4 °C for 45 s at low speed in a Waring Blendor in 0.5 ml of polymerization buffer (PM: 50 mM Pipes–KOH, pH 6.9; 0.5 mM $MgSO_4$; 1 mM EGTA; 0.5 mM GTP) per g wet weight, and the homogenate was centrifuged at 130 000*g* for 75 min at 4 °C. The supernatant was diluted 1:1 with PM containing 8 M glycerol and incubated at 37 °C for 30 min to assemble microtubules. The microtubules were then sedimented at 130 000*g* for 75 min at 25 °C, and the pellet was resuspended in cold PM (0.20–0.25 the volume of the crude supernatant) using a Dounce homogenizer and incubated at 4 °C for 30 min. The solution was then centrifuged at 130 000*g* for 30 min at 4 °C to sediment any remaining microtubules and aggregates not dissociated by the cold treatment. The supernatant from this centrifugation was made 8 M in glycerol and stored for a maximum of 3 to 4 days at −20 °C before use.

For a typical experiment, an appropriate aliquot of the stored solution was diluted 1:1 with PM containing 2 mM GTP and incubated at 37 °C for 30 min to assemble microtubules. The microtubules were collected as described above, resuspended and depolymerized in column buffer (see chromatography section), and incubated at 4 °C for 30 min to depolymerize the microtubules. The solution was then clarified by centrifugation at 130 000*g* for 30 min at 4 °C. The supernatant from this centrifugation was designated as 2×-microtubule protein and was used in all the experiments reported here.

Column Chromatography. Tubulin dimers were isolated by molecular sieve chromatography on Bio-Gel A-1.5m (200–400 mesh). Approximately 100–150 mg of 2×-microtubule protein in 10–12 ml of column buffer (CB: 50 mM Pipes–KOH, pH 6.9; 0.5 mM $MgSO_4$; 1 mM EGTA; 0.1 mM GTP) was applied to a 55 × 2.5 cm column equilibrated in CB at 4 °C. Protein was eluted at a hydrostatic pressure of 100 cm with a flow rate of 1.2 ml/min; 3.3-ml fractions were collected.

Phosphocellulose (Whatman P11) was precycled by suspending in 0.5 N KOH for 30 min (1 g of phosphocellulose/15 ml); the exchanger was allowed to settle, the supernatant was decanted, and the phosphocellulose was washed with distilled water until the effluent was pH 8. The exchanger was then suspended in 0.5 N HCl for 30 min (1 g/15 ml), allowed to settle, the supernatant decanted, and this step was repeated. The phosphocellulose was then washed with distilled water until the effluent was near neutrality, suspended in 50 mM Pipes-KOH (pH 6.9), and stored at 4 °C until use.

Phosphocellulose columns, 25 × 1.5 cm, were equilibrated by washing with CB, 2×-microtubule protein was run into the column (3 mg of protein/ml bed volume), and the column was washed with 1–2 bed volumes of CB at a hydrostatic pressure of 100 cm to elute unbound protein. Adsorbed material was eluted with either a continuous gradient of 0–1 M KCl in CB, as described by Weingarten et al. (1975), or with a single-step elution of 0.8 M KCl in CB; 2.7-ml fractions were collected, and the protein was monitored by absorbance at 280 nm.

Appropriate fractions were pooled and concentrated by pressure dialysis on Amicon PM 10 filters and desalted by passing through Sephadex G-25 columns (7 × 1.5 cm) equilibrated in CB. Protein was quantitated by the Schacterle and Pollack (1973) modification of the method of Lowry et al. (1951).

Turbidity Assay. Microtubule polymerization was monitored by an increase in absorbance (Gaskin et al. 1974) at 350 nm with a Gilford Model 240 recording spectrophotometer equipped with a temperature controlled cuvette chamber. Assembly took place in CB in which the GTP concentration was adjusted to 1 mM, and the reaction was initiated by raising the temperature of the reaction mixture from 4 to 37 °C. The presence of microtubules, as indicated by the absorbance increase, was verified by negative-stain electron microscopy.

Electron Microscopy. For the length distribution studies, aliquots from the spectrophotometer cuvettes were fixed by the addition of an equal volume of 2% glutaraldehyde in PM. A drop of the fixed material was placed on a carbon-over-formvar-coated grid which was then rinsed with 0.2% cytochrome *c* in 1% amyl alcohol, stained with 1% uranyl acetate, and drained to near dryness with filter paper. Microtubules were photographed and enlarged tracings were made from the negatives and measured with a map measurer. For thin sectioning, in vitro-assembled microtubules were sedimented at 36 000*g* for 30 min at 25 °C. The pellets were fixed for, 1 h in 2% glutaraldehyde in PM and postfixed, embedded, and sectioned as previously described (Dentler et al., 1975). All electron microscopic observations were made with a Philips 201 electron microscope.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8% acrylamide slab gels (20 × 15 × 0.15 cm) according to the method of Laemmli (1970), the acrylamide gels were fixed in 12.5% Cl_3CCOOH overnight and then stained in 0.2% Coomassie blue in ethanol–acetic acid–water (45:10:45) at 50 °C for 30 min. The gels were destained at 50 °C in two 30-min changes of ethanol–acetic acid–water (25:10:65), and the destaining was completed overnight at room temperature in 10% acetic acid. Alternatively, cylindrical gels (10 × 0.6 cm) were run and quantitatively stained with Fast green (Gorovsky et al., 1970) as described previously (Sloboda et al., 1975). The gels were scanned at 650 nm with a Gilford Model 240 recording spectrophotometer equipped with a linear transport and the areas under the peaks were determined by planimetry.

