Separation and Characterization of Microtubule Proteins from Calf Brain†

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ABSTRACT: Electrophoresis of microtubule preparations purified from calf brain by repeated cycles of assembly and disassembly shows that they contain many proteins in addition to α- and β-tubulin. These additional proteins constitute about 17% of the total material present after five cycles of assembly and disassembly. Both one-dimensional and two-dimensional (P. H. O'Farrell (1975), *J. Biol. Chem. 250,* 4007) electrophoretic techniques have been used to characterize them. They can be divided into two groups: one that contains proteins which remain in constant quantitative ratio to tubulin during the purification cycles, and one composed of proteins which are removed during purification, although inefficiently. Gel-filtration chromatography of cold-depolymerized microtubule preparations yields a polydisperse fraction of high molecular weight containing most of the non-tubulin proteins. This fraction contains flexible filaments about 100 Å in diameter similar to those reported by R. A. B. Keats and R. H. Hall ((1975), *Nature (London) 247,* 418). It is suggested that these fibers are neurofilaments, and that they may be the major source of the group of inefficiently removed proteins.

The discovery by Weisenberg (1972) of the conditions necessary for the in vitro assembly of cytoplasmic microtubules has stimulated biochemical studies of the protein composition of these organelles. Early reports (Weisenberg, 1972; Shelanski et al., 1973; Weingarten et al., 1974) implied that tubulin, a protein heterodimer having a molecular weight of 110 000 (Lee et al., 1973), was the only protein required to form microtubules. Recently Erickson (1974), Borisy et al. (1975), Burns and Pollard (1974), Gaskin et al. (1974), Sloboda et al. (1975), Kuriyama (1975), Haga and Kurokawa (1975), Keats and Hall (1975), Weingarten et al. (1975), and Sandoval and Cuatrecasas (1976a) have indicated that several other proteins called microtubule associated proteins, MAPs[1] (Sloboda et al., 1975), copurify with tubulin when microtubules are isolated by in vitro polymerization–depolymerization procedures. In addition, several enzymatic activities have been found in microtubule preparations so isolated (Daleo et al., 1974; Soifer et al., 1975; Sloboda et al., 1975; Sandoval and Cuatrecasas, 1976b; Shigekawa and Olsen, 1975).

Analytical electrophoresis of cytoplasmic microtubules isolated in vivo (Kirkpatrick et al., 1970) indicates that these structures contain several high molecular weight proteins in addition to tubulin. Ultrastructural studies in vivo (Wuerker and Palay, 1969; Burton and Fernandez, 1973) and in vitro (Dentler et al., 1975; Murphy and Borisy, 1975) have shown fibrous projections on cytoplasmic microtubules. These organelles are thus apparently complex in morphology and composition.

Various MAPs have been implicated as necessary cofactors of microtubule assembly in vitro (Weingarten et al., 1975; Murphy and Borisy, 1975; Keats and Hall, 1975; Bryan et al., 1975; Sloboda et al., 1976). On the other hand, Lee and Timasheff (1975), Himes et al. (1976), and Herzog and Weber (1977) have reported that highly purified tubulin can be made to polymerize into microtubules without the presence of other proteins. The significance of the polymerization-stimulating activity possessed by the MAPs is further rendered complex by the fact that similar activities have been reported to reside in various polycations such as DEAE-dextran, ribonuclease, lysozyme, and polylysine (Behnke, 1975; Jacobs et al., 1975; Levi et al., 1975; Erickson and Voter, 1976). It is thus unclear whether the MAPs are necessary elements of cytoplasmic microtubules, or whether they simply alter either the kinetics of polymerization, the equilibria involved, or both, but are not essential to the formation of the microtubular structure.

In this paper we report the characterization of microtubule preparations made by the assembly–disassembly procedure. We have assessed the number and relative amounts of those proteins which co-purify with tubulin during repeated cycles of polymerization and depolymerization of microtubules and have determined whether they exist free in solution under depolymerizing conditions or are associated with larger structures. We have found that long flexible fibers, about 100 Å in diameter and apparently similar to those initially reported by Keats and Hall (1975), are present in our preparations. We suspect that these filaments are neurofilaments, and that they contain many of the MAPs.

Materials and Methods

Microtubule Preparation. In vitro assembled microtubules from calf brains were purified by a modification of the method described by Shelanski et al. (1973). Brain tissue, 100 g at a time, was homogenized at 4 °C with 75 mL of PM 4M (100 mM Pipes–NaOH, 2 mM EGTA, 1 mM $MgSO_4$, 4 M glycerol, pH 6.9 at 23 °C) buffer in a Sorvall Omni-Mixer for 50 s at speed 3 and 10 s at speed 9. The homogenate was then centrifuged in a Sorvall GSA rotor at 8000 rpm for 15 min at 3 °C. The supernatant was decanted and centrifuged in a Beckman T35 rotor at 35 000 rpm for 70 min at 3 °C. The supernatant (S_0) was decanted, made 0.5 mM GTP, and in-

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[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); DEAE, diethylaminoethyl; MAP, microtubule associated protein; HMW-MAP, high molecular weight microtubule associated protein; NQ-MAP, nonquantitatively purifying microtubule associated protein; LMW-MAP, low molecular weight microtubule associated protein; GTP, guanosine triphosphate.

cubated at 32 ± 2 °C for 45 min to allow assembly of microtubules. The material was then centrifuged in a T35 rotor by spinning at 35 000 rpm for 45 min at 25 °C. The resulting pellets were resuspended in 4 to 5 pellet volumes of cold PM (same as PM 4M, but containing no glycerol) buffer containing 0.5 mM GTP and spun in a T35 rotor at 35 000 rpm for 30 min at 3 °C to remove undissociated material. The supernatant (1XMT) was decanted, diluted 1:1 with PM 8M (same as PM 4M, but containing 8 M glycerol) buffer containing 0.5 mM GTP and either stored at −20 °C or carried through additional cycles of polymerization and depolymerization to give 2XMT, 3XMT, 4XMT, and 5XMT cold, high speed supernatants. Before any stored microtubule preparation was used, it was cycled through a round of polymerization, centrifugation, depolymerization, and centrifugation. Protein concentrations were determined by the method of Lowry et al. (1951) or by measurement of A_{280}. The extinction coefficient of 0.72 mL/mg of Kirschner et al. (1974) was employed.

