

THE PERIODIC ASSOCIATION OF MAP₂ WITH BRAIN MICROTUBULES IN VITRO

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ABSTRACT

Several high molecular weight polypeptides have been shown to quantitatively copurify with brain tubulin during cycles of in vitro assembly-disassembly. These microtubule-associated proteins (MAPs) have been shown to influence the rate and extent of microtubule assembly in vitro. We report here that a heat-stable fraction highly enriched for one of the MAPs, MAP₂ (mol wt ~300,000 daltons), devoid of MAP₁ (mol wt ~350,000 daltons), has been purified from calf neurotubules. This MAP₂ fraction stoichiometrically promotes microtubule assembly, lowering the critical concentration for tubulin assembly to 0.05 mg/ml. Microtubules saturated with MAP₂ contain MAP₂ and tubulin in a molar ratio of ~1 mole of MAP₂ to 9 moles of tubulin dimer. Electron microscopy of thin sections of the MAP₂-saturated microtubules fixed in the presence of tannic acid demonstrates a striking axial periodicity of 32 ± 8 nm.

KEY WORDS brain microtubule · brain tubulin · in vitro assembly · microtubule arm microtubule-associated protein · microtubule ultrastructure · thermostable protein

Brain tubulin copurifies with at least two high molecular weight proteins which have been shown to maintain a constant stoichiometry to tubulin through several cycles of assembly and disassembly (4, 30, 44, 52). These high molecular weight proteins, called MAPs (44) for microtubule-associated proteins, have been shown to influence both the initiation and elongation processes of microtubule assembly in vitro (30, 31, 43). By fluorescence microscopy, using fluorescein-labeled MAP antibodies, the MAPs have been shown to be distributed all along the lengths of microtubules *in situ* (9, 41). The MAPs have also been visualized by electron microscopy as a filamentous coating on the surfaces of microtubules

assembled in vitro (10, 29, 41, 42). The filamentous coating resembles the filaments observed on neuronal microtubules *in situ* (14, 45, 46), and sodium dodecyl sulfate (SDS) electrophoresis has shown isolated, intact neurotubules to consist, in part, of polypeptides of molecular weights similar to those of the MAPs (20).

The in vivo functions of these filamentous MAPs are unknown, but several hypotheses can be proposed: (a) they may impart structural stability, by bridging microtubules to each other, to 100 Å filaments, or to other structures such as the cell membrane; (b) they may have a motile function and be directly involved in the translocation of particles and organelles; or (c) they may provide structural support for an actomyosin system which in turn may be directly involved in translocation of particles. There is ultrastructural or biochemical evidence for the first two possibilities (references 14, 45, 46, see 11 for others) but little

evidence for the third, although actin and myosin are present in neurons (5, 7, 18, 21, 22, 23).

Because microtubules are composed of subunits assembled in a regular periodic fashion (2, 12), it is reasonable to expect that structures associated with them will also show a periodicity which reflects some multiple of the subunit periodicity. The dynein arms and radial links, periodically arranged on the outer doublet microtubules of cilia and flagella, are examples of this (16). Recently, Amos (2), using negatively stained preparations of *in vitro* assembled neurotubules, was able to show by optical diffraction a longitudinal periodicity of 32 nm attributed to the MAPs. This periodicity, however, was difficult to observe directly in the negatively stained preparations.

This report demonstrates that a heat-stable fraction highly enriched for one of the MAPs, MAP₂, will stoichiometrically promote the assembly of microtubules, decorating them with projections whose periodicity can be visualized in thin-sectioned material by use of improved staining procedures.

MATERIALS AND METHODS

Preparation of Microtubule Protein

Twice-cycled microtubule protein (2 × MT) was prepared from calf brain tissue by a modification (4) of the Shelanski et al. (39) procedure, except that the second polymerization was done without glycerol. The protein was frozen as microtubule pellets in liquid nitrogen and stored at -20°C.

Purification of Tubulin Dimers

Tubulin dimers were obtained by molecular sieve chromatography of 2 × MT as described by Sloboda et al. (43), using a Bio-Gel A 1.5 M column (Bio-Rad Laboratories, Richmond, Calif.) (gel bed dimensions 2.5 × 25 cm) equilibrated in column buffer (CB): 50 mM piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES), pH 6.9, 0.5 mM MgSO₄, 1 mM ethylene glycol-bis-(β aminoethyl ether) N,N'-tetraacetic acid (EGTA), 0.1 mM Guanosine-5'-triphosphate (GTP). Tubulin dimers eluted in the peak corresponding to an apparent molecular weight of 100,000 daltons (43); this peak was pooled and concentrated to 4-8 mg/ml by pressure dialysis using an Amicon Ultrafiltration apparatus and a PM30 filter (Amicon Corp., Lexington, Mass.). The protein either was used immediately or was stored as droplets in liquid nitrogen.

Purification of MAPs

Unfractionated MAPs were obtained by ion-exchange chromatography of 2 × MT on phosphocellulose accord-

ing to a modification (43) of the method of Weingarten et al. (53), and clarified by centrifugation at 160,000 g for 1 h at 4°C in a Type 65 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The clarified supernate was desalted into CB without GTP by centrifugation through Sephadex G-25 (34) and stored as droplets in liquid nitrogen.

