

Purification of Tau, a Microtubule-associated Protein that Induces Assembly of Microtubules from Purified Tubulin

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Tau, a microtubule-associated protein which copurifies with tubulin through successive cycles of polymerization and depolymerization, has been isolated from tubulin by phosphocellulose chromatography and purified to near homogeneity. The purified protein is seen to migrate during electrophoresis on acrylamide gels as four closely spaced bands of apparent molecular weights between 55,000 and 62,000. Specific activity for induction of microtubule formation from purified tubulin has been assayed by quantitative electron microscopy and is seen to be enhanced three- to fourfold in the purified tau when compared with the unfractionated microtubule-associated proteins. Nearly 90% of available tubulin at 1 mg/ml is found to be polymerizable into microtubules with elevated levels of tau. Moreover, the critical concentration for polymerization of the reconstituted tau + tubulin system is seen to be a function of tau concentration and may be lowered to as little as 30 μ g of tubulin per ml. Under depolymerizing conditions, 50% of the tubulin at only 1 mg/ml may be driven into ring structures. A separate purification procedure for isolation of tau directly from cell extracts has been developed and data from this purification suggest that tau is present in the extract in roughly the same proportion to tubulin as is found in microtubules purified by cycles of assembly and disassembly. Tau is sufficient for both nucleation and elongation of microtubules from purified tubulin and hence the reconstituted tau + tubulin system defines a complete microtubule assembly system under standard buffer conditions. In an accompanying paper (Cleveland *et al.*, 1977) the physical and chemical properties of tau are discussed and a model by which tau may function in microtubule assembly is presented.

1. Introduction

The development of procedures for the assembly of microtubules *in vitro* (Weisenberg, 1972; Borisy & Olmsted, 1972; Shelanski *et al.*, 1973) has made possible the investigation of specific aspects of the polymerization process. Thus far it has been reported that under depolymerizing conditions (temperatures below 10°C or in the presence of millimolar concentrations of calcium) the 110,000 molecular weight, 6 S tubulin dimer and a faster sedimenting (30 to 36 S) tubulin oligomer (seen by electron microscopy to consist of single and double rings) are both present in preparations of microtubule protein purified by successive cycles of polymerization and depolymerization (Kirschner *et al.*, 1974; Erickson, 1974; Kirschner & Williams, 1974; Olmsted *et al.*, 1974; Rebhun *et al.*, 1974). The tubulin dimer, when separated from the ring fraction by high speed centrifugation (Borisy & Olmsted, 1972; Shelanski *et al.*, 1973)

or by gel filtration (Kirschner *et al.*, 1974; Erickson, 1974), has been shown either to fail to polymerize or to polymerize with greatly reduced efficiency, whereas the fraction enriched in rings remains competent for rapid and efficient polymerization. The further observation (Weingarten *et al.*, 1974; Haga *et al.*, 1974; Dentler *et al.*, 1975) that most, if not all, of the observable non-tubulin proteins which copurify with tubulin during cycles of assembly and disassembly are present in this polymerizable ring fraction, has directed attention to the role of these non-tubulin components in the assembly process. Two discoveries have recently demonstrated the importance of the associated proteins in microtubule assembly. In this laboratory Weingarten *et al.* (1975) have found a protein factor (named tau) which can be dissociated from tubulin with high salt and which can be isolated from tubulin by phosphocellulose chromatography. Under depolymerizing conditions, tau confers upon tubulin the ability to form rings and, under polymerizing conditions, it induces microtubule formation. Concurrently, Murphy & Borisy (1975) separated microtubule-associated proteins from tubulin utilizing DEAE-Sephadex and have reported microtubule-inducing activity similar to that of tau to be present in the associated protein fraction. Additional reports on the presence and properties of these or similar proteins have now appeared from these and other investigators (Dentler *et al.*, 1975; Keates & Hall, 1975; Penningroth *et al.*, 1976; Sloboda *et al.*, 1976a,b; Witman *et al.*, 1976; Murphy *et al.*, 1977).

Our initial paper on tau factor (Weingarten *et al.*, 1975) reported simply that the removal of associated proteins from tubulin by phosphocellulose rendered the tubulin totally incompetent for self-assembly even at high protein concentrations, and that the addition of adsorbed tau protein back to the tubulin restored polymerizability. Since this initial observation appeared, we have reported (Penningroth *et al.*, 1976) the partial purification and characterization of the tau activity and have shown (Witman *et al.*, 1976) that tau is required not only for initiation of assembly but also, in experiments testing competence of neutral tubulin for polymerization onto flagellar seeds, we have demonstrated the requirement for tau in the elongation process. Moreover, from the kinetics of the assembly reaction as a function of tau concentration, we have concluded that tau is required in stoichiometric rather than catalytic amounts during microtubule formation.

In the present paper we report on the final purification and characterization of tau from the standpoint of its ability to induce microtubule formation. The purified tau activity is seen to migrate during electrophoresis on sodium dodecyl sulfate/polyacrylamide gels as four closely spaced bands (two major, two minor) of apparent molecular weights between 55,000 and 62,000. Evidence for the close relationship of these four polypeptides is presented in the following paper (Cleveland *et al.*, 1977), as well as a description of the physical properties of the purified tau protein and a model by which tau may function in the assembly of microtubules.

2. Materials and Methods

(a) Preparation of microtubule protein, tubulin and tau

Microtubule protein was purified from porcine brain by cycles of polymerization and depolymerization according to the method of Shelanski *et al.* (1973), as modified by Weingarten *et al.* (1974). Purified tubulin and tau were prepared by chromatography of microtubule protein on phosphocellulose (Whatman P11, batch 511 or 359) as before (Witman *et al.*, 1976). After complete elution of the tubulin (hereafter call PC-tubulin)

from the phosphocellulose, crude tau was obtained by washing the column with PC buffer (0.025 M-*N*-morpholinoethane sulfonate (pH 6.4 with NaOH), 1 mM-2-mercaptoethanol, 0.5 mM-MgCl₂, 0.1 mM-EDTA) containing 0.8 M-NaCl. The tau fraction was then desalted into P buffer (0.1 M-*N*-morpholinoethane sulfonate (pH 6.4 with NaOH), 1 mM-2-mercaptoethanol, 0.5 mM-MgCl₂, 0.1 mM-EDTA, 1 mM-EGTA†) on Bio-Gel P6 (Bio-Rad). Hydroxyapatite and ammonium sulfate used in the further purification of tau were purchased from Bio-Rad (lot 3656) and Schwartz-Mann (ultrapure), respectively. Sepharose 6B used in the gel filtration of tau was from Pharmacia (lot 5073).

(b) *Quantitative electron microscopic assay for tau activity*

Quantitative electron microscopy was performed by the method of Kirschner *et al.* (1975). In a typical assay, PC-tubulin was mixed with tau to final concentrations of 1 mg/ml and 0.15 mg/ml, respectively, in a final volume of 75 μ l.

(c) *Polyacrylamide gel electrophoresis*

Gel electrophoresis in the presence of sodium dodecyl sulfate was performed using polyacrylamide gels in a slab gel apparatus according to the method of Laemmli (1970). Gels were stained for 1 to 3 h in a solution of 0.25% Coomassie blue R250, 45% methanol and 9% acetic acid. Destaining was achieved by diffusion in 7.5% acetic acid, 5% methanol.

