# Separation of Active Tubulin and Microtubule-Associated Proteins by Ultracentrifugation and Isolation of a Component Causing the Formation of Microtubule Bundles†

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ABSTRACT: A new method for separating microtubule-associated proteins (MAPs) and tubulin, appropriate for relatively large-scale preparations, was developed. Most of the active tubulin was separated from the MAPs by centrifugation after selective polymerization of the tubulin was induced with 1.6 M 2-(*N*-morpholino)ethanesulfonate (Mes) and GTP. The MAPs-enriched supernatant was concentrated and subsequently clarified by prolonged centrifugation. The supernatant (total soluble MAPs) contained almost no tubulin, most of the nucleosidediphosphate kinase activity of the microtubule protein, good activity in promoting microtubule assembly in 0.1 M Mes, and proteins with the electrophoretic mobility of MAP-1, MAP-2, and $\tau$ factor. The pellet, inactive in supporting microtubule assembly, contained denatured tubulin, most of the ATPase activity of the microtubule protein, and significant amounts of protein with the electrophoretic mobility of MAP-2. Insoluble material at this and all previous stages, including the preparation of the microtubule protein, could be heat extracted to yield soluble protein active in promoting microtubule assembly and containing MAP-2 as a major constituent. The total soluble MAPs were further purified by DEAE-cellulose chromatography into bound and unbound components, both of which induced microtubule assembly. The bound component (DEAE-MAPs) contained proteins with the electrophoretic mobility of MAP-1, MAP-2, and $\tau$ factor. The polymerization reaction induced by the unbound component (flow-through MAPs) produced very high turbidity readings. This was caused by the formation of bundles of microtubules. Although the flow-through MAPs contained significantly more ATPase, tubulin-independent GTPase, and, especially, nucleosidediphosphate kinase activity than the DEAE-MAPs, preparation of a MAPs fraction without these enzymes required heat treatment.

Microtubules isolated from brain tissue homogenates consist not only of tubulin but also of a number of minor components termed microtubule-associated proteins (MAPs).[1] Generally separated from tubulin by ion-exchange chromatography (Weingarten et al., 1975; Murphy & Borisy, 1975), the MAPs markedly enhance in vitro microtubule assembly, particularly at low ionic strengths and low divalent cation and tubulin concentrations. Although on polyacrylamide gel electrophoresis a large number of bands are usually visualized in MAP preparations, most attention has thus far been directed at only three protein components. These are the high molecular weight proteins termed MAP-1 and MAP-2, which usually form the most prominent non-tubulin bands on gels of microtubule protein (Murphy & Borisy, 1975; Sloboda et al., 1976; Berkowitz et al., 1977), and a group of intermediate molecular weight, the $\tau$ proteins (Weingarten et al., 1975; Cleveland et al., 1977), which become more notable on gels after MAPs and tubulin are separated.

In our own studies we have been routinely disappointed by low yields of MAPs obtained by chromatography of microtubule protein on DEAE-cellulose with subsequent heat treatment (Hamel et al., 1981). In an effort to increase the amounts of MAPs we could obtain, we began to explore the selective polymerization of tubulin by high concentrations of organic anions (Waxman et al., 1981; Hamel et al., 1982) as a means to separate tubulin and MAPs.

## Materials and Methods

*Materials.* Mes, ATP, GTP, and GDP were obtained from Sigma; [$^3$H]colchicine was from Amersham; [$^{32}$P]$P_i$ was from ICN; monosodium glutamate was from Grand Island. The method of Walseth & Johnson (1979) was used to prepare [$\gamma$-$^{32}$P]ATP and [$\gamma$-$^{32}$P]GTP. Purified calf brain tubulin was prepared as described previously (Hamel & Lin, 1981). Stock solutions of Mes were adjusted to pH 6.4 or 6.9 with NaOH and of monosodium glutamate to pH 6.6 with HCl. Solid, neutralized Mes was obtained by lyophilization of a 2 M solution of Mes (pH 6.9).

*General Methods.* Protein concentrations were determined by the method of Lowry et al. (1951). Beckman rotors were used in all centrifugations. In a "warm centrifugation", the rotor was prewarmed to 37 °C, and the centrifuge's refrigeration system was off. In a "cold centrifugation", the rotor was prechilled, and the centrifuge's refrigeration system was set at 0 °C. All centrifugations were in a Ti 45 rotor at 40000 rpm, except as indicated. If a heat treatment was performed, the protein solution was placed in a boiling water bath and maintained at 90 °C for 10 min. Solution A was 0.1 M Mes (pH 6.4), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, and 0.1 mM GTP. Solution B was 0.5 M Mes (pH 6.9) and 2 mM dithiothreitol. Solution C was 0.1 M Mes (pH 6.9) and 2 mM dithiothreitol. Protein solutions, in 1-cm dialysis tubing, were concentrated with dry Sephadex G-200. Protein recovery was at least 95%. All proteins were stored in liquid nitrogen.

*Preparation of Microtubule Protein.* Forty calf brains (14.06 kg) were homogenized in a Waring blender in 0.75 mL/g of solution A containing 4 M glycerol. The homogenate was centrifuged in the cold at 32000 rpm in Ti 15 zonal rotors for 1 h. The supernatant was accumulated (9800 mL) and

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[1] Abbreviations: MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonate; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; Pipes, 1,4-piperazinediethanesulfonate.

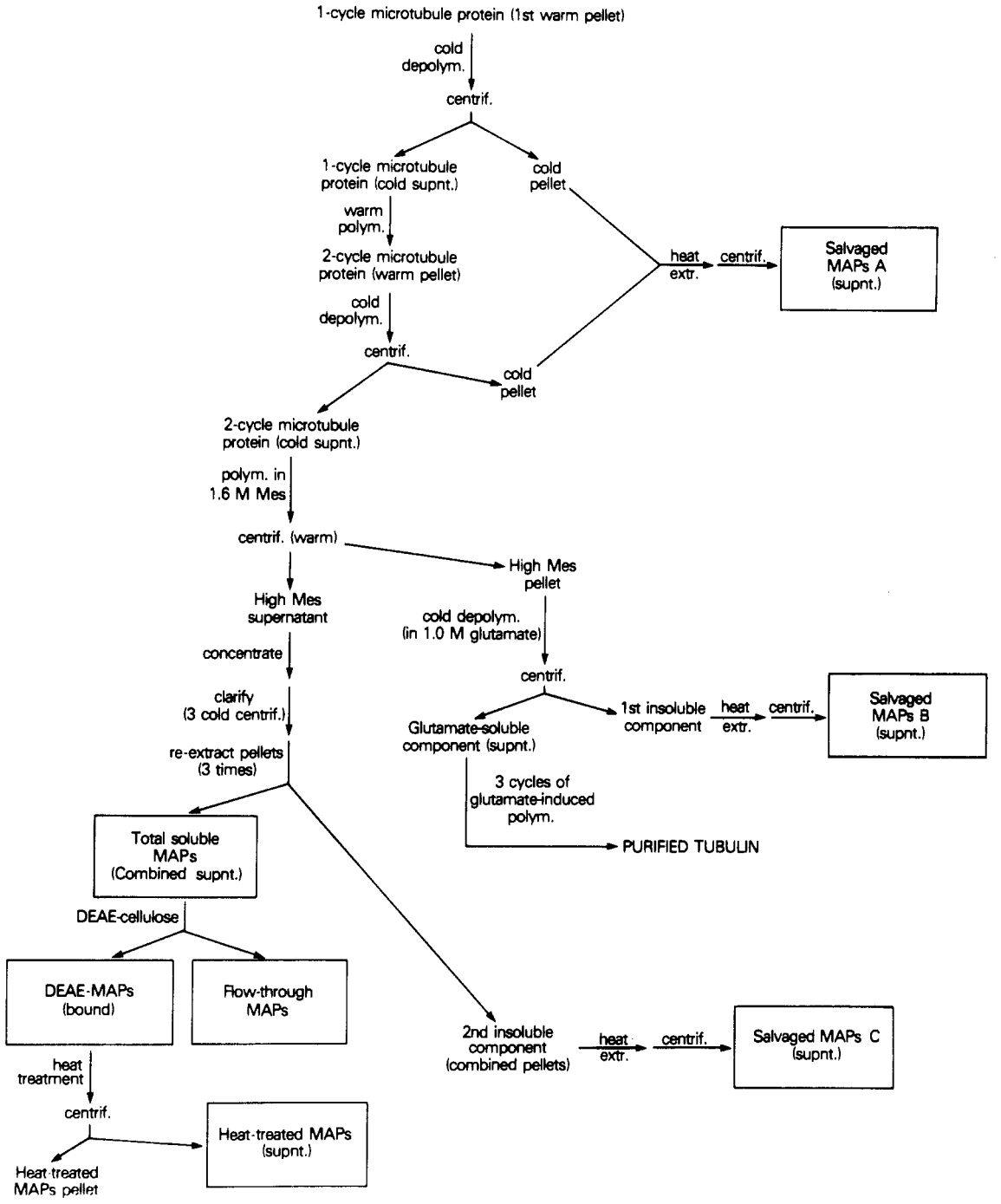


FIGURE 1: Scheme for the preparation of MAPs. Details of the procedure and nomenclature are described in the text. Components in boxes are free of active tubulin and induce purified tubulin to form microtubules.

divided into four aliquots. ATP and GTP were added to final concentrations of 1 mM and 0.3 mM. Each aliquot was warmed to 37 °C, incubated for 40 min, and centrifuged in the warm at 32000 rpm for 1 h in Ti 15 zonal rotors. The supernatants were discarded, and the pellets were homogenized in 400 mL of cold solution A.

