Identification of the Major 68 000-Dalton Protein of Microtubule Preparations as a 10-nm Filament Protein and Its Effects on Microtubule Assembly in Vitro†

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ABSTRACT: The major 68 000-dalton protein present in cycled microtubule preparations from bovine brain can be isolated in a rapidly sedimenting fraction consisting of filaments 10 nm in diameter. This 68 000-dalton protein remains in the filament fraction after gel filtration, phosphocellulose chromatography, or salt extraction of microtubule protein. Microtubule protein devoid of 10-nm filaments contains ring structures under depolymerizing conditions, and it polymerizes into microtubules with a characteristically low critical concentration, although all of the 68 000-dalton protein has been removed from it. When cycled microtubule protein is subjected to chromatography on phosphocellulose, the tubulin fraction (PC-tubulin) assembles into microtubules only at concentrations greater than 2 mg/mL. The other fraction, eluted from phosphocellulose at high ionic strength, contains the major 68 000-dalton protein and can be further resolved into two components by centrifugation. The supernatant, which consists mainly of high molecular weight microtubule-associated proteins, stimulates low concentrations of PC-tubulin to assemble. The pellet contains all of the 68 000-dalton protein, consists of 10-nm filaments, and does not stimulate assembly of PC-tubulin. Boiling of purified filaments, however, releases several proteins, including the 68 000-dalton protein, and these released proteins stimulate the assembly of PC-tubulin. The morphology and protein composition of the filaments isolated from microtubule preparations by these techniques are very similar to those of mammalian neurofilaments. These results suggest that the major 68 000-dalton protein in cycled microtubule preparations, which may correspond to tubulin assembly protein [Lockwood, A. H. (1978) *Cell 13*, 613–627], is a constituent of neurofilaments.

Since the development of procedures for the assembly of microtubules in vitro (Weisenberg, 1972), the nontubulin proteins present in microtubule preparations have been the subject of intensive investigation. Various laboratories have studied the role of microtubule-associated proteins (MAPs)[1] in microtubule assembly in vitro (Burns & Pollard, 1974; Erickson, 1974; Haga et al., 1974; Borisy et al., 1975; Gaskin et al., 1975; Kuriyama, 1975; Murphy & Borisy, 1975; Sloboda et al., 1975; Weingarten et al., 1975; Sloboda et al., 1976; Cleveland et al., 1977a,b; Fellous et al., 1977; Murphy et al., 1977). One group of MAPs, the high molecular weight MAPs, which have molecular weights of 250 000 to 350 000 (Burns & Pollard, 1974; Borisy et al., 1975; Murphy & Borisy, 1975; Sloboda et al., 1976), have been shown to copurify quantitatively with tubulin (Berkowitz et al., 1977; Murphy et al., 1977). These proteins stimulate microtubule assembly under polymerizing conditions and induce the formation of ring structures under depolymerizing conditions (Murphy & Borisy, 1975; Sloboda et al., 1976; Murphy et al., 1977). τ, another group of polypeptides ranging in molecular weight from 56 000 to 70 000 (Weingarten et al., 1975; Cleveland et al., 1977a,b; Fellous et al., 1977), is reported to have similar properties. A single 68 000-dalton protein designated tubulin assembly protein (TAP) (Lockwood, 1978) has recently been purified from τ-containing fractions obtained by phosphocellulose chromatography. TAP stimulates microtubule assembly and apparently is the only heat-stable protein[2] in crude τ fractions which shows a high affinity for purified tubulin (Lockwood, 1978). Although most microtubule preparations appear to contain each of these proteins in at least small amounts, no explanation is available for why the amounts of these proteins differ considerably in different preparative schemes. Furthermore, there is disagreement over which MAP (or MAPs) is responsible for the low critical concentrations observed for the assembly of cycled microtubule proteins.

We report here evidence that the major 68 000-dalton protein present in microtubule preparations is a component not of microtubules but of contaminating filaments (Keats & Hall, 1975; Berkowitz et al., 1977) of 10-nm diameter. We present further evidence that these filaments may be neurofilaments (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976; Schlaepfer, 1977a,b; Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978) and that the protein may be TAP. We show that the assembly-stimulating activity of the protein is not seen unless it is released from the filaments by heat treatment.

Materials and Methods

Reagents. Pipes, EGTA, DTE, and GTP (type II-S) were obtained from Sigma Biochemicals, St. Louis, MO. Bio-Gel A-150m was from Bio-Rad Laboratories, Richmond, CA, and phosphocellulose was Whatman P-11 (control no. 2111-62).

Experimental Conditions. All experiments involving microtubule protein were performed in 0.1 M Pipes (pH 6.9), 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM GTP, 2 mM DTE (referred to as PM buffer) at 4 °C, unless otherwise specified. The 10-nm filaments purified directly from crude brain homogenate were buffered in PM without GTP.

Microtubule Preparations. Cycled microtubule protein[3] was

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[1] Abbreviations used: DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; GTP, guanosine 5'-triphosphate; HMW, high molecular weight; MAPs, microtubule-associated proteins; PC, phosphocellulose; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); $NaDodSO_4$, sodium dodecyl sulfate; TAP, tubulin assembly protein.

[2] Throughout this paper "heat-stable proteins" will refer to those polypeptides which remain soluble following boiling of a protein fraction. We do not intend to imply that these proteins retain a native conformation following heat treatment.

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prepared by the assembly–disassembly method of Shelanski et al. (1973) as modified by Berkowitz et al. (1977). The amount of material present as 10-nm filaments could be controlled by the length of the clarification centrifugation which followed resuspension and depolymerization of microtubules: 15 min at 4 °C and at 35 000 rpm in a Beckman T35 rotor (95500*g*) produced microtubule preparations containing substantial amounts of 10-nm filaments, while 60 min under the same conditions produced microtubule preparations with minimal amounts of 10-nm filaments. Protein concentrations were determined by the method of Bradford (1976), standardized with tubulin as described (Detrich & Williams, 1978).

Isolation of 10-nm Filaments from Cycled Microtubule Preparations. The 10-nm filaments, free of ring structures, were isolated from microtubule preparations by three techniques: (i) Bio-Gel A-150m chromatography of twice cycled microtubule preparations as described by Berkowitz et al. (1977); (ii) centrifugation of cycled microtubules in 1 M NaCl at 4 °C, as described by Keats & Hall (1975); or (iii) elution (with PM buffer containing 0.7 M NaCl) of the fraction of microtubule proteins bound to phosphocellulose, followed by centrifugation of the eluate to obtain 10-nm filaments. Phosphocellulose chromatography was performed by the method of Weingarten et al. (1975) as modified by Detrich & Williams (1978).

Preparation of 10-nm Filaments from Brain Homogenate. Bio-Gel A-150m chromatography of a high speed supernatant of bovine brain homogenate was performed as described by Berkowitz et al. (1977). Brain was homogenized at 4 °C in PM buffer without GTP, and the homogenate was subjected to centrifugation, first at 6000*g* for 15 min and then at 95500*g* for 75 min, to remove particulate matter. The resulting glycerol-free high speed supernatant was applied to a column of Bio-Gel A-150m and eluted with PM buffer without GTP. Void volume fractions were pooled and centrifuged in a T35 rotor at 17 000 rpm (19500*g*) for 30 min, 4 °C, to remove large aggregates. The supernatant was decanted and centrifuged in a T35 rotor at 35 000 rpm (95500*g*) for 120 min, 4 °C, to pellet 10-nm filaments. The pellet was resuspended into PM buffer without GTP by means of a Dounce homogenizer and frozen dropwise in liquid nitrogen. The 10-nm filaments were further purified after thawing and just before use by centrifugation in a 20–40% linear sucrose gradient for 3.5 h in a Beckman SW27.1 rotor at 25 000 rpm, 4 °C. The 10-nm filament pellet was resuspended into PM buffer without GTP as described above.