MAP₂ Purification

A heat-stable fraction was obtained from 2 × MT by the following modification of the method of Fellous et al. (13): 2 × MT pellets were resuspended in CB containing 2 mM dithiothreitol (DTT) and 0.75 M NaCl, depolymerized for 30 min at 0°C, and then immersed in a 100°C water bath for 5 min. A clear supernate was obtained by centrifugation of the heated solution at 40,000 g for 30 min at 4°C. This supernate was concentrated to ~10 mg/ml by pressure dialysis with an Amicon filter. To further enrich for MAP₂, the supernate was chromatographed on a Bio-Gel A 1.5 M column (gel bed dimensions: 1.5 × 55 cm) equilibrated in the same buffer used for the heating. 1.5-ml fractions were eluted with a hydrostatic pressure head of 100 cm and monitored for absorbance at 280 nm. The fractions were pooled as shown in Fig. 2, concentrated in dialysis tubing with Aquacide (Calbiochem, San Diego, Calif.), and then desalted into CB without GTP. These fractions were frozen and stored as droplets in liquid nitrogen.

Protein Determination

Protein concentrations were determined by a modification (38) of the method of Lowry et al. (27), using bovine serum albumin as the standard.

Electrophoresis

SDS-urea polyacrylamide slab gel electrophoresis was carried out according to the method of Laemmli (24), except that the gels contained a 4-16% acrylamide gradient, a 1-8 M urea gradient, and no SDS in either the stacking or the separating gel (J. Jarvik, unpublished data). The gels (20 × 15 × 0.15 cm) were stained with Coomassie Brilliant Blue R. For quantitation, samples were electrophoresed on 8% Laemmli slab gels (8 × 16 × 0.075 cm), which were stained and fixed in 0.5% Fast Green (17) in 50% methanol-7% acetic acid for 1 h at 55°C, and then destained in 5% methanol-7% acetic acid for 1-2 h at 55°C; appropriate channels were cut out of the slab and scanned at 650 nm on a Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) equipped with a linear transport. The peak areas were determined by planimetry.

Polymerization Assay

Microtubule polymerization was monitored by continuously recording the increase in absorbance at 350 nm (15) in a Gilford spectrophotometer equipped with a temperature-controlled cuvette. All polymerization was

carried out in CB, with the GTP adjusted to 1 mM. All components for the reactions were mixed at 4°C, and polymerization was initiated by raising the temperature from 4° to 37°C.

Electron Microscopy

Negative-stain electron microscopy was performed as described previously (37). Thin-section electron microscopy was carried out using a modification of procedures developed by Tilney et al. (48) and Begg and Rebhun (3): Microtubules were sedimented at 40,000 g for 30 min at 25°–30°C. The pellets were fixed overnight at room temperature in 1% glutaraldehyde, 1% tannic acid, 10 mM NaH₂PO₄, pH 7.0. The pellets were postfixed for 30 min at 4°C with 0.5% osmium tetroxide in 10 mM NaH₂PO₄, pH 6.0, and stained en bloc for at least 6 h with 1% aqueous uranyl acetate. The samples were dehydrated with acetone, embedded in Spurr's medium (47), and sectioned on a Porter-Blum MT-2 ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.). Sections were stained for 30 min in 2% uranyl acetate in 12.5% methanol and 35% ethanol, then for 30 min in 0.4% lead citrate in 0.1 N NaOH. Electron microscopy was done with a Philips 201 electron microscope, calibrated with a carbon replica of an optical diffraction grating.

RESULTS

Purification of MAP₂ Fraction

Calf brain microtubules purified by two cycles of assembly-disassembly contained tubulin and the high molecular weight MAPs 1 and 2, as well as a number of other minor polypeptides (Fig. 1*a*). It should be noted that both MAPs 1 and 2, which normally migrate as single bands on 8% Laemmli SDS gels, were each resolved into two distinct polypeptides on the acrylamide-urea gradient gels described here. When 2× microtubule protein was heated to 100°C for 5 min, no change in the electrophoretic composition of the total protein was noted (Fig. 1*b*); however, subsequent centrifugation sedimented all of the MAP₁, all of the tubulin¹, and many of the minor species present in the original preparation (Fig. 1*c*). The clear supernate, which contained ~15% of the total protein, was greatly enriched for MAP₂, but still contained other polypeptides of molecular weight lower than that of MAP₂ (Fig. 1*d*).

A further enrichment for MAP₂ was accom-

¹ Although there were two bands in the supernate with molecular weights similar to those of α and β tubulin, these species have been determined not to be tubulin by the 2-dimensional gel electrophoresis technique of O'Farrell (35) (data not shown).

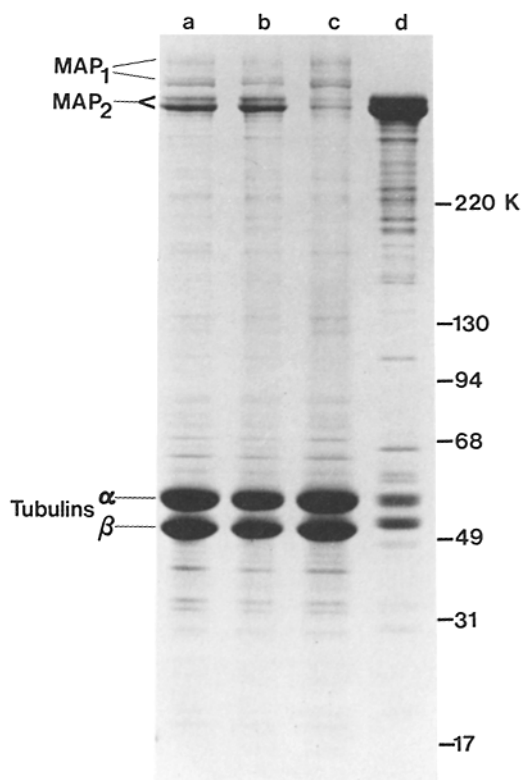


FIGURE 1 SDS-urea polyacrylamide gel electrophoresis showing the purification of the heat-stable fraction from calf brain microtubule protein. (*a*) 40 μg of twice-cycled microtubule protein before exposure to 100°C; (*b*) same as *a*, but after 5-min exposure to 100°C; (*c*) 40 μg of pellet obtained upon centrifugation of *b*; (*d*) 20 μg of supernate obtained upon centrifugation of *b*. The numbers on the right indicate molecular weight standards.