Electrophoresis. $NaDodSO_4$-polyacrylamide electrophoresis was carried out on 8% cylindrical or slab gels according to the method of Laemmli (1970). Gels used for quantitative studies were stained and fixed with 1% (w/v) Fast Green (Gorovsky et al., 1970) in 50% methanol-7% acetic acid (v/v) for 24 h. Gels were then destained in 5% methanol-7% acetic acid (v/v) in a diffusion destainer. Amounts of protein were determined by scanning the gels with a Gilford Model 240 spectrophotometer equipped with a linear transport and then measuring the areas of appropriate peaks in the resulting scans. Gels stained with Coomassie Brilliant Blue R were first fixed in a 12.5% (w/v) Cl_3CCOOH solution for about 3 h at 50 °C or overnight at room temperature. Fixed gels were then stained with 0.2% (w/v) Coomassie in ethanol-acetic acid-water (45:10:45) for 30 min at 50 °C and then destained twice in ethanol-acetic acid-water (25:10:65) for 30 min at 50 °C. Gels were further destained at room temperature over a period of about 2 days in multiple changes of the destaining solution. All destained gels were stored in an 8% (v/v) acetic acid solution.

Two-dimensional gel electrophoresis procedures were similar to those described by O'Farrell (1975). Isoelectric focusing on 3% polyacrylamide was performed in the first dimension on cylindrical (12 cm × 4 cm) gels containing 8 M urea (ultrapure, Schwarz/Mann) and 1% (v/v) ampholine (LKB) pH 3.5-10.0 at room temperature. Samples containing 8 M urea, 1% ampholine, 10% glycerol were applied to gels at the cathode end and overlayered with 0.2 mL of solution containing 8 M urea and 2% (v/v) ampholine. The anode and cathode buffers were 0.2% (v/v) sulfuric acid and 0.4% (v/v) ethanolamine, respectively. Runs were started at a constant current of 1 mA/tube. When the voltage reached 240 V the run was continued at constant voltage. Total running time was about 16 h. Gels were removed, equilibrated for approximately 2 h with a $NaDodSO_4$ buffer (containing 5% (v/v) 2-mercaptoethanol, 2% (w/v) $NaDodSO_4$, and 0.0625 M Tris-HCl, pH 6.8) and run in the second dimension on a $NaDodSO_4$ Laemmli slab gel. A warm 1% (w/v) agarose solution containing 0.0625 M Tris-HCl, pH 6.8, was employed to couple the two gels. The pH gradient in the first dimension was determined by cutting duplicate gels into 3-mm sections, eluting ampholine from each section with 1 mL of water overnight and measuring the pH of the resulting solution at room temperature (23 °C). Molecular weight standards were run in the second dimension of each gel by placing 0.10 mL of a solution containing lactate dehydrogenase, ovalbumin, catalase, serum albumin, β-galactosidase, and myosin in specially formed wells on each end of the slab gel.

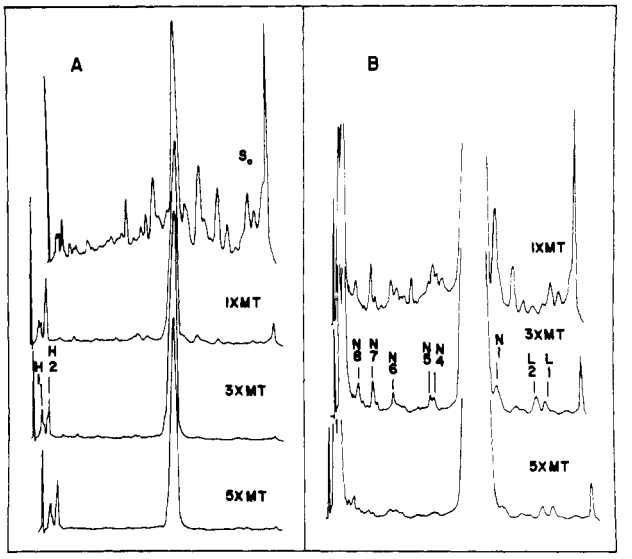

FIGURE 1: Densitometric scans of Fast Green stained sodium dodecyl sulfate gels of (A) samples containing about 200 μg of S_0 and 60 μg of 1XMT, 3XMT, and 5XMT, and of (B) the same samples, but eight times more concentrated. Electrophoretic migration was from left to right. The large central peak is tubulin. Peaks or regions are labeled according to the nomenclatures of Tables I and II: H1 and H2 are HMW-MAPs 1 and 2; N1 through N8 are NQ-MAPs 1 through 8; and L1 and L2 are the two LMW-MAPs. Bands corresponding to NQ-MAP2 and NQ-MAP3 are buried under the tubulin band.

