Microtubules from Mammalian Brain: Some Properties of Their Depolymerization Products and a Proposed Mechanism of Assembly and Disassembly

(electron microscopy/sedimentation analysis/protofilaments/tubulin/colchicine)

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ABSTRACT Depolymerization products of purified microtubules from porcine brain were examined by sedimentation analysis and electron microscopy. The complete depolymerization mixture exhibited 36S and 6S components in concentration-dependent equilibrium, whether depolymerization was caused by low temperature or by calcium ion. These components were recognized by electron microscopy as spirals and rings, and small particles. Agarose column chromatography yielded two major fractions, a leading one comprising mostly 36S and some 6S material and a trailing one of solely 6S material. The latter had high specific colchicine-binding activity and no tendency to polymerize. For the leading peak material these properties were the converse. It is proposed that tubulin molecules (of mass 110,000 daltons) exist in two states, here called X and Y, with those of the X-state equivalent to the material found predominantly in the trailing fraction, and those of the Y-state equated with the material in the leading fraction. Participation of tubulin molecules in microtubule assembly and disassembly is discussed, based on the assumption that those of both states have longitudinal and lateral binding domains whose strengths differentially depend upon temperature and calcium-ion concentration.

Microtubules are found in a variety of cytoplasmic structures involved with the morphology and movement of eukaryotic cells (1, 2). They are semi-rigid, hollow cylinders, about 30 nm in diameter, made up of 10–14 coaxial protofilaments. Recent information about the composition and arrangement of their protein subunits, called "tubulin," makes it likely that the appearance and disappearance of microtubules within cells result from polymerization–depolymerization reactions of the tubulin molecules (3, 4).

Weisenberg (5) discovered that microtubules could be polymerized from tubulin-containing extracts of mammalian brain if calcium ion was removed with chelators. Borisy and Olmsted (6) found that polymerization of tubules could occur only if the tubulin preparation contained material that sedimented into the pellet fraction during high-speed centrifugation. Recently Shelanski *et al.* (7) developed a process for purifying large quantities of microtubules from mammalian brain, thus allowing depolymerization–polymerization reactions to be performed on material better defined than the previously used crude extracts.

In this report, we present evidence that microtubules can release two distinct products upon depolymerization, and consider their possible role in microtubule assembly.

MATERIALS

Preparation. Microtubules were purified by the method of Shelanski *et al.* (7) from fresh hog brains. Storage was at −20°C, 5 mg/ml of protein, in MES reassembly buffer (0.1 M sodium morpholinoethane sulfonate–1 mM GTP–1 mM EGTA–0.5 mM MgCl₂) containing glycerol added to 8 M. Before use, 1 ml of stored material was diluted 1:1 with MES buffer, warmed at 37°C for 20 min, and centrifuged at 75,000 × *g* for 20 min. The pellet was resuspended in 0.5 ml of MES buffer and treated to three strokes of a loose-fitting Dounce homogenizer to yield microtubules at 5–10 mg/ml. For electron microscopy, the pellet was resuspended in 2.5 ml of a solution, at pH 6.4, containing 0.2 M ammonium acetate, 1 mM GTP, 1 mM EGTA, and 5 mM 2-mercaptoethanol, followed by centrifugation at 75,000 × *g* for 20 min. The pellet was resuspended in 0.5 ml of the same solution, chilled at 0°C for 15 min, and clarified by centrifugation at 0°C for 15 min at 15,000 × *g*. The supernatant was warmed at 37°C for 10 min to reassemble the microtubules.

RESULTS

Sedimentation Analysis of Depolymerization Products. The microtubule preparation in MES buffer, centrifuged at 25°C, contained no detectable material sedimenting at <200 S. This result is to be expected, since in the preparation of tubules for sedimentation analysis all smaller particles had been eliminated.