Preparation of Antiserum. Microtubule proteins isolated from three day old chick brains as described (Sloboda et al., 1975) were separated by electrophoresis on 8% polyacrylamide preparative slab gels (15 × 9 × 0.3 cm) in the presence of 0.1% sodium dodecyl sulfate after the method of Laemmli (1970). The gels were stained with Coomassie blue, and the band corresponding to MAP 2 was cut into small pieces and washed in phosphate buffer (pH 7.4) until the pH was greater than 6.0. A quantity of the gel equal to approximately 120 μg of protein was homogenized in 1 ml of phosphate-buffered saline (pH 7.4) in a glass–Teflon homogenizer. Three milliliters of Freund's complete adjuvant was then added and the mixture was homogenized thoroughly in two hypodermic syringes

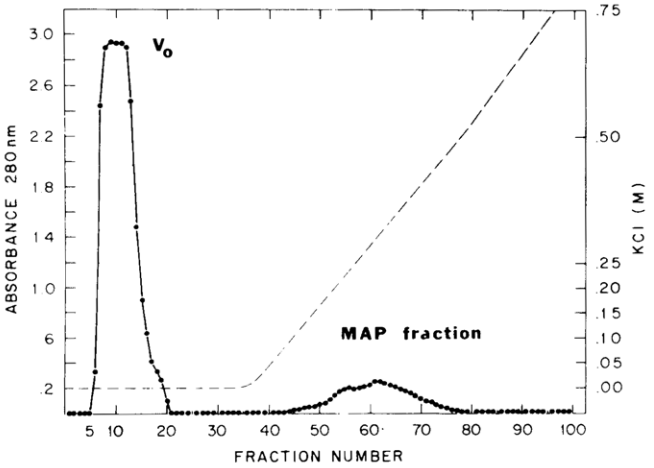

FIGURE 1: Fractionation of 2X-microtubule protein by chromatography on phosphocellulose (25 × 1.5 cm). The majority of the starting material does not adsorb to the column and elutes with the void volume (V_0). The remaining protein that is adsorbed to the column elutes as a broad peak between 0.15 and 0.6 M KCl, and is referred to as the MAP fraction. See Figure 2b,c for the protein composition of these fractions.

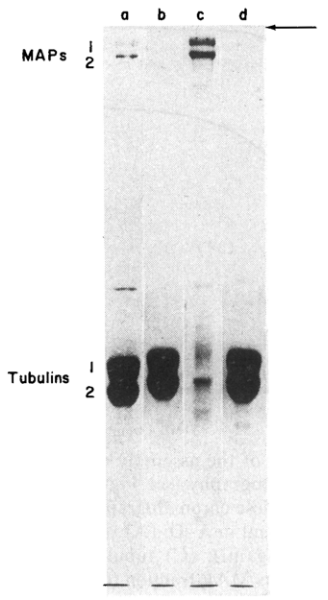

FIGURE 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing the separation of the MAPs from tubulin by ion-exchange and molecular-sieve chromatography as described in the text: (a) 2X-microtubule protein; (b) protein that eluted with the void volume (V_0) of the phosphocellulose column (see Figure 1); (c) protein (MAP fraction) adsorbed to the phosphocellulose column (see Figure 1); (d) tubulin purified by molecular-sieve chromatography on Bio-Gel A-1.5m. The arrow indicates the interface between the stacking gel and the separating gel. The gels were stained with Coomasie blue. Each sample has been overloaded to show minor components.

connected by a right-angle stopcock. Rabbits were injected intramuscularly with a total of 3 ml of the antigen homogenate. An identical subcutaneous booster injection was given after 2 weeks. Five weeks after the initial immunization, 30 ml of blood was collected from each animal, the blood was allowed to clot at 4 °C overnight, and then centrifuged at 35 000*g* for 20 min at 4 °C to clarify the serum. The immunoglobulin fraction was partially purified by precipitation with a final concentration of 50% ammonium sulfate. The precipitate was allowed to form at 4 °C for 30 min, collected by centrifugation at 35 000*g* for 15 min at 4 °C, resuspended in 50 mM Pipes–KOH (pH 6.9), 0.5 mM $MgSO_4$, and 1 mM EGTA, and dialyzed against this same buffer overnight at 4 °C.

The presence of antibodies was determined by double diffusion in 1% agarose in CB. Alternatively, protein samples were separated by electrophoresis on polyacrylamide gradient slab gels (see above) in the presence of 0.1% sodium dodecyl sulfate and immunoelectrophoresis was then performed on the resolved proteins after the method of Converse and Papermaster (1975).

Results

Calf brain microtubules, purified by two cycles of asssembly and disassembly in vitro (2X-microtubule protein), were composed of four major proteins, microtubule-associated proteins 1 and 2 and tubulins 1 and 2, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2a). In addition, several other proteins that migrated faster than MAPs 1 and 2 were present in minor amounts. The 2X-microtubule protein preparation was separated by phosphocellulose chromatography into two protein-containing fractions (Figure 1). Approximately 80% of the starting material eluted with the void volume and was composed primarily of tubulins 1 and 2, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2b). The adsorbed fraction, which eluted as a broad peak between 0.15 and 0.6 M KCl, was composed of MAPs 1 and 2 and many proteins in lesser amounts migrating faster than MAPs 1 and 2 on sodium dodecyl sulfate–polyacrylamide gels (Figure 2c). This adsorbed fraction was referred to as the MAP fraction (Figure 1).

Kinetics of in Vitro Assembly. Tubulin dimers isolated by molecular sieve chromatography assembled poorly[2] when warmed to 37 °C but could be stimulated to assemble upon the addition of the MAP fraction. In order to study the kinetics of this stimulated assembly, tubulin dimers, isolated by Bio-Gel A-1.5m molecular sieve chromatography (whose purity is shown electrophoretically in Figure 2d), were induced to assemble by the addition of aliquots of the desalted MAP fraction obtained by phosphocellulose chromatography (Figures 1 and 2c).