Gel Electrophoresis and Peptide Mapping. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) on 8% cylindrical or slab gels. Gels were stained with Coomassie Brillant Blue R (Detrich & Williams, 1978). Separation of α- and β-tubulin was carried out preparatively [after reduction and alkylation according to Crestfield et al. (1963)] by the method of Yang & Criddle (1970) on 1 × 17 cm cylindrical gels. Isoelectric focusing of cyanogen bromide fragments from isolated α- and β-tubulin was performed according to the method of Bibring & Baxandall (1978).

Polymerization Assays. Microtubule polymerization was monitored at 34 ± 1 °C in a Cary 118 C recording spectrophotometer with water-jacketed cuvette holders. The final polymerization buffer was PM supplemented with 1.0 mM GTP for polymerizations involving phosphocellulose-purified tubulin.

Electron Microscopy. Samples for electron microscopy were applied dropwise to freshly carbon-coated collodion grids and negatively stained with 1% aqueous uranyl acetate. One drop of sample was deposited on a grid for 30 s, excess solution was drawn off with filter paper, and several drops of uranyl acetate were deposited on top of the grid. The last drop was removed with filter paper, and the grids were allowed to air dry. Samples were examined with a Hitachi HU-11B electron microscope. When grids were prepared from depolymerized solutions of microtubules, a drop of the solution (at 4 °C) was placed on a grid at room temperature. Stain was applied within 30 s. The resulting distribution of components does not differ from that obtained by deposition of solution on a cold (4 °C) grid. When grids were made from polymerized tubules, aliquots were taken from the warm (34 °C) solution and an identical protocol was followed.

Results

Centrifugation of Cycled Microtubule Preparations at 4 °C. Figure 1A shows an electron micrograph of a negatively stained sample of assembled microtubules prepared by two cycles of the polymerization–depolymerization procedure. The time of centrifugation at 4 °C was 15 min at each cycle. The two main structures apparent are microtubules and 10-nm filaments. Figure 1B shows a sample of the same preparation after it had been held at 4 °C to bring about depolymerization. Two formed elements are present: rings and 10-nm filaments.

Centrifugation of a cycled microtubule preparation at 4 °C (106000*g* for 20 min) yielded the pellet represented in Figure 1C. The pellet was composed almost exclusively of 10-nm filaments, while the supernatant (not shown) contained both filaments and rings. Centrifugation for a longer period (106000*g* for 90 min) produced a final supernatant, shown in Figure 1D, that contained numerous rings but was apparently devoid of filaments.

A preparation of twice cycled microtubule protein was carried through a third cycle of polymerization and depolymerization and was then divided into several aliquots and subjected to centrifugation at 106000*g* and 4 °C, for progressively longer periods of time. The protein contents of the supernatants and pellets were measured and each was examined with the electron microscope. The results are shown in Table I. Examination of that table shows that the filaments sediment faster than the rings, that they can be recovered essentially completely from depolymerized microtubule protein without removing substantial numbers of rings, and that the filaments comprise probably less than 20% of this preparation.

A sample of each pellet and supernatant from Table I was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis with the results shown in Figure 2. Both the pellet and supernatant which resulted from short durations of centrifugation contained tubulin (see below), the major 68 000-dalton protein, HMW-MAPs, and several other minor proteins. It is evident that, as the duration of centrifugation was increased, only tubulin and HMW-MAPs remained in the supernatant. The nonquantitatively copurifying MAPs identified by Berkowitz et al. (1977), including the 68 000-dalton protein, are found almost exclusively in the pelleted fraction, which, as shown above, contains the 10-nm filaments as its primary morphological constituent. The material which pelleted at 60 and 90 min also contained substantial amounts of HMW-MAPs. Electron microscopy showed increasing amounts of rings in the pellet at these late times, and it may be inferred

[3] In this paper microtubule protein refers to tubulin plus all associated proteins (MAPs) which copurify with tubulin through cycles of in vitro assembly and diassembly. Tubulin refers specifically to the $\alpha\beta$ dimer of the main microtubule protein.