After 30 min on ice, the very viscous suspension was centrifuged in the cold for 30 min at 24000 rpm in a Ti 45 rotor. The pellet was rehomogenized sequentially 2 times in 200 mL of solution A, and the cold centrifugation was repeated each time. The three supernatants, containing 10.3, 6.5, and 2.7 g of protein, were pooled (840 mL). The cold pellets from the third centrifugation were set aside.

To each 100 mL of the combined supernatant were added 55.3 g of glycerol (4 M) and 5 mL of a solution containing Mes (pH 6.4), EGTA, $MgCl_2$, 2-mercaptoethanol, and EDTA to maintain the concentrations of these components and ATP and GTP for final concentrations of 1 and 0.3 mM. The mixture was incubated at 37 °C for 1 h and centrifuged in the warm in a Ti 15 rotor for 1 h at 32000 rpm. The warm supernatant ("second warm supernatant") was set aside. The pellet was homogenized in 250 mL of solution A. The suspension was left on ice for 30 min and centrifuged in the cold for 30 min at 24000 rpm in a Ti 45 rotor. Very small cold pellets are usually obtained at this stage, and they were combined with the previous cold pellets. The cold supernatant contained 14.2 g of "two-cycle microtubule protein". Preparation of the microtubule protein and further steps in the purification of MAPs and tubulin are outlined in Figure 1. Yields at each step are summarized in Table I.

*Heat Treatment of the Cold Pellet and the Second Warm Supernatant.* The combined cold pellet was homogenized in

Table I: Preparation of MAPs Components[a]

| procedure | protein component | amount of protein (g) | | yield (mg/g of microtubule protein) |
|---|---|---|---|---|
| (step I) Mes-induced polymerization of microtubule protein | microtubule protein | 8.80 | | |
| | high-Mes supernatant | 3.27 | | 372 |
| | glutamate-soluble component | 4.42 | 9.01 | 502 |
| | first insoluble component | 1.32 | | 150 |
| | heat-extractable protein (salvaged MAPs B) | 0.046 | | 5.2 |
| (step II) centrifugal separation of aggregated and soluble protein in high-Mes supernatant | high-Mes supernatant | 3.21 | | |
| | first supernatant | 1.04 | | |
| | second supernatant | 0.20 | | |
| | third supernatant | 0.14 | | |
| | fourth supernatant | 0.08 | | |
| | total soluble MAPs (all supernatants) | 1.46 | 3.19 | 169 |
| | 2-h pellet | 0.59 | | |
| | first overnight pellet | 0.93 | | |
| | second overnight pellet | 0.21 | | |
| | second insoluble component (all pellets) | 1.73 | | 200 |
| | heat-extractable protein (salvaged MAPs C) | 0.168[b] | | 19.4 |
| (step III) DEAE-cellulose chromatography of total soluble MAPs | total soluble MAPs | 0.922 | | |
| | flow-through MAPs | 0.144 | 0.765 | 26.4 |
| | DEAE-MAPs | 0.621 | | 114 |
| (step IV) heat treatment of DEAE-MAPs | DEAE-MAPs | 0.304 | | |
| | heat-treated MAPs | 0.112 | 0.274 | 41.9 |
| | heat-treated MAPs pellet | 0.162 | | 60.7 |

[a] Experimental details and nomenclature are described in detail in the text. [b] This value assumes that the entire second insoluble component had been heat extracted, rather than the 0.75 g that was actually used.

200 mL of 0.1 M Mes (pH 6.9) containing 0.2 M NaCl and 2 mM dithiothreitol. Heat treatment was performed, and the preparation was clarified by a 2-h cold centrifugation. The supernatant ("salvaged MAPs A") was concentrated 20-fold, dialyzed against solution B, and clarified by a 4-h cold centrifugation. This yielded 362 mg of protein (equivalent to 25.5 mg/g of two-cycle microtubue protein).

NaCl (0.2 M) and dithiothreitol (2 mM) were added to part of the second warm supernatant. This was heat treated and clarified by a 1-h cold centrifugation. The supernatant was dialyzed against solution C, concentrated 50-fold, dialyzed against solution B, and clarified again. This "heat-extracted warm supernatant" contained protein equivalent to 31.0 mg/g of two-cycle microtubule protein.

*High-Mes Polymerization.* The large-scale procedure has been reproducibly performed 4 times. Two-cycle microtubule protein (8.8 g) was adjusted to 27.5 mg/mL with solution A, and GTP (1 mM), dithiothreitol (2 mM) and solid, neutralized Mes (1.6 M, 530 mg/mL) were added. The mixture was incubated at 37 °C for 1 hour and centrifuged in the warm for 1 h. The "high-Mes supernatant" was set aside.

The pellets were homogenized in 60 mL of cold 1 M glutamate. The homogenate was left on ice for 2 h and centrifuged in the cold for 1 h. The supernatant ("glutamate-soluble component") was almost pure tubulin.

The pellets ("first insoluble component") were homogenized in 50 mL of solution A, and NaCl (0.2 M) and dithiothreitol (2 mM) were added. The mixture was heat treated and clarified by a 5-h cold centrifugation. The supernatant ("salvaged MAPs B") was concentrated 6-fold and dialyzed against solution B.

*Concentration and Centrifugation of the High-Mes Supernatant.* The high-Mes supernatant (380 mL) was dialyzed against 840 mL of 2 mM dithiothreitol, to reduce the nominal Mes concentration to 0.5 M, and concentrated 5-fold. Initially somewhat turbid, after concentration the high-Mes supernatant had a protein concentration of 41.4 mg/mL and was moderately turbid and viscous.

About 20% of the protein in the high-Mes supernatant was tubulin, as compared to about 70% in the two-cycle microtuble protein. We were unable to induce a second polymerization reaction, but most of the residual tubulin could be removed by high-speed centrifugation in the cold. The high-Mes supernatant was therefore centrifuged in the cold for 2 h. A large pellet was obtained, but the supernatant remained turbid. The pellet was homogenized in 50 mL of solution B and centrifuged again for 2 h in the cold. This was repeated twice more, for a total of four supernatants. The final pellet ("2-h pellet") was set aside.

Because the first supernatant remained turbid, it was centrifuged for 12 h in the cold. A second large pellet was obtained, and this was homogenized sequentially in the above second, third, and fourth supernatants and centrifuged each time for 12 h in the cold. The final pellet ("first overnight pellet") was set aside.

The first supernatant remained slightly turbid, and it was centrifuged a third time for 12 h in the cold. A small pellet was obtained that was homogenized sequentially in the second, third, and fourth supernatants and centrifuged for 12 h in the cold each time. The final pellet ("second overnight pellet") was set aside.

The 2-h pellet and both overnight pellets were homogenized in solution B, and the four supernatants and three pellet homogenates were examined by polyacrylamide gel electrophoresis. All three pellet homogenates were enriched in tubulin, while the tubulin content of the four supernatants was minimal. The pellet homogenates were pooled as the "second insoluble component".

The supernatants were pooled ("total soluble MAPs") and concentrated 4-fold. Two-thirds of the preparation was further processed by DEAE-cellulose chromatography. The other one-third was applied to a 5 × 20 cm Sephadex G-50 (superfine) column equilibrated and developed with solution B. Protein-containing fractions were pooled, concentrated 6-fold, and dialyzed against solution B.

*Heat Extraction of the Second Insoluble Component.* A

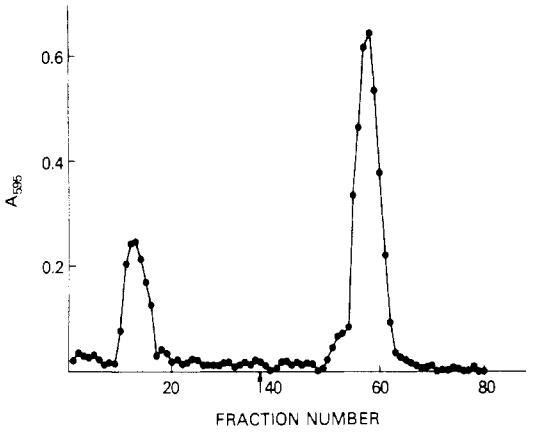


FIGURE 2: DEAE-cellulose chromatography of total soluble MAPs. Fraction volume was 20 mL. Protein in 5 μL of each fraction was determined as described by Bradford (1976). The arrow on the abscissa indicates the point at which the NaCl gradient was applied to the column.

total of 750 mg of the second insoluble component was diluted with solution B to 14 mg/mL, and NaCl and dithiothreitol were added for final concentrations of 0.2 M and 4 mM. After heat treatment, the preparation was clarified by a 10-h cold centrifugation. The supernatant ("salvaged MAPs C") was concentrated 12-fold, dialyzed against solution B, and clarified by a 2-h cold centrifugation.