Gel Filtration Chromatography. Gel filtration chromatography was conducted with Bio-Gel A-15m (100-200 mesh) and with Bio-Gel A-150m (100-200 mesh) obtained from Bio-Rad Laboratories. In nearly all cases columns contained a short layer of Sephadex G-25 medium (Pharmacia), placed on top of A-15 columns and on the bottom of A-150 columns. The G-25 served to separate small solutes (GTP and residual free glycerol) from the proteins (Detrich et al., 1976). All columns were equilibrated in PM buffer made 0.1 mM in GTP (Type II-S, Sigma) and 2 mM in dithiothreitol (Sigma). All samples applied to gel filtration columns had protein concentrations between 10 and 20 mg/mL.

Analytical Sedimentation. Sedimentation velocity experiments were carried out in a Beckman Model E ultracentrifuge. All sedimentation experiments were conducted at 5 °C with double-sector aluminum-filled Epon centerpieces of 30-mm optical path. The Bridgman equation (Bridgman, 1942; Fujita, 1975)

$$g(s) = (1/c_0)(dc_0/ds) \quad (1)$$

where c_0 is the initial concentration, was employed to evaluate the distribution of sedimentation coefficients, $g(s)$. Procedures used to compute $g(s)$ are described by Schumaker and Schachman (1957).

Electron Microscopy. Samples for electron microscopy were negatively stained with 1% aqueous uranyl acetate (Rosenbaum et al., 1975) and observed in a Phillips 201 electron microscope.

Results

Electrophoresis. Densitometric scans of $NaDodSO_4$ gels of dilute and concentrated samples of microtubule solutions are shown in Figure 1 at four stages of purification. A number of nontubulin proteins are visible. Most of these proteins are seen to decrease in amount as the preparation is carried through more cycles of purification. The bands labeled H1 and H2 (of molecular weight 250 000 to 350 000 and here called

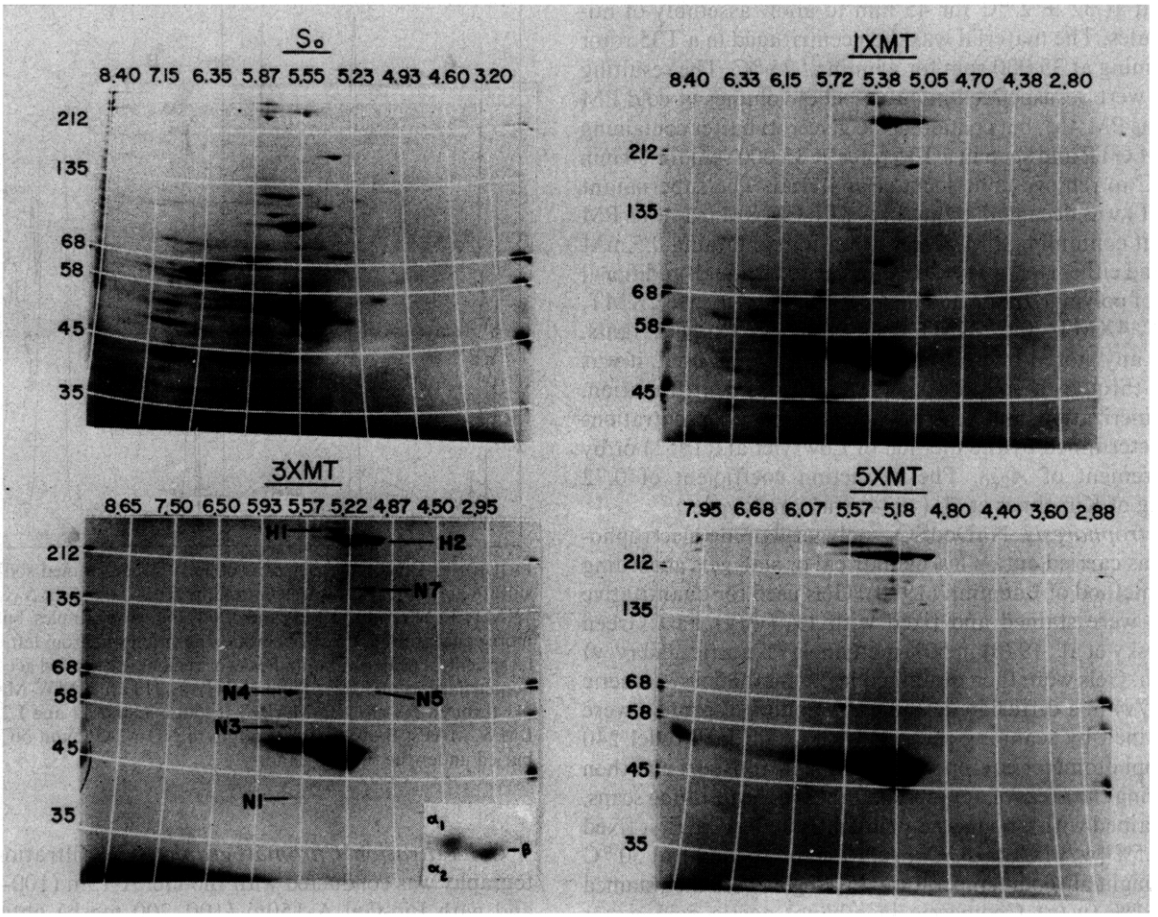

FIGURE 2: Two-dimensional gels, stained with Coomassie Blue, of samples containing about 1.2 mg of S_0 and 0.5 mg of 1XMT, 3XMT, and 5XMT. The insert in the 3XMT gel represents the tubulin region from a dilute gel showing the splitting of α-tubulin. Isomolecular weight lines were drawn by connecting corresponding molecular weight standards run at each end of the gel, taking into account vertical curvature. Isop*I* lines were drawn by superimposing the measured pH profile on the slab gel, taking into account lateral curvature. Spots or regions are labeled as in Figure 1.