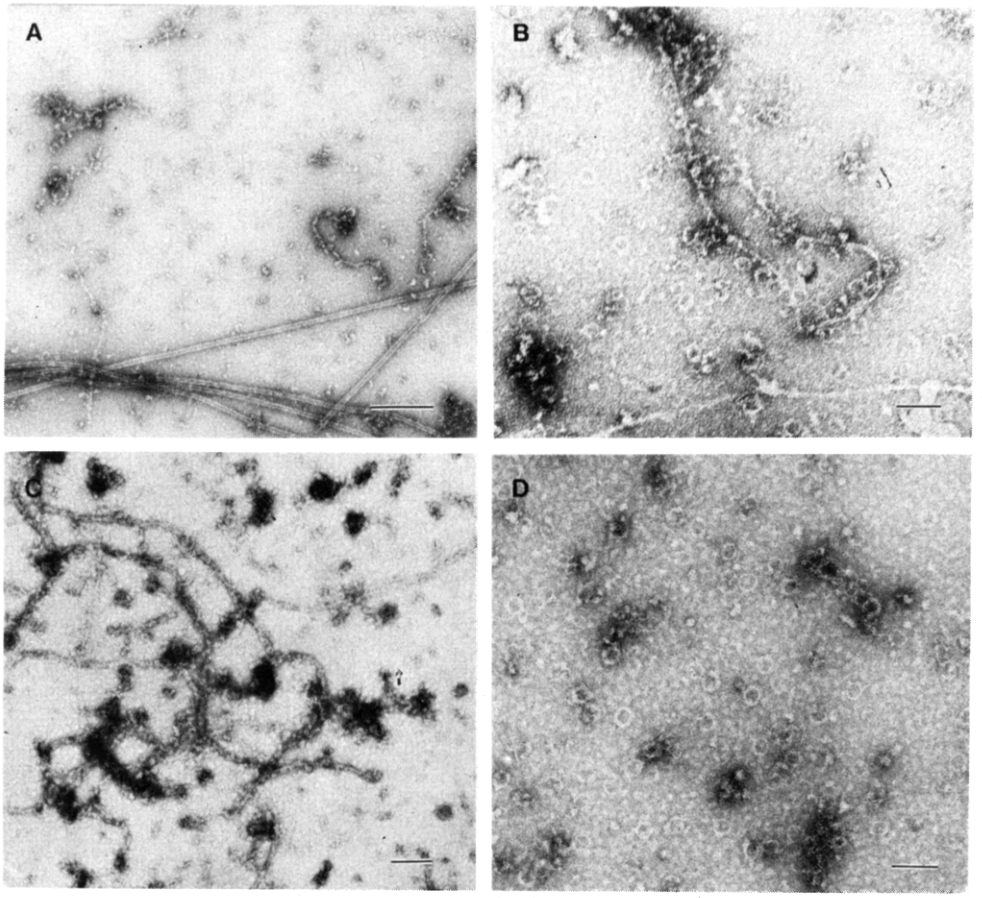

FIGURE 1: Electron microscopy of microtubules and of fractions prepared by centrifugation. (A) Microtubules prepared by two cycles of polymerization and depolymerization were polymerized once more by incubation at 34 °C, sedimented (106000*g* for 60 min), resuspended in cold PM, depolymerized by incubation at 4 °C for 30 min, centrifuged (106000*g* for 15 min), and polymerized at 24 °C. Bar = 0.2 μm. (B) An aliquot of the same sample, taken just before the final polymerization. Bar = 0.1 μm. (C) Microtubules prepared by two cycles of polymerization and depolymerization (as in A) were carried through a third cycle of polymerization, centrifugation, resuspension, and depolymerization. The resulting microtubule protein was subjected to centrifugation at 4 °C in a Beckman type 40 rotor at 106000*g* for 20 min. A sample of the resuspended pellet is shown. Bar = 0.1 μm. (D) Supernatant from a sample prepared identically with that in C, except the final centrifugation, instead of 20 min, was for 90 min at 4 °C and 106000*g*. Bar = 1 μm.

that the majority of the HMW-MAPs are associated not with the filaments but with the rings.

The pellet also contained proteins of approximately 55 000 daltons which appeared to be tubulin (seen as a single band in Figure 2). These were found to be separable from each other by electrophoresis according to the method of Yang & Criddle (1970). When these two electrophoretic fractions were cut from preparative gels and were subjected to peptide mapping and amino acid analysis, they were found to be extremely similar, if not identical, to α- and β-tubulin (data not shown).

To demonstrate that 10-nm filaments are not generated upon storage of cycled microtubule protein, the following experiment was performed. The 10-nm filaments were removed from a sample of cycled microtubule protein by high-speed (95500*g* for 120 min) centrifugation at 4 °C. Electron microscopy showed the supernatant contained rings but no filaments. $NaDodSO_4$–polyacrylamide gel electrophoresis showed only the presence of tubulin and HMW-MAPs. After storage at −30 °C for 4 weeks in PM with 4 M glycerol, careful examination by electron microscopy showed rings but no filaments. $NaDodSO_4$–polyacrylamide gel electrophoresis showed that only the HMW-MAPs and tubulin were present, and no detectable pellet was obtained after centrifugation at 95500*g* for 120 min.

We have also examined the possibility that 10-nm filaments are generated as an aberrant assembly product during assembly of filament-free microtubule protein solutions. Ten-nanometer filaments were removed from a cycled microtubule preparation as described in the preceding paragraph. Following polymerization at 37 °C for 30 min, microtubules but not 10-nm filaments were observed by electron microscopy.

Association of the Major 68 000-Dalton Protein with 10-nm Filaments. The 10-nm filaments were purified directly from crude brain homogenate as described in Materials and Methods. The filaments obtained are shown in Figure 3. They are distinct from filaments obtained from cycled microtubule preparations (cf. Figure 1C) due to their lack of "knobs" or irregular protrusions. In other respects, i.e., diameter, flexibility, and staining characteristics, they are indistinguishable in appearance. They are also morphologically indistinguishable from authentic neurofilaments isolated from other mammalian sources (Yen et al., 1976; Davidson et al., 1977; Schlaepfer, 1977a,b). Following removal of the minor membrane contaminants by sucrose density gradient centrifugation and electrophoretic analysis of the resulting filaments, the pattern shown in Figure 4A was obtained. The four major protein bands visible there are also seen prominently in the filaments isolated from cycled microtubule preparations (cf. Figure 2, pellets at 20–60-min centrifugation, and Figure 4B). These four bands are apparently identical in molecular weights and similar in relative amounts to the four proteins found in purified mammalian neurofilaments (Schlaepfer 1977a,b; Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978).

Table I: Centrifugation of Depolymerized Microtubule Protein[a]

sample	vol (mL)	spin time (min)	protein concn (mg/mL)	% total protein	EM observation
starting material	3		1.30	100	10-nm fil. & rings
pellet I	0.5	10	0.40	5	10-nm fil.
super. I	3	10	1.23	95	10-nm fil. & rings
pellet II	0.5	20	1.07	13	10-nm fil.
super. II	3	20	1.18	87	fewer 10-nm fil., rings
pellet III	0.5	35	1.20	15	10-nm fil., a few rings
super. III	3	35	1.14	85	some 10-nm fil., rings
pellet IV	0.5	60	1.58	20	10-nm fil., more rings seen
super. IV	3	60	1.05	80	very few 10-nm fil., many rings
pellet V	0.5	90	1.80	22	10-nm fil., rings
super. V	3	90	1.06	78	no 10-nm fil. seen, many rings

[a] Microtubules prepared by two cycles of polymerization-depolymerization (short cold spins) were carried through a third cycle of polymerization (30 min at 30 °C), centrifugation (60 min at 106000*g* and 28 °C), resuspension in PM buffer at 4 °C, and depolymerization (30 min at 4 °C). Six 3-mL aliquots were subjected to centrifugation at 106000*g* in a Beckman T40 rotor at 4 °C for the times indicated. All samples were spun for 10 min; then one was removed for analysis. The remaining samples were spun for another 10 min; then another was removed for analysis, etc. Protein concentrations of the supernatants were measured directly, and the concentrations of the filaments were determined following resuspension in 0.5 mL of PM buffer. Grids were made directly from the supernatants and from the pellets after their resuspension.

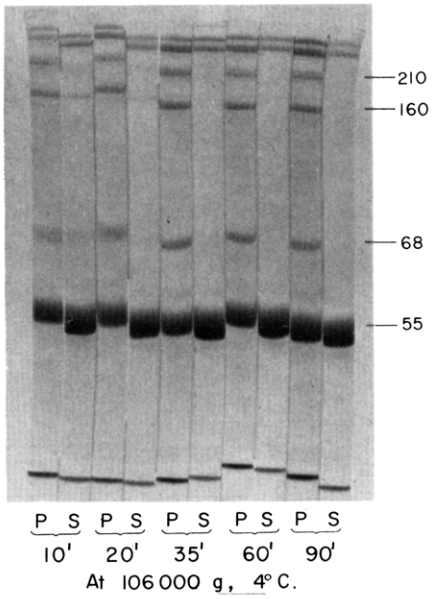

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of the centrifuged fractions of Table I. An aliquot containing 40 μg of total protein was applied to each gel and electrophoresis was carried out as described in the text. Labeling of the gels corresponds to Table I. P (pellet) and S (supernatant) at 10 min are the pellet and supernatant from the first centrifugation, P and S at 20 min are the pellet and supernatant from the second centrifugation, etc. Direction of migration was from top to bottom. The single dark band near the bottom of each gel is the tracking dye. Gels were stained with Fast Green (Gorovsky et al., 1970).

The 55 000-dalton band could be resolved into two bands by electrophoresis in the presence of $NaDodSO_4$–urea, and it is most probably tubulin. From quantitative staining of these gels, it was found that >85% of the total protein in the filaments is accounted for by the four major polypeptides.