plished by molecular sieve chromatography of the heat-stable supernate, as shown in Fig. 2. ~75% of the MAP₂ in the column load (Fig. 3*a*) eluted in the void volume (Fig. 3*b*). Quantitation of SDS gels of this void volume showed it to be 80% MAP₂ by weight, with no species smaller than 145,000 daltons (Fig. 4). For purposes of discussion, this fraction (Fig. 4) will be referred to as MAP₂.

In Vitro Stimulation of Microtubule Assembly by the MAP₂ Fraction

At a tubulin dimer concentration of 1 mg/ml, where there was no self-assembly, MAP₂ stoichiometrically promoted the assembly of microtubules (Fig. 5). Above an initial MAP₂:tubulin

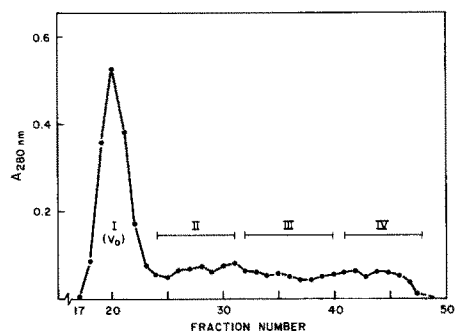


FIGURE 2 Molecular sieve chromatography of supernate of heated $2 \times$ MT (Fig. 1d) on Bio-Gel A 1.5 M. Each fraction is 1.5 ml. Peak I eluted at the void volume; fractions eluting subsequent to the void volume were pooled and concentrated into groups II, III, and IV, as indicated.

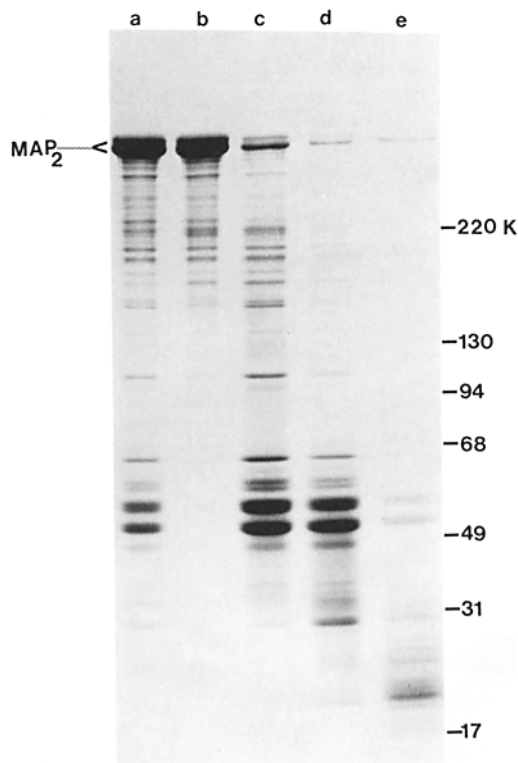


FIGURE 3 SDS-urea gel showing fractionation of heated supernate of $2 \times$ MT on Bio-Gel A 1.5 M. (a) $20 \mu\text{g}$ of column load (heated supernate); (b) $10 \mu\text{g}$ of void volume (Fig. 2, peak I); (c-e) $40 \mu\text{g}$ each of pooled fractions II, III, and IV (see Fig. 2).

mass ratio of 1:1, the total amount of polymer assembled at the apparent equilibrium did not increase, indicating that the system was saturated with respect to MAP_2 . In the presence of saturat-

ing concentrations of MAP_2 , the critical concentration for tubulin assembly was 0.05 mg/ml .

To determine how MAP_2 saturation of tubulin, as indicated turbidimetrically, would be reflected in the assembled microtubules, two samples with initial MAP_2 :tubulin mass ratios of 1:1 and 2:1, both saturated with respect to MAP_2 , were polymerized, pelleted, and analyzed by SDS electrophoresis (Fig. 6). Quantitation of the gels revealed that the mass ratio of MAP_2 :tubulin in both pellets was the same, 0.29 ± 0.018 , or, $\sim 1 \text{ mol MAP}_2/9 \text{ mol tubulin dimer}$, using molecular weights of 300,000 daltons for MAP_2 (44) and 110,000

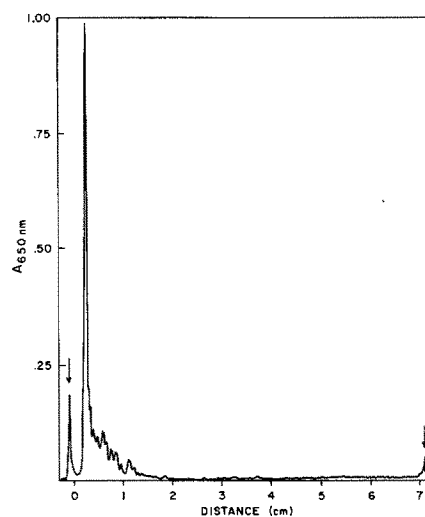


FIGURE 4 Densitometric scan of the MAP_2 fraction after electrophoresis on an 8% Laemmli gel and staining with Fast Green. Left and right arrows indicate top of gel and tracking dye, respectively. $10 \mu\text{g}$ of protein was loaded.