Figure 3 shows the kinetics of assembly, measured by an increase in apparent absorbance at 350 nm, obtained when a constant concentration of tubulin dimers was stimulated to assemble by the addition of increasing concentrations of the MAP fraction. Two characteristics of this assembly reaction were evident. First, as increasing concentrations of the MAP fraction were added to a constant concentration of tubulin, the total amount of microtubule protein assembled at the apparent equilibrium point of the reaction increased, and, second, the initial rate of the assembly reaction also increased.

Increase in Total Amount of Assembly with MAP Addition. The increase in the total amount of assembly obtained when the concentration of MAP fraction was increased relative to a constant concentration of tubulin dimers indicated that the tubulin dimer–polymer equilibrium was being shifted toward the production of polymer. The total mass of microtubules assembled at equilibrium increased linearly (Figure 4a) until a ratio was reached after which further linear increases in the total mass of assembled microtubules were no longer obtained, presumably because the amount of tubulin dimers present became limiting. This occurred at an average MAP fraction: tubulin ratio of 0.3:1 on a weight basis (see Discussion). However, even at those MAP fraction:tubulin ratios that sat-

[2] No assembly was detectable by turbidity measurements, but a few long microtubules could be seen by negative stain electron microscopy.

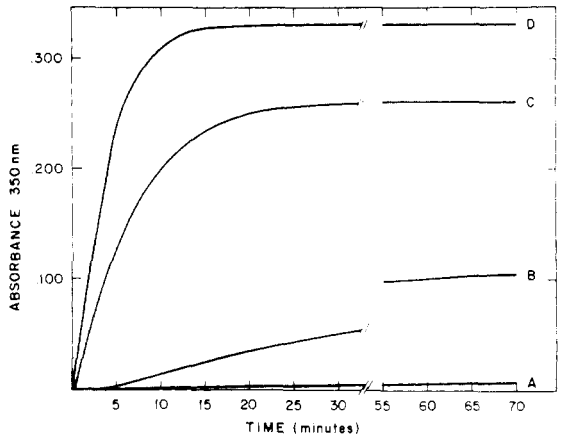

FIGURE 3: Stimulation of the assembly of tubulin dimers isolated by molecular-sieve chromatography (see Figure 2d) by the MAP fraction isolated by phosphocellulose chromatography. The tubulin concentration was constant at 1.8 mg/ml in A–D; (A) tubulin alone; (B) tubulin plus MAP fraction (0.26 mg/ml); (C) tubulin plus MAP fraction (0.52 mg/ml); (D) tubulin plus MAP fraction (0.78 mg/ml). Initial A_{350} was normalized to zero in A–D.

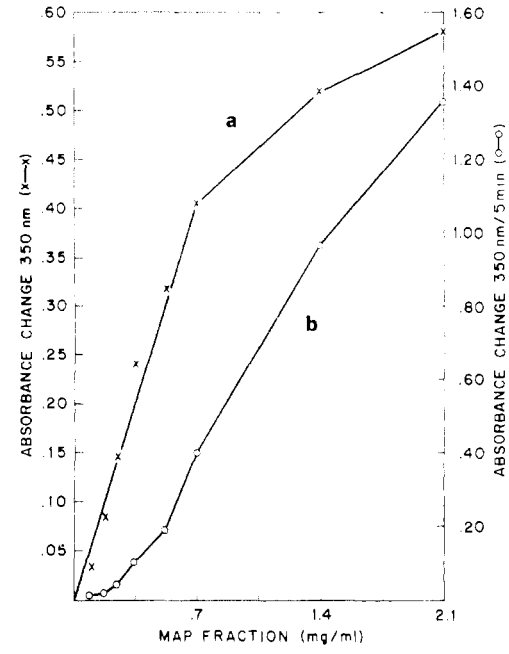

FIGURE 4: Increase in the total amount of assembly (a, x—x) and initial rate of assembly (b, o—o) for a constant concentration of tubulin dimers (3.4 mg/ml) stimulated to assemble by increasing concentrations of the MAP fraction. Total amount of assembly was determined by the change in absorbance between the initial A_{350} and the apparent equilibrium A_{350} values.

urated the available tubulin dimers with respect to the total amount of assembly obtained (Figure 4a), the initial rate of the assembly reaction still continued to increase (Figure 4b).

Increase in Initial Rate of Assembly with MAP Addition. In order to determine if the increase in initial rate of assembly shown in Figures 3 and 4b was due to the formation of increasing numbers of initiation centers, increasing concentrations of the MAP fraction were added to a constant concentration of tubulin dimers and, after an apparent equilibrium was reached, microtubule lengths were determined (see Materials and Methods). As shown in Figure 5, as the concentration of the MAP fraction was increased, the average length of the microtubules assembled at the apparent equilibrium point of the reaction decreased from 39 to 5 μm. These results indicated that the tubulin available for assembly was being distributed over an increasing number of polymer ends as the MAP fraction:tubulin ratio was increased.

Stimulation of Ring Formation by the MAP Fraction. The MAP fraction appeared to act in the initiation process by inducing the formation of rings from tubulin dimers (Murphy and Borisy, 1975; Weingarten et al., 1975; Granett et al., 1975), a process which occurred at 4 °C. Indeed, as shown in Figure 6a, as increasing concentrations of the MAP fraction were added to a constant concentration of tubulin dimers at 4 °C, the number of rings formed in the preparation increased proportionally to the concentration of MAP fraction added. This increase in the number of rings, as the MAP fraction: tubulin ratio was increased, was also reflected in a proportional increase in the initial rate of assembly (Figure 6b).