3. Results

(a) *Microtubule proteins from successive cycles of polymerization*

Three proteins, two high molecular weight (270,000 to 300,000) components (Borisy *et al.*, 1975; Dentler *et al.*, 1975) and a cyclic AMP-stimulated protein kinase (Sloboda *et al.*, 1975) have been reported to remain associated at a fixed ratio to tubulin with microtubules through successive cycles of assembly and disassembly. In view of these reports and our initial observation, which suggested that non-tubulin microtubule proteins were essential for the polymerization of purified tubulin, microtubule protein was taken through five cycles of polymerization to ascertain which, if any, of the non-tubulin proteins in our preparations would continue to copurify with tubulin. A portion of the pellet from each warm centrifugation in the cycling procedure was chromatographed on phosphocellulose to separate the tubulin and non-tubulin components. This was necessary because tubulin is the major protein and its presence might have obscured minor components of comparable electrophoretic mobilities. After each cycle of polymerization, the microtubule protein, the PC-tubulin and the phosphocellulose-adsorbed associated proteins were examined separately (Fig. 1). It is clear (Fig. 1(a) to (l)) that little additional purification of tubulin was achieved after the second cycle of assembly. From the second through the fifth cycle, the fraction of the applied protein which adsorbed to the phosphocellulose (seen in Fig. 1(m) to (r) to consist of non-tubulin proteins) remained constant at 10% as judged by the Lowry assay for protein (Lowry *et al.*, 1951). This represents a higher value than was previously estimated from densitometry of stained gels of microtubule protein (Weingarten *et al.*, 1975). With the exception of a 70,000 molecular weight band which, while easily visible in the extract, was lost with successive cycles, all of the major non-tubulin proteins which were present after two cycles of assembly were retained through additional cycles in roughly constant stoichiometries with tubulin. Most prominent were some high molecular weight components at the top of the gel

† Abbreviation used: EGTA, ethyleneglycol-bis-(β -aminoethyl ether)*N,N'*-tetraacetic acid.

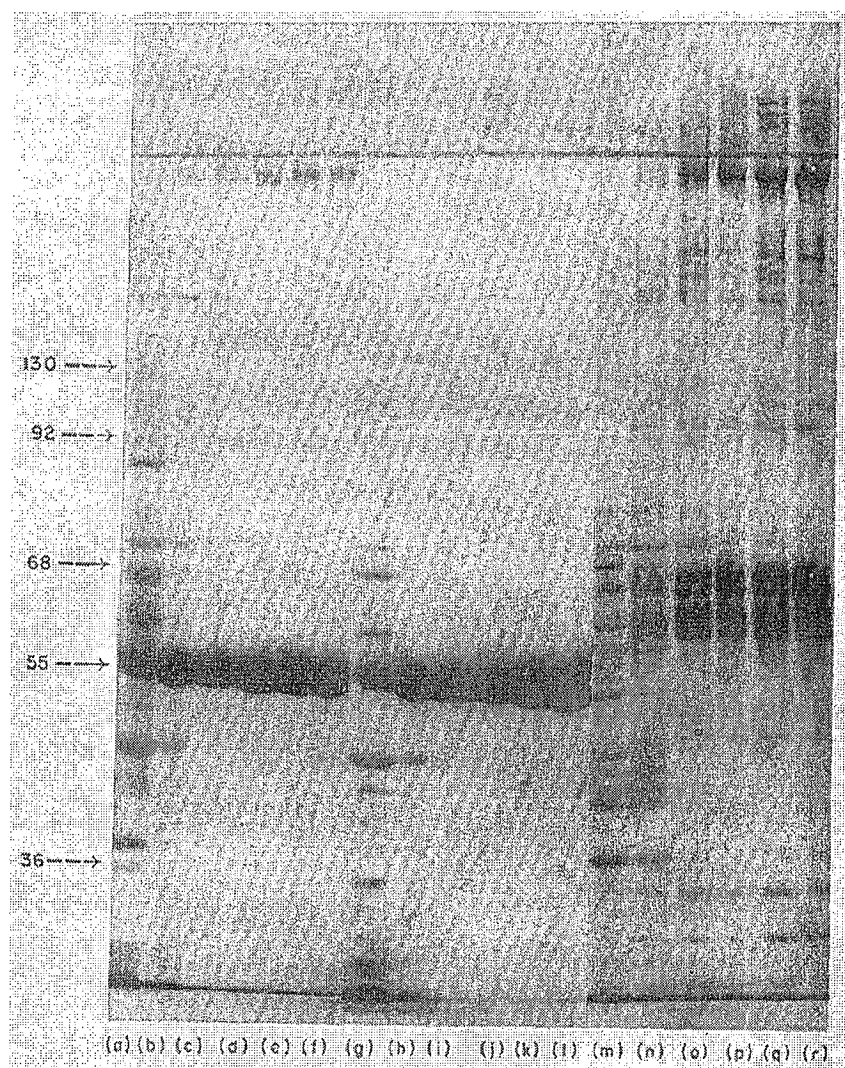


FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of microtubule protein, phosphocellulose-purified tubulin, and the non-tubulin protein components after 1 to 5 cycles of polymerization and depolymerization. Portions of the pellets from successive warm centrifugations in the alternating warm/cold (assembly/disassembly) purification procedure were resuspended in PC buffer, depolymerized on ice for 30 min, and clarified by centrifugation at 4°C at 12,000 *g* for 15 min. Portions of the supernatants of these centrifugations, representing microtubule protein prepared by 1 to 5 cycles of assembly, were applied immediately to a phosphocellulose column equilibrated with PC buffer. After complete elution of unabsorbed protein (as monitored by absorbance at 280 nm), bound proteins were eluted with a single wash of 0.8 M-NaCl in PC buffer. For electrophoresis, appropriate amounts (15 to 20 μ g) of the microtubule protein samples and unadsorbed phosphocellulose fractions were diluted 1:1 with gel sample buffer as given by Laemmli (1970). Bound proteins were precipitated after elution by addition of trichloroacetic acid to a final concentration of 20% and pelleted by centrifugation at 12,000 *g* for 10 min. Pellets were washed twice with diethyl ether and resuspended in gel sample buffer which had been diluted 1:1 with distilled water. All samples were boiled for 1 min in the presence of 10% 2-mercaptoethanol immediately prior to loading on the gel. The resolving gel was 9% in acrylamide; the stacking gel was 6%. (a) Porcine brain extract, i.e. the supernatant recovered from the initial cold centrifugation of the purification of microtubule protein; (b) to (f) microtubule protein following 1 to 5 cycles of assembly, respectively; (g) to (l) fraction of the extract which did not adsorb to phosphocellulose following 1 to 5 cycles of assembly; (m) fraction of the extract which adsorbed to phosphocellulose; (n) to (r) fraction of microtubule protein which adsorbed to phosphocellulose following 1 to 5 cycles of assembly, respectively. The positions of standard proteins of known molecular weights are indicated in units of 1000.

and a series of proteins of electrophoretic mobilities between bovine serum albumin and tubulin (shown below to contain the tau activity).