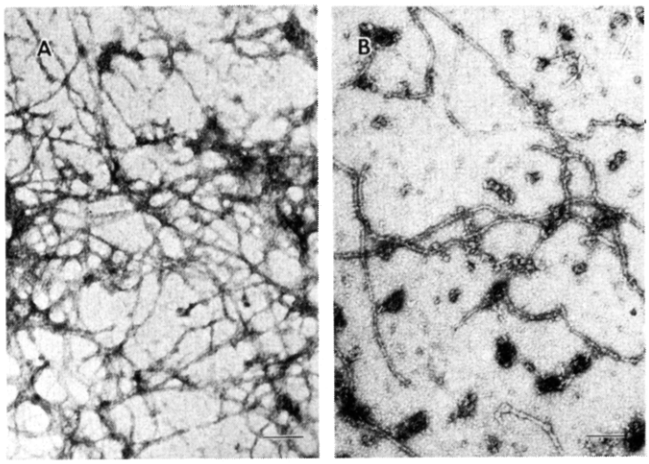

FIGURE 3: Electron micrographs of 10-nm filaments purified directly from brain. The 10-nm filaments were isolated from crude brain homogenate by chromatography on Bio-Gel A-150m and further purified as described in Materials and Methods. The protein composition of this fraction is shown in Figure 4A. (A) Low magnification field showing 10-nm filaments as the major morphological constituent. Bar = 0.2 μm. (B) High magnification field (cf. Figure 1C). Bar = 0.1 μm.

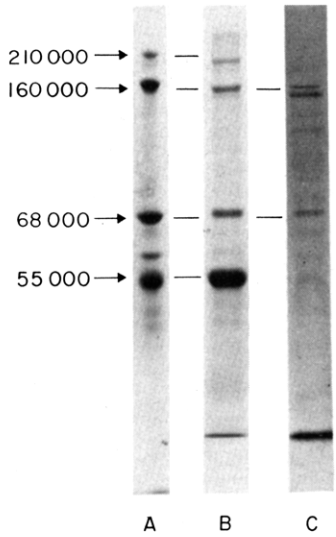

FIGURE 4: Protein composition of 10-nm filaments. (A) Ten-nanometer filaments isolated directly from brain by chromatography of crude brain homogenate on Bio-Gel A-150m followed by further purification as described in Materials and Methods; (B) 10-nm filaments isolated from twice-cycled microtubule preparations by chromatography on Bio-Gel A-150m as described in Materials and Methods; (C) 10-nm filaments, prepared as in B, heated to 100 °C for 7 min in PM buffer without GTP and centrifuged at 4 °C and 120000*g* for 15 min to remove heat-denatured material. An aliquot of the supernatant is shown. Approximate apparent molecular weights are shown. Direction of migration is from top to bottom. Molecular weights marked are approximate.

Filament Proteins in Microtubule Preparations. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed on samples from each step in a preparation of cycled microtubules in which long cold centrifugations (60 min at 95500*g*) were employed. The results are shown in Figure 5. It is apparent that the pellets from the cold centrifugations contain the bulk of the 10-nm filament proteins (55 000, 68 000, 160 000, and 210 000 daltons). Examination of the resuspended pellets from the first and second cold centrifugations in the electron microscope showed the presence of 10-nm filaments.

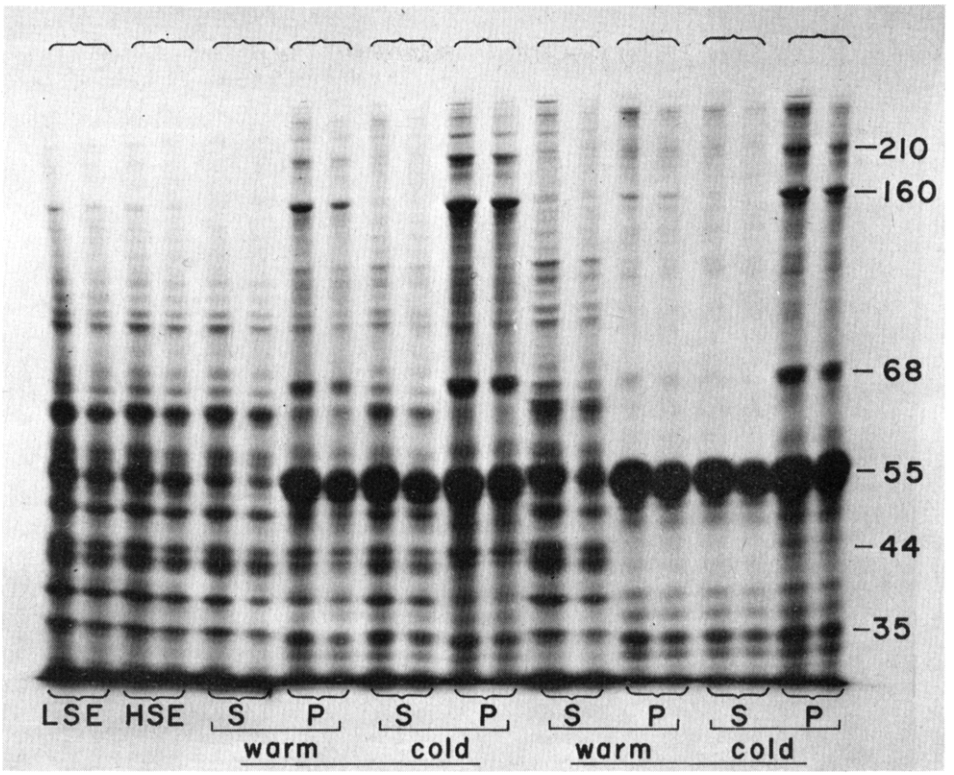

FIGURE 5: Microtubule purification by in vitro assembly/diassembly. Approximately 50- and 33-μg aliquots from each fraction (grouped as indicated by the brackets) throughout the microtubule preparative scheme were loaded on an 8% $NaDodSO_4$–polyacrylamide slab gel and electrophoresis was performed as described in Materials and Methods. LSE, low speed extract; HSE, high speed extract; supernatants (S) and pellets (P) from warm and cold centrifugations are indicated, left to right, through two cycles of assembly and diassembly. In this preparation, 60-min clearing centrifugations at 4 °C were employed. Approximate apparent molecular weights are marked.

It is interesting to note that after one cycle of polymerization the microtubule pellet (i.e., the pellet from the first warm centrifugation) contained four closely spaced bands between 56 000 and 64 000 daltons, likely candidates for τ (Cleveland et al., 1977a). These proteins remained in the supernatant (with the majority of tubulin) after resuspension and depolymerization of microtubules and centrifugation at 4 °C. However, when this fraction was warmed (for microtubule assembly) and microtubules were pelleted, the four closely spaced bands were greatly enriched in the supernatant of this second warm centrifugation and were not visible in the microtubule pellet.

Removal of Proteins from 10-nm Filaments. Preliminary attempts have been undertaken to dissociate the 68 000-dalton protein and other proteins from the 10-nm filaments. Either 1 M NaCl, 0.7 M KCl, 5 mM $CaCl_2$, or 0.5 mM colchicine was added to aliquots of filaments suspended in PM buffer without GTP at 4 °C. After incubation for 30 min, the samples were centrifuged for 90 min at 106000*g*. $NaDodSO_4$–polyacrylamide gels of the pellet and supernatant of each sample showed that no detectable 68 000-dalton protein remained in the supernatant.