Analysis of the Stability of MAPs 1 and 2 in the MAP Fraction. In order to study the stability of the MAP fraction obtained by phosphocellulose chromatography, the fraction was stored at 4 °C for various times after which aliquots containing equal amounts of protein were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. As shown in Figure 7, with increasing storage time at 4 °C the bands corresponding to MAPs 1 and 2 broke down almost completely into a heterogeneous population of polypeptides migrating faster than MAP 2. The inclusion of NaN_3 and thymol in the MAP fraction to inhibit bacterial growth during storage at 4 °C had no effect on the breakdown of MAPs 1 and 2.

Analysis of the breakdown of MAPs 1 and 2 by quantitative staining of the gels with Fast green is shown in Figure 8. Before storage at 4 °C, MAPs 1 and 2 accounted for approximately 36% of the total protein in the MAP fraction (Figure 8a). However, after 157 h at 4 °C, this value had decreased to approximately 13% of the total protein because of the breakdown of MAPs 1 and 2 (Figure 8d). The products of this hydrolysis became distributed through the gel primarily in the region between molecular weights of 75 000–165 000.

An antibody, specific to MAP 2 as determined by immunoelectrophoresis (see below), was prepared and reacted with the MAP fraction at various stages of breakdown of MAP 2. When judged by double diffusion in 1% agarose, the antibody reacted equally well with the MAP fraction before and after MAP 2 had broken down. Resolution of the proteins in the intact MAP fraction by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoelectrophoresis of the MAP fraction after the method of Converse and Papermaster (1975), showed that the antibody reacted specifically with MAP 2 (Figure 9a). However, immunoelectrophoresis of the MAP fraction, which had been stored at 4 °C for 8 days and from which MAPs 1 and 2 had almost completely disappeared, showed that the antibody reacted with proteins that migrated more slowly than tubulin but faster than MAP 2 on sodium dodecyl sulfate–polyacrylamide gels (Figure 9b). Therefore, during storage of the MAP fraction at 4 °C new polypeptides appeared that had the same antigenic sites as MAP 2, suggesting that they resulted from the breakdown (hydrolysis) of MAP 2.

Effect of Breakdown of MAPs 1 and 2 on the Stimulation of Tubulin Assembly by the MAP Fraction in Vitro. Several proteins are known to retain their biological activity after enzymatic cleavage to lower molecular weight polypeptides. For example, the specific cleavage of the muscle protein myosin by trypsin results in the production of a peptide fragment, heavy meromyosin (HMM), that retains the ATPase activity

