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THE MECHANISM OF MICROTUBULE ASSEMBLY IN VITRO

Marc W. Kirschner* and Robley C. Williams†

**Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540*
†Department of Molecular Biology, University of California, Berkeley, California 94720

The mechanism of microtubule polymerization and depolymerization has been studied with protein purified from extracts of porcine brain. Under polymerizing conditions characteristic microtubules composed of parallel protofilaments are observed in the electron microscope. Under depolymerizing conditions three forms are observed: double rings of outside diameter 49 nm, spirals, and 7-nm globular subunits. Under the same conditions two boundaries are observed in the analytical ultracentrifuge at 6S and 36S, whether depolymerization is accomplished by cooling to 0°C, by addition of 1 mM $CaCl_2$ at 25°C, or by removal of GTP. On polymerization all of the 36S and most of the 6S is converted to a fast-sedimenting form which the electron microscope reveals to be microtubules.

The depolymerization mixture may be fractionated by gel chromatography into two fractions, one consisting solely of 6S and the other mostly 36S. Neither fraction regenerates the original equilibrium mixture. The 36S form may be reversibly dissociated into 6S subunits by addition of NaCl. From these and other considerations we have postulated that microtubule protein is composed of two different types of tubulin, both of which participate in polymerization. Studies are reported showing that colchicine does not dissociate microtubule rings but blocks polymerization by interfering with their proper lateral association into a protofilament array within microtubules. The role of GTP in polymerization is also discussed. Electron micrographic evidence is presented suggesting the conversion of protofilaments directly into rings and spirals, and a pathway for microtubule assembly is proposed.

INTRODUCTION

Microtubules are a major structural component of higher cells. They are involved in both establishing and maintaining form in a variety of stable, semistable, and ephemeral cellular structures such as cilia, nerve axons, and the mitotic apparatus. To form such structures they must be capable of polymerizing and depolymerizing in response to certain developmental and environmental signals and this polymerization must occur at specific

locations and in specific orientations. In the last two years the study of the mechanism of microtubular polymerization has been facilitated by the discovery of conditions which allow for the polymerization of microtubules from extracts of mammalian brain (1). Microtubules were identified by their characteristic appearance in the electron microscope. Recently a purification for microtubules has been devised based on their ability to polymerize from brain extracts by warming to 37° and depolymerize by cooling to 0°C (2). This has allowed sufficient quantities of better characterized microtubular protein to be available for physical-chemical characterization of the mechanism of assembly.

Working with extracts of porcine brain, Borisy and Olmsted, (3) deduced that the assembly of microtubules was not due to a simple aggregation of monomeric units into a polymer but required "nucleating centers" which could be removed by high speed centrifugation. Centrifugation was accompanied by the removal of disc-shaped objects seen in the electron microscope. It was suggested that these discs might nucleate the addition of monomeric units to form tubules in analogy with models for tobacco mosaic virus assembly (4).

We have examined the products obtained by depolymerizing purified microtubules from porcine brain. We have found that microtubules contain two functionally different forms of microtubular protein, both of which participate in the polymerization process but which differ in their ability to form lateral and longitudinal bonds in microtubular structures (5, 6). In this paper we will review some of the evidence for the existence of these two different forms of the protein and extend the study to the mechanism by which these depolymerization products participate in the polymerization process.

METHODS

Microtubular protein was purified from fresh hog brain extracts by three cycles of polymerization and depolymerization, using the method of Shelanski et al. (2).

Typically from an extract containing 7 gm of protein prepared from 6 pig brains (wet weight 500 gm), about 400 mg of purified protein is obtained. The purified protein is stored at −20°, in 8 M glycerol 0.1 M sodium N-morpholino-ethane sulfonate (Mes) buffer, pH 6.4, containing 10 mM mercaptoethanol, 1 mM ethylene glycol bis (β-aminoethyl ether) tetraacetic acid (EGTA), 1 mM GTP, 0.5 mM $MgCl_2$, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). It is polymerized by dilution in reassembly buffer (0.1 M Mes, pH 6.4, containing the above concentration of GTP, EGTA, and $MgCl_2$) and warming to 37° for 20 min. The microtubules are spun out at 75,000 × g for 30 min and the pellet resuspended in reassembly buffer. In some experiments residual glycerol was removed by dialysis or by a subsequent repolymerization and resuspension in reassembly buffer. The residual glycerol was found to have no effect on any of the experiments. For electron microscopy the microtubule pellet is resuspended in 0.2 M ammonium acetate containing 1 mM Mes and 1 mM EGTA, 1 mM GTP, and 0.5 mM $MgCl_2$, pH 6.4, at 0°C and aggregates removed by sedimenting at 0°C at 15,000 × g for 15 min. The depolymerized mixture is then repolymerized by warming to 37° for 10 min and diluted 1 to 1 with the same buffer and centrifuged at 4,500 × g in a swinging bucket rotor for 5 min. The tubules are found in the bottom third of the tube. Ultracentrifuge, electron microscopic, and colchicine binding procedures are described in Kirschner et al. (6). Polyacrylamide gel electrophoresis was performed using the discontinuous buffer system containing sodium dodecyl sulfate and containing 10% acrylamide according to the method of Laemmli (7).

RESULTS

A microtubular protein preparation purified by three cycles of polymerization and depolymerization from extracts of porcine brain is composed of 95% α and β tubulin as judged by densitometry of SDS polyacrylamide gels of the type shown in Fig. 1A. Electron microscopic examination of the purified protein under polymerizing conditions shows material to be either in the form of characteristic microtubules 290 Å in diameter and composed of arrays of parallel protofilaments or in the form of globular units about 70 Å in diameter (Fig. 2A). When examined in the analytical ultracentrifuge under polymerizing conditions most of the purified protein sediments at greater than 300S with a small amount which sediments at 6S.

Under a variety of depolymerizing conditions the microtubules dissociate into two types of structures as demonstrated in the ultracentrifuge and the electron microscope. As shown in Fig. 3A, cooling the microtubules to 0° produces two components, one with an $s_{20,w}$ of 6S and one with 30S. Similar components are produced at 25° in the absence of GTP (Fig. 3B) or at 25° in the presence of GTP and in the presence of 2 mM $CaCl_2$ (Fig. 3C). In the latter case a shoulder is seen on the faster sedimenting species. The presence of the shoulder depends on the Ca^{2+} or Mg^{2+} concentration and it includes material from both the faster and slower components as determined by measuring the areas under the peaks, and as can be seen by comparing Fig. 3C with 3D where the effect has been augmented by increasing the Ca^{2+} concentration to 5 mM. Under all these conditions no other components are observed in the ultracentrifuge.