*DEAE-cellulose Chromatography of Total Soluble MAPs.* The total soluble MAPs (57 mL) was dialyzed against 4 volumes of 2 mM dithiothreitol to reduce the nominal Mes concentration to 0.1 M. The solution was applied to a 5 × 15 cm column of DEAE-Sephacel equilibrated with solution C. The column was washed with 600 mL of solution C and developed with a 1000-mL gradient from 0 to 1 M NaCl in solution C. Two protein peaks were obtained (Figure 2), one not bound to the resin ("flow-through MAPs") and the other eluting with the NaCl gradient ("DEAE-MAPs"). Fractions in each peak were pooled. The flow-through MAPs pool was concentrated 10-fold and dialyzed against solution B. Half the DEAE-MAPs pool was concentrated 7-fold and applied to a 5 × 20 cm column of Sephadex G-50 (superfine) equilibrated and developed with solution B. The protein-containing fractions were concentrated 6-fold and dialyzed against solution B.

*Heat Treatment of DEAE-MAPs.* Dithiothreitol (final concentration 4 mM) was added to the other half of the DEAE-MAPs pool. The preparation was heat treated and clarified by a 12-h cold centrifugation. The pellets were homogenized in 12 mL of solution B ("heat-treated MAPs pellet") and dialyzed against solution B. The supernatant ("heat-treated MAPs") was concentrated 6-fold and applied to a 5 × 20 cm Sephadex G-50 (superfine) column equilibrated and developed with solution B. The protein-containing fractions were concentrated 6-fold and dialyzed against solution B.

*Further Purification of Tubulin, the Glutamate-Soluble Component.* The glutamate-soluble component was diluted to 37 mg/mL with 1 M glutamate. GTP was added (1 mM), and the solution was incubated at 37 °C for 1 h (second polymerization) and centrifuged in the warm for 40 min. The pellet was homogenized in 90 mL of cold 1 M glutamate. After 1 h on ice, the homogenate was clarified by a 40-min cold centrifugation. GTP was added (1 mM) to the supernatant, and the mixture was incubated for 1 h at 37 °C (third polymerization). The polymerized tubulin was recovered by a 40-min warm centrifugation, and the pellet was homogenized in 90 mL of cold 1 M glutamate. The homogenate was left overnight on ice and clarified as before. GTP was added (1 mM) to the supernatant, and a fourth polymerization was performed. The pellet was homogenized in 40 mL of cold 1 M glutamate. The homogenate was left on ice for 1 h and clarified as before. The supernatant will be referred to as "purified tubulin (new)".

*Other Procedures.* Polyacrylamide gel electrophoresis was performed on disk gels by the procedure of Stephens (1975) and on 5–10% gradient slab gels by that of Laemmli & Favre (1973). Densitometric scans of gels, obtained with a Gilford Model 2410S linear-transport scanner, were used to determine the percent of β-tubulin. GTPase, ATPase, and nucleoside-diphosphate kinase activities were determined as described previously (Hamel & Lin, 1981). Tubulin polymerization was followed turbidimetrically (Gaskin et al., 1974) in a Gilford Model 2400S recording spectrophotometer equipped with a Gilford Thermoset electronic temperature-control unit. Base lines were established with cuvette contents at 0 °C. At zero time, the temperature was set at 37 °C, and the temperature of the cuvette contents increased about 0.5 °C/min. Electron microscopy was performed as described previously (Hamel et al., 1981). Negatively stained specimens were prepared from samples diluted with 4 parts of an isothermic solution containing 50% sucrose, 0.1 Mes (pH 6.9), and 0.5 mM $MgCl_2$ (Terry & Purich, 1980). The binding of [$^3$H]colchicine to tubulin was quantitated by the DEAE-cellulose filter method (Borisy, 1972), as described previously (Hamel & Lin, 1982).

## Results

*Microtubule Protein.* In our studies on interactions of tubulin with nucleotides we have routinely used heat-treated MAPs. They retain their ability to support tubulin polymerization (Weingarten et al., 1975; Bryan et al., 1975; Fellous et al., 1977), but heat treatment destroys tubulin-independent GTPase (David-Pfeuty et al., 1978) and ATPase and nucleosidediphosphate kinase activities (Hamel et al., 1981). We have prepared these MAPs either by DEAE-cellulose chromatography of microtubule protein followed by heat treatment or by the reverse procedure, with comparable results in terms of activity, band patterns on polyacrylamide gel electrophoresis, and yield (Hamel et al., 1981). We routinely obtained 10–20 mg of heat-treated MAPs/g of microtubule protein (or per kilogram of brain tissue). Since our method of tubulin purification yields up to 650 mg/kg of brain tissue (Hamel & Lin, 1981), MAPs have been in short supply, and we wanted to obtain increased amounts of these proteins.

We began by adapting the glycerol-enhanced, cyclical polymerization procedure of Shelanski et al. (1973) for the large-scale preparation of microtubule protein, made possible by the use of zonal rotors. The electrophoretic appearance of the two-cycle microtubule protein obtained is shown in Figure 3A. Besides tubulin, MAP-1 and MAP-2 are prominent protein components. Densitometric analysis of the gels presented in Figure 3 indicate that α-tubulin and β-tubulin represent 38 and 34% of the total protein in the microtubule protein.

During this study we found that heat extraction of the cold pellets obtained during the polymerization cycles yielded the equivalent of 25.5 mg of protein/g of two-cycle microtubule protein. This protein (salvaged MAPs A) could induce tubulin to polymerize (see Table II) and on gels had a prominent band with the mobility of MAP-2 (Figure 3A).

Since significant amounts of active MAPs can be lost in the cold pellets, we wondered whether comparable amounts of

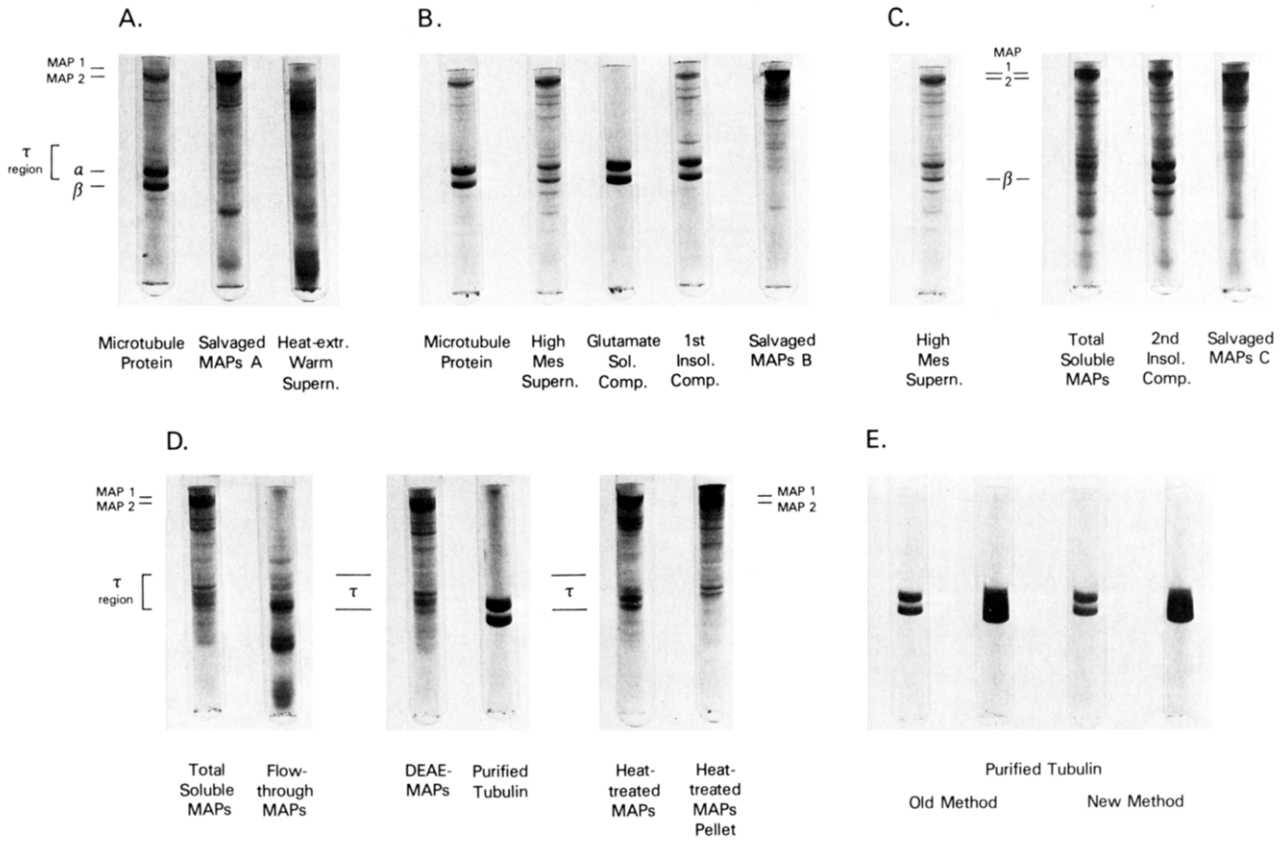


FIGURE 3: Polyacrylamide gel electrophoresis of microtubule protein, tubulin, and MAPs components. Sample nomenclature is described in detail in the text. Polyacrylamide gel electrophoresis was performed as described by Stephens (1975), using 6% acrylamide. Each gel contained 20 μg of protein, except as follows: group D, fourth gel, 30 μg; group E, second and fourth gels, 80 μg each.