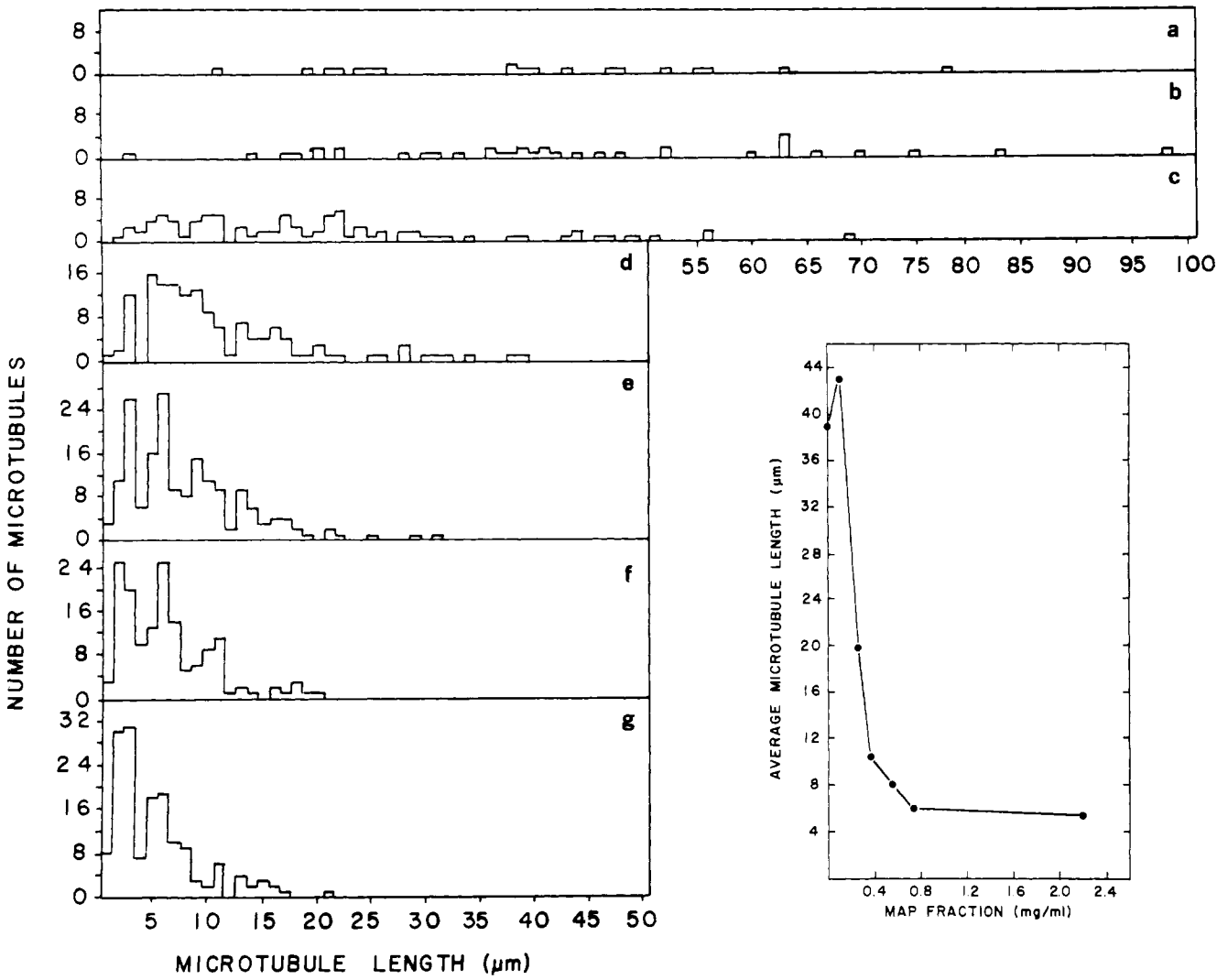

FIGURE 5: Histogram showing the number of microtubules (ordinate) of various lengths (abscissa) obtained at apparent equilibrium as a function of the MAP fraction concentration. Tubulin concentration was held constant at 3.4 mg/ml. MAP fraction concentrations were: (a) 0 mg/ml; (b) 0.11 mg/ml; (c) 0.26 mg/ml; (d) 0.37 mg/ml; (e) 0.55 mg/ml; (f) 0.74 mg/ml; (g) 2.20 mg/ml. Inset: data plotted to show mean lengths of microtubules as a function of MAP fraction concentration.

and actin binding ability of native, intact myosin (Szent-Gyorgyi, 1953). Similarly, a tryptic fragment (fragment A) of sperm tail dynein can be prepared that retains most of the ATPase activity of the original molecule (Ogawa, 1973), and an antibody prepared against fragment A reacts with both fragment A and intact dynein (Ogawa and Mohri, 1975).

Since the major proteins in the MAP fraction (MAPs 1 and 2) were hydrolyzed into smaller molecular weight components during storage at 4 °C, it was of interest to determine if the MAP fraction also lost its ability to stimulate the initiation of tubulin dimer assembly and/or to affect the total amount of microtubules assembled. In order to test this, tubulin dimers were stimulated to assemble by aliquots of the MAP fraction that had been stored for various times at 4 °C. The effect of the breakdown of MAPs 1 and 2 on the total amount of assembly and the initial rate of assembly for three different MAP fraction:tubulin ratios is shown in Figure 10. During the breakdown of MAPs 1 and 2 the total mass of microtubules stimulated to assemble by the MAP fraction remained constant (Figure 10a); however, the initial rate of the assembly reaction decreased with the breakdown of MAPs 1 and 2 (Figure 10b) at each MAP fraction:tubulin ratio tested. Thus, storage at 4 °C differentiated between the two activities of the MAP fraction; i.e., the initiating activity of the MAP fraction de-

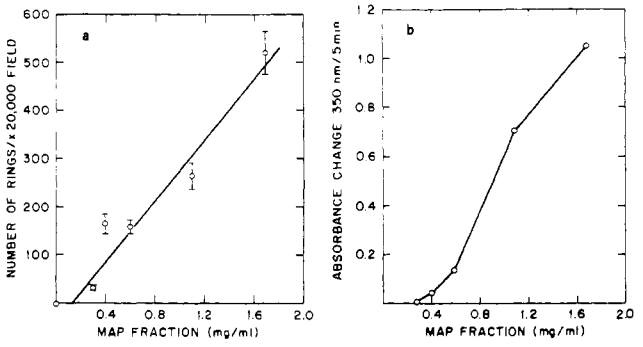

FIGURE 6: Effect of increasing the concentration of the MAP fraction with a constant concentration of tubulin (3.1 mg/ml) on (a) the number of rings in randomly selected ×20 000 fields and (b) the initial rate of assembly in the same preparation. Error bars in (a) denote + and − 1 standard error of the mean.

cayed with storage at 4 °C, while the ability of the MAP fraction to affect the total amount of assembly remained the same.

Trypsin Digestion of the MAP Fraction. Trypsin has been used to effect the controlled hydrolysis of the MAP fraction in an attempt to reproduce the breakdown of MAPs 1 and 2. In these experiments, aliquots of the MAP fraction (3 mg/ml)

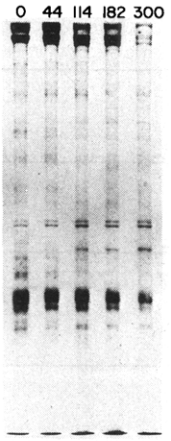

FIGURE 7: Breakdown of MAPs 1 and 2 during storage at 4 °C, as monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Aliquots of the MAP fraction were prepared for electrophoresis after increasing times of storage at 4 °C, indicated in hours above each gel slot, and equal volumes of these samples were then separated by electrophoresis on an 8% polyacrylamide slab gel. The gel was stained with Coomasie blue.

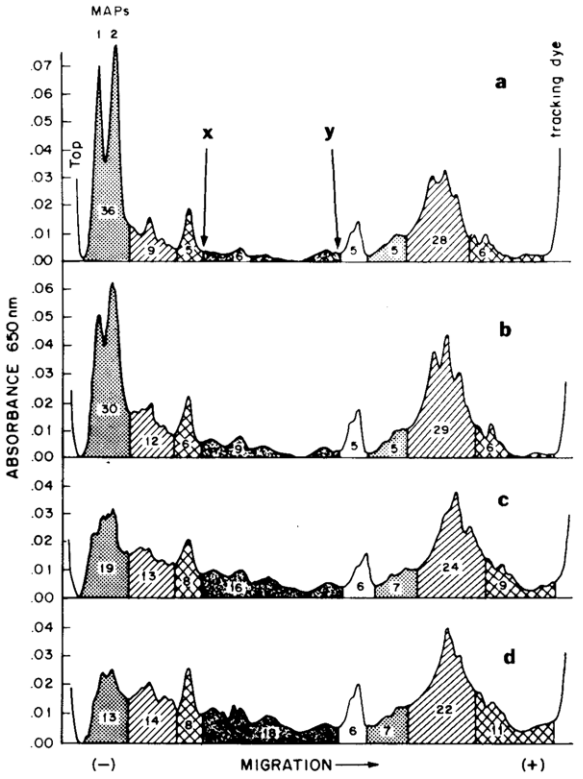

FIGURE 8: Quantitation of the breakdown of MAPs 1 and 2 by sodium dodecyl sulfate–polyacrylamide gels stained with Fast green. The length of storage of the MAP fraction at 4 °C was (a) 0, (b) 63, (c) 109, and (d) 157 h. The gel scans (650 nm) are divided into eight sections on the basis of R_f values, and the percent of total protein is indicated in each section. Arrow X indicates a molecular weight of approximately 165 000 and arrow Y a molecular weight of approximately 75 000.