Fig. 1 shows schlieren patterns obtained after exposure of a microtubule preparation to low temperature; prominent boundaries at $s_{20,w} = 5.8$ S and $s_{20,w} = 23$ S are seen, with no intervening material. Over 80% of the starting protein was accounted for in the two boundaries. The remaining material was heterogeneous and ran ahead of the leading boundary. An aliquot was similarly analyzed after depolymerization by addition of 2 mM CaCl₂. The sedimentation values were $s_{20,w} = 6.0$ S and $s_{20,w} \approx 26$ S, with at least 85% of the starting material accounted for. The similarity in sedimentation behavior of the depolymerization products, after quite different treatments, indicates that they arise from structures intrinsic to microtubules.
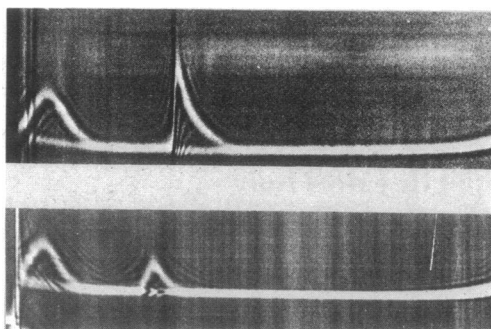
FIG. 1. Sedimentation behavior of products released from microtubules at low temperature. Samples were introduced into matched double-sector centrifuge cells, either plane or wedge-window, with 12-mm optical pathlength, mounted in an AnD rotor. Sedimentation was in a Spinco-Beckman model E ultracentrifuge equipped with schlieren optics. Microtubules in MES reassembly buffer were depolymerized by chilling to 0°C for 20 min. (*Upper*) 6 mg/ml, 6.2°C, photographed with phaseplate setting of 80°, 16 min after rotor had reached operating speed of 47,660 rpm. (*Lower*) 3 mg/ml, 7.7°C, phaseplate setting of 75°, 8 min after a speed of 47,660 rpm had been reached.

Table 1 shows that the $s_{20,w}$ values and the relative amounts of the two depolymerization products are dependent upon protein concentration. At the highest concentration examined, 12 mg/ml, 66% of the material dissociated by low temperature sedimented at $s_{20,w} \approx 17$ S, while at the lowest concentration about 32% sedimented at $s_{20,w} \approx 30$ S. Similar results were obtained with calcium-dissociated material. A supplementary test showed that the equilibrium distribution was not time-dependent, at least over a 24-hr period. The changes in the relative amounts of the components indicate that they are in concentration equilibrium. The concentration-dependent decrease in the observed s-value of the faster-sedimenting com-

FIG. 2. Binding of colchicine to microtubule protein of leading and trailing peak fractions from an Agarose 15m column after Ca^{++} depolymerization. Assays were performed in duplicate with samples of leading peak and trailing peak material diluted to 0.17 mg/ml and 0.27 mg/ml, respectively. The samples, 2.0 ml in volume, contained 0.1 M MES buffer at pH 6.4, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM GTP, and, when present, 1.0 M NaCl. The binding reaction was started by the addition of colchicine to a final concentration of 50 μM and a specific activity of 5×10^7 cpm/μmol. Incubation was at 23°C. At the times indicated 0.1-ml aliquots were withdrawn and assayed for bound colchicine by a modification of the DEAE–filter paper assay of Wilson (10). To the aliquot was added 1 ml of a 2% slurry of TEAE–cellulose (Whatman) suspended in 20 mM sodium phosphate buffer, pH 7.0, and 1 mM $MgCl_2$. After 5 min of adsorption at 0°C, the slurry was collected on a glass-fiber filter (Whatman GF 83) and washed five times with 1-ml aliquots of the same phosphate buffer. Radioactivity was determined by liquid scintillation counting. Duplicate binding assays agreed within 10%, and the background did not exceed 0.02% of the total radioactivity. Amount of protein was determined by the Lowry reaction. (*Open symbols*) NaCl-treated; (*closed symbols*) untreated. (*Circles*) leading fraction; (*triangles*) trailing fraction.

TABLE 1. *Sedimentation characteristics of depolymerization products from microtubules*

Total concentration (mg/ml)	$s_{20,w}$		Amount (%) in leading boundary
	Leading boundary	Trailing boundary	
Ca⁺⁺ dissociation			
2.3	32.5	5.9	59
7.0	29.3	5.3	72
14.0	18.7	4.7	85
Low-temperature dissociation			
3.0	30.0	5.4	32
6.0	25.8	5.2	45
12.0	16.8	4.5	66