From the second cycle of assembly and beyond, the protein which does not bind to phosphocellulose is entirely tubulin. No other bands are visible, even in highly overloaded gels (Fig. 1(j) to (l)). As noted previously (Weingarten *et al.*, 1975), such PC-tubulin will not polymerize. Using quantitative electron microscopy at protein concentrations as high as 10 mg/ml, no microtubules are observable from this tubulin after incubation for 30 minutes at 37°C in P buffer supplemented with 1 mM-GTP. Some care, however, must be exercised in obtaining such tubulin reproducibly. The efficiency with which the associated proteins are removed from tubulin, though consistent within a given lot of phosphocellulose, varies considerably from batch to batch of resin even from the same manufacturer. Moreover, the highest purity tubulin preparations can only be achieved in our experience on columns which are loaded with no more than 3 mg of protein per ml of resin and the flow-rates of which are maintained at roughly one column volume per hour. The failure to observe such procedures may result, as Erickson (1976) has reported, in less highly purified tubulin preparations which will still support low levels of polymerization.

(b) Assay for tau activity

The ability of tau to restore polymerizability to PC-tubulin and the development of quantitative electron microscopy has afforded us an unambiguous assay with which to monitor the purification of the tau activity. Fractions obtained from a standard biochemical purification technique (e.g. an ion-exchanging or gel filtration column, etc.) may be mixed with purified tubulin, brought to 1 mM-GTP, incubated at 37°C and then analysed for the formation of microtubules by electron microscopy. Such an assay, however, requires freshly prepared tubulin. The polymerizability of PC-tubulin (brought to 0.1 M-sodium *N*-morpholinoethane sulfonate and 1 mM-GTP after elution from phosphocellulose and maintained on ice throughout) when assayed with sufficient added crude tau to drive initially greater than 75% of the PC-tubulin into microtubules, falls (in a roughly exponential fashion) more than 35% within nine hours after elution from the phosphocellulose and no microtubules whatsoever are observable after 20 hours. Similarly, PC-tubulin which has been frozen in PC buffer, P buffer, or in P buffer with 1 M-sucrose is also completely inactive when tested after thawing. Therefore, throughout the experiments reported in this paper PC-tubulin was used within five hours of its preparation.

(c) Fractionation of native tau factor on hydroxyapatite

Fractionation of tau factor was achieved by chromatography on hydroxyapatite (Fig. 2). Crude tau adsorbed to a column of hydroxyapatite was eluted with a linear phosphate gradient. So as not to bias the assay towards fractions of higher protein concentrations, a constant concentration of protein from each fraction was assayed for ability to promote microtubule formation with PC-tubulin. As is visible in Figure 2, two peaks of activity and two peaks of protein elution are observable. The major peak of activity, which we call tau-I, comprises roughly two-thirds of the total recovered activity and elutes near 0.09 M-phosphate. The second peak, which we call tau-II, elutes at higher ionic strength and represents the remaining tau activity. Its specific activity, however, is only one-quarter that of tau-I.

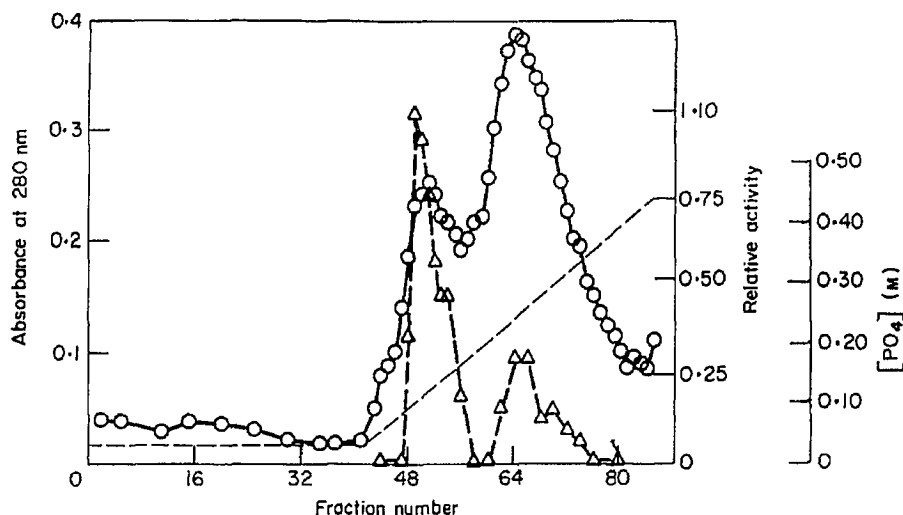


FIG. 2. Hydroxyapatite fractionation of crude tau. A 6-ml column of hydroxyapatite was equilibrated with P buffer and 22 ml of crude tau (1.1 mg/ml) in P buffer was applied. Adsorbed protein was eluted with a linear gradient of 0 to 0.5 M-potassium phosphate (pH 6.4) in P buffer. Fractions of 1 ml were collected and elution of protein was monitored by measuring absorbance at 280 nm (—○—○—). Phosphate concentration was determined by measuring conductivity (-----). For assay of tau activity, fractions were desalted into P buffer on Bio-Gel P6 and concentrations of protein were determined by the method of Lowry *et al.* (1951). Freshly prepared PC-tubulin was mixed with a portion from each fraction to final concentrations of 1.25 mg tubulin/ml and 0.17 mg added non-tubulin protein/ml. GTP was then added to 1 mM and samples were incubated for 15 min at 37°C. Microtubule formation was analyzed by quantitative electron microscopy. --△--△--, Relative specific tau activity of the fractions.

Gel electrophoresis of the two fractions does not reveal common polypeptides. The tau-I fraction contains major bands between $M_r = 55,000$ and 70,000 and the tau-II fraction contains significant amounts of both high and low molecular weight proteins (see Fig. 9 of Penningroth *et al.*, 1976). We report that the two activities may also be distinguished by differential heat sensitivity. Incubation at 75°C for up to one hour leads to little, if any, diminution in the ability of tau-I to induce polymerization. No turbidity is generated during heating and, after the 60-minute incubation, no protein may be pelleted by low-speed centrifugation. Tau-II, on the other hand, loses activity very rapidly, falling to half initial stimulation of microtubule formation after only five minutes. Protein aggregation is evident even at early times of incubation. It is of course possible that the precipitous loss of activity is not an inherent property of the tau-II component, but merely reflects heat denaturation of other proteins in the tau-II fraction and coaggregation of the tau activity with these proteins. Elucidation of this question must await further fractionation of the tau-II activity.

(d) Native molecular weight estimate of tau-I activity

An estimate of the native molecular weight of the tau-I activity purified by chromatography on hydroxyapatite has been obtained by combining measurements of the diffusion coefficient of the tau activity as indicated by gel permeation chromatography with the apparent sedimentation coefficient indicated by sucrose gradient centrifugation. Both of these techniques are zonal in nature and hence when combined with activity assays may be used to measure the appropriate properties of an individual (active) protein even from an initially heterogeneous mixture of proteins. In

these experiments tau was examined in 0.3 M-NaCl to minimize the possibility of aggregation. Parallel experiments in the presence of 1 M-urea showed identical results.