Table II: Properties of MAPs Components and Purified Tubulin

| protein prepn | polymerization[c] $\Delta A_{350}$ | polymerization[c] $\Delta A_{350}$ with tubulin | polymerization[c] cold reversible? | tubulin content: % protein comigrating with β-tubulin[d] | tubulin content: colchicine bound (pmol/μg of protein)[e] | enzymatic activities[f]: nucleosidediphosphate kinase[g] | enzymatic activities[f]: ATPase[h] | enzymatic activities[f]: GTPase[i] | enzymatic activities[f]: GTPase with tubulin[i] | enzymatic activities[f]: Δ[j] |
|---|---|---|---|---|---|---|---|---|---|---|
| purified tubulin (old)[a] | 0.011 | | | 49 | 5.3 | 0 (0) | 0 | 0.6 | | |
| microtubule protein | | | | 34 | 2.9 | 21 | 81 | 77 | | |
| salvaged MAPs A | 0.016 | 0.110 | yes (90%) | <1 | 0 | 0 (0) | 0 | 0.1 | 7.3 | 7.2 |
| high-Mes supernatant | | | | 11 | | | | | | |
| purified tubulin (new)[b] | 0.006 | | | 47 | 5.9 | 0 (0) | 0 | 1.1 | | |
| first insoluble component | | | | 28 | | | | | | |
| salvaged MAPs B | 0.005 | 0.195 | yes (90%) | <1 | 0 | 0 (0) | 0.4 | 0.6 | 16 | 15.4 |
| second insoluble component | 0.002 | 0.033 | no | 24 | 0 | 16 | 87 | 67 | 50 | −17 |
| salvaged MAPs C | 0.009 | 0.203 | yes (95%) | <1 | 0 | 0 (0) | 0 | 0 | 14 | 14 |
| total soluble MAPs | 0.008 | 0.100 | yes (90%) | 3 | 0 | 40 | 22 | 21 | 46 | 25 |
| flow-through MAPs | 0.044 | 0.860 | yes (65%) | 3 | 0 | 26 | 18 | 7.5 | 26 | 18.5 |
| DEAE-MAPs | 0.016 | 0.124 | yes (85%) | 2 | 0 | 0.7 (26) | 10 | 15 | 41 | 26 |
| heat-treated MAPs | 0.016 | 0.161 | yes (90%) | 1 | 0 | 0 (0) | 0 | 0 | 13 | 13 |
| heat-treated MAPs pellet | 0.002 | 0.027 | partial (40%) | <1 | 0 | 0 (0) | 0 | 0 | 18 | 18 |

[a] Tubulin prepared as described previously, including DEAE-cellulose chromatography (Hamel & Lin, 1981). [b] Tubulin prepared from microtubule protein by cycles of Mes- and glutamate-induced polymerization only (no ion-exchange chromatography step). [c] All 250-μL reaction mixtures contained 1 mM GTP, 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, and, as indicated, 1.0 mg/mL purified tubulin and 0.5 mg/mL indicated MAPs component. In the experiments with both tubulin + MAPs component, tubulin prepared by the old method was used. Turbidity readings from triplicate samples were averaged, and readings after 30 min at 37 °C are presented in the table. [d] Obtained from densitometric analysis of the gels presented in Figure 3. [e] Each 100-μL reaction mixture contained 500 μg of the indicated protein, except as follows: tubulin and microtubule protein, 10 μg; salvaged MAPs B, 250 μg; heat-treated MAPs, 440 μg. [f] All 100-μL reaction mixtures contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, and, as indicated, 1.0 mg/mL purified tubulin and 0.5 mg/mL MAPs component. All incubations were for 30 min at 37 °C. In the experiments with both tubulin + MAPs component, tubulin prepared by the old method was used. Further reaction components are described for the individual assays. In all experiments, the data are expressed as the percent radiolabeled reactant converted to radiolabeled product. [g] Reaction mixture contained 1 mM GDP and 1 mM [$\gamma$-$^{32}$P]ATP. The conversion of ATP to GTP was quantitated. The numbers in parentheses refer to an experiment in which high specific activity [$\gamma$-$^{32}$P]ATP was used as phosphate donor (concentration about 100 nM). [h] Reaction mixtures contained 0.1 mM [$\gamma$-$^{32}$P]ATP. The formation of [$^{32}$P]$P_i$ was quantitated. [i] Reaction mixtures contained 0.1 mM [$\gamma$-$^{32}$P]GTP. The formation of [$^{32}$P]$P_i$ was quantitated. [j] The reaction without tubulin is subtracted from the reaction with tubulin.

MAPs might be in the warm supernatant remaining after the second cycle of polymerization. Heat-extracted protein, however, was unable to support tubulin polymerization and had a heterogeneous appearance on polyacrylamide gels (Figure 3A), indicating that few MAPs were lost in the warm supernatant.

*Preparation of MAPs.* If heat-treated MAPs are prepared by an initial heat treatment of microtubule protein, the tubulin is destroyed. On the other hand, we have had great technical difficulty with DEAE-cellulose chromatography of larger amounts of microtubule protein (3–4 g). We therefore decided to explore alternate methods for the separation of MAPs and tubulin. Himes et al. (1977) reported that tubulin was selectively polymerized in 10% dimethyl sulfoxide. We subsequently reported the selective polymerization of tubulin with high concentrations of glutamate (Hamel & Lin, 1981), Mes, and Pipes (Waxman et al., 1981). While our initial studies were with lower concentrations of microtubule protein, the large-scale preparation of MAPs might be possible if similar selective polymerization occurred at higher protein concentrations.

In trial experiments we examined Mes, Pipes, and glutamate at microtubule protein concentrations of 20–30 mg/mL. We obtained polymer pellets that were homogenized and clarified by centrifugation at 0 °C. With both Pipes and glutamate somewhat more MAPs remained in the tubulin-enriched supernatant than with Mes, and the Mes reaction was chosen for preparative studies. The largest preparation, with 8.8 g of microtubule protein, was described above. The high-Mes supernatant was enriched in MAPs and depleted in tubulin (Figure 3B), which was reduced about 3-fold (Table II). The protein in the cold supernatant obtained from the polymer pellet was practically pure tubulin.

The cold insoluble protein (first insoluble component) in the polymer pellet was not simply denatured tubulin. Its band pattern was similar to that of microtubule protein (Figure 3B). Heat treatment of the first insoluble component yielded 46 mg (5.2 mg/g of two-cycle microtubule protein) of soluble protein highly enriched in protein(s) with the electrophoretic mobility of MAP-2 (Figure 3B) and active in promoting microtubule assembly (Table II).

Following Mes-induced polymerization, 36% of the protein was in the high-Mes supernatant, 49% in the tubulin fraction, and 15% in the first insoluble component (Table I).

We were disappointed by the significant amount of tubulin remaining in the high-Mes supernatant. Since Mes-induced polymerization of tubulin has a relatively high critical concentration for the protein (Waxman et al., 1981), we next concentrated the supernatant. We planned to use a second cycle of polymerization to further reduce its tubulin content, but various methods of inducing such a second cycle of polymerization were unsuccessful. While significant warm pellets enriched in tubulin were obtained, they were poorly soluble.

Moreover, the concentrated high-Mes supernatant was distinctly turbid in the cold, and when small amounts were centrifuged in the cold without GTP, a firm pellet and clear supernatant were obtained. Virtually all of the tubulin, with some of the MAPs, was found in the pellet. We therefore repetitively centrifuged the entire high-Mes supernatant in the cold until the final supernatant was almost clear and the pellet small. Since MAPs were present in the pellets, we tried to resolubilize them by three successive extractions. Table I (step II) presents the total protein recovered in the extracted pellets, the final supernatant, and the three extracts. Over 80% of the protein originally present in the pellets could not be resolubilized, and each extract had less protein than the preceding one. Compared to the original high-Mes supernatant, the pellets were all enriched in tubulin, and the supernatants all contained little tubulin. All supernatants and all pellet homogenates were therefore separately pooled as, respectively, the total soluble MAPs and the second insoluble component. The appearance of these preparations on polyacrylamide gels is presented in Figure 3C.

The second insoluble component still contained significant amounts of MAPs, as it was only about 50% tubulin (Table II). The tubulin in the second insoluble component was inert. It did not bind colchicine (Table II), and we were unable to find associated bound guanine nucleotide when it was dissolved in 8 M urea and subjected to gel filtration chromatography. Suspension of the second insoluble component in 2 M NaCl solubilized less than 5% of the protein. When 75 mg of the insoluble protein was applied to a column of either DEAE-cellulose or phosphocellulose, less than 2% of the protein was eluted with either a low or high (1 M NaCl) ionic strength solution. The second insoluble component remained precipitated at the top of these columns. Nevertheless, the MAPs in the second insoluble component are not inactive. Heat treatment released 19.4 mg of protein/g of two-cycle microtubule protein (salvaged MAPs C). This protein was enriched in a component with the electrophoretic mobility of MAP-2 (Figure 3C) and was highly active in supporting microtubule assembly (Table II).

The component of greatest interest to us was the total soluble MAPs. Besides minimal tubulin content, the preparation was enriched in MAP-1. To compare the total soluble MAPs to our previously prepared heat-treated MAPs, part of the preparation was subjected to DEAE-cellulose chromatography and subsequent heat treatment. Unlike the minimal recovery of protein from the second insoluble component after DEAE-cellulose chromatography, 83% of the applied protein was recovered when the total soluble MAPs were chromatographed (Table I). About one-fifth of the recovered protein did not bind to the resin (Table I, the flow-through MAPs), while four-fifths was recovered by NaCl gradient elution (Table I, the DEAE-MAPs). The flow-through MAPs consist of lower and intermediate molecular weight proteins, some with the mobility of $\tau$ factor (Figure 3D). The DEAE-MAPs contain MAP-1, MAP-2, proteins with the mobility of $\tau$ factor, and several protein bands between MAP-2 and $\tau$ factor (Figure 3D). The DEAE-MAPs differ little in their electrophoretic appearance from MAPs we obtained by direct DEAE-cellulose chromatography of microtubule protein [cf. Figure 1 of Hamel et al. (1981)].