Another solution of 10-nm filaments was heated at 100 °C for 7 min in PM buffer without GTP and then centrifuged at 4 °C and 120000*g* for 15 min. Assay of the protein concentration in the supernatant showed that 10–15% of the filament proteins remained in the clarified supernatant. $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4C) showed that the 68 000-dalton protein and the 160 000-dalton protein, as well as traces of others, composed this heat-stable fraction.[2] When examined in the electron microscope, this supernatant contained no visible 10-nm filaments.

Polymerization of Cycled Microtubules in the Presence and Absence of 10-nm Filaments. Polymerization of an aliquot of cycled microtubule protein (which contains 10-nm filaments) and of another aliquot of the same material from which the filaments had been centrifugally removed is shown in

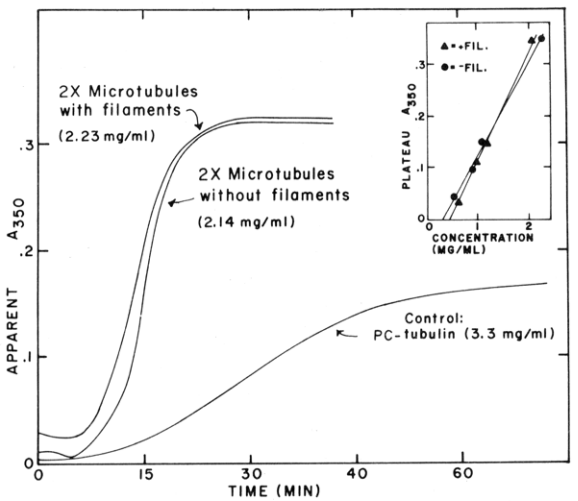

FIGURE 6: Microtubule polymerization in the presence and absence of filaments. Microtubule protein, prepared by two cycles of the polymerization–depolymerization procedure, was prepared as described in the caption to Figure 1C. The resulting microtubule protein was centrifuged for either 15 or 90 min at 106000*g* and 4 °C. The material subjected to the shorter spin (labeled "with filaments") contained numerous 10-nm filaments, while the material spun for 90 min (labeled "without filaments") contained no detectable filaments. The protein concentration in each fraction is shown. The difference represents filaments removed. The solutions were warmed from 4 to 34 °C at zero time, and apparent absorbance was observed as described in Materials and Methods. The control sample is an aliquot of PC-tubulin in PM buffer with 1 mM GTP. Inset: measurement of plateau turbidity as a function of total protein concentration for other diluted aliquots of the same two solutions.

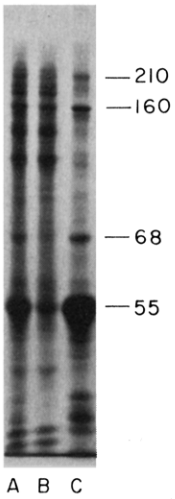

FIGURE 7: Phosphocellulose microtubule-associated protein fractions. Chromatography of microtubule proteins on phosphocellulose was performed as described in Materials and Methods. The fraction of bound protein was eluted with PM + 0.7 M NaCl. The protein-containing fractions were pooled and an aliquot was dialyzed against PM to remove salt and subjected (A) to electrophoresis. The remainder of this whole MAP fraction, in PM + 0.7 M NaCl, was centrifuged. The supernatant (B) and pellet (C) (PC-MAP-P) are represented. Approximate apparent molecular weights are shown. Direction of migration is from top to bottom.

Figure 6. It is apparent that the removal of the filaments had no appreciable effect on either the rate or extent of polymerization of microtubules at the concentration shown. The inset shows plateau turbidities obtained with different concentrations of the same protein preparations. Critical concentrations extrapolated for each of the two preparations show little, if any, significant differences. The curve labeled PC-tubulin is included for comparison. This protein showed a lower rate and extent of assembly despite its higher concentration. Thus, although the filaments are removed by centrifugation, residual nonsedimentable factors must be acting to favor polymerization in the cycled microtubule protein.

Stimulation of Assembly of Phosphocellulose-Purified Tubulin. Chromatography of cycled microtubule proteins on phosphocellulose produced a nonadsorbed peak which was about 95% tubulin and a second fraction of bound protein (referred to as whole PC-MAP fraction) which was eluted with PM + 0.7 M NaCl. Its protein composition is shown in Figure 7A. This second fraction was further resolved into two components by centrifugation at 106000*g* for 60 min, 4 °C. (The supernatant is referred to below as PC-MAP-S and the pellet as PC-MAP-P.) As shown in Figure 7B the supernatant (PC-MAP-S) was enriched in proteins of high molecular weight, while the pellet (PC-MAP-P) shown in Figure 7C contained the bulk of the 68 000-dalton protein and other 10-nm filament proteins. When examined by electron microscopy, the major morphological constituent of the pellet fraction was seen to be 10-nm filaments, while the supernatant contained none. In addition, both PC-MAP-S and PC-MAP-P fractions were found to contain heat-stable polypeptides. These polypeptides were isolated by boiling each of the two fractions (PC-MAP-S and PC-MAP-P) for 5 min in PM buffer + 0.75 M NaCl, removing denatured protein by centrifugation (106000*g* for 20 min), and dialyzing the supernatant against PM buffer. The resulting fractions are designated PC-MAP-S_B (boiled supernatant) and PC-MAP-P_B (boiled pellet), respectively.

To assess the effect of each of these fractions on the polymerization of phosphocellulose-purified tubulin, an aliquot of each was added to a sample of PC-tubulin, and polymerization was assayed by measurement of turbidity at 34 °C. The overall fractionation scheme is delineated in Figure 8A. Amounts of each fraction, tubulin, and buffer were adjusted to produce a tubulin concentration of 1.0 mg/mL and a total protein concentration of 1.5 mg/mL (Figure 8B) or 1.18 mg/mL (Figure 8C).

It is apparent from Figure 8B that phosphocellulose-purified tubulin at 1.0 mg/mL showed little polymerization. Polymerization was enhanced by addition of whole PC-MAP fraction (experiment 1). After resolution of PC-MAPs into PC-MAP-S and PC-MAP-P, addition of PC-MAP-S to PC-tubulin stimulated assembly (experiment 3), while PC-MAP-P, which contained 10-nm filaments, did not stimulate assembly (experiment 2). Figure 8C compares the stimulatory effect of PC-MAP-S_B and PC-MAP-P_B, experiments 4 and 5. The PC-MAP-P_B fraction (which before boiling contained no assembly stimulating activity) after boiling stimulated assembly in a fashion very similar to that of the PC-MAP-S_B fraction (which before boiling also stimulated assembly). It is thus apparent that the proteins which are liberated by boiling the 10-nm filaments are capable of stimulating microtubule assembly after they are released from the filament but not before.

Heat-stable polypeptides have also been prepared from 10-nm filaments isolated directly from crude brain homogenates. An aliquot of 10-nm filaments purified directly from crude brain homogenate was boiled for 7 min and centrifuged to remove denatured material as described previously. Whereas the filaments before boiling do not stimulate PC-tubulin (1 mg/mL) to assemble into microtubules, the heat-stable protein fraction, consisting of polypeptides of molecular weight 68 000, 160 000, and 210 000, stimulates assembly in a manner similar to that of the PC-MAP-P_B fraction.