TABLE I: Amounts of Proteins that Copurify with Tubulin.[a]

	S_0		1XMT		3XMT		5XMT		
Protein	% Total protein	Ratio to tubulin	% Total protein	Ratio to tubulin	% Total protein	Ratio to tubulin	% Total protein	Ratio to tubulin	Mol wt
Tubulin	22.3 ± 0.1	1.0	65.7 ± 0.7	1.0	82.2 ± 0.8	1.0	82.4 ± 3.2	1.0	54 000
HMW-MAPs	2.3 ± 0.1	0.103 ± 0.005	8.5 ± 0.7	0.129 ± 0.011	9.3 ± 0.8	0.113 ± 0.010	10.2 ± 1.0	0.124 ± 0.013	250 000–350 000[d]
LMW-MAPS			0.7 ± 0.1	0.011 ± 0.002	0.9 ± 0.1	0.011 ± 0.001	1.0 ± 0.1	0.012 ● 0.001	30 000–35 000[c]
NQ-MAPs[b]			25.1 ± 1.5	0.38 ± 0.02	7.6 ± 1.7	0.09 ± 0.02	6.4 ± 4.3	0.08 ± 0.05	

[a] Results are expressed both as percent of the total protein present on the gel and as the ratio of the amount of a given protein to the amount of tubulin. The specified limits of uncertainty reflect all expected sources of experimental error. Complete analyses were performed on three different preparations. [b] NQ-MAPs includes the nonquantitatively purifying proteins NQ-MAP1 through NQ-MAP8 (see text and Table II). [c] Approximate molecular weight. [d] Sloboda et al. (1975) and Murphy and Borisy (1975).

HMW-MAPs, for high molecular weight microtubule-associated proteins) and those labeled L1 and L2 (of molecular weight between 30 000 and 35 000 and here called LMW-MAPs, for low molecular weight microtubule-associated proteins) appear to remain constant in amount. Table I shows the fraction of the total protein comprising each of these two groups, as well as the fraction made up of those proteins that decrease in amount during purification. It will be seen that both the HMW-MAPs and the LMW-MAPs quantitatively copurify with tubulin: i.e., from 1XMT through 5XMT there is a constant weight ratio of MAPs to tubulin. The proteins, comprising eight distinct electrophoretic bands with apparent molecular weights ranging from 45 000 to 220 000, as well as numerous faint bands, decrease in relative amount by a factor of approximately four between 1X and 5X microtubules. We consider this latter behavior to represent nonquantitative copurification and call these proteins NQ-MAPs.

Results of analysis by two-dimensional electrophoresis of a microtubule preparation are shown in Figure 2 at four stages of purification. This method reveals complexity not seen in the one-dimensional gels. First, α-tubulin is seen to be slightly less acidic (p*I* = 5.4 ± 0.1) than β-tubulin (p*I* = 5.3 ± 0.1). In

TABLE II: Proteins Seen on Two-Dimensional Electrophoresis.

Protein	App mol wt[b]	App p*I*[c]	Quant copurification with tubulin
NQ-MAP1	45 000	5.7 ± 0.1	No
NQ-MAP2[a]	50 000		No
NQ-MAP3	60 000	5.8 ± 0.1	No
NQ-MAP4	66 000	5.7 ± 0.1	No
NQ-MAP5	67 000	4.9 ± 0.1	No
NQ-MAP6[a]	100 000		No
NQ-MAP7	160 000	5.1 ± 0.1	No
NQ-MAP8[a]	220 000		No
HMW-MAP1	300 000–350 000[d]	5.4 to 5.7	Yes
HMW-MAP2	250 000–300 000[d]	5.0 to 5.4	Yes
α_1-Tubulin	56 000	5.4 ± 0.1	
α_2-Tubulin	55 000	5.4 ± 0.1	
β-Tubulin	54 000	5.3 ± 0.1	

[a] NQ-MAP2, 6, and 8 do not appear on two-dimensional gels. They are listed here for completeness. [b] Apparent molecular weights as judged by mobility in the sodium dodecyl sulfate electrophoresis dimension. The accuracy of these values is estimated to be 10%. [c] Apparent p*I* as judged from position in the isoelectric focusing direction. [d] Sloboda et al. (1975) and Murphy and Borisy (1975).

addition, the α-tubulin has usually been observed to split into two spots in the $NaDodSO_4$ direction (see insert in Figure 2). Second, the lowest molecular weight proteins in the HMW-MAP group, HMW-MAP2 (see Figure 1), are present as several spots, usually three, covering a p*I* range of 5.00 to 5.35. Third, the highest molecular weight protein in the HMW-MAP group, HMW-MAP1 (see Figure 1), focuses broadly over a p*I* range of 5.4 to 5.7. Fourth, spots which might correspond to the LMW-MAPs are not seen in these gels. Fifth, five of the eight major bands that make up the bulk of the NQ-MAPs, which do not copurify in constant ratio to tubulin, can be identified on two-dimensional gels, as summarized in Table II.

Gel Filtration Chromatography. Results have been obtained by gel-filtration chromatography which show that the MAPs exist as components of aggregated particles in cold-depolymerized microtubule preparations. Chromatography on an A-150 column at 4 °C of microtubules prepared by polymerization–depolymerization (4XMT) is shown in Figure 3, along with a $NaDodSO_4$ slab gel showing electrophoresis of several fractions from the column eluate. It will be seen that the NQ-MAPs and the LMW-MAPs are eluted almost entirely in peak I, near the void volume. Quantitative electrophoresis showed this peak to contain about 40% tubulin, 12% HMW-MAPs, 2% LMW-MAPs, and 46% NQ-MAPs. This peak contains all of NQ-MAPs 5 and 7, and almost all of NQ-MAPs 3 and 8. The HMW-MAPs appear in both peaks. Their distribution (cf. Figure 5A) suggests that they are parts of at least two kinds of aggregates: those large aggregates comprising peak I and the smaller ones which comprise the left-hand edge of peak II.