When the DEAE-MAPs were heat treated and centrifuged overnight, 40% of the recovered protein (Table I) was in the supernatant (heat-treated MAPs) and 60% in the pellet (heat-treated MAPs pellet). The major difference between the gel patterns of the DEAE-MAPs and the heat-treated MAPs was the disappearance of the MAP-1 band following heat treatment (Figure 3D). The heat-treated MAPs pellet contained the MAP-1 as well as significant amounts of MAP-2 (Figure 3D).

Since the gels presented in Figure 3 resolved MAP-1 and MAP-2 poorly, we examined the components described above in another system (Figure 4). This gel confirmed the presence of MAP-1 in the high-Mes supernatant, the total soluble MAPs, and the DEAE-MAPs, its loss by heat treatment, as it is present in the insoluble heat-treated MAPs pellet but not the heat-treated MAPs, and the relative absence of MAP-1 in the first and second insoluble components.

*Preparation of Tubulin.* The 4.4 g of tubulin in the glutamate-soluble component was further purified by three cycles of glutamate-induced polymerization. The final preparation had no contaminants visualized by polyacrylamide gel electrophoresis and was identical in electrophoretic appearance with tubulin purified by batch DEAE-cellulose chromatog-

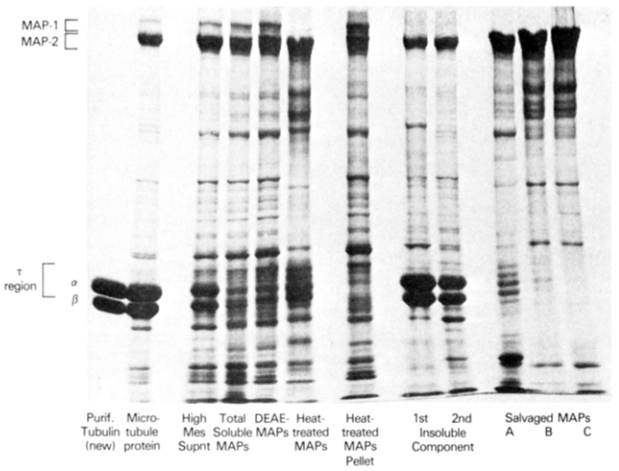


FIGURE 4: Polyacrylamide gel electrophoresis to separate MAP-1 and MAP-2. Sample nomenclature is described in detail in the text. Polyacrylamide gel electrophoresis (5–10% acrylamide gradient) was performed as described by Laemmli & Favre (1973). Each gel slot contained 30 μg of protein, except for purified tubulin (20 μg), microtubule protein (40 μg), and the first insoluble component (40 μg).

raphy and glutamate-induced polymerization (Figure 3E). After the final cold centrifugation, 2.6 g of tubulin was recovered (300 mg/g of two-cycle microtubule protein). The two tubulin preparations were free of nucleosidediphosphate kinase and ATPase activities (Table II), they had nearly equivalent activity in binding colchicine (Table II), and their polymerization in the presence of MAPs or glutamate was identical (data not presented). Both preparations had minimal GTPase activity in the absence of MAPs in 0.1 M Mes (pH 6.9) –0.5 mM $MgCl_2$ (Table II).

*Properties of MAPs Components.* Significant cold-reversible polymerization was observed with the total soluble MAPs, the DEAE-MAPs, the flow-through MAPs, the heat-treated MAPs, and salvaged MAPs A–C. The absolute turbidity reading, with the amount of MAPs held constant, steadily increased with each step of the primary purification route (total soluble MAPs → DEAE-MAPs → heat-treated MAPs). Salvaged MAPs A were less active than the heat-treated MAPs, but salvaged MAPs B and C were more active. A very high turbidity reading was obtained with the flow-through MAPs (see below).

The maximum tubulin content of the preparations was estimated by densitometric analysis of the gels presented in Figure 3. Only the protein comigrating with β-tubulin was measured since some of the τ proteins overlapped with α-tubulin. No MAPs component nor the second soluble component contained tubulin able to bind colchicine.

No heat-treated preparation had detectable nucleosidediphosphate kinase activity. More activity was found in the total soluble MAPs than in the second insoluble component. After DEAE-cellulose chromatography of the total soluble MAPs, the nucleosidediphosphate kinase activity was almost entirely in the flow-through MAPs [cf. Jacobs & Huitorel (1979)]; but residual nucleosidediphosphate kinase activity in the DEAE-MAPs was clearly demonstrated with high specific activity [$\gamma$-$^{32}$P]ATP as the phosphate donor.

Although the ATPase and tubulin-independent GTPase may not represent different activities, since they are always found together, heat treatment destroys them (David-Pfeuty et al., 1978; Hamel et al., 1981). Most of the phosphatase activity in the microtubule protein was recovered in the second insoluble component, but ample activity was also present in the total soluble MAPs. After DEAE-cellulose chromatography

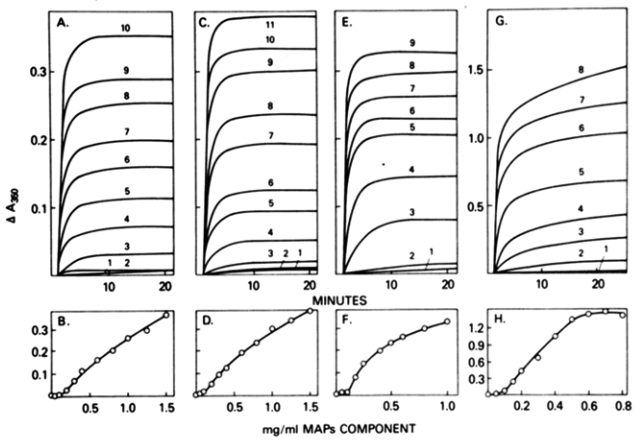


FIGURE 5: Effects of increasing amounts of MAPs components on polymerization. Each 0.25-mL reaction mixture contained 1.5 mg/mL tubulin, 0.5 mM $MgCl_2$, 0.1 M Mes (pH 6.9), either 1.0 mM (panels A–D) or 0.5 mM (panels E–H) GTP, and MAPs components as indicated below. In panels B, D, F, and H, the turbidity reading at 20 min is plotted against the concentration of MAPs component. (A and B) Total soluble MAPs. Each reaction mixture contained the indicated amount of total soluble MAPs in mg/mL: (for panel A) curve 1, none; curve 2, 0.1; curve 3, 0.2; curve 4, 0.3; curve 5, 0.4; curve 6, 0.6; curve 7, 0.8; curve 8, 1.0; curve 9, 1.25; curve 10, 1.5. (C and D) DEAE-MAPs. Each reaction mixture contained the indicated amount of DEAE-MAPs in mg/mL: (for panel C) curve 1, none; curve 2, 0.05; curve 3, 0.1; curve 4, 0.2; curve 5, 0.3; curve 6, 0.4; curve 7, 0.6; curve 8, 0.8; curve 9, 1.0; curve 10, 1.25; curve 11, 1.5. (E and F) Heat-treated MAPs. Each reaction mixture contained the indicated amount of heat-treated MAPs in mg/mL: (for panel E) curve 1, none; curve 2, 0.1; curve 3, 0.18; curve 4, 0.25; curve 5, 0.4; curve 6, 0.5; curve 7, 0.6; curve 8, 0.8; curve 9, 1.0. (G and H) Flow-through MAPs. Each reaction mixture contained the indicated amount of flow-through MAPs in mg/mL: (for panel G) curve 1, none; curve 2, 0.1; curve 3, 0.15; curve 4, 0.2; curve 5, 0.3; curve 6, 0.4; curve 7, 0.5; curve 8, 0.6.

of the total soluble MAPs, both the flow-through MAPs and the DEAE-MAPs hydrolyzed ATP and GTP; but ATPase activity was greater in the flow-through MAPs, and GTPase activity was greater in the DEAE-MAPs.

In the presence of tubulin, significant hydrolysis of GTP occurred with all heat-treated preparations. In addition, substantial stimulation of hydrolysis occurred with the total soluble MAPs, the flow-through MAPs, and the DEAE-MAPs. There was, however, no clear correlation between the amount of tubulin-dependent GTP hydrolysis and the extent of cold-reversible turbidity development.

The heat-treated MAPs pellet was particularly notable in this regard. It was originally saved to document that it contained the MAP-1 present in the DEAE-MAPs prior to heat treatment (see Figures 3D and 4), for we assumed that this component consisted only of denatured protein. Even though it had only feeble activity in supporting cold-reversible tubulin polymerization, the heat-treated MAPs pellet stimulated a more extensive tubulin-dependent hydrolysis of GTP than did the heat-treated MAPs.

*Polymerization Reactions Supported by MAPs Components.* An initial characterization of the polymerization reactions induced by the total soluble MAPs, DEAE-MAPs, heat-treated MAPs, and flow-through MAPs was performed. These preparations and the tubulin used in these studies were freed of unbound nucleotide by gel filtration chromatography (GDP partially coelutes from DEAE-cellulose with the DEAE-MAPs), except for the flow-through MAPs, which were assumed to be nucleotide free.