Preparations in MES reassembly buffer were analyzed as described in the legend of Fig. 1. Dilute samples were contained in single-sector, 30-mm cells, and centrifuged in an AnE rotor. Concentrated samples were sedimented in 12-mm cells. Low-temperature sedimentation was at 3° to 8°C; Ca^{++} (2 mM)-treated samples at 20° to 24°C. Protein concentration was varied as indicated. Relative amounts of sedimenting material were estimated from areas under schlieren peaks after measurement of photographs by trapezoidal summation of ordinates with a Nikon comparator.
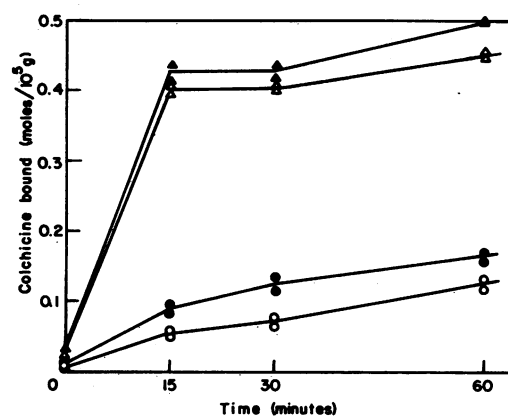
ponent may not signify a decreased molecular weight; the weight might even increase but the effect on the s-value could be more than offset by the hydrodynamic effects of greater concentration.

The s-value of the faster-sedimenting species, extrapolated to infinite dilution, was found to be $s_{20,w}^0 = 36$ S. We shall call it the "36 S" component, although it is probable that it is somewhat heterogeneous. The small material will be called "6 S"; it seems to have the same sedimentation coefficient as that found by Weisenberg *et al.* (8) for purified tubulin ($s_{20,w}^0 = 5.8$ S).

The equilibrium distribution between 36S and 6S material was examined after repeated polymerization and depolymerization. A preparation was brought through six cycles of incubation at 37°C for 15 min and chilling to 0°C for 15 min, ending with the chilling step. The GTP level was kept constant, by dialysis, at 1 mM throughout the experiment. Comparison of this preparation with one that underwent a single depolymerization showed no change in the 36S/6S ratio.

Microtubule preparations, when dissociated by either calcium ion or low temperature, and then treated with 1 M NaCl, showed no 36S component. Since the amount of 6S material accounted for the total protein, it may be concluded that the 36S structures are polymers of 6 S.

Column Fractionation of Depolymerization Products. Microtubular material, depolymerized by 2 mM CaCl₂, was fractionated on a column of Agarose A-15m. Two major fractions were recovered, each comprising about half the total protein, one eluting immediately after the void volume and the other just before the column volume, with recovery of over 85% of the material applied to the column. Sedimentation analysis of the center portion of the trailing fraction showed that it was solely 6S material, even when concentrated to 5 mg/ml. The leading fraction contained material sedimenting at 34 S (80%) and 6 S (20%), when analyzed at 2 mg/ml. This 80:20 ratio contrasts with the 55:45 ratio in the whole depolymerization mixture at the same concentration (Table 1). Thus, the fractionated components did not have the equilibrium distribution that existed in the mixture before fractionation. The two fractions were also analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Both showed the α and β polypeptides of tubulin (9). Less than 5% of the protein in the gels was at other positions, as determined by absorbance analysis of stained gels.

Absorbance spectroscopy of the two components showed that, after correction for light scatter, the A_{280}/A_{260} ratio was 1.20 for the leading peak and 1.50 for the trailing one. Respective extinctions, $E_{280\,nm}^{1\,mg/ml}$, were 0.84 and 0.72 (protein concentration determined by Lowry assay). The difference spectrum (leading fraction *minus* trailing) resembled the absorbance spectrum of nucleotides, with an extinction equivalent to about one molecule of nucleotide-like chromophore per 10^5 daltons of protein in the leading component. It cannot be excluded, however, that this result arises from contamination by RNA that accompanied the purified microtubules and was preferentially eluted in the leading fraction.

Fig. 2 shows the rates of colchicine binding of the two fractionated components. Binding rate, per mg of protein, was at least seven times as rapid in the trailing fraction as in the leading one, with saturation at 0.5 colchicine molecules per 110,000 daltons of protein. Colchicine binding was also assayed after addition of 1 M NaCl to both fractions. The binding rate, after NaCl addition, continued to be about seven times as great as that of the NaCl-treated leading fraction. Since 1 M NaCl completely depolymerizes the 36S structures to 6 S, the poor binding ability of this fraction seems not to be attributable to its state of polymerization. The results indicate that the 36S component can release a 6S species differing in colchicine-binding properties from the 6S material found in the trailing fraction. Hence, the 6S component found in the *whole* depolymerization mixture seems to contain two species, distinguishable by their colchicine-binding activity.