Cosedimentation of Heat-Stable Polypeptides with Tubulin upon Polymerization. Fractions containing heat-stable polypeptides from 10-nm filaments were obtained by boiling PC filaments and removing denatured protein by centrifugation. A second fraction was obtained by boiling 10-nm filaments isolated directly from crude brain homogenate and removing denatured protein by centrifugation. The third fraction was obtained by boiling twice-cycled microtubules (prepared with 15-min, cold, clearing centrifugations) and removing denatured protein by centrifugation. The fourth fraction was obtained from the third by passing an aliquot over phosphocellulose in PM buffer and then eluting the protein bound to phosphocellulose with a gradient of PM buffer containing 0–0.5 M KCl. This fourth fraction was greatly enriched in the 68 000-dalton protein.

In each case the assembly stimulating fractions contained the 68 000-dalton protein, bands at 160 000 and 210 000 daltons, and a number of less prominent bands. Aliquots of each fraction (in PM buffer) were added to PC-tubulin (in PM + 1 mM GTP), and volumes were adjusted such that the final tubulin concentration was 1.0 mg/mL and the total protein concentration was 1.1 mg/mL. These solutions were warmed to 34 °C for 30 min and microtubules were subsequently pelleted by centrifugation (106000*g* for 30 min, 27 °C). The centrifugations used to pellet microtubules in these experiments were insufficient to pellet any of the polypeptides from the heat-stable protein fractions before incubation with PC-tubulin. Aliquots of each supernatant and pellet were saved for electron microscopy and gel electrophoresis.

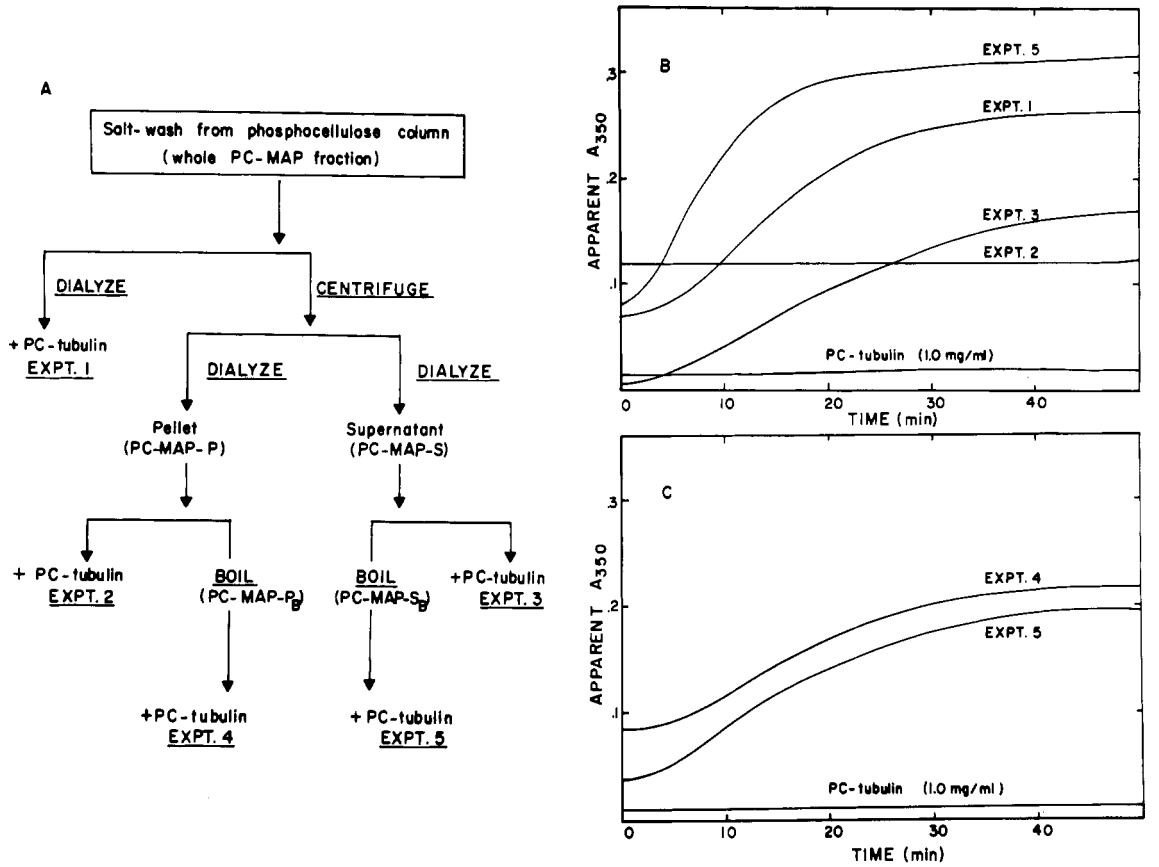

FIGURE 8: Stimulation of microtubule polymerization by subfractions of the whole PC-MAP fraction. (A) Fractionation scheme employed in the preparation of subfractions tested at right. Experiment numbers correspond to those shown on the graphs. (B) Polymerization of PC-tubulin to which had been added either nothing (labeled "PC-tubulin") or the fraction indicated by the experiment number. Amounts of each fraction, of tubulin and of buffer, were adjusted to produce a tubulin concentration of 1.0 mg/mL and a total protein concentration of 1.5 mg/mL. (C) Same as above but with concentrations of tubulin of 1.0 mg/mL and a total protein concentration of 1.18 mg/mL. The zero level of absorbance was established before each experiment with buffer in the sample cuvette. Because fractions containing 10-nm filaments are noticeably turbid, the base line was much higher for polymerization mixtures (experiment 2) than for mixtures containing only nonsedimentable MAPs and tubulin.

Observations of negatively stained samples from each pellet in the electron microscope showed that the major product present was intact microtubules. Analysis of pellets and supernatants by $NaDodSO_4$–polyacrylamide gel electrophoresis showed that the 68 000-, 160 000-, and 210 000-dalton proteins were greatly enriched in the pellet fractions. Other heat-stable proteins present in the stimulating fractions did not appear to be enriched in the microtubule pellets.

Discussion

The data presented above show that about 20% of the total protein present in microtubule preparations made by two cycles of the assembly–disassembly procedure can be removed by a 90-min centrifugation at 106000*g* and 4 °C. The final pelleted material consists mainly of filaments 10 nm in diameter, but includes a substantial number of rings (the relative amount of ring material present is negligible with short centrifugation and increases with longer centrifugation times). These findings are in accord with those of Berkowitz et al. (1977) and of Keats & Hall (1975). They found that filaments of the same protein composition and general appearance are present in microtubule preparations. In the present work, depolymerized cycled microtubule preparations and the MAP-containing fractions eluted from phosphocellulose were observed to be markedly turbid or hazy in appearance. In the experiments reported in Table I, the supernatant became clear after a 35-min centrifugation, before substantial numbers of rings had been removed from it. Centrifugation of the phosphocellulose-prepared MAP fractions of Figures 7 and 8 removed both the 10-nm filaments and the haziness. These results suggest that 10-nm filaments, not rings, are the primary source of the characteristic turbid appearance of depolymerized microtubule preparations.