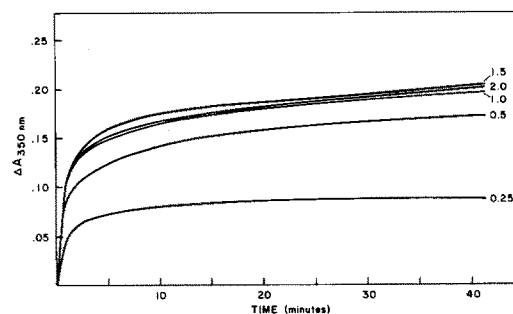


FIGURE 5 Microtubule assembly stimulated by the purified MAP_2 fraction. The tubulin concentration was held constant at 1 mg/ml . The MAP_2 fraction concentration in mg/ml is indicated by the numbers next to the turbidity curves.

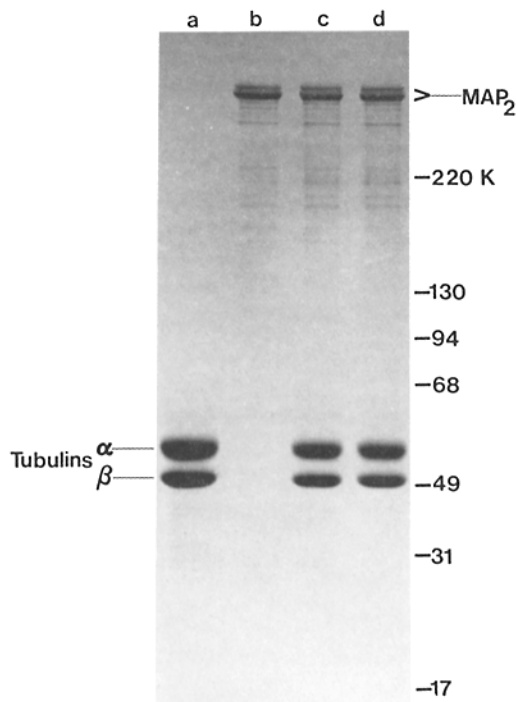


FIGURE 6 SDS-urea gel showing amounts of MAP₂ and tubulin in two different microtubule pellets, both saturated with respect to MAP₂. (a) 30 μ g of the tubulin used in these experiments; (b) 10 μ g of the MAP₂ fraction used in these experiments; (c) 30 μ g of a pellet of microtubules polymerized with an initial MAP₂:tubulin mass ratio of 1:1; (d) 30 μ g of a pellet of microtubules polymerized with an initial MAP₂:tubulin mass ratio of 2:1.

daltons for tubulin (25). For this quantitation, only the dominant MAP₂ bands, indicated in Figs. 1, 3, and 6, were taken into account.

MAP₂ Axial Periodicity

When thin sections of MAP₂-saturated microtubules were examined by electron microscopy, periodic filamentous projections could be seen along the lengths of many of the microtubules (Fig. 7a, 8, and 9c). Direct measurement of spacings between adjacent projections yielded an axial periodicity of 29 ± 2 nm. Because of the morphological nature of the projections, however, direct measurement of spacings often could only