Tau-I protein was applied to a column of Sepharose 6B that had been calibrated by chromatographing proteins of known diffusion coefficients (Fig. 3). Microtubule-forming activity eluted in a single peak corresponding to an effective Stokes radius of 56 Å or a diffusion coefficient of $3.8 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (not shown) of the various fractions showed most high molecular weight proteins to elute somewhat earlier than the tau activity, and the residual low molecular weight proteins in the initial tau-I (less than 50,000 by gel) to be retarded with respect to tau. The major bands which eluted in the fractions of peak activity were between 55,000 and 70,000 molecular weight, despite the fact that the Stokes radius corresponds to a molecular weight of 600,000 for a globular protein.

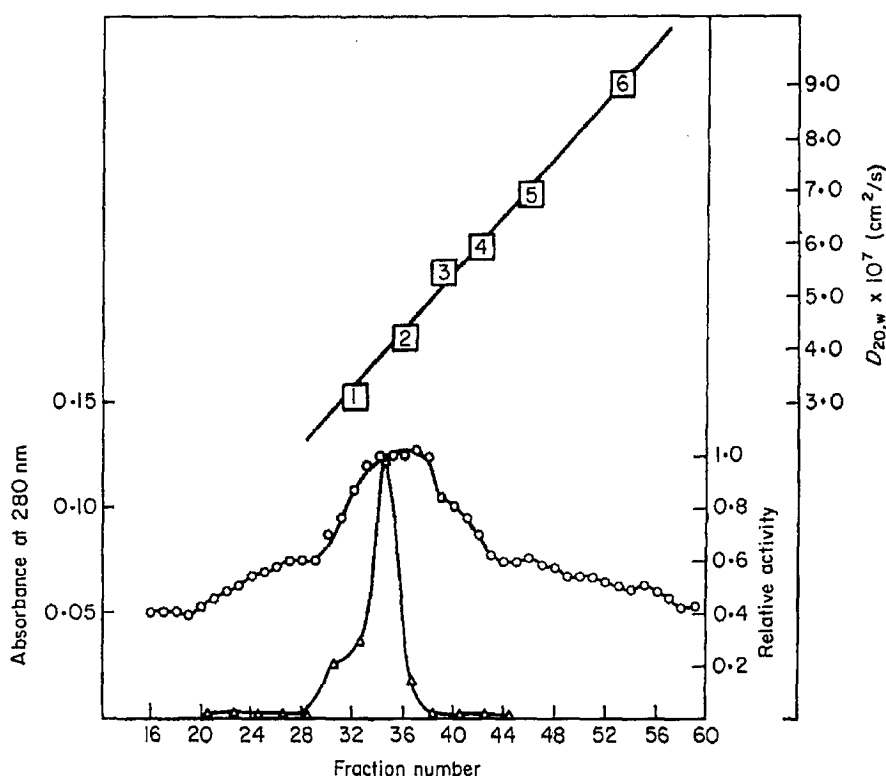


FIG. 3. Sepharose 6B chromatography of tau-I. A column (0.7 cm \times 30 cm) of Sepharose 6B was equilibrated with P buffer containing 0.3 M-NaCl. Tau-I protein (1.3 mg) was precipitated by addition of an equal volume of saturated ammonium sulfate. The precipitate was pelleted by centrifugation at 12,000 g for 15 min, resuspended in 0.2 ml of P buffer plus 0.3 M-NaCl, and applied to the column. Fractions (7 drops, approx. 250 μ l) were collected. The column was run at 4°C at 3 ml/h. For determination of microtubule-simulating activity, adjacent fractions were pooled, desalted on Bio-Gel P6 into P buffer, and assayed by quantitative electron microscopy for microtubule formation after addition of PC-tubulin to a final concentration of 1.0 mg/ml and incubation at 37°C. Proteins used in the calibration were chromatographed separately and their elution positions monitored by measuring absorbance at 280 nm (—○—○—), with the exception of catalase, whose elution position was determined catalytically by the loss of absorbance at 240 nm in a peroxide-containing solution. Values for the diffusion coefficients of these proteins were taken from the tabulation of Smith (1970). —△—△—, Relative microtubule-stimulating activity. The numbers in squares show the positions of elution of the proteins used in the column calibration: 1, *Escherichia coli* β -galactosidase; 2, bovine liver catalase; 3, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase; 4, bovine serum albumin; 5, human hemoglobin; 6, soybean trypsin inhibitor.

Sucrose gradient centrifugation experiments have been reported and yield a sedimentation coefficient of 2.7 S for tau activity (Penningroth *et al.*, 1976). Globular proteins which sediment at 2.7 S should have a molecular weight of only about 21,000. Using the values for s and D obtained from the measurement of tau activity and utilizing the Svedberg equation ($s = M(1 - \bar{v}\rho)/Nf = M(1 - \bar{v}\rho)D/RT$) with \bar{v} taken to be 0.722 cm³/g (calculated by the method of Cohn & Edsall (1943) from the amino acid composition given by Cleveland *et al.*, 1977), we have calculated a native molecular weight for tau-I of 61,000. When combined with the staining pattern from acrylamide gels, this indicates that the native tau-I is a monomer. The data also imply a frictional ratio f/f_0 of roughly 2, which translates into an axial ratio of greater than 20:1 using a prolate ellipsoidal model. The ellipsoid may not be a good model, but in any case tau-I must be decidedly asymmetric.

(e) *Final purification of tau-I activity*

We now report the successful final purification of the tau-I activity. Microtubule protein was chromatographed on phosphocellulose as before with the exception that bound protein was eluted with 0.35 M-NaCl in PC buffer instead of 0.8 M-NaCl in PC buffer. Although the trail of stimulatory activity which elutes at ionic strengths higher than 0.35 M-NaCl has been found to be more significant with the current lot (Whatman, 359) of phosphocellulose than was reported using Whatman lot 511 (Weingarten *et al.*, 1975), roughly two-thirds of total recoverable microtubule stimulatory activity and one-half of total bound protein was eluted by this 0.35 M-NaCl wash. Recovered protein was desalted on Bio-Gel P6 into P buffer, diluted to an optical density of 0.18 at 280 nm, and brought to 35% saturation in ammonium sulfate by addition of saturated ammonium sulfate. The protein was left on ice for 30 minutes and then was centrifuged at 4°C for 20 minutes at 12,000 g to pellet precipitated protein. The supernatant was recovered and brought from 35% to 45% saturation in ammonium sulfate. Again, the mixture was left for 30 minutes on ice and the protein precipitate was pelleted by centrifugation. When both the 0 to 35% and the 35 to 45% pellets were resuspended in P buffer and desalted to remove residual ammonium sulfate, Lowry assays showed roughly equal amounts of protein in each fraction, with less than 10% of the initial protein remaining in the final 45% ammonium sulfate supernatant.

The 35 to 45% protein fraction, found to contain most of the tau activity, was applied to an hydroxyapatite column equilibrated in P buffer. Protein was loaded at 2 mg per ml of column and the flow-rate was maintained at one column volume per hour. Tau-I protein was eluted from the column by application of a solution of 0.15 M-phosphate in P buffer: 75% of recovered protein eluted in this salt wash. The overall yield of protein from the column was 70%.

A sodium dodecyl sulfate/acrylamide gel of the purification procedure beginning with two-cycle microtubule protein and ending with purified tau-I from hydroxyapatite is shown in Figure 4. High molecular weight polypeptides are seen to be present initially in significant amounts by weight, only to be lost during purification in both the ammonium sulfate and the hydroxyapatite steps. The final tau-I preparation consists of four closely spaced bands of apparent molecular weights of 55,000, 56,000 and 62,000†. The relatedness of these four bands is demonstrated by a variety of methods in the following paper (Cleveland *et al.*, 1977).