Characterization of Depolymerization Products by Electron Microscopy. Microtubules appeared as cylindrical bundles of parallel protofilaments, generally protruding unequally at the ends. After depolymerization by either calcium ion or low temperature, three characteristic forms were seen: spirals, rings, and small particles (Fig. 3A) with practically no structures of intermediate size. The rings were usually double, but some (<10%) single rings, and broken and segmented ones, were encountered. Both the rings and the spirals showed signs of uniformly spaced subunit structure (Fig. 3C and D). The subunits, spaced at 3–4 nm, have a size such as to indicate that they are the α and β polypeptides of tubulin. About 40 of them are estimated to compose the outer ring
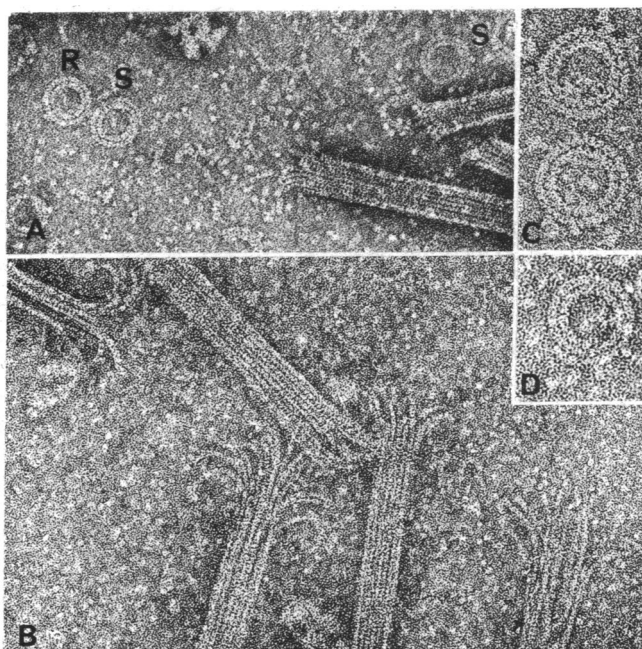

FIG. 3. Electron micrographs of depolymerization states of microtubules. Specimens were prepared for electron microscopy in two steps: (*1*) a mixture of 2% uranyl acetate (pH 4.6) and 0.02% cytochrome *c* was spray-deposited from a nebulizer upon carbon-coated collodion specimen films; (*2*) the microtubular preparation, at 2–4 mg/ml, was then spray-deposited upon the films. (*A*) Specimen chilled to 0°C for 15 sec. A double ring (*R*) and two spirals (*S*) are marked; portions of intact tubules and numerous small particles are also seen. ×130,000. (*B*) Specimen after room-temperature dilution in water to 1 mg/ml for 1 hr. ×130,000. (*C*) A double ring, showing subunit structure. (*D*) A spiral containing slightly more than two turns. Both (*C*) and (*D*) (×250,000) were obtained from microtubules depolymerized by chilling.

and about 30 the inner ring. Size measurements, made on 179 double rings that had been produced by chilling microtubules to 4°C for 24 hr, yielded a mean diameter of 43.0 nm for the outer ring and 29.4 nm for the inner one. Thus, the interring spacing was close to 7 nm. The spirals were commonly found to contain 1.5–3.5 turns; their interturn separation was similar to that of the double rings. The small particles were judged to represent free tubulin molecules, since their diameter of 6–7 nm is compatible with that of a spherical protein molecule of about 10^5 daltons mass. Material from the trailing (6 S) fraction from the Agarose column was seen to contain only small particles, while that from the leading fraction consisted mostly of rings and spirals, with numerous small particles.

It seems most likely that the structures seen in the electron microscope are the 36S and 6S components identified by sedimentation analysis. The large diameter of the rings and their characteristic double nature preclude the possibility that they are cross-sections of microtubules.

A study was made of the relative proportions of rings and spirals, and their contour lengths. (The "contour length" of a double ring was taken as the sum of the circumference of its outer and inner rings; the contour length of a spiral was determined by use of a map reader.) Comparison was made of two preparations that were: (*A*) chilled to 0°C for 15 sec or 60 sec and, (*B*) briefly chilled to 0°C followed by

storage at 4°C for 24 hr. Preparation A yielded a spiral/ring number ratio of 2.1 (85 spirals, 40 rings) while preparation B showed a ratio of 0.4 (76 spirals, 179 rings). Thus, there was a 5-fold change of the ratio of the spiral form to the double-ring form as chilling continued. The average contour length of 179 double rings was 230 nm with a standard deviation of only ±14 nm, and with no systematic difference between the rings in the preparations A and B. The average contour length of the spirals was 229 ± 35 nm (85 spirals) in preparation A and 233 ± 49 nm (76 spirals) in preparation B. The near-identity of the average contour lengths of rings and spirals, and the broad distribution of the contour lengths of the latter, will be commented upon later.