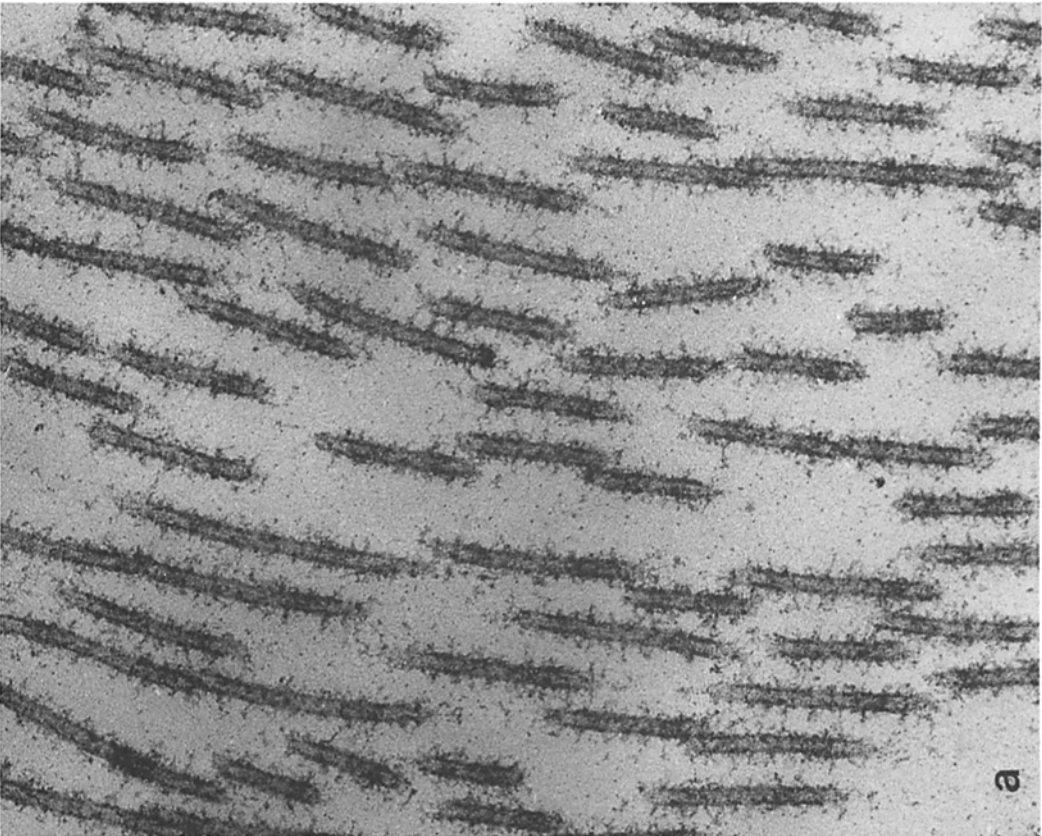
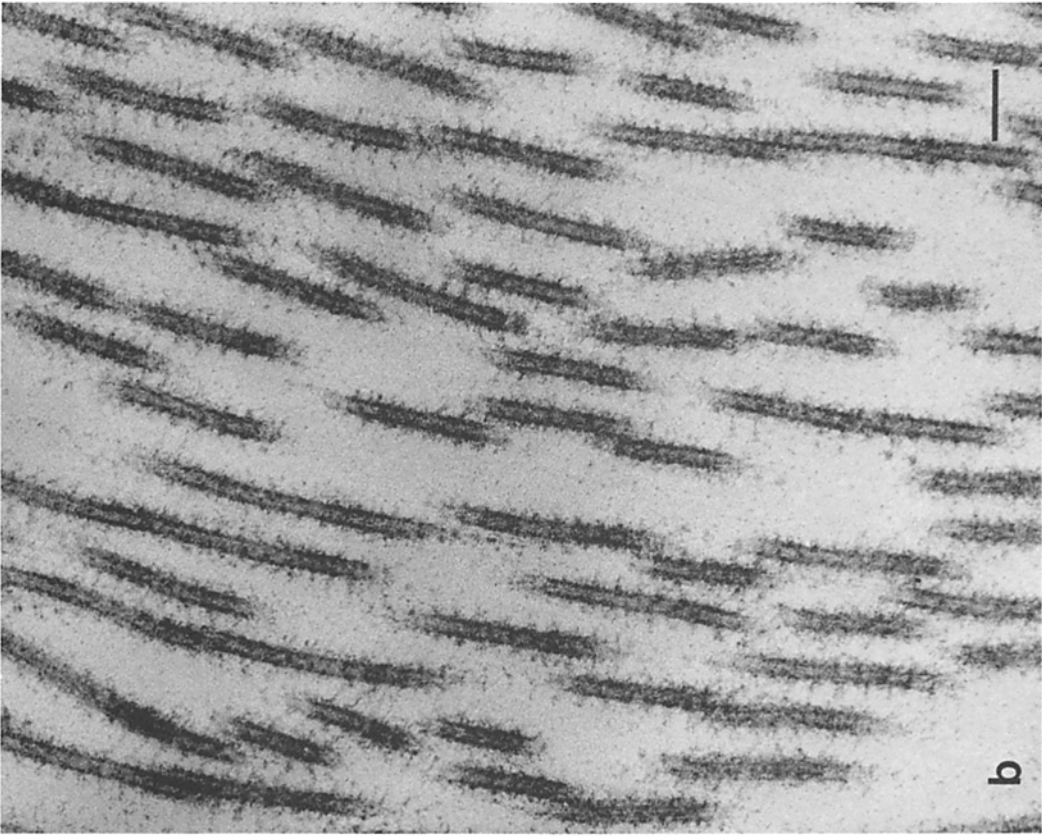
be made along short distances of microtubules. To enhance the MAP₂ axial periodicity, two types of translational superpositioning procedures (29) were employed.

The first procedure involved the superimposition of duplicate negatives containing fields of microtubules oriented parallel to one another. One negative was moved along the microtubule axis while keeping the other negative stationary until a periodicity was enhanced. The negatives were then taped in this position and printed, resulting in whole fields of microtubules with projections whose axial periodicity had been obviously enhanced (Fig. 7b). This method yielded an average periodicity of 36 ± 4 nm. The second method was to project an individual microtubule image, measure a spacing between two projections, and then print multiple exposures on the photographic paper, moving the paper along the microtubule axis the same, measured spacing between exposures. Typical results of this type of analysis are shown in Fig. 8; the average axial periodicity obtained from this method was 30 ± 2 nm. Weighting the three techniques of measurement equally, the axial periodicity was 32 ± 8 nm. Microtubules assembled from tubulin without MAPs appeared smooth-walled (Fig. 9a), and no enhancement of a similar axial periodicity due to projections was obtained, using the translation techniques described.

Analysis of Transverse Sections

Electron microscopy of transverse sections of three different microtubule populations (tubulin alone, tubulin plus saturating levels of MAP₂, and tubulin plus saturating levels of unfractionated MAPs) revealed that, when subjected to identical centrifugation conditions (see Materials and Methods), the different populations of microtubules sedimented with different center-to-center spacings. Center-to-center spacing here refers to the distance from the center of one microtubule to the center of another. Microtubules polymerized without MAPs tended to sediment very closely together, in clusters, with a center-to-center spacing of 44 ± 18 nm (Fig. 9a). Microtubules polymerized with unfractionated MAPs (predom-

FIGURE 7 Thin sections of microtubules saturated with respect to MAP₂. (b) was produced by moving duplicate negatives of a relative to each other until the best reinforcement of an axial periodicity on the microtubules was evident. In this case, the distance moved was equivalent to 33 nm. Bar, 0.2 μ m.