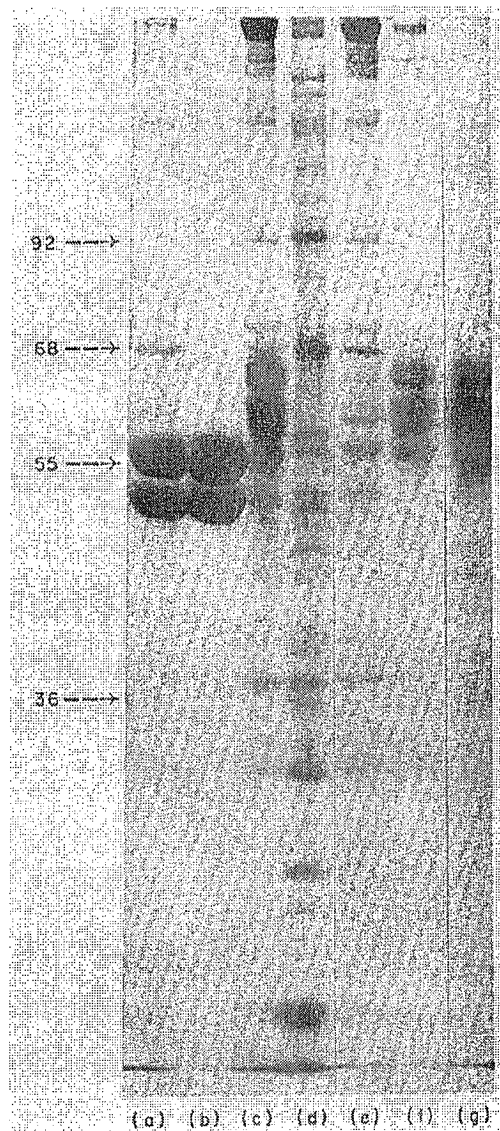


FIG. 4. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of fractions from the tau-I purification. The resolving gel was cast from 8.5% acrylamide and the stacking gel from 4.0%. (a) Microtubule protein purified by 2 cycles of polymerization and depolymerization; (b) phosphocellulose-purified tubulin; (c) microtubule-associated proteins adsorbed to phosphocellulose and eluted with 0.35 M-NaCl; (d) 1.0 M-NaCl wash of phosphocellulose following the 0.35 M-NaCl wash; (e) protein fraction from the 0.35 M-NaCl elution of phosphocellulose which precipitated in 35% saturated ammonium sulfate; (f) protein fraction eluted from phosphocellulose in the 0.35 M-NaCl wash which precipitated in the 35 to 45% saturation ammonium sulfate step; (g) purified tau-I obtained by application of the 35 to 45% ammonium sulfate-precipitable protein to a hydroxyapatite column which was then eluted with 0.15 M-phosphate in P buffer. Electrophoretic positions of proteins of known molecular weights are shown in units of 1000.

† The electrophoretic mobilities of the various tau bands differ somewhat from gel to gel, giving rise to variable estimates for their respective molecular weights. This situation, we believe, is not unlike that of α and β -tubulin, whose relative mobilities are well-known to produce molecular weight estimates which may differ by several thousand depending on the type of gel system and the precise conditions of electrophoresis used. In this paper we have used molecular weight estimates for the tau bands deduced from Fig. 4, with molecular weights of 55,000 and 53,000 taken for α and for β -tubulin.

The ability of fractions from successive stages in the purification procedure to induce microtubule assembly from PC-tubulin was measured by quantitative electron microscopy. The amount of microtubules formed as a function of the amount of tau preparation which was added is shown in Figure 5. It is clear, when compared with crude tau, that three to four times less of the final hydroxyapatite-purified tau-I needs to be added to a given concentration of tubulin to effect comparable levels of polymerization. Hence, specific activity has been enhanced by three- to fourfold. At levels of purified tau-I of roughly 10% by weight, as much as 90% of the PC-tubulin at a concentration of 1 mg/ml may be driven into microtubules. The specific activities and the total recovered activities of the various fractions from the purification are summarized in Table 1. Activity recovered in tau-I represents roughly 40% of total crude tau activity, and shows a specific activity about 3.7 times that of crude tau. The 0 to 35% ammonium sulfate precipitable fraction containing primarily the high molecular weight peptides (Fig. 4) is seen to have roughly 10% the specific activity of tau-I and to contain only 10% of initial total activity. In addition, this fraction

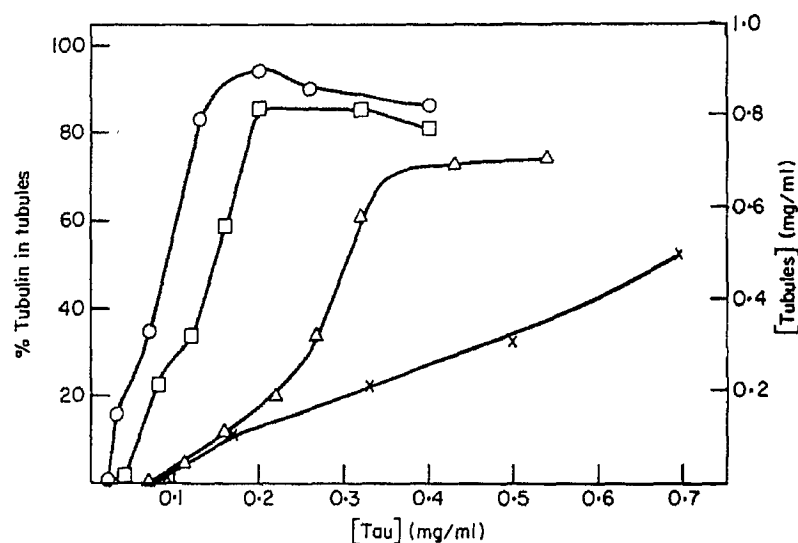


FIG. 5. Assay of fractions from the tau-I purification by quantitative electron microscopy. Crude tau-I protein was prepared by elution with 1 M-NaCl wash of phosphocellulose to which had been applied microtubule protein purified by 2 cycles of assembly and disassembly. Recovered crude tau was desalted on Bio-Gel P6 into P buffer. Purification of tau-I was begun with a 0.35 M-NaCl in PC-buffer elution of phosphocellulose again to which had been applied 2-cycle microtubule protein. After desalting into P buffer and dilution with P buffer to an optical density of 0.18 at 280 nm, saturated ammonium sulfate was added to a final concentration of 35%. After 30 min on ice, precipitated protein was pelleted by centrifugation at 4°C for 15 min at 12,000 g. The pellet was resuspended and desalted into P buffer and is referred to as 0 to 35% protein. The supernatant from the centrifugation was brought to 45% saturation in ammonium sulfate, left on ice for 30 min, and the precipitate pelleted by centrifugation as before. The pelleted protein was resuspended and desalted into P buffer and is referred to as 35 to 45% protein. A portion of the 35 to 45% protein was saved for the activity assay and the remainder applied to an hydroxyapatite column equilibrated in P buffer. Adsorbed protein was eluted in a single wash of 0.15 M phosphate in P buffer and, after desalting into P buffer, represents purified tau-I. Quantitative electron microscopy was performed with final concentrations of 0.95 mg phosphocellulose-purified tubulin/ml and varying concentrations of each of the 4 fractions from this purification procedure. Samples were incubated for 15 min at 37°C with 1 mM-GTP before addition of bushy stunt virus to a final concentration of 0.155 mg/ml. Samples were then loaded into a double nebulizer and sprayed immediately onto an electron microscope grid. —○—○—, Purified tau-I; —□—□—, 35 to 45% protein; —△—△—, crude tau; —×—×—, 0 to 35% protein.