Measurements of sedimentation patterns observed as a function of protein concentration show that the ratio of material in the fast boundary relative to the slow boundary decreases with decreasing concentration. At a protein concentration of 6 mg/ml at 25° and in the absence of GTP 56% of the protein is in the fast sedimenting boundary and 44% in the slow boundary. At 2 mg/ml the ratio becomes 35% in the fast sedimenting boundary to 65% in the slower boundary. The sedimentation coefficient at infinite dilution obtained by extrapolating the $s_{20,w}$ values to zero concentration is 36 ± 2S for the fast boundary and 5.8 ± 0.4S for the slower boundary for the Ca^{2+}, cold and minus-GTP-induced depolymerization products (6, 8).*

Examination of depolymerized microtubules in the electron microscope reveals three types of structures, 70 Å globular units, double rings, and spirals as shown in Fig. 2B. The double rings, composed of two concentric rings, are homogeneous in size with an overall contour length of 2300 ± 100 Å. The spirals have the same average contour length but are more heterogeneous, 2300 ± 400 Å. No structures intermediate in size between the rings and spirals and the 70 Å units are seen in the electron microscope.

A model for the double ring is shown in Fig. 4. Its structure was deduced by combining the dimensions from the electron microscope with sedimentation data (8). It is composed of two concentric rings of subunits of molecular weight 55,000 corresponding to the individual α and β chains. These are spaced at 54 Å along the circumference. A good fit to the dimensions of the double ring would have 24 subunits in the outer ring and 18 subunits in the inner ring. Such a radial arrangement of course does not allow each subunit on the inner circle to pair in the same manner with a subunit on the outer circle. The

*Throughout the rest of this article we will designate the faster sedimenting component as 36S and the slower component as 6S. It should be remembered that the actual sedimentation coefficient will depend on concentration owing to the effect of the protein on the viscosity and density of the solution, as well as to the effects of backward flow in the ultracentrifuge cell.

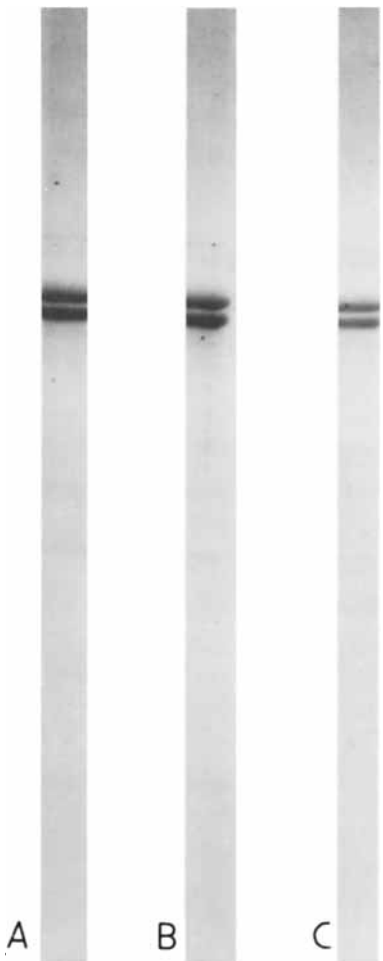

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of microtubular fractions. A. Microtubular protein purified by 3 cycles of polymerization-depolymerization. B. Leading fraction from agarose column of Ca^{2+} dissociated microtubules. C. Trailing fraction of agarose column.

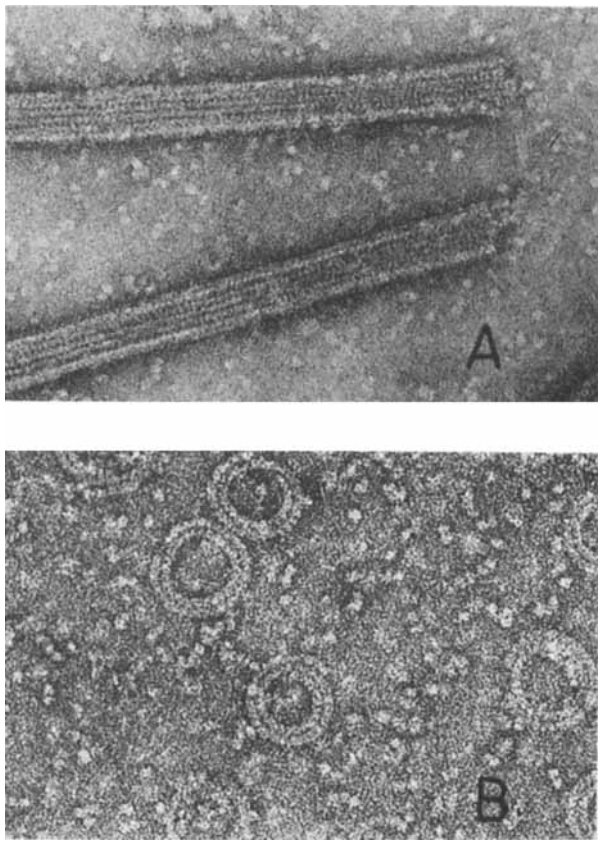

Fig. 2. Electron micrograph of forms exhibited by microtubules. A. Microtubules in ammonium acetate reassembly buffer (X 180,000). B. Rings, spirals, and 70 Å globular units from microtubules depolymerized by exposure to 0°C (X 280,000).

molecular weight of the whole structure is 2.3×10^6.

The existence of double rings, spirals, and 70 Å globular subunits raises a number of interesting questions. First, how do these structures arise from microtubules during depolymerization? Second, what is the relationship of the globular protein to the protein subunits comprising the rings and spirals? Third, what is the role of the rings and globular subunits in polymerization of microtubules? The diameter of the outer ring is 485 Å and the existence of spirals with which the rings apparently interconvert make it extremely unlikely that these structures could represent cross sections or a cross-sectional piece of microtubules whose diameters are typically 250 Å.