The turbidity of similar MAP (or crude τ) fractions has been reported (Cleveland et al., 1977a; Lockwood, 1978) and it seems likely that 10-nm filaments are often a component of these fractions. Berkowitz et al. (1977) have noted that the distribution of sedimentation coefficients of these filaments is such that the majority of them will not be pelleted in the centrifugation steps (95500*g* for 15 min in this work) applied at 4 °C to remove nondissociable materials. Their presence is therefore not unexpected in any preparation where short, cold centrifugations are employed. That the 10-nm filaments can be removed by longer cold centrifugations is shown by Table I. This fact is also apparent from comparison of the preparation shown in Figure 5, where cold centrifugations of 60 min at 95500*g* were employed, with that shown in Figure 2, where conditions were identical except that cold centrifugations during the assembly–disassembly procedure were 15 min at 95500*g*. The low-temperature supernatant after the second cycle of polymerization–depolymerization shown in Figure 5 is virtually devoid of filament marker proteins, while the preparation employed for the experiments in Figure 2 contains substantial amounts of the major filament proteins.

In agreement with the results of Berkowitz et al. (1977) it was found that most of the nonquantitatively copurifying microtubule-associated proteins sediment with the 10-nm filaments (Table I; Figures 2 and 5). This finding does not

demonstrate that all of the cosedimenting proteins are structural parts of the 10-nm filaments. However, the proper filament proteins must be among them. The filaments of similar morphology isolated directly from brain (rather than from microtubule preparations) were found to have associated with them four proteins common to them and to the microtubule-derived filaments. One of the proteins is almost certainly tubulin, which may not be part of the essential structure of the filaments.

Of particular interest among these proteins is the prominent one of 68 000 daltons. All observed properties of this protein suggest that it is one of the τ peptides originally described by Weingarten et al. (1975) and that it is most likely identical with the TAP protein isolated from bovine brain by Lockwood (1978). This protein appears to be the only one of molecular weight near 68 000 present in the once- and twice-cycled microtubule preparations made with short, cold centrifugation steps (Figure 2). The preparative conditions employed above are quite similar to those employed in preparation of the microtubules from which τ and TAP are eventually prepared (Cleveland et al., 1977a; Lockwood, 1978). This major 68 000-dalton protein is present in the fraction adsorbed to phosphocellulose and it is eluted by 0.7 M NaCl, as are τ and TAP. It is stable to heating for 5 min at 100 °C, as are τ and TAP. The solutions containing the heat-treated 68 000-dalton protein stimulate assembly of phosphocellulose-purified tubulin, as do τ and purified TAP. Although the possibility remains that a component of this assembly-stimulating solution other than the 68 000-dalton protein is responsible for its activity, the strong similarity of the purification scheme of the 68 000-dalton protein to those of τ and to the limited published steps of TAP preparation makes it seem most likely that it is TAP and that assembly is stimulated by it. This strong likelihood cannot be further investigated at this time because of the lack of a published procedure for preparation of TAP.

It is difficult to prove that a given protein component of an observed structure is an intrinsic part of that structure. The 68 000-dalton protein is not easily dissociated from the 10-nm filaments. It sediments with them in PM buffer (Figures 2 and 5) and is not dissociated from them by high ionic strength, high concentrations of Ca^{2+}, or by colchicine. Boiling, an extreme treatment which probably disrupts the filament structure, does release some of the 68 000-dalton protein (Figure 4). It is thus certain that the 68 000-dalton protein is strongly associated with the 10-nm filaments, and it is possible that it is an intrinsic component of them. Murphy et al. (1977) have noted that chromatography of microtubule-associated proteins on 4% agarose yields a turbid void volume fraction which contains substantial amounts of a 68 000-dalton protein as well as tubulin and bands at apparent molecular weights 160 000 and 210 000. Keates & Hall (1975) have noted a 68 000 molecular weight protein not dissociable by high ionic strength from filaments similar in appearance to those described here. Recent reports on the protein composition of mammalian neurofilaments show that antibodies to a 68 000-dalton protein decorate purified neurofilaments (Schlaepfer, 1977b).

Comparison of evidence presented under Results with evidence in the literature suggests that the 10-nm filaments are neurofilaments. The 10-nm filaments (Figure 1C) isolated from microtubule preparations are similar in appearance to isolated neurofilaments from several mammalian sources (Yen et al., 1976; Davison et al., 1977; Schlaepfer, 1977a,b; Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978), differing only by the presence of "knobs". Electron microscopy of the filaments prepared directly from crude brain homogenate without the use of glycerol shows them to have appearances identical with those mentioned above. (Their lack of "knobs" is accompanied by a reduced amount of tubulin and HMW-MAPs. It can be speculated that the "knobs" are composed either of tubulin and/or of HMW-MAPs.) The protein composition of the filaments is also similar or identical with that of neurofilaments from several different mammalian sources. The three proteins of apparent molecular weights 210 000, 160 000, and 68 000, as well as tubulin, are characteristic of the neurofilaments prepared by Schlaepfer (1977a,b), by Schlaepfer & Freeman (1978), and by Micko & Schlaepfer (1978). Proteins of these molecular weights have been identified as moving together in slow axonal transport (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976). The possibility has been raised that more than one kind of mammalian neurofilament is present in the nervous system (Gilbert et al., 1975; Lasek & Hoffman, 1976; Gilbert, 1978). There are reports (Yen et al., 1976; Davison et al., 1977) that neurofilaments can be prepared which are primarily composed of a protein or proteins (not tubulin) of 50 000–55 000 daltons. The 10-nm filaments described here are not related to these filaments. The 55 000-dalton protein found in association with them is almost certainly tubulin.

That the 10-nm filaments are not an artifact of microtubule preparation or of storage of microtubule protein was suggested by Berkowitz et al. (1977). Further evidence for this is provided by the fact that the protein triad (210 000, 160 000, and 68 000 daltons) is characteristic of the filaments present in microtubule preparations from their early stages (Figure 5). In addition, the filaments were shown not to be generated during storage or assembly of microtubule protein. The significance of the large amount of tubulin present in the filament fraction is not clear at present. The protein is tightly associated with the filaments, but there is no evidence that it is a part of their structure.

Despite the fact that they appear to contain the major 68 000-dalton protein and that this protein is probably one of the assembly-stimulating τ proteins, the removal of the filaments, along with all detectable amounts of this material, has little or no effect on polymerization (Figure 6). The difference in apparent absorbance observed at zero time is attributable to the presence of the filaments in one sample and their absence in the other. The small observed difference in critical concentration is not significant within the precision of the measurements. The control indicates that, even at higher tubulin concentrations than those present in the cycled microtubule protein, the rate and extent of assembly of PC-tubulin was substantially less. Therefore, it may be inferred that some component(s) of the cycled microtubule preparation other than 10-nm filaments were responsible for a substantial stimulation of assembly. The preparation still contains HMW-MAPs after the filaments have been sedimented away, and it seems probable that they are responsible for the observed stimulation (Borisy et al., 1975; Murphy & Borisy, 1975; Sloboda et al., 1976). It appears that the intact filaments play little or no role in stimulation of assembly and that the 68 000-dalton protein, when it is incorporated into the filaments, is of little or no importance in this assembly, at least under the present conditions of assay. The possibility remains that the 68 000-dalton protein may be reversibly inactivated or "masked" in the filaments as they are isolated and that it may have assembly-stimulating activity in vivo.