TABLE I

Purification of tau†

| | Total protein‡ (mg) | % Initial protein | Specific tau activity§ | % Initial total activity |
|--|---------------------|-------------------|------------------------|--------------------------|
| Crude tau | 50 | 100 | 1 | 100 |
| 0-35% ammonium sulfate-precipitable protein following 0.35 M-NaCl wash of phosphocellulose | 9.5 | 19 | 0.4 | 8 |
| 35-45% ammonium sulfate-precipitable protein following 0.35 M-NaCl wash of phosphocellulose | 9.5 | 19 | 2.1 | 40 |
| Tau-I, 0.15 M-phosphate wash of hydroxyapatite following 35-45% ammonium sulfate precipitation | 5 | 10 | 3.7 | 39 |
| Tau-II, 0.20-0.28 M-phosphate wash of hydroxyapatite chromatography of crude tau, as in Fig. 4 | 17.5 | 35 | 0.55 | 19 |

† Preparation of fractions is described in the text.

‡ From 500 mg of microtubule protein purified by 2 cycles of assembly.

§ Defined as the ratio of the concentration of crude tau needed to induce 50% polymerization in 0.95 mg of PC-tubulin/ml to the corresponding concentration of the fraction being assayed.

does not induce polymerization of all of the tubulin, even at high concentrations. The tau-II fraction, obtained by hydroxyapatite chromatography as in Figure 2, is also seen to have half the specific activity of crude tau and to account for just under 30% of total crude activity.

(f) *Effect of tau on the critical concentration for microtubule polymerization*

Purified tau-I was assayed for its ability at constant concentration of tau to induce microtubules in varying concentrations of PC-tubulin. Figure 6 shows the results over a sixfold range of tau concentrations and a 300-fold range (0.02 to 6.5 mg/ml) of tubulin concentrations. Ideally, if tau is required stoichiometrically (Witman *et al.*, 1976), for each level of tau increasing concentrations of microtubules should be induced from increasing initial concentrations of tubulin until a point is reached at which the tau supply is exhausted, and above which additional levels of tubulin should remain unpolymerized. This expectation is essentially verified in Figure 6(a), although a slow continuous rise in the level of polymerization at the highest levels of tubulin is also evident.

Figure 6(b) shows an expanded portion of Figure 6(a) for low tubulin concentrations. At the highest level of tau (0.5 mg/ml), microtubules were observed from tubulin levels as low as 0.04 mg/ml, whereas a sixfold decrease in the amount of tau yielded microtubules only at tubulin concentrations of 0.2 mg/ml and above. These results indicate extrapolated critical concentrations of tubulin necessary for polymerization ranging from 0.03 mg/ml to 0.18 mg/ml for the different concentrations of tau.

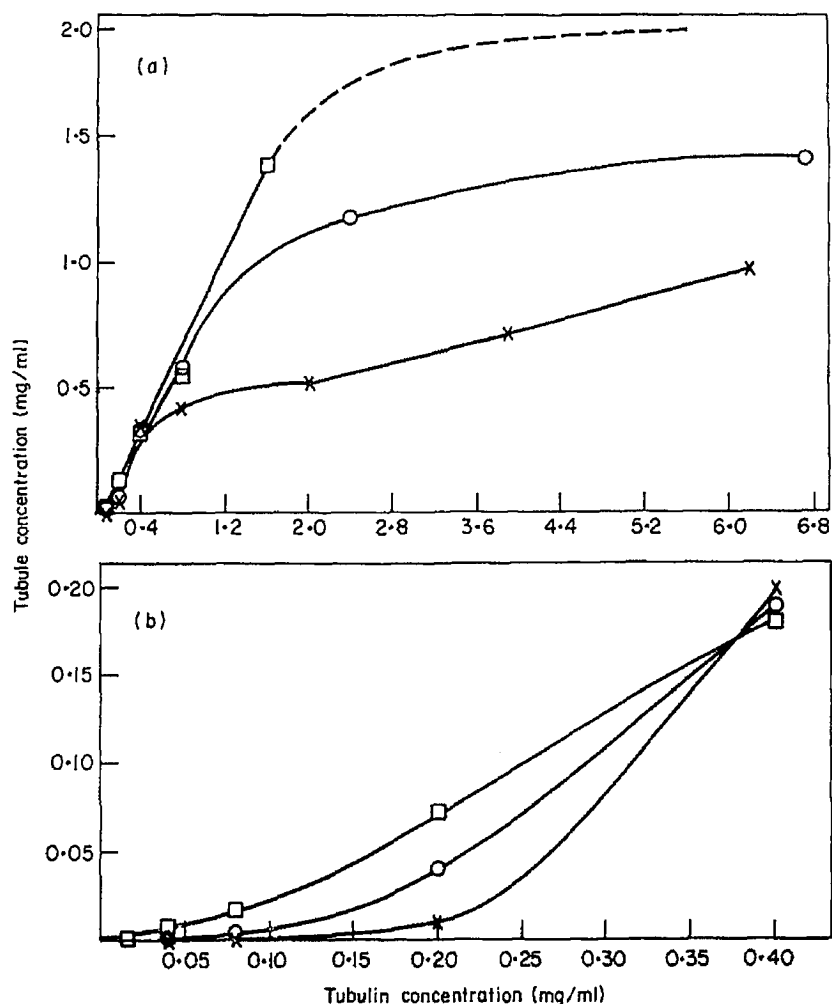


FIG. 6. Levels of microtubules formed from different concentrations of purified tau-I shown as a function of initial concentration of PC-tubulin. Assay for microtubules was by quantitative electron microscopy following addition of GTP to 1 mM and incubation of the tau-tubulin samples at 37°C for 30 min. (a) Data up to 6.5 mg PC-tubulin/ml and (b) data at lower PC-tubulin concentrations. (a) and (b) Final concentration of tau-I: —x—x—, 0.08 mg/ml; —o—o—, 0.2 mg/ml; —□—□—, 0.5 mg/ml.

(g) *Stimulation of ring formation with purified tau*

The ability of purified tau-I protein to stimulate PC-tubulin to form ring structures under depolymerizing conditions was investigated. Increasing amounts of tau-I were added to a constant concentration of PC-tubulin and the number concentration of rings was measured by quantitative electron microscopy. The results are shown in Figure 7 along with the equivalent assay for crude tau. The only structures observable were double ring or spiral structures and possibly a few triple rings. As can be seen in Figure 7, at a level of purified tau-I roughly 10% that of added tubulin, somewhat over 13% of the tubulin is present in ring structures. Ring-forming activity in crude tau when compared with tau-I is seen to be three- to sixfold lower, with essentially no rings observed with crude tau when it is added at a concentration of 10% of the tubulin. The unfractionated microtubule protein from which both the tubulin and the tau were prepared is itself comprised of 10% non-tubulin proteins, and when assayed at a total protein concentration of 1.0 mg/ml yields a fast-sedimenting species of double

procedure avoids incubating the protein at 37°C for extended periods of time in crude or partially purified extracts. The previous purification involving initial cycles of polymerization and depolymerization to isolate the initial microtubules may render the protein more susceptible to proteolytic action.