Evidence for Two Different Types of 6S Subunit

To find out whether the 36S subunit was simply a polymer of 6S subunits and in equilibrium with them we attempted to fractionate the depolymerization mixture. If there were a simple equilibrium between 6S and 36S, each fraction would regenerate the equilibrium mixture specified by that concentration. Microtubular protein depolymerized

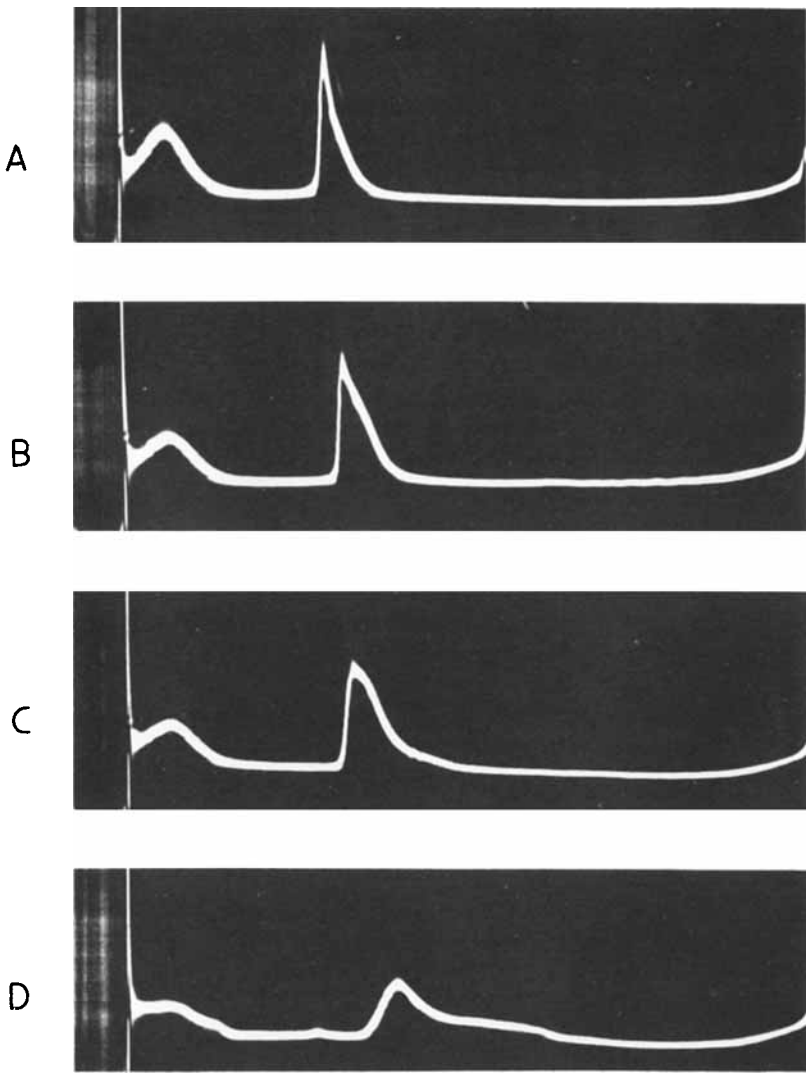

Fig. 3. Ultracentrifuge patterns of depolymerized microtubules. Microtubules at 7.5 mg/ml were depolymerized by exposure to 0°C and examined in the ultracentrifuge in 0.1 M Mes, 0.5 mM $MgCl_2$, and 1 mM EGTA, under the following conditions: A. At 0°C with addition of 1 mM GTP, photograph taken 28 min after reaching an operating speed of 47,660. B. At 25°C in the absence of GTP, photograph taken 16 min after reaching an operating speed of 47,660. C. At 25°C with the addition of 2 mM $CaCl_2$ and 1 mM GTP, photograph taken 16 min after reaching the operating speed of 47,660. D. At 25°C with the addition of 5 mM $CaCl_2$ and 1 mM GTP, photograph taken 16 min after reaching the operating speed of 47,660. The four experiments were examined at approximately identical effective sedimentation times. The schlieren phase plate was set at an angle of 75° in each experiment.

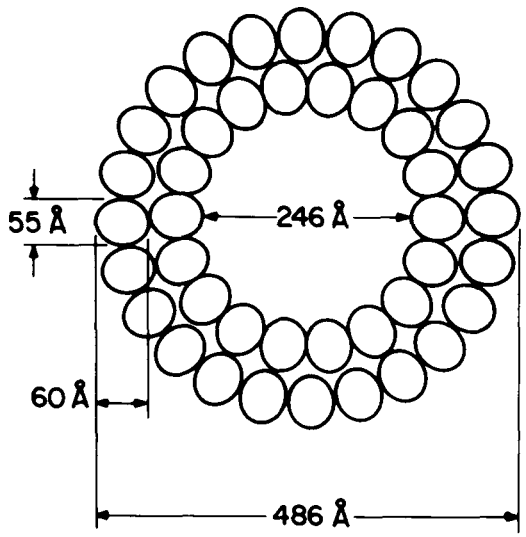

Fig. 4. Model for microtubule double ring.

with 2 mM $CaCl_2$ can be fractionated into two components on an agarose 15 m column at room temperature in the presence of 0.1 mM $CaCl_2$ as shown in Fig. 5. Depolymerization with Ca^{2+} at 25° was chosen in preference to depolymerization by exposure to 0° because at the same protein concentration the equilibrium is shifted more toward the 36S structure with Ca^{2+} treatment than with cold treatment. The leading fraction elutes in the void volume; the trailing fraction elutes just before the included volume. Both fractions contain about the same amounts of protein and together they account for 90% of the protein applied on the column.

In Table I the properties of the leading and trailing fractions from the agarose column are compared to the properties of the unfractionated depolymerized mixture. Neither fraction regenerates the original equilibrium distribution of 6S and 36S. At 2 mg/ml the leading fraction is composed of 80% 36S and 20% 6S while the unfractionated mixture at that concentration contains 45% 36S and 55% 6S. At this and a higher concentration which favors the formation of the 36S polymer only 6S is found in the trailing fraction. Electron microscope observation of the leading fraction reveals that it contains rings, while no rings or other polymers are found in the trailing fraction. The two fractions demonstrate the correspondence between the 6S component found in the ultracentrifuge with the 70 Å globular structures seen in the electron microscope and the correspondence of the 36S component with the rings and spirals. Both methods reveal that a simple equilibrium between 6S and 36S is not established in the fractions.