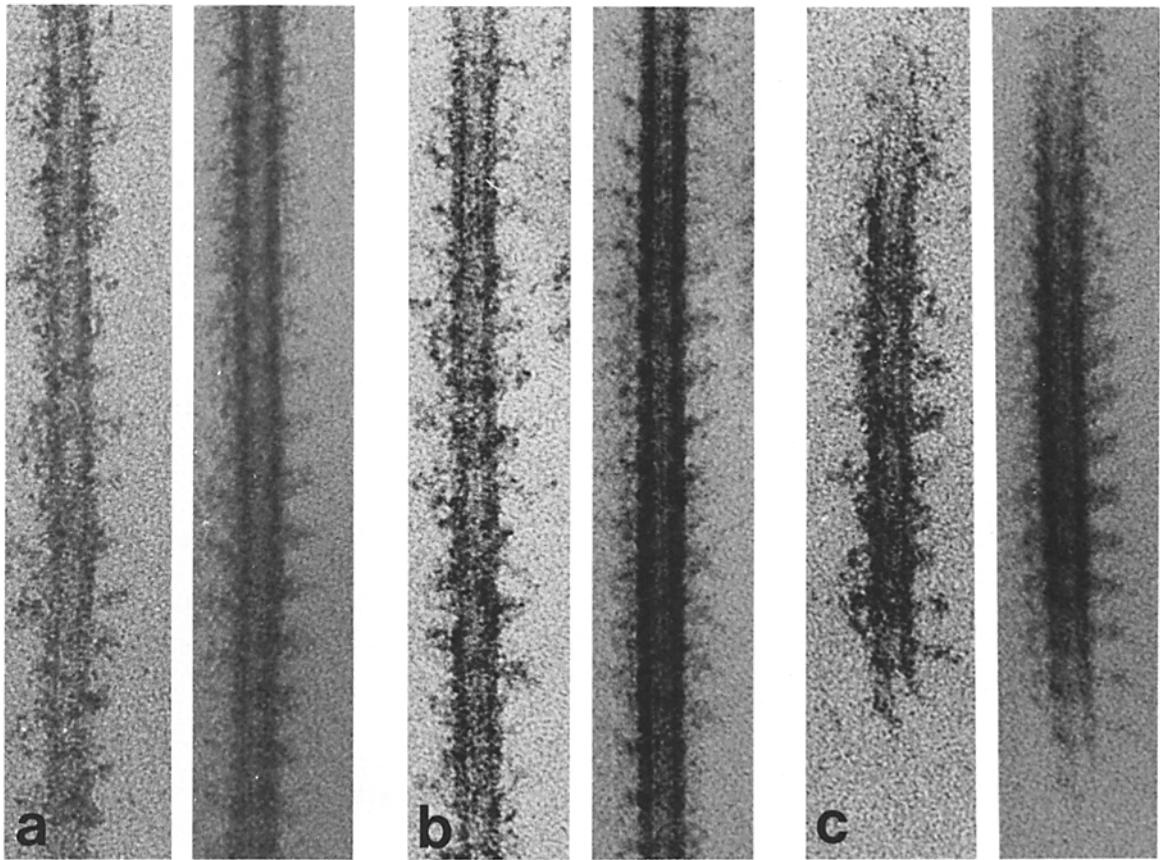


FIGURE 8 Electron micrographs of thin sections of MAP₂-saturated microtubules before and after translational superposition. In each pair of microtubule images, the right one was produced by translational superposition along the axis of the microtubule by a distance determined by measuring a spacing between projections on the original microtubule in the left image. (a) was photographed 3 times, with the translation increment equivalent to 29 nm; (b) was photographed 3 times, with the translation increment equivalent to 28.2 nm; (c) was photographed 4 times, with the translation increment equivalent to 27.5 nm.

inantly MAPs 1 and 2) sedimented with a center-to-center spacing of 75 ± 12 nm (Fig. 9*b*). For microtubules polymerized with the purified MAP₂ fraction, the center-to-center spacing was 89 ± 19 nm (Fig. 9*c*). Assuming a microtubule diameter of 25 nm, then the average distance from one microtubule wall to another was 19 ± 18 nm for microtubules without MAPs, 50 ± 12 nm for microtubules saturated with unfractionated MAPs,