Figure 5 presents studies in which turbidity development was examined as a function of the concentration of MAPs component. In all cases, increasing amounts of MAPs resulted

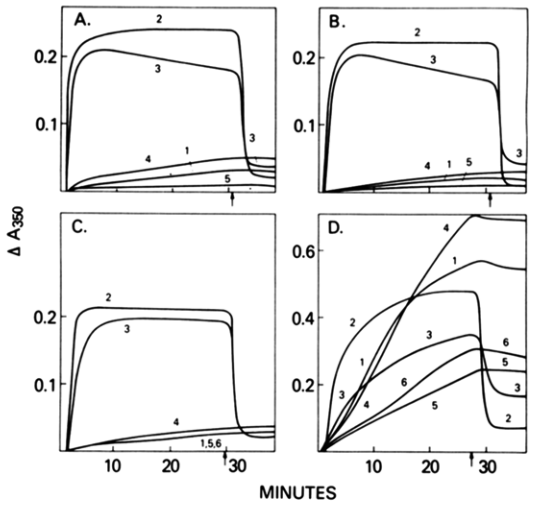


FIGURE 6: Requirements for polymerization induced by MAPs components. Each 0.25-mL reaction mixture contained 1.5 mg/mL tubulin, 0.1 M Mes (pH 6.9), if indicated either 1.0 mM (panels A and B) or 0.1 mM (panels C and D) GTP, the MAPs component indicated below, and the following additions: curves 1, 0.5 mM $MgCl_2$ (no GTP); curves 2, GTP and 0.5 mM $MgCl_2$; curves 3, GTP; curves 4, GTP, 0.5 mM $MgCl_2$, and 2 mM $CaCl_2$; curves 5, GTP, 0.5 mM $MgCl_2$, and 50 $\mu$M colchicine; curves 6 (panels C and D only), GTP, 0.5 mM $MgCl_2$, and 1.0 mM GDP. At the times indicated by the arrows on the abscissa, the Thermoset unit was set at 0 °C. MAPs components were added as follows: (A) total soluble MAPs, 0.9 mg/mL; (B) DEAE-MAPs, 0.75 mg/mL; (C) heat-treated MAPs, 0.5 mg/mL; (D) flow-through MAPs, 0.2 mg/mL.

in increased turbidity development (Figure 5A,C,E, G); but turbidity development as a function of MAPs concentration was sigmoidal, with minimal reactions at lower concentrations (Figure 5B,D,F,H). Specific activity (e.g., turbidity reading per microgram of protein) increases with each step in the sequence from total soluble MAPs to DEAE-MAPs to heat-treated MAPs. The turbidity readings obtained with the flow-through MAPs Figure 5G,H) were much higher than those obtained with the other preparations (see below).

Figure 6 presents studies with these four components that demonstrate the GTP requirement for polymerization, the cold reversibility of the GTP-dependent reactions, and the effects of $Mg^{2+}$, $Ca^{2+}$, GDP, and colchicine on polymerization. The patterns obtained with the total soluble MAPs (Figure 6A), DEAE-MAPs (Figure 6B), and heat-treated MAPs (Figure 6C) were similar. Little turbidity development occurred without GTP, and the reaction with GTP was inhibited by $Ca^{2+}$, colchicine, and GDP. Added $Mg^{2+}$ was not required for the reaction, but had a minimal stimulatory effect. Without $Mg^{2+}$, the turbidity plateau was unstable with the total soluble MAPs and the DEAE-MAPs but was completely stable with the heat-treated MAPs. The reason for this difference is not known.

With the flow-through MAPs (Figure 6D), GTP induced a rapid, cold–reversible reaction. Without GTP, there was slower, but extensive, turbidity development. This reaction was temperature dependent but not cold reversible. The GTP-dependent reaction was inhibited by $Ca^{2+}$, GDP, and colchicine and significantly stimulated by $Mg^{2+}$. Turbidity development with GTP and either GDP or colchicine was substantially less than that obtained in the GTP-independent reaction, while with $Ca^{2+}$ and GTP turbidity development was similar to that obtained without the nucleotide.

With the total soluble MAPs and the DEAE-MAPs, the turbidity plateaus obtained with GTP concentrations of 100 $\mu$M or less were unstable, unlike those observed at 1 mM GTP. This is shown in Figure 7, where reactions with the four

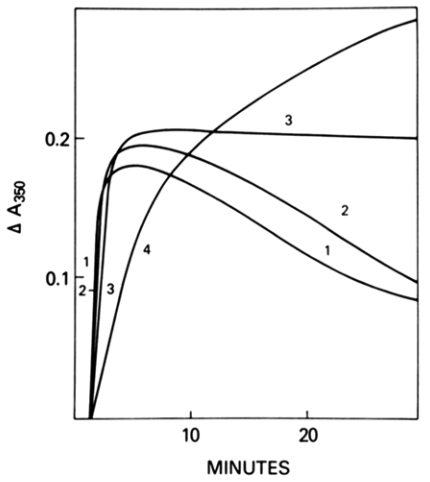


FIGURE 7: Instability of the turbidity plateau induced by the total soluble MAPs and DEAE-MAPs at low GTP concentrations. Each 0.25-mL reaction mixture contained 1.5 mg/mL tubulin, 0.5 mM $MgCl_2$, 0.1 M Mes (pH 6.9), 50 $\mu$M GTP, and the indicated MAPs component: curve 1, 0.9 mg/mL total soluble MAPs; curve 2, 0.75 mg/mL DEAE-MAPs; curve 3, 0.5 mg/mL heat-treated MAPs; curve 4, 0.15 mg/mL flow-through MAPs.

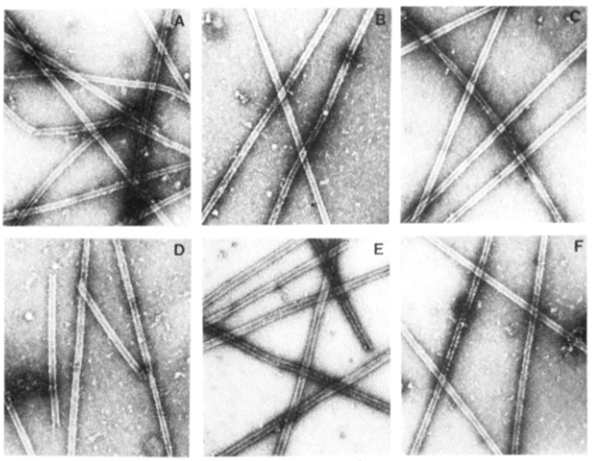


FIGURE 8: Morphology of polymer induced by MAPs components. Negatively stained specimens were prepared after a 30-min incubation at 37 °C of reaction mixtures containing 1.5 mg/mL tubulin, 1 mM GTP, 0.5 mM $MgCl_2$, 0.1 M Mes (pH 6.9), and MAPs components as follows: (A) total soluble MAPs, 0.9 mg/mL; (B) DEAE-MAPs, 0.75 mg/mL; (C) heat-treated MAPs, 0.5 mg/mL; (D) salvaged MAPs A, 0.75 mg/mL; (E) salvaged MAPs B, 0.5 mg/mL; (F) salvaged MAPs C, 0.5 mg/mL. All magnifications are ×24 500.

components at 50 $\mu$M GTP are compared. In contrast, the turbidity plateau with the heat-treated MAPs was stable. With the flow-through MAPs, no plateau was reached, and the turbidity reading continued to rise throughout the experiment. The unstable plateaus with the total soluble MAPs and the DEAE-MAPs probably result from GTP degradation caused by their tubulin-independent GTPase activity, for the polymer formed with the heat-treated MAPs can be rapidly disrupted by the addition of fructose 6-phosphate and phosphofructokinase [data not presented; cf. Margolis (1981)].

The morphology of the polymer formed with the different MAPs components was examined in the electron microscope. In all cases, microtubules were formed, as demonstrated in Figure 8 for the total soluble MAPs, DEAE-MAPs, heat-treated MAPs, and salvaged MAPs A–C.