Two other important properties distinguish the leading and trailing fractions. The leading fraction will polymerize into microtubules on removal of Ca^{2+} with EGTA and on addition of GTP. Under similar conditions the trailing fraction will not. The trailing fraction, however, has a much higher affinity for colchicine. The lower affinity for colchicine of the leading fraction is not due to the fact that the protein is polymerized into rings and spirals, since the difference in colchicine binding persists in 1 M NaCl, a condition under which the 36S component is completely broken down into 6S subunits as discussed below. Both fractions contain the α and β polypeptides of tubulin as shown in Fig. 1B and C.

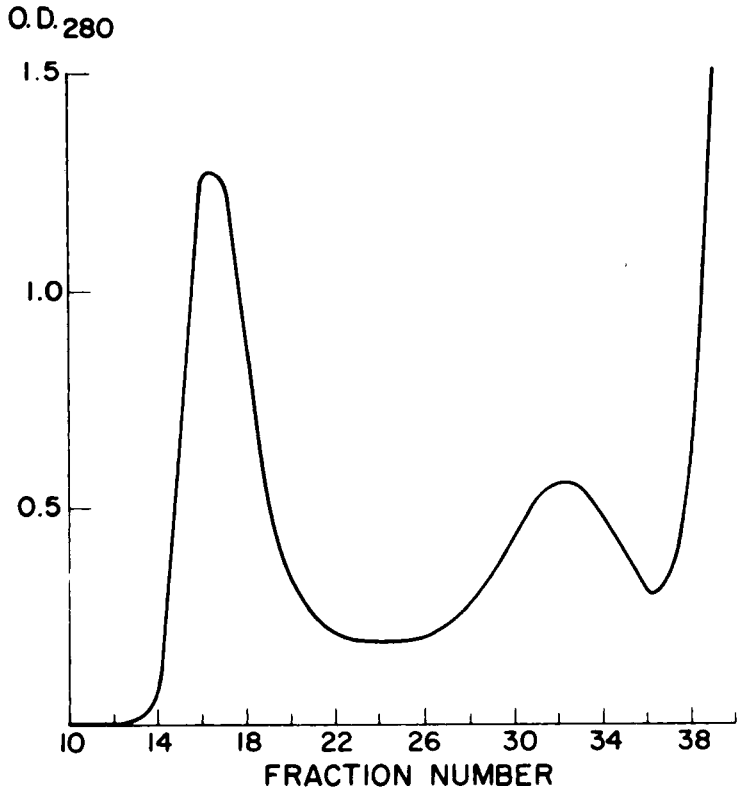

Fig. 5. Agarose column fractionation of Ca^{2+} depolymerized microtubules. Microtubular protein (1.5 ml of 10 mg/ml) was depolymerized by addition of 2 mM $CaCl_2$ and applied onto a 1.5 X 25 cm agarose 15 m (100–200 mesh) column equilibrated at 22° with 0.1 M Mes, pH 6.4, containing 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$, eluted with the same buffer, and 1 ml fractions collected. The void volume is at fraction 14 and the included volume at fraction 40. The high $O.D_{280}$ after fraction 38 represents free GTP.

The 36S component can be reversibly formed from its 6S subunits. Conditions under which 36S dissociates into 6S and then reassociates to form 36S will not cause the material in the trailing fraction to form 36S. This is demonstrated by the experiment shown in Fig. 6. Addition of 0.75 M NaCl to depolymerized tubules dissociates the 36S component completely into 6S subunits as shown in Fig. 6A. A 6S preparation with properties similar to the trailing fraction can be produced by sedimenting Ca^{2+} dissociated tubules at 180,000 X g for 15 min at 20°. This yields only 6S protein in the supernatant judged by analytical sedimentation. This 6S protein still sediments as 6S in 0.75 M NaCl as shown in Fig. 6B. After dialysis to remove the NaCl, the 36S component is not re-formed in the sample originating from the 6S preparation obtained from high speed centrifugation (Fig. 6C); 36S component is reformed in the sample originating from depolymerized microtubules (Fig. 6D). The latter sample will form microtubules under polymerizing conditions while the former will not. Colchicine has no effect on the relative distribution of 6S and 36S components at a given concentration of protein. In addition, the reassociation to form 36S from NaCl dissociated tubules is not inhibited by 0.5 mM colchicine.

TABLE I. Comparison of Microtubule Fractions*

	Leading Fraction	Trailing Fraction	Whole Mixture
36S at 2 mg/ml (%)	80	0	45
6S at 2 mg/ml (%)	20	100	55
36S at 5 mg/ml (%)	–	0	70
6S at 5 mg/ml (%)	–	100	30
Colchicine binding (moles/10^5 gm)	.07	0.43	
Colchicine binding (+NaCl) (moles/10^5 gm)	0.04	0.40	
Ability to form tubules	+	–	+
Polypeptide chain composition	α and β	α and β	α and β
$S_{20,w}$ in 1 M NaCl	6S	6S	6S
Appearance in electron microscope	rings and spirals and 70 Å units	70 Å units	rings and spirals and 70 Å units

*Properties of fractions obtained from Ca^{2+} depolymerized microtubules and separated on an agarose A 15 m column as shown in Fig. 5. "Leading fraction" corresponds to material pooled from fractions 14 to 18, "trailing fraction" to fractions 28 to 35.

Data obtained from Kirschner et al (6). All results except electron microscopy obtained using 0.1 M Mes, pH 6.4, containing 1 mM EGTA, 1 mM GTP, and 0.5 mM $MgCl_2$. For electron microscopy the buffer used was 0.2 M ammonium acetate containing 1 mM EGTA, 1 mM GTP, and 0.5 mM $MgCl_2$. Colchicine binding results are reported as amount bound in 15 min per 10^5 gm of protein at 20°C.

The assays were performed at a colchicine concentration of 5×10^{-4} M using ^{3}H-colchicine e with a specific activity of 5×10^7 cpm/μm.