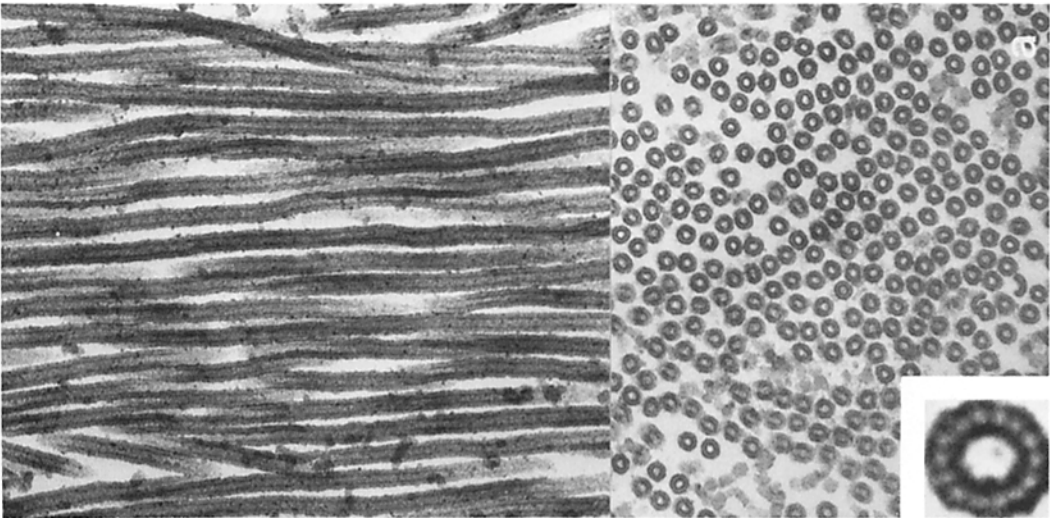
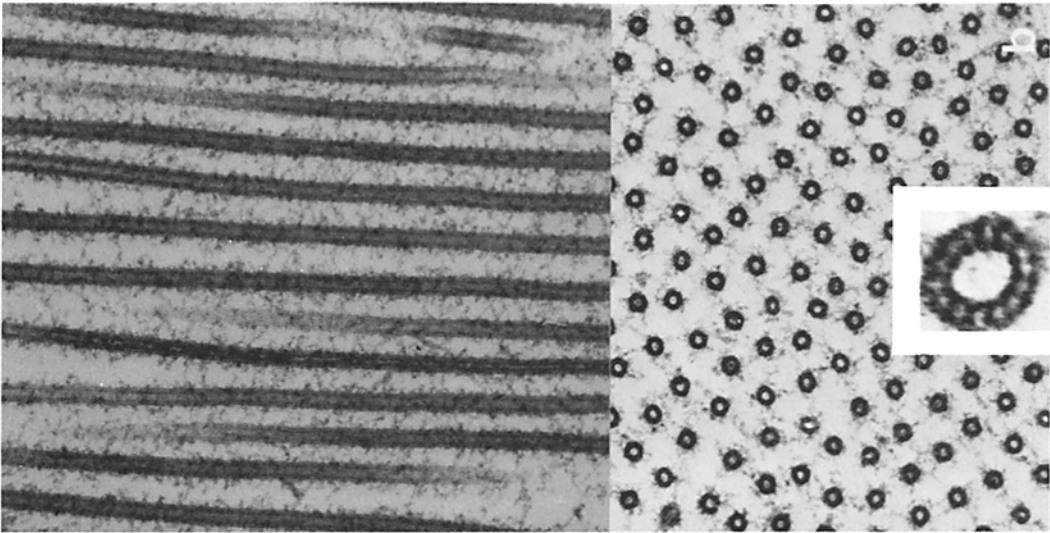
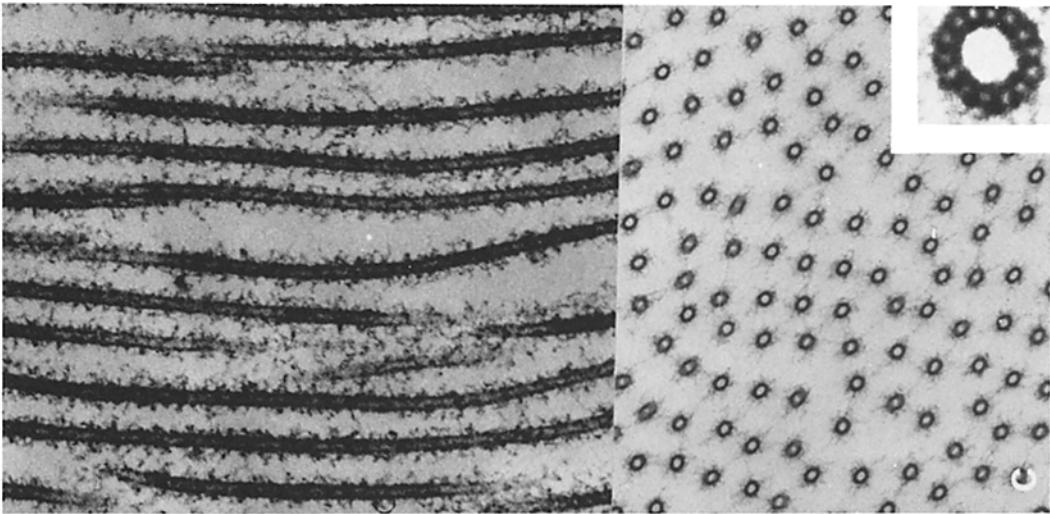
and 64 ± 19 nm for microtubules saturated with the MAP₂ fraction.

It should be noted that 95% of the microtubules polymerized under the experimental conditions described in this report contained 14 protofilaments rather than 13 (Fig. 9, insets).

DISCUSSION

The literature reports many observations of

FIGURE 9 Thin sections of microtubules assembled with and without MAPs. All samples were identically centrifuged for 30 min at 40,000 *g*, at 25°–30°C. Insets are enlargements of representative transverse sections. (a) microtubules polymerized from tubulin only; (b) microtubules saturated with unfractionated MAPs; (c) microtubules saturated with the purified MAP₂ fraction. $\times 76,400$.