*Flow-Through MAPs.* The unusually high turbidity plateaus produced by the flow-through MAPs led us to examine this component in further detail. One possibility was that the flow-through MAPs were simply much more efficient in

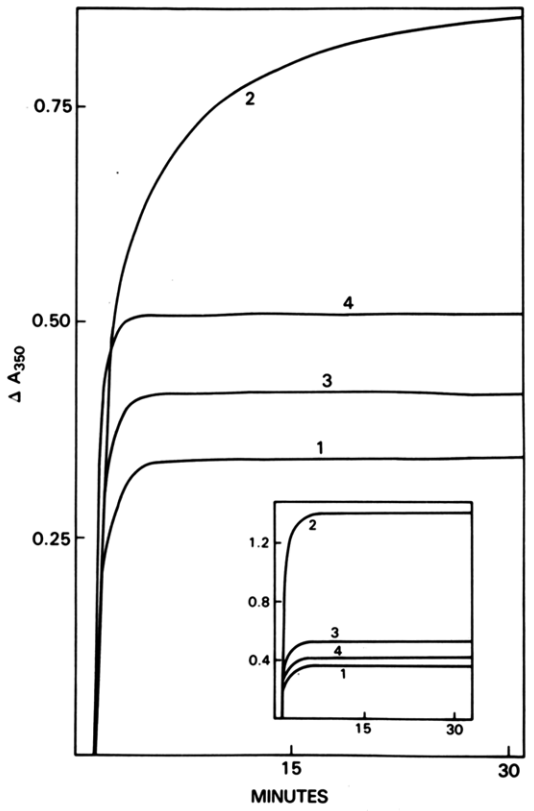


FIGURE 9: Suppression by the DEAE-MAPs of the high turbidity plateau obtained with the flow-through MAPs. Each 0.25-mL reaction mixture contained 1.5 mg/mL tubulin, 0.1 M Mes (pH 6.9), 1 mM GTP, 0.5 mM $MgCl_2$, and the following additions: curve 1, 1.2 mg/mL DEAE-MAPs; curve 2, 0.3 mg/mL flow-through MAPs; curve 3, 0.3 mg/mL flow-through MAPs and 1.2 mg/mL DEAE-MAPs; curve 4, 0.8 mg/mL flow-through MAPs and 1.2 mg/mL DEAE-MAPs. (Inset) (Curve 1) 1.2 mg/mL DEAE-MAPs; (curve 2) 0.8 mg/mL flow-through MAPs; (curve 3) 0.8 mg/mL flow-through MAPs and 1.2 mg/mL DEAE-MAPs; (curve 4) 0.3 mg/mL flow-through MAPs and 1.2 mg/mL DEAE-MAPs.

promoting microtubule assembly than the other components. This was excluded by determining the amount of polymer formed by centrifugal analysis. With all MAPs components examined, over half the protein in the reaction mixtures was recovered in the pellet following centrifugation, even though the flow-through MAPs produced a turbidity reading 3–4 times that obtained with the other preparations (data not presented).

The turbidity plateaus obtained with the total soluble MAPs and the DEAE-MAPs are not very different. We therefore wondered whether the plateau observed with the total soluble MAPs could be reconstituted. Since the flow-through and DEAE-MAPs were obtained in a weight ratio of 1:4, we examined 0.3 mg/mL flow-through MAPs and 1.2 mg/mL DEAE-MAPs separately and mixed together (Figure 9). The DEAE-MAPs were dominant, as the high reading observed with the flow-through MAPs alone was suppressed. Increasing the amount of flow-through MAPs to 0.8 mg/mL, with the DEAE-MAPs held constant at 1.2 mg/mL, resulted in a small further rise in turbidity; but the reading with DEAE-MAPs plus 0.8 mg/mL flow-through MAPs was only about one-third as great as the reading obtained with 0.8 mg/mL flow-through MAPs alone (Figure 9, inset).

The high turbidity readings obtained with the flow-through MAPs led us to anticipate that structures of aberrant morphology would be formed with these proteins. Instead, we found that the polymer consisted almost entirely of microtubules (Figure 10). Examination of micrographs of negatively

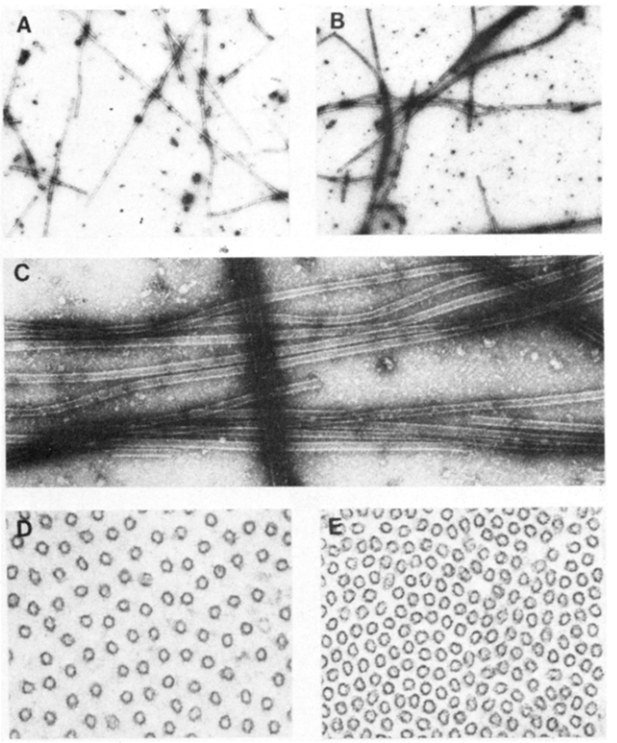


FIGURE 10: Morphology of polymer formed with 0.4 mg/mL flow-through MAPs. Each reaction mixture contained 1.5 mg/mL tubulin, 1.0 mM GTP, 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, and MAPs components as follows: (A) heat-treated MAPs, 0.5 mg/mL, magnification ×5250; (B) flow-through MAPs, 0.4 mg/mL, magnification ×5250; (C) flow-through MAPs, 0.4 mg/mL, magnification ×35 000; (D) DEAE-MAPs, 0.75 mg/mL, magnification ×52 500; (E) flow-through MAPs, 0.4 mg/mL, magnification ×52 500. Negatively stained specimens (A–C) and pellets for thin sections (D and E) were prepared after a 30-min incubation at 37 °C.

stained specimens did indicate a significant difference between the microtubules formed with the flow-through MAPs as compared to the other components. Figure 10A presents a lower power view of microtubules formed with heat-treated MAPs, and most of the microtubules are single and well separated. With the flow-through MAPs, substantial numbers of microtubules were in clusters of various sizes, ranging from doublets and triplets (Figure 10B) to groups of six to eight tubules (Figure 10C).

The negatively stained specimens indicated that the microtubules formed with the flow-through MAPs were clumped together, rather than true doublets (or larger groups) sharing walls. To confirm this, thin sections of polymer harvested by centrifugation were prepared. Panels D and E of Figure 10 compare typical groups of cross-sectioned microtubules formed with respectively DEAE-MAPs and flow-through MAPs. In both cases, single microtubules were the primary polymerization product, but the microtubules formed with the flow-through MAPs are packed much closer together than those formed with the DEAE-MAPs.

In performing the studies presented above in Figure 5, we observed significant turbidity at 0 °C when tubulin was mixed with higher concentrations of flow-through MAPs in the presence of GTP. Warming such a mixture to 37 °C resulted in a further substantial increase in turbidity. Since it seemed possible that at sufficiently high concentrations of flow-through MAPs microtubules might be formed in the cold, we examined such a reaction mixture in the electron microscope before and after the 37 °C incubation (Figure 11). In the cold (Figure 11A), no microtubules were present, but large numbers of rings, both single and double, were observed. These seem to

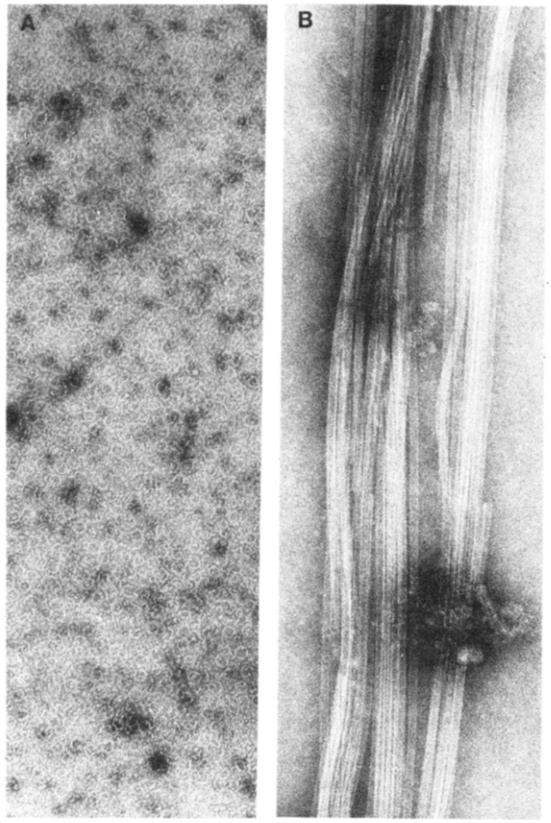


FIGURE 11: Morphology of polymer formed with 1.0 mg/mL flow-through MAPs at 0 and 37 °C. The reaction mixture contained 1.5 mg/mL tubulin, 1.0 mg/mL flow-through MAPs, 1.0 mM GTP, 0.1 M Mes (pH 6.9), and 0.5 mM $MgCl_2$. Negatively stained specimens were prepared at 0 °C (A) and after 30 min at 37 °C (B). Magnification ×35 000.

form an aggregate, but this appearance may simply result from the large number of rings in the preparation. Upon being warmed to 37 °C, the rings disappeared and were replaced by microtubules. Many of these were grouped in relatively massive bundles (Figure 11B), significantly larger than those observed at a lower flow-through MAPs concentration (Figure 10C).

We also attempted to determine whether the temperature-dependent increase in turbidity with tubulin plus flow-through MAPs in the absence of GTP represented the formation of discrete structures. Negatively stained specimens with 0.4 mg/mL flow-through MAPs and 1.5 mg/mL tubulin were prepared. Although single rings and small groups of rings were observed at 37 °C without GTP, such rings were also present at 0 °C, both in the presence and absence of GTP. We therefore tentatively conclude that the turbidity increase without GTP represents the formation of aggregates without defined structure.