From the above considerations we have postulated that mammalian brain microtubular protein is composed of two different types of tubulin which differ in a number of properties. The tubulin which comprises most of the trailing fraction from the agarose column and which has high colchicine affinity and is incapable of forming 36S aggregate or tubules is here called X tubulin. The form of tubulin found principally in the leading fraction which can spontaneously form 36S, has little or no colchicine affinity, and can form microtubules possibly by itself or in the presence of X tubulin we have called tubulin.

In microtubules two different binding domains for subunits can be distinguished. Longitudinal binding domains represent interactions between subunits along protofilament lengths. Presumably, this is the same bond which runs circumferentially in the rings and spirals. Lateral interactions represent bonding between protofilaments and possibly between the inner and outer rings. The Y tubulin presumably has strong longitudinal interactions which are not inhibited by colchicine, removal of GTP, cooling to 0°, and exposure to Ca^{2+}. X tubulin lacks these bonds since it does not form 36S material. In tubules, however, it may have lateral interactions which are inhibited by colchicine, cold, and Ca^{2+}. Agents which break down 36S also dissociate tubules. They include high salt, high pH, and reagents which react with sulfhydryl groups on proteins such as N-ethylmaleimide, p-hydroxymercuribenzoate and dithiobis-2-nitrobenzoate. These agents break longitudinal bonds in rings, although they may also affect lateral interactions in tubules.

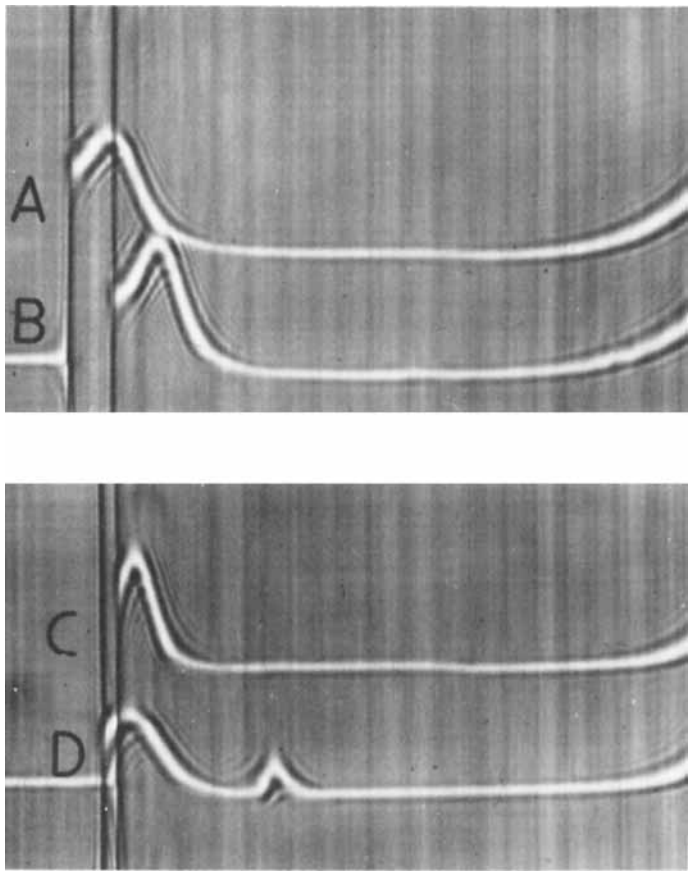

Fig. 6. Reversible dissociation of 36S by NaCl. A. Microtubular protein,1.25 mg/ml in reassembly buffer plus 0.75 M NaCl and 2 mM $CaCl_2$ at 22°C. The photograph was taken 19 min after reaching an operating speed of 47,660. B. The "supernatant" 6S protein obtained by centrifuging cold depolymerized microtubules for 2¼ hour at 180,000 X g at 1.25 mg/ml in Mes reassembly buffer plus 0.75 M NaCl and 2 mM $CaCl_2$ at 22°C. C. Sample B, after dialysis against two volumes of Mes reassembly buffer plus 2 mM $Cacl_2$ at 22°C for a total of 3½ hours. D. Sample A after dialysis against two changes of 50 ml of Mes reassembly buffer plus 2 mM $CaCl_2$ for a total of 4 hr. The photograph was taken 6 min after reaching an operating speed of 47,660.

The above experiments were performed in paired double sector cells with an optical path length of 30 mm using an AnE aluminum rotor. The phase plate was set at an angle of 75°.

Capacity of X Tubulin to be Copolymerized into Tubules

Since the trailing fraction from the column did not itself polymerize into microtubules, it was important to determine whether this fraction had irreversibly lost its ability to be incorporated into microtubules. In other words, X tubulin might merely be some inactive or denatured form of tubulin. A number of experiments mitigate against this interpretation. First, the trailing fraction has a much higher colchicine binding capacity than the leading fraction. Second, it is unclear at what stage the trailing fraction would become inactive. The ratio of 6S to 36S protein in a depolymerization mixture remains

unchanged for over 48 hours at 0° or over 24 hours at 20°. The absolute and relative amounts of 6S and 36S are independent of the number of times the protein is polymerized and depolymerized. Third, as shown in Fig. 7, on repolymerization all of the 36S and 65% of the 6S protein disappear into rapidly sedimentable material which electron microscopy reveals as microtubules. The remaining 6S is not necessarily inactive but may represent the concentration of 6S subunits in equilibrium with tubules. Finally, recent experiments (Weingarten and Kirschner, unpublished results) with radioactively labeled microtubular protein purified from chick brain indicate that more than 50% of the 6S protein in the trailing fraction is reincorporated into microtubules after it is added to a mixture of cold dissociated microtubules and subsequently polymerized.

Abortive Polymerization in the Presence of Colchicine

It has already been shown that colchicine binds preferentially, if not solely, to X tubulin and has no observed effect on the structure of double rings. Polymerization might be expected to occur in the presence of colchicine if the participation of the X tubulin was not required. In the following experiment microtubule protein was incubated with colchicine in the absence of GTP and warmed to 37°. The result shown in Fig. 8A is identical to what would be observed in the absence of colchicine (Fig. 3B). Both 6S and 36S species are present and there is no material sedimenting faster than 36S. If polymerization is initiated by the addition of GTP all of the 36S disappears and there is a small increase in the amount of the 6S protein corresponding to 20% of the original 36S, as shown in Fig. 8B. The rest of the material sediments in a heterogeneous manner at greater than 50S. When the results of a similar experiment were examined in the electron microscope, it was found that most of the double rings have been replaced by half-moon shaped aggregates, as shown in Fig. 9. The rings and spirals have broken open and have aggregated in curved arrays containing between 3 and 6 protofilament fragments. The 70 Å subunits were also present.