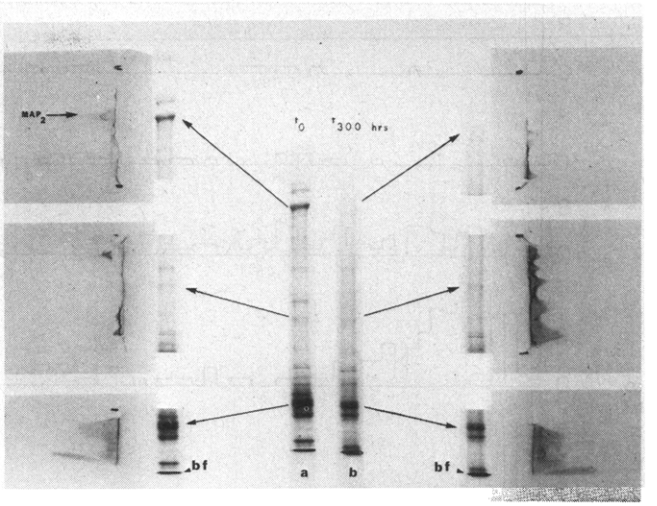

FIGURE 9: Immunoelectrophoresis of the MAP fraction, using an antibody to MAP 2, before (a, 0 h) and after (b, 300 h) breakdown of MAPs 1 and 2. Aliquots of each sample were separated on a 4–10% acrylamide gradient slab gel in the presence of 0.1% sodium dodecyl sulfate and immunoelectrophoresis was performed in the second dimension after the method of Converse and Papermaster (1975). Note that in the 0-h sample the antibody reacts specifically with MAP 2, while in the 300-h sample, in which MAPs 1 and 2 have almost completely broken down, the antibody reacts with several polypeptides with molecular weights less than MAP 2. The sample buffer for the first dimension contained 2% sodium dodecyl sulfate, and thus the unbound sodium dodecyl sulfate that moved with the buffer front (bf) passed through the protecting Lubrol layer and nonspecifically precipitated antibody in the area of the buffer front.

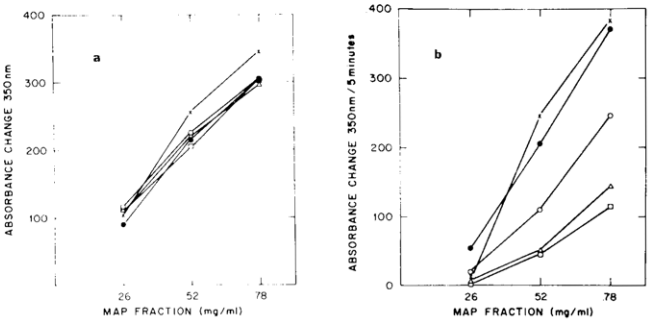

FIGURE 10: (a) Effect of storage of the MAP fraction at 4 °C on the total amount of assembly obtained with a constant concentration of tubulin (1.8 mg/ml). Three different concentrations of the MAP fraction were used. Total assembly was determined turbidimetrically as the difference between the initial A_{350} and the apparent equilibrium A_{350} values. Increasing times of storage of the MAP fraction at 4 °C are denoted as follows: (x—x) 0 h, (●—●), 37 h, (○—○), 60 h, (□—□) 130 h, and (△—△) 177 h. (b) Effect of storage of the MAP fraction at 4 °C on the initial rate of assembly of a constant concentration of tubulin (1.8 mg/ml) stimulated by three different concentrations of the MAP fraction. Symbols are the same as in (a).

were incubated with trypsin (1 μg/ml) at 25 °C for 1–5 min. Initially, tryptic fragments of the MAPs appeared, having molecular weights of 200 000–250 000, with a concomitant decrease in the amount of MAPs 1 and 2. After longer digestion times, MAPs 1 and 2 were completely broken down and the tryptic fragments that resulted ranged in size from approximately 80 000–200 000 daltons. When MAP fractions digested for varying lengths of time were tested for their ability to stimulate the assembly of tubulin dimers, it was observed that the initial rate of the reaction decreased with increased digestion. This suggested that the trypsin-digested MAP fraction was less efficient at stimulating the initiation of microtubule assembly. However, the total amount of assembled material obtained for any given MAP fraction:tubulin ratio was much less affected by trypsin digestion. Thus, tryptic digestion of the MAP fraction closely paralleled the effects of storage at 4 °C (see above) on both the hydrolysis of MAPs 1 and 2 and on the ability of the MAP fraction to stimulate tubulin dimer assembly. These results will be reported in more detail elsewhere (R. D. Sloboda and J. L. Rosenbaum, manuscript in preparation).

TABLE I: Effect of Storage of the MAP Fraction on Microtubule-Associated Filaments.[a]

Hours of MAP Storage at 4 °C	No. of Filaments/μm of Microtubule	Average Filament Length (nm)
0	14 ± 5	41 ± 13
63	5 ± 2	28 ± 11
157	5 ± 2	24 ± 11

[a] Tubulin dimers were stimulated to assemble into microtubules by a MAP fraction that was stored at 4 °C for varying lengths of time. The microtubules were then sedimented and thin sectioned. The number and length of microtubule-associated filaments were determined from electron micrographs.