The 100-Å Filaments. The exclusion limit of A-150 corresponds to particle weights of about 150 000 000 for globular particles, and peak I of Figure 3 emerges close to the void volume of the column. This peak must therefore be composed of particles in the size range of many tens of millions molecular weight, evidently aggregates of the proteins seen by $NaDodSO_4$ electrophoresis. Electron microscopic observation of this material shows that it contains numerous flexible filaments of diameter approximately 100 Å, decorated with globular particles and linear projections. A representative field of peak

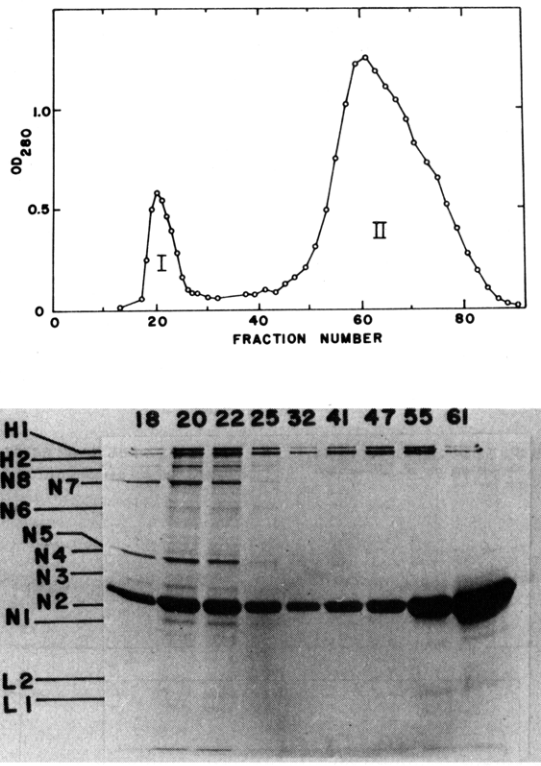

FIGURE 3: (Above) Fractionation of 4XMT on a column (2.5 cm × 24 cm) containing A-150. (Below) Sodium dodecyl sulfate slab gel appropriate fractions from the chromatogram. Bands or regions are labeled as in Figure 1.

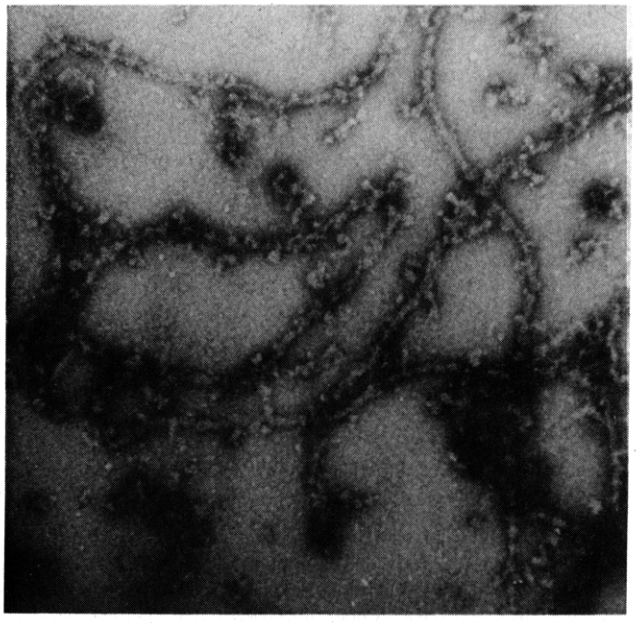

FIGURE 4: Electron micrograph of material from peak I from an A-150 column loaded with 4XMT. A 1-μm bar is present at the bottom of the micrograph. The sample was kept at 4 °C before and during its preparation for electron microscopy.

I material is shown in Figure 4. Material from peak II readily polymerizes to give structures recognized as microtubules in the electron microscope.

Two experiments were performed to assess the possibility that the 100-Å filaments might be artifacts of the polymerization and depolymerization process in vitro. In the first experiment, a 2XMT preparation was subjected to chromatography on an A-150 column with the results shown in Figure