Purified preparations of microtubules examined at room temperature infrequently contained structures having a splayed-out "ram's-horn" appearance at one end. Preparations, examined after dilution to about 1 mg/ml for an hour or more, exhibited many such forms, as seen in Fig. 3B. Here, the majority of protofilaments are coiled near their ends. Such micrographic evidence, and the universal occurrence of spirals in completely depolymerized material, strongly suggest that microtubular disassembly proceeds by release of segments of protofilaments which coil into spirals. At the least, it shows that the protofilaments of disintegrating microtubules retain a linear continuity and have a tendency to coil into generally spiral or circular form.

Competence of Depolymerization Products for Tubule Formation. A microtubule preparation was depolymerized at 0°C for 30 min, and one aliquot at 6 mg/ml, used as a control, was sedimented at 3°C. It contained the characteristic species 36 S (45%) and 6 S (55%). Another aliquot, repolymerized at 37°C for 15 min, was sedimented at 25°C. About 75% of the material in the latter showed an $s > 200$ S, with 6S material accounting for all of the remaining 25%. Thus, about half the 6S molecules in the depolymerization mixture was not incorporated into microtubules. The repolymerized samples were examined by electron microscopy and found to contain intact tubules, some small particles, and no rings nor spirals. Similar structures were found in a sample of calcium-depolymerized tubules, after repolymerization by the addition of EGTA to a final concentration of 8 mM and of GTP to 1 mM, followed by incubation at 37°C for 10 min.

Calcium-depolymerized microtubules, fractionated on an Agarose column, were tested for competence to repolymerize readily. The trailing peak fraction (6 S), at 3 mg/ml, was supplemented with 1 mM GTP and 8 mM EGTA and incubated at 37°C for 10 min. Sedimentation analysis showed it to contain as much 6S material as a control aliquot not exposed to repolymerization conditions, and to contain no 36S or >200S species. Fractions of the leading peak, after identical incubation, exhibited tubules when examined in the electron microscope.

DISCUSSION

Three kinds of observations are reported above: (1) the number ratios and the dimensions of spiral and ring structures in material from depolymerized microtubules, (2) the sedimentation characteristics and the relative amounts of the products of depolymerization, both unfractionated and separated by column chromatography, and (3) the relative colchicine-binding activities in the two major fractions of depolymerized material after column separation.

(1) The near-identity of the average contour lengths of the spirals and of the total circumference of the double rings strongly suggests that these two forms are in equilibrium. Since the number ratio of spirals to rings significantly decreased as time of chilling continued, we conclude that equilibrium is established by a net, but not instantaneous, conversion of spirals to rings. We visualize the mechanism of the equilibrium process to be one in which a spiral, constantly changing its contour length by addition or subtraction of tubulin molecules at its free ends, can convert reversibly to the double-ring form when its length amounts to almost exactly two turns, perhaps by a breaking and rejoining mechanism. The maximum length of a spiral, as its outer arm increases its radius of curvature, would be determined by the consequences of increasing mismatch of subunits in adjacent turns. The minimum length of a spiral would approach the circumference of the inner member of a double ring. Within this range the lengths of spirals would vary, accounting for their broad distribution of length.

(2 and 3) The fraction of 6S material in a whole depolymerization mixture was found to vary from 34% to 68% after chilling to 0°C, and from 15% to 41% after addition of 2 mM calcium ion, depending upon protein concentration. This dependence suggests a monomer–polymer equilibrium between the 6S and the 36S species. If this equilibrium were due to linear aggregation of monomers of a single species, a column fractionation of the particle population should yield preparations which would re-establish the original equilibrium distribution of 6 S and 36 S. But neither of the two major fractions from the column re-established the original distribution. The trailing fraction contained exclusively 6S material, while the leading fraction had a 36 S/6 S ratio of 80%:20%, when examined under conditions in which the whole depolymerization mixture exhibited a 55%:45% ratio. Thus, we think it likely that two or more species of 6 S are present in the original, unfractionated mixture. In further support of this notion is the fact that the fractionated materials differed greatly in their colchicine-binding activity; the material in the trailing peak bound colchicine at about seven times the rate evinced by the leading fraction.