Initiation of Microtubule Assembly after Storage of the MAP Fraction. It appeared from the results above that during storage at 4 °C the MAP fraction lost its ability to initiate microtubule assembly. In order to determine this more directly, tubulin dimers (1.5 mg/ml) were stimulated to assemble by aliquots of the MAP fraction (1.7 mg/ml) stored at 4 °C for varying periods of time, and microtubule lengths were determined after equilibrium was reached. The results showed that as the MAP fraction decayed with storage at 4 °C, the average length of the microtubules assembled at the apparent equilibrium point increased from 9 to 49 μm. This indicated that the same amount of tubulin dimers was being distributed over a decreasing number of polymer ends and, therefore, that the ability of the MAP fraction to initiate microtubule assembly was being lost. Presumably, the number of initiation centers (rings) induced to form by the MAP fraction at 4 °C would also decrease with increasing storage times, and this would be reflected in the longer average lengths obtained at the apparent equilibrium point of the reaction.

Breakdown of MAPs 1 and 2 and the Ultrastructure of in Vitro-Assembled Microtubules. Since it has been demonstrated that MAPs 1 and 2 form the filaments that coat the surface of in vitro-assembled microtubules (Dentler et al., 1975; Murphy and Borisy, 1975), it was of interest to study the filaments on microtubules stimulated to assemble by MAP fractions that had decreased amounts of MAPs 1 and 2. Microtubules stimulated to assemble by MAP fractions containing normal and decreased amounts of MAPs 1 and 2 both had a filamentous coating (Figure 11), but the filaments were shorter and fewer in number on the microtubules stimulated by MAP fractions in which MAPs 1 and 2 were present in decreased amounts. The quantitation of these results (Table I) showed that the microtubule-associated filamnts decreased in length by at least 50% and that the number of filaments/μm of microtubule also decreased concomitant with the decrease in the amount of MAPs 1 and 2 in the MAP fraction as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

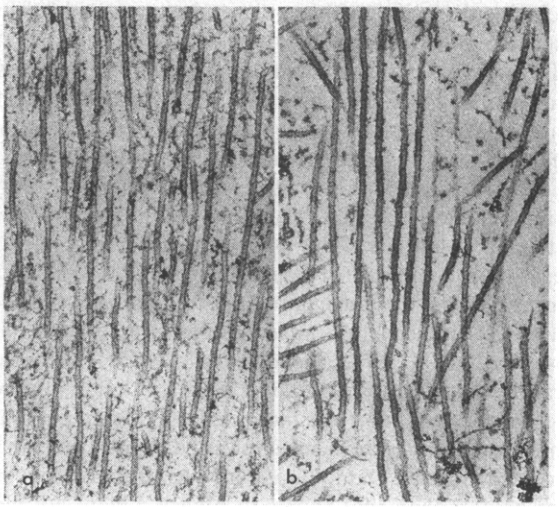

FIGURE 11: Thin sections of microtubules stimulated to assemble by (a) freshly prepared MAP fraction and (b) MAP fraction that was stored at 4 °C for 157 h. Compare figure with the data on average length and number of microtubule-associated filaments shown in Table I.

Discussion

Preparations of in vitro-assembled brain microtubules have been separated into two fractions by ion-exchange chromatography on phosphocellulose (Figure 1; see also Weingarten et al., 1975). One fraction contained primarily tubulin (Figure 2b) while the other was composed of several microtubule-associated proteins (MAPs; see Sloboda et al., 1975), the main components of which were MAP 1 and MAP 2 (Figure 2c). Neither fraction alone formed microtubules when warmed to 37 °C, but when the tubulin-free MAP fraction was combined with tubulin dimers (Figure 2d) and warmed to 37 °C, microtubules assembled (Figure 3).

This stimulation of tubulin dimer assembly appeared to result from the ability of the MAP fraction to induce the tubulin dimers to form rings which acted as initiation centers for the polymerization of microtubules when the solution was subsequently warmed to 37 °C. This sequence of events was suggested by the following evidence: (1) it was previously reported that brain supernatants from which the rings were removed by high-speed centrifugation did not assemble microtubules when warmed to 37 °C (Borisy and Olmsted, 1972) or did so only after a long lag period (Borisy et al., 1974; Rosenbaum et al., 1975); (2) it was also shown that the rings disappeared within the first 2–3 min of an assembly reaction even though the polymerization continued until a plateau was reached 15–20 min later (Borisy et al., 1975; Dentler and Rosenbaum, 11975; Kirschner et al., 1975); (3) in this report, as the MAP fraction concentration was increased relative to a constant concentration of tubulin, the number of rings formed in the preparation at 4 °C increased proportionately (Figure 6a), and the initial rate of the reaction also increased with the amount of MAP fraction added (Figure 4b and 6b); also, as the MAP fraction concentration was increased relative to a constant concentration of tubulin, the average length of the microtubules formed at the apparent equilibrium point of the reaction decreased (Figure 5), a direct result of increased initiation. Taken together, the results summarized above strongly suggest, although they do not conclusively prove, that the rings are intermediates in the initiation of microtubule polymerization in vitro and that the MAP fraction functions in initiation through its ability to stimulate the formation of rings from tubulin dimers.

In addition to inducing the initiation of microtubule assembly, the MAP fraction also affected the apparent tubulin dimer–polymer equilibrium, since increased amounts of polymer were assembled as the MAP fraction:tubulin ratio was increased. In this discussion, this effect is referred to as the "elongation" activity of the MAP fraction. However, this effect could also be thought of as microtubule "stabilization", since Haga and Kurokawa (1975) have shown that microtubules assembled in the presence of the MAPs are more stable to

colchicine dissociation than microtubules assembled in the absence of the MAPs.