As shown in Figure 8B, and in agreement with numerous previous observations (Weingarten et al., 1975; Cleveland et

al., 1977a; Fellous et al., 1977; Herzog & Weber, 1977; Lockwood, 1978), at 1 mg/mL, phosphocellulose-purified tubulin showed little or no assembly as observed by A_{350}. In further agreement with previous observations (Weingarten et al., 1975; Cleveland et al., 1977a; Fellous et al., 1977), when the whole MAP fraction from phosphocellulose chromatography of cycled microtubules was added to PC-tubulin, assembly proceeds in a manner very similar to that observed for cycled microtubules (low critical concentration). Fractionation of the whole PC-MAP fraction by centrifugation yielded (i) a supernatant fraction which showed higher assembly stimulating activity/mg of protein than did the whole PC-MAP fraction and (ii) a pellet which when resuspended was turbid, contained 10-nm filaments and the major 68 000-dalton protein, and did not activate microtubule assembly. Thus it seems that the assembly-stimulating factors in the whole phosphocellulose-derived MAP fraction remain in the supernatant, while the 68 000-dalton protein is pelleted.

These two fractions were further subfractionated by boiling each of them and then centrifuging each to remove denatured protein. The boiled PC-MAP supernatant fraction (PC-MAP-S_B) still stimulated assembly and the boiled PC-MAP pellet (PC-MAP-P_B) also stimulated assembly in a similar manner. Thus, the 10-nm filament fraction, which contains the major 68 000-dalton protein, did not stimulate microtubule assembly. But following boiling (which released some of the 68 000-, 160 000-, and 210 000-dalton proteins, as well as very small amounts of others), this fraction showed as high a degree of assembly stimulation per mg of protein as any fraction tested. When heat-stable polypeptide fractions (prepared in several different ways) are mixed with PC-tubulin, and the solution is warmed to 34 °C, and assembled microtubules are collected by centrifugation, then in each case the 68 000-dalton protein is enriched in the microtubule pellet. In addition, the 160 000- and 210 000-dalton polypeptides are also enriched in the microtubule pellets. It is possible that any or all three are responsible for the assembly stimulation seen. This may not be unexpected in light of recent evidence for immunological cross-reactivity among neurofilament proteins of similar molecular weights (Liem et al., 1978). If, as seems likely, the 68 000-dalton protein corresponds to the TAP element of the τ proteins and does contribute to the assembly stimulation seen, its assembly-stimulating activity can be seen in vitro only when it is released from its association with the 10-nm filaments. The heat-treatment step applied here is similar to heat-treatment steps employed in the preparation of both τ (Cleveland et al., 1977a,b; Fellous et al., 1977) and TAP (Lockwood, 1978).

Another observation recorded in Figure 8B is that heat treatment apparently purifies or activates components of the HMW-MAPs (compare PC-MAP-S_B with PC-MAP-S). This observation accords with similar experiments of Kim et al. (1979), who used boiling as a step in the purification of MAP_2 from the centrifuged PC-MAP supernatant.

The major 68 000-dalton protein in these microtubule preparations is probably a component of the contaminating 10-nm filaments. It is not responsible for the low critical concentrations observed for assembly of cycled microtubules, and it stimulates assembly of PC-tubulin (if at all) only when released from the filaments by heat treatment. The reported fact that TAP, which is probably identical with the major 68 000-dalton protein in these preparations, has high affinity for tubulin (Lockwood, 1978) suggests that the 68 000-dalton protein present in 10-nm filaments may be masked by tubulin bound to it. If so, it is possible that 10-nm filaments (devoid of tubulin) may interact with tubulin and microtubules in vitro, and such interactions may occur in vivo as well.

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Assembly of Nonneural Microtubules in the Absence of Glycerol and Microtubule-Associated Proteins†

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ABSTRACT: Microtubule protein from Ehrlich ascites tumor cells purified by an in vitro polymerization process in the absence of glycerol and calcium chelators contains several accessory proteins but lacks the high molecular weight proteins which are present in neurotubulin. DEAE-Sephadex chromatography of two-times cycled tubulin removes these nontubulin proteins, resulting in pure tubulin, as critically examined by sodium dodecyl sulfate gel electrophoresis. This tubulin can readily assemble into microtubules in assembly buffer, at low magnesium concentrations, without glycerol and at tubulin concentrations above 0.8 mg/mL. Electron microscopy shows that the tubules are identical with normal microtubules. When the purified tubulin fraction was reduced and carboxymethylated, a significant minor protein component could be observed electrophoretically, migrating between α- and β-tubulin. At present, the identity and function of this protein are not known. The results demonstrate that the in vitro assembly of tubulin from Ehrlich ascites tumor cells does not require high molecular weight proteins or τ-like factor(s) as has been proposed for the neurotubulin system.

To experimentally analyze the mechanisms of microtubule assembly in living cells, it is necessary to study the biochemistry of microtubules in vitro and to correlate these findings with the in vivo role of microtubules. Until recently, almost all of the information about microtubule assembly in vitro came from experiments with either partially purified tubulin or crude cytosol of mammalian brain. Microtubule-associated proteins (MAPs)[1] have been described in brain tissue that stimulate the in vitro assembly of microtubules (Gaskin et al., 1974; Dentler et al., 1975; Murphy & Borisy, 1975; Weingarten et al., 1975; Sloboda et al., 1976; Erickson, 1974; Borisy et al., 1974). These MAPs copurify with neurotubules through several cycles of polymerization and depolymerization and can be separated from tubulin by ion-exchange chromatography. The protein(s) necessary for stimulation of microtubule assembly has been designated either the high molecular weight [M_r(h)] MAPs (Murphy & Borisy, 1975; Borisy et al., 1975; Keates & Hall, 1975; Murphy et al., 1977) or the τ factor (Weingarten et al., 1975; Cleveland et al., 1977a,b). In some reports it has been postulated that MAPs are absolutely required for the in vitro assembly of neurotubulin (Weingarten et al., 1975; Sloboda et al., 1976; Bryan et al., 1975). However, in others it has been shown that purified tubulin can readily assemble into microtubules in the absence of nontubulin proteins and under appropriate conditions (Murphy et al., 1977; Lee & Timasheff, 1975, 1977; Erickson & Voter, 1976; Himes et al., 1976; Wheland et al., 1977; Herzog & Weber, 1977; Lee et al., 1978). Several investigators have described the presence of ring forms in neurotubulin complexed with MAPs (Erickson, 1974; Kirschner et al., 1974; Doenges et al., 1976; Marcum & Borisy, 1978). It has been reported that these rings may be intermediates in the assembly process and that they are probably essential for tubulin polymerization (Olmsted et al., 1974; Erickson, 1974; Kirschner et al., 1974).

Recently, the reversible in vitro polymerization of microtubules from sources other than brain has been reported (Wiche & Cole, 1976; Wiche et al., 1977; Weber et al., 1977; Doenges et al., 1977; Nagle et al., 1977; Weatherbee et al., 1978; Farrell & Wilson, 1978). It has been found that microtubule protein from nonneural and cultured cells (Doenges et al., 1977; Nagle et al., 1977; Weatherbee et al., 1978) and from sea urchin sperm tail outer doublet microtubules (Farrell & Wilson, 1978) lacks M_r(h) MAPs but contains some proteins of intermediate molecular weight associated with

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[1] Abbreviations used: EAT, Ehrlich ascites tumor; M_r(h), high molecular weight components; Pipes, 1,4-piperazinediethanesulfonic acid 5625-37-6; RB, reassembly buffer; M_r, molecular weight; MAPs, microtubule-associated proteins.

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