The Role of GTP in Microtubule Polymerization

Both in the presence and absence of colchicine GTP is required for the disappearance of the 36S component into higher molecular weight aggregates, as shown in Figs. 7 and 8. Since the rings and spirals probably represent coiled segments of protofilaments, one can argue that GTP is required for the conversion of linear arrays of Y tubulin from a conformation with a small radius of curvature (rings and spirals) to conformations having a larger radius of curvature (half-moon shaped aggregates in the presence of colchicine or linear protofilaments in microtubules).

Intermediates in Polymerization and Depolymerization

When microtubules are diluted to 1 mg/ml and allowed to stand for 1 hr at room temperature the tubules appear splayed out at their ends and many "ram's horn" forms are found (6). These forms were interpreted to mean that disintegrating microtubules retain a linear continuity and a tendency to coil and strongly suggest that segments of protofilaments are released which coil into spirals. Similar forms are found when preparations are examined at a short time after polymerization is initiated, as shown in Fig. 10. Since

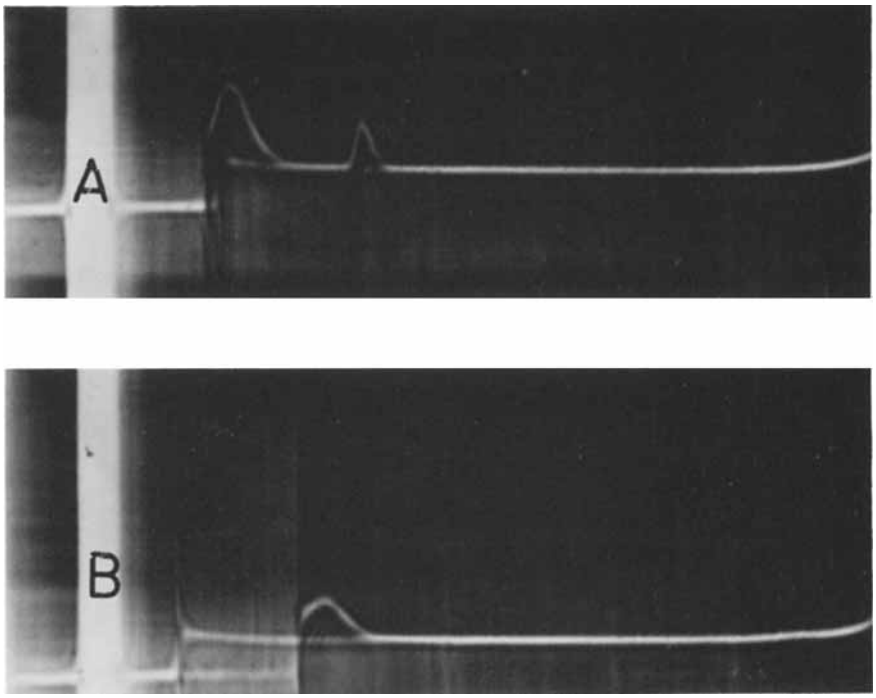

Fig. 7. Repolymerization of depolymerized microtubules. Microtubules in Mes reassembly buffer plus 1 mM GTP depolymerized at 0°C and then sedimented in 12 min cell at 7.2°C (A). The photograph was taken at a bar angle of 75° 13 min after the centrifuge reached an operating speed of 47,660. Another sample was repolymerized by warming to 37°C for 20 min. This sample (B) was then sedimented at 23°C. The photograph was taken 18 min after the centrifuge reached an operating speed of 47,660.

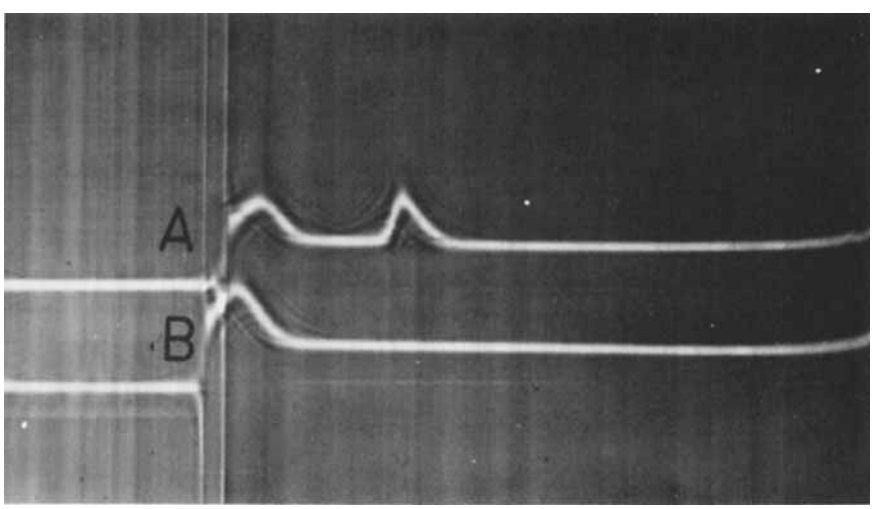

Fig. 8. Effect of colchicine on microtubule polymerization. Microtubular protein at 4 mg/ml was depolymerized by cooling to 0°C and then dialyzed vs two 200 ml volumes of Mes reassembly buffer not containing GTP. Colchicine was then added to a final concentration of 0.5 mM. The protein was then warmed to 37°C for 20 min and examined in the centrifuge at 23°C (A). To an aliquot of the same protein GTP was added to give a final concentration of 1 mM and the sample warmed to 37°C and examined in the centrifuge at 23°(B). The photographs were taken 14 min after reaching an operating speed of 47,660 with a phase plate angle of 75°.