The increase in total amount of microtubules assembled was a linear function of the amount of MAP fraction added until a ratio of MAP fraction to tubulin was reached beyond which linear increases no longer occurred (Figure 4a). Saturation of the tubulin in this manner with respect to total assembly occurred at an average MAP fraction:tubulin ratio of approximately 0.3:1 on a weight basis, which corresponded to a preparation composed of approximately 77% tubulin. This value fell within the range of tubulin composition reported for in vitro-assembled brain microtubules (Borisy et al., 1975; Rosenbaum et al., 1975; Sloboda et al., 1975). Furthermore, it can be seen from the data in Figure 4a that, as the MAP fraction concentration was increased, the maximum amount of microtubule assembly tended toward a plateau while the rate increased linearly. Data points for MAP fraction concentrations greater than that shown in Figure 4 were not plotted because of complicated overshoot kinetics that occurred at high MAP fraction:tubulin ratios (0.8:1 and greater on a weight basis). This behavior made it difficult to accurately determine when an apparent equilibrium had been reached.

In contrast to the total amount of assembly, the increase in initial rate of assembly did not become saturated above MAP fraction:tubulin ratios of 0.3:1 but rather continued to increase approximately linearly (Figure 4b). This behavior suggested that fewer tubulin dimers interacted with the MAP fraction during the formation of initiation centers than the number that interacted with the MAP fraction during microtubule elongation. However, at low ratios of MAP fraction:tubulin (less than 0.1:1 on a weight basis) the curve showing initial rate as a function of MAP fraction concentration departed from linearity (Figure 4b). This may be indicative of a cooperative effect between a component of the MAP fraction and the tubulin dimers that is less efficient at low MAP fraction:tubulin ratios for the formation of initiation centers (rings).

The proteins in the MAP fraction responsible for affecting microtubule elongation may be MAPs 1 and 2, which have been shown to copurify with (Borisy et al., 1975; Sloboda et al., 1975) and to form the filaments which coat the surfaces of in vitro-assembled brain microtubules (Dentler et al., 1975: Murphy and Borisy, 1975). Although the gel bands corresponding to MAPs 1 and 2 disappeared from the MAP fraction during storage at 4 °C (Figures 7–9), the breakdown products were still capable of associating with the assembled tubulin. This conclusion was based on the following biochemical and morphological evidence: (1) microtubules induced to assemble by the MAP fraction, in which MAPs 1 and 2 were almost completely broken down, still had filaments associated with them (Figure 11); however, the filaments were of shorter length and of lower density (fewer filaments/μm) than the filaments on microtubules induced by MAP fractions in which MAPs 1 and 2 were still intact (Table I); (2) an antibody made against intact MAP 2 reacted with polypeptides of molecular weights less than MAP 2 but greater than tubulin only after the breakdown of MAPs 1 and 2 as shown by immunoelectrophoresis (Figure 9); the progressive breakdown of MAPs 1 and 2 into lower molecular weight components was further supported by quantitative gel electrophoresis (Figure 8).

The results showing that the products of the hydrolysis of MAPs 1 and 2 were still capable of associating with the microtubules during elongation might be possible if only a portion of the filament were active in promoting elongation, that portion being the end of the filament associated with the wall of the microtubule. The fact that the number of filaments/μm of microtubule decreased (Table I), and yet the same total amount of assembly was obtained from MAP fractions that had lost MAPs 1 and 2, further supported the suggestion that the association of only a portion of MAPs 1 and 2 possibly too small to be visualized as a filament in the electron microscope, was all that was necessary to promote elongation.

It has been shown here that MAPs 1 and 2 are involved in *both* the initiation and elongation processes, but that after their breakdown they are less efficient at initiation (Figure 10b), but equally effective at stimulating elongation (Figure 10a). This dual function might be possible if MAPs 1 and 2 formed a bipolar filament, each end of which could insert into the microtubule wall to promote elongation. After breakdown, there would still be sufficient ends of the filaments to stimulate the elongation process and the total amount of assembly would remain constant (Figure 10a). On the other hand, the intact bipolar filament might be the most efficient form for bringing tubulin dimers together to form an initiation center, and breakdown into monopolar filaments might cause a decrease in the initiation capacity of MAPs 1 and 2 (Figure 10b). It will be important, therefore, to know the stoichiometry of MAPs 1 and 2 in the filaments as well as the capacity of the ends of the filament to attach to the microtubule wall.

The protein tau, with a molecular weight of ca. 70 000 (Cleveland et al., 1975), reported by Weingarten et al. (1975) to be necessary for tubulin assembly in vitro, may be a breakdown product of MAPs 1 and 2, since the immunoelectrophoresis data (Figure 9) and quantitative gel electrophoresis (Figure 8) showed that the breakdown products of MAPs 1 and 2 which were still active in stimulating assembly were polypeptides with molecular weights between approximately 75 000 and 165 000. However, further purification and characterization of MAPs 1 and 2 will be necessary to determine this.

It has recently been demonstrated that a variety of positively charged proteins (e.g., lysozyme, RNAase, histone, and nerve growth factor) can initiate microtubule assembly from tubulin dimers in vitro (Behnke, 1975; Erickson, 1975; Jacobs et al.,1975; Levi et al., 1975), although it is not clear that these microtubules have normal ultrastructure. Because of these observations, one still should be cautious about the possible in vivo role of MAPs in microtubule initiation and elongation. However, in support of their role in the assembly of microtubules in vivo are results showing (1) that MAPs 1 and 2 copurify with tubulin at a constant ratio through at least five cycles of assembly–disassembly in vitro (Borisy et al., 1975; Sloboda et al., 1975), and that (2) similar high-molecular-weight proteins are found associated with intact microtubules isolated from brain homogenates (Kirkpatrick et al., 1970).

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