Porcine brain was homogenized in a blender and subjected as before to centrifugation in the cold to pellet cellular debris. The supernatant (15 to 20 mg/ml) of this centrifugation, i.e. the extract, was brought to 30% saturation in ammonium sulfate by addition of the appropriate volume of saturated ammonium sulfate. After 30 minutes on ice the mixture was centrifuged at 12,000 *g* for 20 minutes to pellet precipitated protein. The pellet was discarded and the supernatant was brought to 45% saturation in ammonium sulfate. Again, after 30 minutes on ice, precipitated protein was collected by centrifugation. This 30 to 45% pellet, representing 25% of the total extract protein, was suspended in PC buffer, clarified by additional centrifugation, and applied at between 5 and 10 mg/ml to a phosphocellulose column. Five mg of protein was loaded per ml of column. Unadsorbed protein containing mostly tubulin was discarded. The bound protein was then eluted with 0.3 M-NaCl in PC buffer (Fig. 8(b)). The 0.3 M-NaCl wash contained 4% of the initial extract protein and after desalting into P buffer showed no ability at 1 mg/ml to stimulate the formation of microtubules from PC-tubulin at 1 mg/ml, although a threefold concentration by an additional ammonium sulfate precipitation step produced a few observable microtubules.

The known heat stability of tau-I suggested that a heat precipitation step might be useful at this point. Heating the desalted 0.3 M-NaCl wash to 85°C for five minutes, followed by centrifugation at 4°C for ten minutes at 12,000 *g* to pellet aggregated protein was sufficient to remove 85% of the initial protein. The remaining supernatant was capable, at a final concentration of 0.41 mg/ml, of stimulating greater than 60% of the PC-tubulin at 1.3 mg/ml to polymerize into microtubules. The tau-I bands were the major surviving bands in this 85°C supernatant (Fig. 8(c)).

Final purification was achieved by a polymerization step with PC-tubulin. The 85°C supernatant at a final concentration of 0.05 mg/ml was incubated with tubulin at 0.80 mg/ml in P buffer containing 4 M-glycerol and 1 mM-GTP. After warming to 37°C for 30 minutes, microtubules were pelleted at 100,000 *g* for 40 minutes at 35°C. Pelleted protein (roughly 10% of the total) was resuspended in PC buffer, depolymerized on ice for 30 minutes, and clarified by centrifugation at 4°C for ten minutes at 12,000 *g*. The tau proteins were then separated from the tubulin by chromatography on phosphocellulose. Protein which adsorbed to the resin represented the purified tau-I and was eluted with 0.3 M-NaCl in PC buffer. Five major bands were visible by gel electrophoresis (shown in Fig. 8(d)), four of which coelectrophoresed with the tau-I bands purified by the conventional hydroxyapatite procedure (Fig. 8(a)). The fifth band ran slightly faster than the tau bands, migrating with an apparent molecular weight of 52,000. Gel electrophoresis of protein adsorbed to a parallel phosphocellulose column loaded with the 100,000 *g* supernatant from the polymerization step demonstrated (Fig. 8(e)) that, whereas virtually all of the five presumptive tau bands had pelleted with the microtubules, most of the other polypeptides visible in the 85°C supernatant (including a large amount of material which electrophoresed in the front) had been lost by the polymerization procedure.

Overall, 0.6% of initial extract protein was retained after the heat precipitation step. Assuming purity after heat precipitation to be between 60% and 75%, this



FIG. 8. Gel electrophoresis of tau-I and fractions from the purification of tau-I directly from extract. (a) Tau-I. For purification of tau-I directly from extract, following an initial 30 to 45% ammonium sulfate precipitation step, extract protein was applied to phosphocellulose and bound protein eluted with a 0.3 M-NaCl in PC-buffer wash. Protein in this wash was then heated to 85°C for 5 min, aggregated protein was pelleted, and the remaining supernatant was polymerized with added PC-tubulin. The microtubules were collected by centrifugation and associated non-tubulin (tau) proteins were recovered by application of the collected microtubules to a second phosphocellulose column. Purified tau was eluted in a 0.3 M-NaCl wash of this second column. Proteins present in the 85°C supernatant which did not pellet with the microtubules in the polymerization step were recovered by chromatography of the supernatant from the microtubule centrifugation step on a third phosphocellulose column. Again bound proteins were eluted with a 0.3 M-NaCl wash. (b) 0.3 M-NaCl wash of the initial phosphocellulose column; (c) 85°C supernatant; (d) final purified tau-I eluted in the salt wash of the second phosphocellulose column; (e) 0.3 M-NaCl wash of the third phosphocellulose column on which was loaded the supernatant of the polymerization step.

indicates the presence of tau-I in the initial extract at a minimum level of approximately 0.4%.

4. Discussion

Like the high molecular weight accessory components previously reported to remain stably associated with microtubules (Borisy *et al.*, 1975; Dentler *et al.*, 1975), tau protein is present in roughly constant stoichiometry with tubulin through multiple cycles of polymerization and depolymerization. Indeed, little if any difference in the relative or total amount of any of the 20 odd microtubule-associated proteins which copurify with tubulin through two cycles of assembly was observed through additional

cycles of polymerization (Fig. 1). Tubulin purity, as contrasted with the 75% value reported for a different purification procedure (Borisy *et al.*, 1975), approaches 90% by the second round of polymerization and remains at this level during additional rounds of assembly†.

Tau protein may be separated from tubulin by chromatography on phosphocellulose. The recovered tubulin is completely incompetent for self-assembly even at protein concentrations of 10 mg/ml. That such tubulin is not simply denatured by the conditions of the chromatography has been demonstrated by the ability of the phosphocellulose-adsorbed tau fraction to restore rapid (see Witman *et al.* (1976) for kinetics) and efficient assembly. However, as can be seen in Figure 4, crude tau, as obtained in a single salt wash of the adsorbed proteins, is needed in amounts near 30% by weight to reconstitute the polymerization properties of unfractionated microtubules. At 0.95 mg of PC-tubulin per ml, 30% by weight of crude tau was required to effect 75% polymerization. Initial unfractionated microtubule protein consisting of only 10% non-tubulin proteins polymerized with comparable efficiency. Thus, crude tau recovered from phosphocellulose contains apparently only one-third of the total initial microtubule-inducing activity when assayed immediately after preparation. Whether this represents the loss or inactivation of two-thirds of the tau activity during chromatography, whether the tubulin once stripped of accessory proteins itself becomes less easily polymerizable, or whether additional factors are involved is not known. We do note that, upon elution from phosphocellulose in high salt, the crude tau preparation is only slightly turbid. Considerable turbidity develops after desalting into P buffer. Some active protein may therefore be denatured in the absence of tubulin or inactivated in buffers of low ionic strength. Purified tau-I does not precipitate in low salt.