## Discussion

Because of dissatisfaction with our yields of MAPs, we decided to attempt to separate tubulin and MAPs by the selective polymerization of tubulin induced by 1.6 M Mes (Waxman et al., 1981). Initially, we had hoped to remove virtually all the tubulin of microtubule protein in one to three cycles of polymerization, leaving the MAPs in the final supernatant. This goal was only partially realized. The major obstacle to achieving a total separation of tubulin and MAPs was the presence of significant amounts of insoluble material in the microtubule protein and at later stages in the preparative procedure. The protein in the first and second insoluble components represented about one-third of the total protein originally present in the two-cycle microtubule protein and contained tubulin and MAPs. It is unclear at this time how much of this protein was originally insoluble and how much precipitated in the course of the preparative procedure.[2]

Although the first and second insoluble components might represent the rings described by other workers (Borisy & Olmsted, 1972; Erickson, 1974), electron microscopy has revealed few discrete structures. Moreover, the first and second insoluble components persisted in high concentrations of NaMes, and the second insoluble component was not dissolved by 1 M NaCl [a condition reported to result in ring disintegration (Kirschner et al., 1974)]. If applied to a column of DEAE-cellulose or phosphocellulose, the insoluble protein was lost, and it is particularly depleted in MAP-1. It is not, however, merely denatured protein. It contains most of the ATPase activity present in the microtubule protein, consistent with recent reports that the ATPase of microtubule protein is associated with membrane vesicles (Murphy et al., 1983; Tominaga & Kaziro, 1983; Prus & Wallin, 1983). Heat extraction of the insoluble components yielded 24.6 mg (per gram of two-cycle microtubule protein) of protein of high specific activity in inducing microtubule assembly. The major protein in these heat extracts has the electrophoretic mobility of MAP-2.

The tubulin in the second insoluble component, however, appeared to be denatured. It has no activity in binding colchicine, and it contained little, if any, bound guanine nucleotide. It may be that this tubulin still has some affinity for active MAPs, and specifically binds the proteins released by heat treatment. Maccioni (1983) reported that denatured tubulin inhibits microtubule assembly in a reconsituted system of tubulin plus MAPs. A possible mechanism could be by its competing with native tubulin for the binding of active MAPs.

We also performed heat extractions of the cold pellets and second warm supernatant usually discarded in the preparation of two-cycle microtubule protein. Although heat extraction of the warm supernatant was unrewarding, a substantial amount of protein active in inducing microtubule assembly was obtained from the cold pellets. Its most prominent protein also had the electrophoretic mobility of MAP-2.

After removal of the Mes-induced tubulin polymer and the insoluble components from the two-cycle microtubule protein, 15–20% of the original protein remained in solution. This preparation (the total soluble MAPs) contained MAP-1, MAP-2, $\tau$ factor, and a number of other proteins.[3] It con-

[2] A subsequent high-Mes polymerization was performed with 4.3 g of the same microtubule protein preparation to answer this question specifically for tubulin. The colchicine binding activity of the total microtubule protein and of the high-Mes supernatant was measured as soon as the initial 1-h centrifugation was completed. The high-Mes supernatant at this point retained some activity in binding colchicine, but it had only 12% of the activity of the microtubule protein. This indicates that about two-thirds of the tubulin in the high-Mes supernatant is initially inactive, since the preparation initially has about one-third the tubulin content of the microtubule protein (Table II). As noted in the text, the high-Mes supernatant retains turbidity after the initial 1-h centrifugation. Rather than immediately concentrating this newly prepared high-Mes supernatant, it was centrifuged in the cold for 39 h. The supernatant had now lost half of its residual colchicine binding activity. This study thus leads us to conclude that although two-thirds of the residual tubulin in the high-Mes supernatant was initially inert, the other one-third was denatured in the course of preparing the total soluble MAPs.

tained little tubulin, as judged by polyacrylamide gel electrophoresis, and no active tubulin, as judged by colchicine binding activity.

The total soluble MAPs preparation was taken through two further steps, DEAE-cellulose chromatography and heat treatment. Each step resulted in a small increase in the specific activity of the MAPs and only minor differences in the properties of the microtubule assembly reaction. DEAE-cellulose chromatography resulted in a moderate decrease in the ATPase activity of the MAPs and a major reduction in their nucleosidediphosphate kinase activity. Total elimination of these enzymes, however, requires either heat treatment or additional purification. In terms of electrophoretic pattern, the most notable effect of these two steps was the disappearance of the MAP-1 band after heat treatment.

In terms of yield, we obtained 2–4 times as much heat-treated MAPs in the procedure described here as in earlier studies when microtubule protein was initially either heat treated or applied to DEAE-cellulose. The reason for this superior yield is not certain but may result from decreased binding of MAPs to denatured tubulin since most of the tubulin is removed early in the purification.

When the total soluble MAPs were applied to DEAE-cellulose, about 20% of the recovered protein did not bind to the resin. Besides containing most of the nucleosidediphosphate kinase activity found in the total soluble MAPs, these flow-through MAPs had a number of unusual properties. Both with and without GTP, when combined with tubulin, the flow-through MAPs produced an intense turbidity. Both reactions were temperature dependent, but that with GTP was significantly more rapid and cold reversible. The GTP-dependent polymer consisted of microtubules. In negatively stained specimens these microtubules tended to form clusters or bundles, and the bundles were larger at higher concentrations of flow-through MAPs. In thin sections of polymer fixed with glutaraldehyde, the microtubules formed with the flow-through MAPs were densely packed. Apparently, microtubules formed with the flow-through MAPs tend to stick together, accounting for the high turbidity plateaus obtained.

In a preliminary characterization (data not presented) of active component(s) in the flow-through MAPs, we have found that its activity in promoting formation and bundling of microtubules is heat stable. The prominent polypeptide bands surviving heat treatment are all of low molecular weight (less than 40 000) with high isoelectric points (about pH 10). Although we cannot at present exclude the possibility that these proteins are proteolytic fragments of higher molecular weight MAPs, we have not had problems with breakdown of MAP-2 under the preparative conditions described here. It is possible that proteolytic enzymes in the two-cycle microtubule protein [cf. Sloboda et al. (1976)] are inactive in the high concentrations of Mes and dithiothreitol that we have used.

We are unaware of any previous report describing a MAPs fraction with the properties of the flow-through MAPs.[4] Our use of DEAE-cellulose was based on the method of Murphy et al. (1977), but these workers did not describe comparable effects with their flow-through fraction. [They also observed a different distribution of $\tau$-type proteins when they passed two-cycle microtubule protein through DEAE-Sephadex. They found that almost all the $\tau$-type proteins were in the flow-through fraction, while we find $\tau$-type proteins in both the bound and unbound components (Figure 3). This has also been our finding when two-cycle microtubule protein was applied directly to DEAE-cellulose (Hamel et al., 1981).] There are, however, many differences in the methods not only of separating MAPs from tubulin but, probably more significantly, also of preparing microtubule protein [the preparation of the microtubule protein used by Murphy et al. (1977) is described in detail by Borisy et al. (1975)], and one of two major differences in the preparation of microtubule protein may account for the different properties of the flow-through MAPs. First, we used glycerol (Shelanski et al., 1973) in both our polymerization cycles, while Borisy et al. (1975) did not. Second, in preparing the initial tissue homogenate, we used the entire brain while Borisy et al. (1975) used only the cortex.

While at this time we cannot define with certainty the cause of the differences in the flow-through MAPs, it is tempting to speculate that it derives from the differences in brain tissue used for the preparation of microtubule protein. Matus et al. (1981) have presented immunologic evidence that MAP-1 and MAP-2 are largely confined to dendritic microtubules. The component in our flow-through MAPs that causes the bundling of microtubules could, analogously, be derived from axons. Since the axons are found predominantly in the white matter discarded by Borisy et al. (1975), this would explain the different properties of their flow-through MAPs (Murphy et al., 1977) as compared to ours.

In summary, in this study we have shown that active tubulin can be largely removed from microtubule protein with a single cycle of Mes-induced polymerization. The residual tubulin, together with most of the ATPase activity and insolubilized but still active MAPs, can be removed by prolonged high-speed centrifugation. The remaining soluble MAPs are active in promoting microtubule assembly and can be further purified as desired. The yield of heat-treated MAPs by the procedure described here is at least twice that of other procedures we have used. At all stages, cold pellets can be heat treated to extract significant amounts of MAPs enriched in MAP-2 and active in promoting microtubule assembly. DEAE-cellulose chromatography of the total soluble MAPs produces a minor fraction, not binding to the resin, which induces tubulin to form bundles of microtubules in the presence of GTP and a still undefined aggregate in the absence of the nucleotide.

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**Registry No.** Mes, 4432-31-9; GTP, 86-01-1; ATPase, 9000-83-3; GTPase, 9059-32-9; nucleosidediphosphate kinase, 9026-51-1.

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[3] Few workers have provided details of their yields of MAPs from microtubule protein. For comparison to the yields reported in Table I [i.e., 169 mg of total soluble MAPs, 114 mg of DEAE-MAPs, and 26 mg of flow-through MAPs per g of two-cycle microtubule protein (starting with 8.8 g)], Murphy et al. (1977) reported yields of 249 mg of MAPs bound to DEAE-Sephadex and 49 mg of unbound MAPs ger g of two-cycle microtubule protein (starting with 0.05 g) and Cleveland et al. (1977) reported a yield of 100 mg of phosphocellulose-bound MAPs/g of two-cycle microtubule protein (starting with 0.5 g).

[4] There have, however, been two recent reports of high molecular weight proteins *not* isolated from microtubules that cause the formation of microtubule bundles (Murofushi et al., 1983; Ishikawa et al., 1983).

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