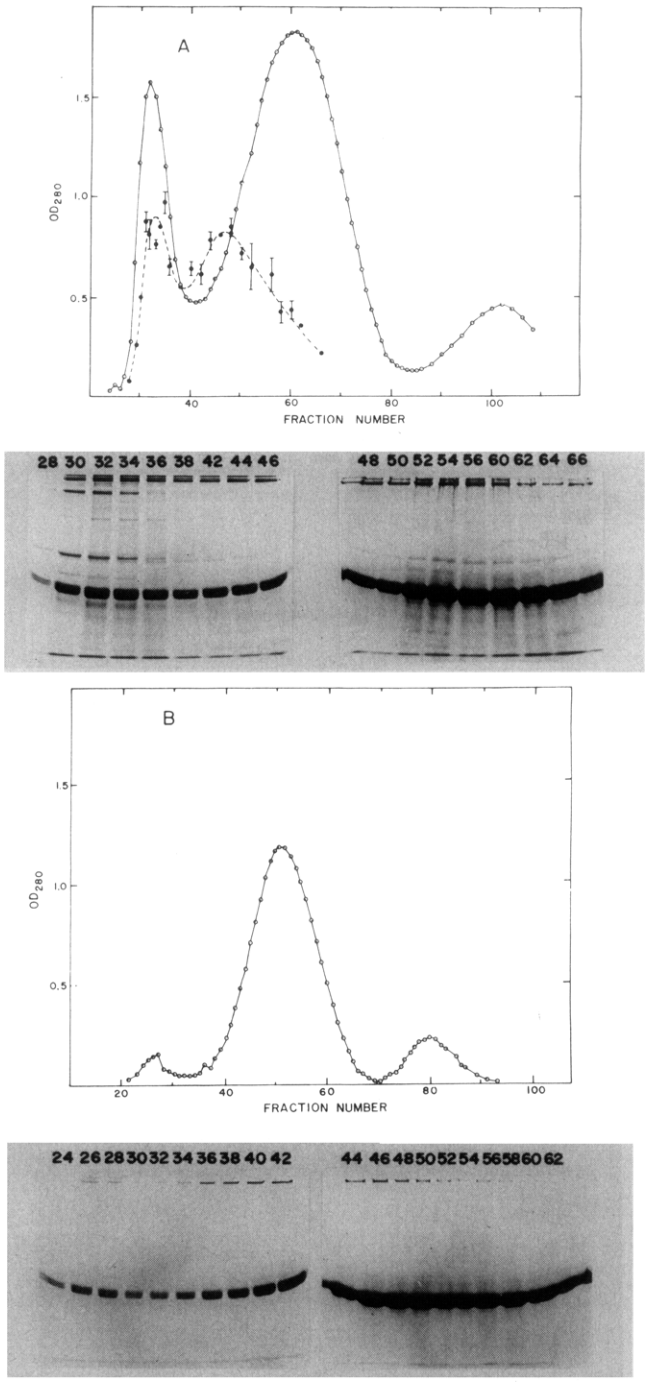

FIGURE 5: (A) (Above) Fractionation of 2XMT on a composite column (2.5 cm × 19 cm) of A-150 (13 cm) and G-25 (6 cm). (Below) Sodium dodecyl sulfate slab gels of indicated fractions are also shown: (●) represent the column profile of the HMW-MAPs expressed in arbitrary units (points were determined by scanning of Fast Green stained sodium dodecyl sulfate cylindrical gels). Fractions 41–76 in this column were pooled with equivalent material from a previous run, concentrated, desalted, and cycled (as described in Materials and Methods) an additional time to give 3XMT. (B) The 3XMT preparation was then fractionated on the same column. The chromatogram and sodium dodecyl sulfate slab gels of various fractions are shown.

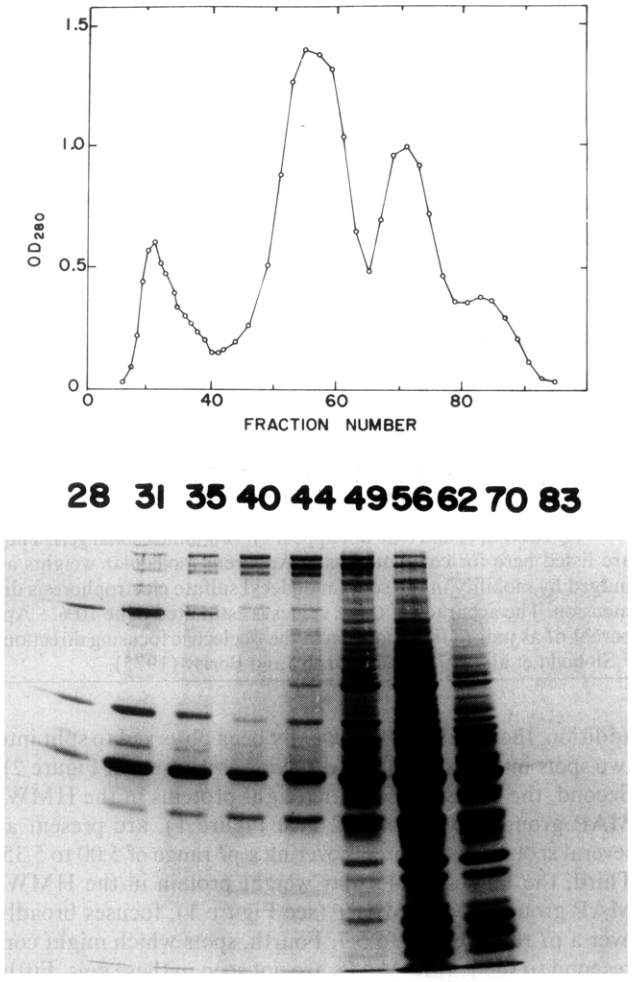

FIGURE 6: Fractionation of S_0 (prepared as described in Materials and Methods, but brain tissue was homogenized with PM instead of PM-4M buffer) on a composite column (2.5 cm × 19 cm) containing A-150 (13 cm) and G-25 (6 cm). Sodium dodecyl sulfate slab gels of indicated fractions are also shown.

5A. Peaks II from two such columns were pooled, concentrated by ammonium sulfate precipitation (solution was brought to 50% saturation by the addition of solid ammonium sulfate), desalted by means of a G-25 column, carried through another round of assembly, centrifugation, disassembly, and centrifugation, and subjected to rechromatography on an A-150 column. The resulting chromatogram is shown in Figure 5B. The $NaDodSO_4$ gels shown there indicate that in peak II the only major protein present besides tubulin is HMW-MAP2. Electron micrographs of material from peak I showed only a small number of short 100-Å filaments. These few filaments were probably present in the fractions initially pooled, since peaks I and II were not completely resolved in the first separation.