Whatever the source of the initial apparent loss in microtubule-inducing activity, purification of the major remaining tau activity, tau-I, has been achieved by ammonium sulfate fractionation and hydroxyapatite chromatography following the phosphocellulose step to remove tubulin. Table 1 summarizes the specific and the total activities recoverable in the various fractions of this purification procedure. Between 7% and 10% of initial crude tau protein is recovered in tau-I, with specific activity for microtubule induction increasing 3.7-fold. As much as 90% of the tubulin at 0.95 mg/ml may be polymerized by addition of just over 10% tau-I. Of the total initial crude tau activity, 40% is recovered in tau-I. The tau-I activity has been found to be stable to heating for 60 minutes at 75°C, to repeated freezing and thawing, to lyophilization, and to storage in excess of one year in P buffer at -20°C.

A second tau activity, tau-II, present in the crude tau preparation along with tau-I and apparently representing about 20% (see Table 1) of the crude microtubule-inducing activity, is differentiable from tau-I by its higher affinity for both phosphocellulose and hydroxyapatite and by its heat lability. Tau-II has not been purified beyond hydroxyapatite chromatography. Whether activity resides in the high or low molecular weight polypeptides seen in sodium dodecyl sulfate/polyacrylamide gels of tau-II fractions is not known. It is possible in view of the relatively low activity

† The ratio of high molecular weight proteins to tubulin (as well as possibly other proteins) in microtubule protein has been shown to depend on the conditions of the microtubule purification (Scheele & Borisy, 1976). Purification in the presence of glycerol produces considerably less of these proteins; moreover, the amounts of these proteins were originally underestimated by us (Weingarten *et al.*, 1975) due to the streaking of these proteins in our stacking gels.

of crude tau and the known tau-II heat lability that this activity initially accounts for a more significant portion of total activity than has been indicated by our assays, and that activity is simply lost quickly after removal from tubulin on phosphocellulose.

In mixing experiments (D. Cleveland & M. Kirschner, unpublished results) no co-operativity between tau-I and tau-II was observed. The levels of microtubules induced when various weight ratios of the two tau fractions were added to PC-tubulin were levels which would have been predicted from a simple sum of the individual tau activities at the appropriate added concentrations.

Gel electrophoresis of tau-I indicates the presence of four closely spaced bands of apparent molecular weights between 55,000 and 62,000. Hydrodynamic measurements monitoring activity yield a sedimentation coefficient of 2.7 S and a diffusion coefficient of 3.8×10^{-7} cm²/s. Taken together, these suggest a native molecular weight of 61,000 for tau-I and a frictional ratio f/f_0 of 2. This would imply a highly asymmetric molecule. When combined with the denatured molecular weight estimates from denaturing gel chromatography (Weingarten, 1975; Penningroth *et al.*, 1976) and from acrylamide gels, these data indicate that the native tau-I molecule is a monomer.

The quantitative electron microscopic assay which has been used throughout to monitor microtubule formation possesses a major advantage over other methods of measurement such as turbidity, viscometry, or pelleting: microtubules (or rings) may clearly and quantitatively be distinguished from other types of aggregates. This asset is not trivial. As noted earlier, the turbidity generated after desalting crude tau is considerable, and when warmed in the absence of added tubulin (i.e. to obtain a scattering background), sufficient additional turbidity is generated by aggregation in this tau fraction to make conclusions drawn from its difference with the tau + tubulin scattering curves somewhat tenuous. Purified tau-I, on the other hand, has no noticeable turbidity.

We have exploited the inherent sensitivity (i.e. low background) in the quantitative microscopic assay to demonstrate that tau-I not only restores polymerizability to purified tubulin, but also possesses the ability with increasing concentration of added tau to lower the critical concentration of the reconstituted tau + tubulin system (see Fig. 6(b)). Shelanski *et al.* (1973) have reported critical concentration values between 0.4 and 0.19 mg/ml, depending on the buffer system used for microtubule protein purified by two cycles of assembly. The reconstituted system yields values for the critical concentration as low as 0.03 mg of tubulin per ml at 0.5 mg of tau-I per ml, and of 0.18 mg of tubulin per ml at 0.08 mg of tau-I per ml. This is the expected result if tau is involved in the nucleation as well as the elongation of microtubule assembly. Moreover, the ability of a sixfold increase in the concentration of tau-I to produce a corresponding sixfold decrease in the apparent critical concentration of tubulin necessary for assembly demonstrates that the critical concentration is not an intrinsic property of tubulin, but rather that it reflects the characteristics of the tau + tubulin interaction.

Tau-I has also been seen to promote ring formation from tubulin under depolymerizing conditions (Fig. 7). When added to 0.95 mg of PC-tubulin per ml to a final concentration (10% by weight) sufficient to effect 80% polymerization, 13% of polymerizable tubulin was present in rings (assuming all rings to be double rings). This compares favorably with unfractionated microtubule protein which, when assayed by centrifugation under similar conditions, has been reported to contain

18% rings (Weingarten *et al.*, 1974). However, with increasing tau concentration, the proportion of rings increases to an unexpected plateau value of 50%. Though this represents a minimum estimate, since at the magnifications used triple and double rings are not easily distinguishable, even if half of the observed rings were triple rings, the plateau value would only approach 65%. A number of explanations for this behavior are possible but no evidence exists to support specifically any particular explanation.

The ability to purify tau-I directly from brain extract has allowed us to estimate the amount of tau present. We believe that tau comprises only 0.4% of the protein in the extract. This represents roughly 2% of the level of the tubulin present in the same extract. This is roughly the same ratio of tau to tubulin as is found in our microtubule protein purified by two cycles of assembly. Purification directly from the extract has also shown that the proteins which coprecipitate with tubulin during ammonium sulfate fractionation of the extract and which bind to phosphocellulose as strongly as does tau-I, do not stimulate assembly of PC-tubulin. Exceedingly high levels of protein which were eluted from phosphocellulose in a 0.3 M-NaCl wash were required to stimulate assembly of a few microtubules from PC-tubulin. This implies that the ability of tau to stimulate microtubule formation is not a general property of proteins (such as basic proteins) which adsorb to phosphocellulose and which are present in the extract.

The results obtained from the purification of tau indicate that tau is required for microtubule assembly. Tau has been shown to remain associated with tubulin through repeated cycles of polymerization and depolymerization. A previous study of the growth of neutral microtubules onto the ends of flagellar nucleation sites has demonstrated the requirement for tau in the elongation process (Witman *et al.*, 1976). In addition, in the same study increasing amounts of tau were shown to produce both an increase in the rate of assembly and an increase in the total level of polymerization. This mandated a stoichiometric, not catalytic, mode of action for tau. Figures 4 and 6(a) given here both support the stoichiometric role of tau, each suggesting that tau-I is incorporated into microtubules in the reconstituted system in a weight concentration of 10% or one tau for every 10 α or β -tubulin chains. The assembly system comprising PC-tubulin and tau produces microtubules of normal morphology whose assembly is subject to the normal *in vitro* conditions for polymerization, such as the presence of GTP and the absence of calcium. Furthermore, tau is both necessary and sufficient for nucleation and elongation of microtubules from PC-tubulin and hence the reconstituted tau + tubulin system defines a complete microtubule assembly system under the usual buffer conditions. In the following paper (Cleveland *et al.*, 1977) we discuss the physical and chemical properties of tau and suggest a model by which it might function in microtubule assembly.

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