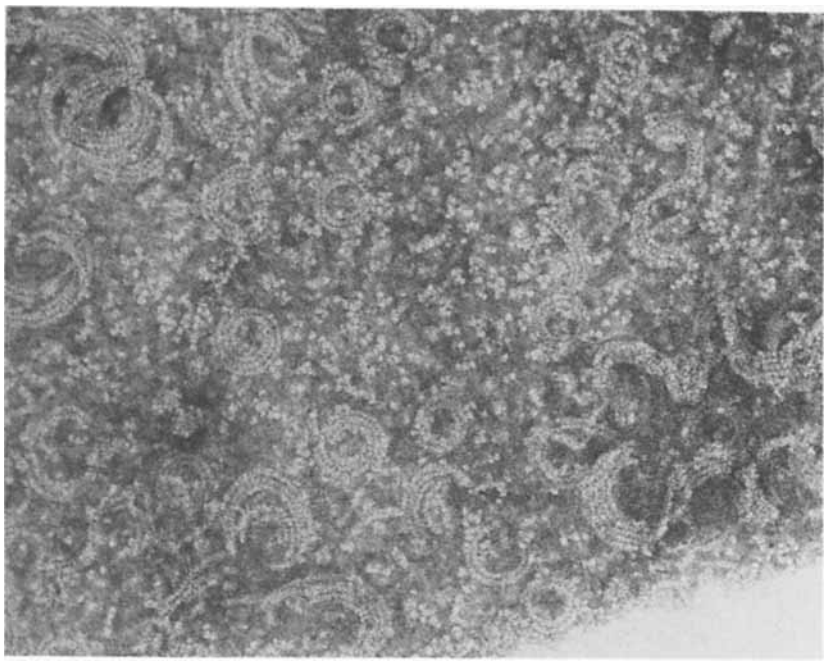

Fig. 9. Electron micrograph of abortive colchicine forms. Microtubular protein at 5 mg/ml was depolymerized by cooling to 0°C. Colchicine at a final concentration of 1.6×10^{-4} M was added and the protein incubated for 6 min at 38°C, and then examined in the electron microscope. Magnification is × 150,000.

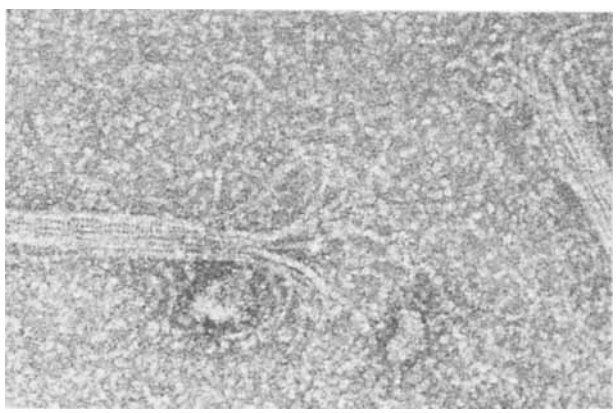

Fig. 10. Electron micrograph of repolymerizing microtubules. Microtubules at 3 mg/ml were depolymerized by cooling to 0°C, then diluted to 0.6 mg/ml and warmed to 30°C and immediately examined in the electron microscope. Magnification is × 150,000.

the same types of spiral structures are seen in both disintegrating microtubules and in repolymerizing microtubules, the experiments suggest that there is a direct interconversion of rings, spirals, and protofilaments during polymerization and depolymerization.

Another form of microtubule polymerization which occurs at low concentration is the formation of helical ribbons of protofilaments, as shown in Fig. 11. These aggregates, composed of 6 to 10 aligned protofilaments, indicate that under certain conditions the protofilaments retain some degree of curvature.

DISCUSSION

The end products of microtubule depolymerization are rings and spirals, sedimenting at about 36S, and tubulin subunits which sediment at 6S. No intermediate forms are found with the ultracentrifuge or the electron microscope. These products arise whether microtubules are depolymerized by cooling or by treatment with Ca^{2+} and are stable if microtubule polymerization is prevented by removing GTP. The exact amount of 36S and 6S in the preparation depends on experimental conditions such as protein concentration, pH, and salt concentration.

The 6S and 36S forms, however, are not in a simple monomer–polymer equilibrium because fractionation of the depolymerized mixture on an agarose column yields two fractions, neither of which re-establishes the original composition. In addition, the two fractions differ in their capacity to form microtubules and in their affinity for colchicine. These two functional criteria are found primarily in the leading and trailing fractions, respectively. We have therefore postulated that two types of tubulin molecules exist in microtubules. The X tubulin, found primarily in the trailing fraction, binds colchicine but will not polymerize or form linear aggregates under any conditions we have studied. The Y tubulin binds colchicine very poorly but aggregates spontaneously into the form of rings and it may have the capacity to polymerize by itself into tubules. It is likely that X tubulin molecules associate with rings and spirals which appear to be augmented in the presence of 2 mM Ca^{2+}. It is therefore possible that the residual colchicine binding ability found in the leading fraction may be due to X tubulin associated with the rings and spirals.

Two types of bonding domains may be distinguished in microtubules: a) longitudinal bonds represented by bonding along protofilaments, rings, and spirals, and b) lateral bonds represented by bonds between protofilaments, and possibly between adjacent turns of spirals and the inner and outer ring in microtubule rings. The two states of tubulin differ in the strengths of their longitudinal bonds. Under our experimental conditions the Y tubulin spontaneously forms strong longitudinal bonds which are not disrupted by cold, Ca^{2+}, or colchicine. These agents disrupt lateral bonds between protofilaments. The X tubulin lacks these strong longitudinal bonds but may function by making lateral bonds under polymerizing conditions. The abortive forms seen in the presence of colchicine, which binds primarily if not exclusively to X tubulin, may indicate that Y tubulin by itself is incapable of forming strong enough lateral interactions for protofilament fragments to aggregate properly into microtubules.

Although little is yet known about what role GTP plays in the polymerization process, we can identify the place in the assembly scheme where GTP is required. Added GTP is not required to stabilize 36S. It has no effect on the 6S–36S equilibrium. It is not required for the formation of 36S from subunits formed by salt dissociation. However,

Fig. 11. Electron micrograph of microtubule polymerization at low concentration. Microtubules were depolymerized at 0°C for 20 min, diluted to 1.5 mg/ml in ammonium acetate reassembly buffer with 1 mM GTP, and then warmed to 38° and examined in the electron microscope. Magnification is × 150,000.

both polymerization into microtubules and the abortive polymerization in the presence of colchicine require GTP. The abortive polymerization proceeds by the opening of the rings and spirals into half-moon shaped particles which have a larger radius of curvature than the rings and spirals. This is accompanied by some side-to-side aggregation of these protofilament segments.