“arms” on microtubules (see McIntosh [29] and Dustin [11] for reviews). Arms have been observed *in situ* connecting microtubules to one another, as in the axostyle of *Saccinobaculus* (28), the mitotic apparatus (19), the feeding organelles of certain ciliates (49, 51), and the flagellar axoneme (47, 48), as well as to other cellular organelles such as mitochondria (46), synaptic vesicles (45), membranes (1, 6, 36, 40, 50), and pigment granules (32). With few exceptions (28, 49), however, an axial periodicity of the arms or bridges *in situ* has not been readily observable, although the precise, periodic arrangement of the tubulin dimers in the microtubule (2, 12) would seem to preclude a random binding of associated molecules.

This report demonstrates the visualization of a 32-nm axial periodicity on *in vitro*-assembled brain microtubules due to binding of a fraction enriched for the high molecular weight MAP₂ protein. Although the filamentous decoration of *in vitro*-assembled brain microtubules by unfractionated MAPs has been shown (10, 30), and a periodicity of 32 nm demonstrated by optical diffraction of negatively-stained microtubules (2), this is the first time that the periodic nature of the projections has been readily observed in thin sections of microtubules assembled *in vitro*. This visualization was facilitated by use of saturating amounts of a purified MAP₂ fraction in the assembly mixture, by the inclusion of tannic acid in the fixative solution, and by *en bloc* staining with uranyl acetate.

Both the 32-nm axial periodicity and the molar stoichiometry of 1 MAP₂/9 tubulin dimers reported here “fit” with the helical MAP superlattice proposed by Amos (2) (see also Vallee and Borisy, reference 52). However, this model presupposes a 13-protofilament microtubule, and 95% of the microtubules analyzed in this report contain 14 protofilaments rather than 13 (see Fig. 9, insets). Any deviations in protofilament number will probably affect the MAP superlattice, but the extent of this effect has yet to be determined.

While conclusive data on *in vivo* functions for the arms and bridges on brain microtubules are lacking, it has been shown that, *in vitro*, the rate and extent of brain microtubule assembly are dependent stoichiometrically on MAP concentrations (30, 31, 43), and that microtubules assembled *in vitro* with MAPs are more stable to cold depolymerization (30, 42). The present report shows that a MAP₂-enriched fraction, without the

higher molecular weight MAP₁ or species smaller than 145,000 daltons, stimulates microtubule assembly stoichiometrically (Fig. 5). That this MAP₂ fraction has been enriched for the stimulatory activity is indicated by the lowering of the critical concentration for tubulin assembly to 0.05 mg/ml. Earlier experiments indicated a critical concentration for tubulin assembly of ~0.2 mg/ml in the presence of less fractionated MAP fractions (31).

Preliminary data indicates that fractions containing lower molecular weight species, resolved from the MAP₂ fraction by molecular sieve chromatography, also promote microtubule assembly. Because of the large number of species in these fractions, however (see Fig. 3, *c-e*), and because the proportions among these species are not reproducible (unpublished data), quantification of the polymerization-inducing activity in these column fractions has been difficult. The present data show only that a MAP₂-enriched fraction, without other factors of lower molecular weight which have been purported to stimulate brain microtubule assembly (8, 13, 26), will promote *in vitro* assembly of brain microtubules, and that the brain microtubules assembled *in vitro* contain a finite number of binding sites for the MAP₂ fraction.

The higher molecular weight MAP₁ (mol wt ~350,000 daltons) (44) has been shown previously to quantitatively copurify with MAP₂ and tubulin (4, 30, 33, 44), yet the data presented here indicate that MAP₁ is not required with MAP₂ for stimulation of assembly *in vitro*. This does not discount the possibility that MAP₁ alone may stimulate microtubule assembly *in vitro*, or that MAP₁ may be required for microtubule function *in vivo*. It remains to be determined how MAP₁ is involved in microtubule assembly and/or function.

It should be noted that the MAP₂ fraction, while greatly enriched for MAP₂, still contains minor amounts of several other high molecular weight species (see Figs 3 *b* and 4), most of which appear to copolymerize or at least cosediment with *in vitro*-assembled microtubules (see Fig. 6). Whether these minor species are proteolytic fragments of MAP₂ that are still able to bind to microtubules (43, 52), or whether they constitute other species that interact with microtubules, is not known.

Electron microscopy of transverse sections of microtubule pellets shows that microtubules, polymerized with either unfractionated MAPs or the more highly purified MAP₂ fraction, are farther

from each other and have a much more uniform center-to-center spacing than microtubules polymerized without MAPs (see Fig. 9). These differences are probably a result of the MAP projections which prevent packing of the microtubules beyond a certain density and which position the microtubules at a precise distance from one another. There also appears to be a slight difference in the center-to-center spacing between microtubules assembled with unfractionated MAPs (50 nm) and those assembled with the purified MAP₂ fraction (64 nm). At present, it is not known what this difference indicates. It is interesting, in this respect, that in the quantitative analysis by Smith et al. (46), the maximum distance between microtubules and mitochondria in axons *in situ* was shown to be 25 nm, which is exactly half the distance between walls of microtubules saturated with unfractionated MAPs (see Fig. 9b).

In summary, a fraction greatly enriched for the major microtubule-associated protein, MAP₂, has been purified from calf brain microtubules by taking advantage of its heat stability and high molecular weight. The results presented here demonstrate that this MAP₂ fraction will promote microtubule assembly *in vitro*, decorating the microtubules with projections that exhibit an axial periodicity of 32 nm.

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