In the second experiment, crude high speed supernatant prepared from brain homogenized in PM buffer (and thus in the absence of glycerol), was subjected to gel filtration on a composite column of A-150 and G-25. The resulting column profile and $NaDodSO_4$ gel analysis are shown in Figure 6. The gel patterns of samples from the first peak (fractions 28–35) are similar to those observed for purified microtubule preparations (cf. Figures 3 and 5A). They differ only in the relative amount of HMW-MAPs seen. Electron microscopic observation of material in this peak shows the presence of the same flexible filaments shown in Figure 4, about 100 Å in diameter and lightly decorated with particles. Both experiments indicate that the 100-Å filaments are not created during microtubule purification.

The sensitivity of the filaments to conditions which affect disassembly or assembly of microtubules has been assessed by measuring the turbidity and sedimentation boundary shapes of peak I material from an A-150 column. No change in either of these properties is detectable upon the addition of 3 mM

$CaCl_2$ or of 1 mM colchicine, or following incubation at 37 °C for 90 min. Some colchicine has been found to bind to material in this fraction, but appears to be at a lower molar ratio of colchicine to tubulin than that expected based on the amount of tubulin present. These filaments do not bind to DEAE-cellulose, but do bind to phosphocellulose and can, to some extent, be eluted from it by high concentrations of salt. This latter property is unexpected since all major proteins in the filament-containing peak have been shown by isoelectric focusing to be acidic proteins.

In order to assess the size and polydispersity of the aggregates fractionated by A-150 columns from cold-depolymerized microtubule preparations, sedimentation velocity experiments were performed at 5 °C. Boundary analysis was carried out with sedimentation runs on samples of peak I from an A-150m column, according to eq 1. The resulting distribution of sedimentation coefficients is shown in Figure 7. It indicates that peak I material is polydisperse, having a modal sedimentation coefficient of about 70 S. The sedimentation coefficients of the 100-Å filaments are included within this distribution, and it is therefore likely that the majority of these filaments have sedimentation coefficients less than about 200 S.

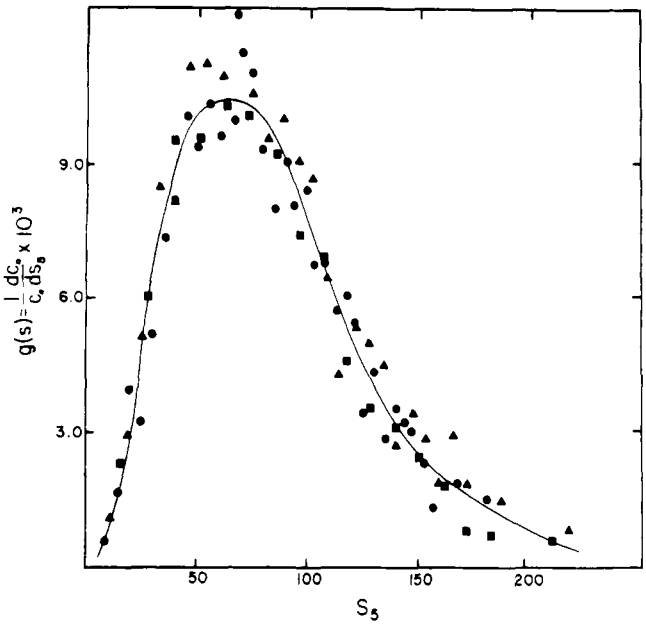

FIGURE 7: Distribution of sedimentation coefficients of a sample (0.56 mg/mL) from peak I of a composite column of A-150 and G-25 loaded with 2XMT (calculated data was taked from scans made 9 min (■), 17 min (▲), and 25 min (●) after reaching speed). Runs were made at 20 000 rpm at 5 °C.

Discussion

The data presented above indicate that the proteins which are found to co-purify with calf-brain tubulin upon repeated cycles of the assembly-disassembly process can be divided into two groups, one of which (the HMW-MAPs and the LMW-MAPs) is quantitatively copurified with tubulin, and the other of which (the NQ-MAPs) is found in relative amounts which decrease as the number of purification cycles increases. The results of the gel-filtration chromatography experiments show that at 4 °C, in the native state, the proteins comprising each of these groups are present predominantly as components of aggregates of high molecular weight. That fraction of the depolymerized microtubule preparation of particle size large enough to be excluded from Bio-Gel A-150m, which has an exclusion limit of 150 000 000 daltons, contains large amounts of 100-Å filaments. This fraction also contains about 50% of the HMW-MAPs and LMW-MAPs, and essentially all of the NQ-MAPs. The results of rechromatography and of direct isolation from crude brain extract indicate that the 100-Å filaments are stable structures, presumably originating in the brain tissue, rather than abnormal assembly products such as those reported by Lee and Timasheff (1975) and by Borisy et al. (1972). When microtubules are polymerized from preparations from which the 100-Å filaments have been removed by gel filtration, they contain tubulin and HMW-MAP 2 as the only major components (Figure 5).

Significance of the MAPs. The probable minimum fraction of a minor protein component detectable in a microtubule preparation by our techniques is approximately 0.1%. This level of detection may be visualized in Figure 1B by noting that the two components of the LMW-MAPs together make up 1% of the total protein applied to the gel. At this level of detection, only HMW-MAP2 is incorporated (in addition to tubulin) into microtubules when they are assembled from a preparation of microtubule protein from which the 100-Å filaments have been removed.