The apparent effect of GTP on the intrinsic curvature of the protofilaments could occur either by changing the conformation of the individual subunits so they will no longer pack into structures with a small radius of curvature, or by increasing the strength of lateral interaction between protofilaments. The increased tendency of the filaments to maximize lateral interactions by packing in a straight array would oppose their inherent propensity to coil. Under certain conditions, as shown in Fig. 11, protofilaments retain some degree of curvature. This configuration increases the amount of lateral interaction while allowing protofilaments to have an intrinsic curvature. Thus one process involved in microtubular polymerization is the straightening of the curved protofilaments from the spirals and rings, a process which may involve GTP and X tubulin.

A model for microtubule disassembly and assembly incorporating data presented in this article is shown in Fig. 12. Microtubules are depicted as an array of parallel protofilaments made up of two types of subunits; X tubulin shown as filled trapezoids, and Y tubulin shown as open trapezoids. Low temperature or Ca^{2+} causes dissociation of the microtubule by breaking lateral bonding interactions. The microtubule releases individual X subunits while runs of Y tubulin coil into spirals. The spirals equilibrate with double rings and individual Y tubulin subunits. The X tubulin subunits may bind to the outside

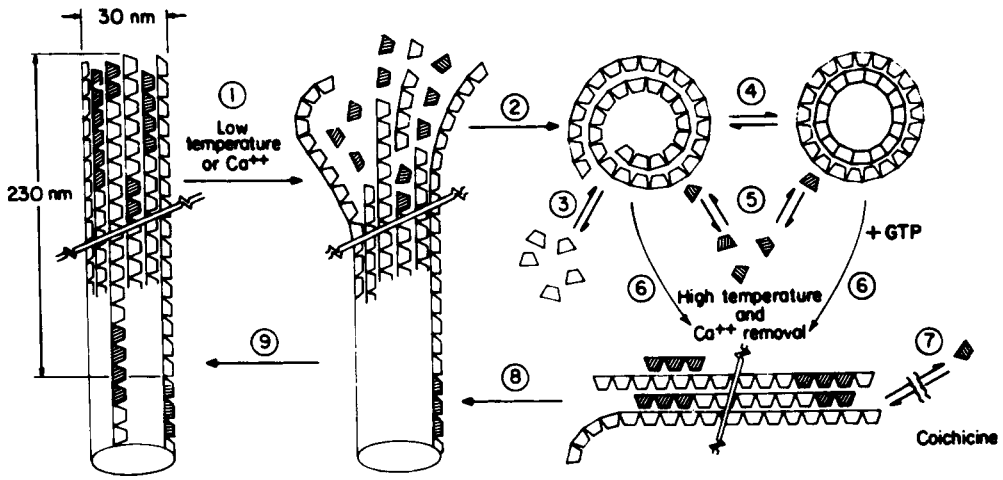

Fig. 12. Model for microtubule polymerization and depolymerization.

of the rings or spirals but show no tendency by themselves to form aggregates. In the absence of GTP the 36S and 6S components are stable. At 37° when GTP is added polymerization is initiated. The rings open up into protofilament segments, possibly by utilizing spirals as an intermediate. If X tubulin is blocked with colchicine, abortive aggregates are formed having a small number of short protofilament lengths aggregated side to side. In the absence of colchicine the X and Y tubulin copolymerize into side by side aggregates of protofilaments. Perhaps, as has been suggested by Erickson (9), these form sheets of protofilaments as an intermediate which then fold over each other to form microtubules.

It is of course, still not clear what the difference is between X and Y tubulin. All of the criteria used so far have been functional such as the ability to form 36S tubules and the ability to bind colchicine. Physical criteria indicate that the proteins are similar in their subunit size and in the size of their two polypeptide chains. It is possible that one or both of the polypeptide chains differs in amino acid sequence. It is possible that phosphorylation of microtubular protein which is known to occur (10–14) or some other post-translational modification might be the source of the difference. Brain tubulin has shown signs of microheterogeneity under isolectric focusing (15). It is possible that the difference is due to a tightly bound cofactor. We are in the process of testing these alternatives.

We cannot flatly state that the double ring structure is a required intermediate in the assembly process. It would be difficult to tell in what manner tubulin subunits are actually transported to the assembling microtubule, though electron microscopic evidence of the type shown in Fig. 11 indicates that coiling protofilaments are associated with both assembling and disassembling microtubules. We do assert, however, that Y tubulin is required in the assembly process. In view of recent experiments using high speed extracts from brain to add to flagellar seeds (16, 17), it is important to consider whether X tubulin is sufficient to add to these seeds. When we have made high speed extracts at 0° from purified hog microtubular protein we find only 6S protein in the supernatant as judged by analytical ultracentrifugation. Upon warming, a small amount of microtubule is formed. Upon warming in the presence of Ca^{2+} a small amount of 36S is also formed. An explana-

tion for this behavior is that cooling shifts the 36S–6S equilibrium (8), thus producing some Y tubulin subunits which will form a small amount of 36S on warming in the presence of Ca^{2+} and, in the absence of Ca^{2+}, will form some microtubules. When these tubules are removed, the 6S remaining will form no further tubules and we predict would not add to flagellar seeds.

Summary

The products of the depolymerization of microtubules have been studied by electron microscopy and ultracentrifugation. Depolymerization by exposure to either low temperature or Ca^{2+} causes tubules to disassemble into 6S subunits. 70 Å in diameter and 36S units composed of double rings and spirals. The ratio of 36S to 6S decreases with decreasing protein concentration. Fractionation of depolymerized tubules by gel filtration yields two fractions which differ in their capacity to form tubules and bind colchicine, and which individually will not reestablish the composition of the initial depolymerization mixture. Both fractions, however, contain α and β tubulin subunits. These results indicate that 6S and 36S tubulin are not in simple monomer–polymer equilibrium. We interpreted the data to mean there are two types of tubulin monomers differing in their capacity to form longitudinal and lateral interactions in microtubules. Studies of the role of GTP and the effect of colchicine reveal that these two binding domains can be distinguished on the basis of their sensitivity to various treatments. A model is proposed depicting the conversion of rings and spirals into protofilaments and involving the participation of both types of tubulin molecules in polymerization.

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