Weingarten et al. (1975), Penningroth et al. (1976), and Witman et al. (1976) have reported that a protein factor (tau) of molecular weight 60 000 to 70 000 is stoichiometrically required for the assembly of hog-brain microtubules and for the formation of 36S rings by hog-brain tubulin. Tau is reported to copurify with tubulin through several cycles of an assembly-disassembly purification procedure. No similar factor seems to be present in our preparations. Only NQ-MAPs 3, 4, and 5 are in the appropriate molecular weight range. NQ-MAPs 3 and 5 are completely removed with the 100-Å filament fraction, and are probably parts of those filaments. NQ-MAP4, although not present in detectable amount in microtubules assembled from non-filament-containing microtubule protein, does not copurify stoichiometrically with tubulin. Unless a protein quantitatively copurifies with tubulin through repeated cycles of polymerization and depolymerization, it is unlikely to be required for microtubule assembly. Even if a protein does copurify quantitatively, however, it may not be a necessary component. Thus, although HMW-MAP2 copurifies quantitatively with calf-brain tubulin, nothing in our results shows that it is necessary for microtubule polymerization. Since various solvent conditions (Lee et al., 1975; Frigon et al., 1974; Rebhun et al., 1975; Lee and Timasheff, 1975; Scheele and Borisy, 1976; Himes et al., 1976; Herzog and Weber, 1977) and polycations (Behnke, 1975; Jacobs et al., 1975; Levi et al., 1975; Erickson and Voter, 1976) have a pronounced effect upon tubulin's ability to form aggregates and microtubules, the ascribed significance of all copurifying proteins should be viewed with caution.

The fractions of MAPs specified in Table I cannot be regarded as specifying definitive properties of the microtubule for two reasons. First, in the cases of the HMW-MAPs and the LMW-MAPs we have found it necessary to lump two or more proteins under one heading because their electrophoretic resolution was insufficient to allow their amounts to be specified separately. Second, there is as yet no independent purification method for any one of the MAPs, and the possibility remains that any of the electrophoretic bands may be composed of more than one protein.

The 100-Å Filaments. It is not clear why the 100-Å filaments are inefficiently removed in the preparative process, although the following explanation seems plausible. From the data of Figure 7, it is apparent that the bulk of the filament-containing fraction from the A-150 column has sedimentation coefficients less than 200 S in PM buffer. Since the cold cen-

trifugation step in the purification procedure is carried out in a medium approximately 0.5 M in glyerol, the sedimentation coefficients are reduced by 27% due to the increase in solvent density and viscosity caused by the glycerol. The sedimentation coefficients of the fibers thus lie in the range of values less than 146S. At 96 000*g* for 30 min, their migration would be less than 3.1 cm; therefore, most of these fibers would be recovered in the supernatant of the cold spin, together with the dissociated microtubule protein. In the warm centrifugation steps, it is less clear why the 100-Å filaments are not removed. We consider it likely, however, that the gel-like network of microtubules entraps these filaments, effectively dragging them down into the pellet.

The major piece of evidence suggesting that these filaments are neurofilaments is their ultrastructural similarity, but additional similarities exist in their physicochemical properties. Qualitative observations indicate that exposure to 1 M NaCl does not eliminate the characteristic turbidity seen in depolymerized microtubule preparations. Since high salt concentrations dissociate ring aggregates (Weingarten et al., 1974; Borisy et al., 1975), residual turbidity must be from the 100-Å filaments. This interpretation is consistent with the work of Keats and Hall (1975), which showed that the 100-Å filaments are stable to high salt treatment. Such insolubility is also characteristic of mammalian neurofilaments (Shelanski et al., 1971; Davison and Winslow, 1974).

The protein composition of the 100-Å filament-containing fraction is compatible with the possibility that these filaments are neurofilaments. Davison and Winslow (1974), Yen et al. (1976), and Jorgensen et al. (1976) have reported that neurofilaments from the white matter of calf brains are predominantly composed of one protein of electrophoretic mobility similar to that of tubulin. Conclusive identification of the observed filaments must await further work.

Two-Dimensional Electrophoresis. Since this is the first report of the application to microtubule preparations of the two-dimensional isoelectric focusing and $NaDodSO_4$ electrophoresis method, some remarks on this useful separation are in order. Microheterogeneity of tubulin has been investigated by several laboratories (Feit et al., 1971; Witman et al., 1972; Olmsted, 1971; Bibring et al., 1976; Lu and Elzinga, 1977). Our two-dimensional electrophoresis results confirm that tubulin derived from cytoplasmic microtubules contains at least three different tubulin polypeptide chains: two α and one β. Therefore, there must be at least two types of $\alpha\beta$ heterodimers in these microtubules. Qualitative observations of two-dimensional gels of fraction from peaks I and II obtained on A-150m and A-15m columns reveal no differences in the number and relative amounts of tubulin spots seen. Hence, the separable aggregates in microtubule preparations which contain tubulin show no preference for one heterodimer or the other. Because urea is required to observe the resolution of the α chains, the normal $NaDodSO_4$ binding of these proteins must be altered by its presence (Weber and Kuter, 1971; Miller and Elgin, 1974), permitting minor differences to be amplified as suggested by Bibring et al. (1976). The detailed explanation for the splitting of α-tubulin will have to await chemical analysis of the polypeptide chains.

The HMW-MAPs and LMW-MAPs show unusual properties upon isoelectric focusing. None of the LMW-MAps penetrate the gel because either their p*I*s are outside the pH range of the experiments or they are part of a structure both too large to enter the gel and resistant to dissociation by urea. Proteins in the HMW-MAP group, similar to those reported by others (Erickson, 1974; Borisy et al., 1975; Burns and Pollard, 1974; Gaskin et al., 1974; Sloboda et al., 1975; Kuriyama, 1975, Haga and Kurokawa, 1975; Keats and Hall, 1975), show a strong tendency to streak and a variability between electrophoretic experiments in the relative intensity of spots which appears to be a function of loading concentration. None of this peculiar behavior is observed in the one-dimensional tube or slab gels. Further work will be required to determine its origin.

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