From the above considerations we postulate that tubulin molecules (6 S) from mammalian brain occur in two states, which we term "X" and "Y". Those in the X-state, existing as the predominant component in the trailing fraction from the Agarose column, are assigned the properties of high colchicine-binding activity and an inability to polymerize into 36S structures. (They may, however, be able to *associate* with them.) The Y-state molecules, predominant in the leading fraction and perhaps occurring as a minor component in the trailing one, are assigned low colchicine-binding activity and an ability to aggregate readily to form 36S polymers. The unfractionated depolymerization mixture would contain molecules of both states, and would display a complex, concentration-dependent equilibrium between the 36S and 6S species, wherein Y-state molecules are in monomer–polymer equilibrium but those of the X-state molecules, while able to associate with 36S structures, are not able to form these structures by polymerization.

We assume further that the molecules of both states may possess two kinds of bonding domains: (A) longitudinal, involved in the assembly of protofilaments, spirals, and rings, and (B) lateral, involved in bonding between the protofila-
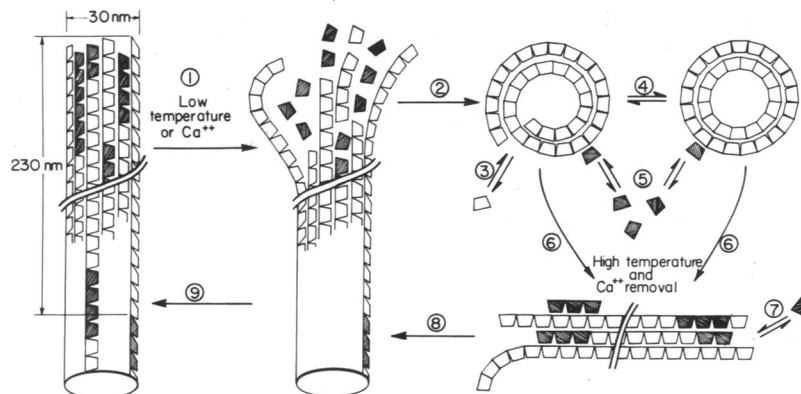
FIG. 4. Schematic representation of disassembly and reassembly of microtubules. An intact microtubule, exposed to conditions that weaken its lateral bonds, separates (step *1*) into protofilaments composed of long runs of Y-state tubulin (□). X-state tubulin molecules (■) are released. The protofilaments coil into spirals (step *2*) whose lengths change by addition or subtraction of tubulin molecules (□) (step *3*). Those spirals almost two turns in length interconvert with double rings (step *4*) by breaking and rejoining. X-state tubulin molecules may associate weakly with the rings and spirals (step *5*). Upon reassembly, the rings and spirals open and associate laterally as segments of protofilaments (step *6*). X-state tubulin (■) is incorporated and forms strong lateral bonds (step *7*). Continuation of this process (steps *8* and *9*) forms the completed microtubule.

ments in intact microtubules and between adjacent turns of spirals and rings. The Y-state molecules would have strong longitudinal bonding domains since they reversibly polymerize into spirals and rings. On the other hand, the molecules of the X-state may lack these bonds, at least in the cold or in the presence of calcium, since they do not form 36S material. It is reasonable to assume that intact microtubules have both longitudinal and lateral bonding. The former would arise mostly from the Y-state tubulin and the latter from that of the X-state; both would be essential for maximizing structural stability.

We suggest the following mechanism of disassembly and assembly of microtubules. Lowering the temperature (or adding calcium) weakens lateral bonds, but not the longitudinal ones between the Y-state tubulin molecules. Segments of protofilament, consisting of runs of consecutive Y-state tubulin, are freed from one another but retain their longitudinal integrity. They coil into spirals which equilibrate with double rings. Residual, weak lateral bonds constrain the released protofilaments to these two general forms. Individual X-state molecules are freed from the disintegrating microtubules. At equilibrium, some free Y-state molecules must also be present. Subsequent warming, or calcium removal, reverses the process, with spirals unwinding to form linear segments of protofilaments. Double rings may directly convert to linear form by undergoing breaks in both their inner and outer members, as well as indirectly by conversion to spirals. These linear segments of protofilaments associate laterally to form tubules, accompanied by colinear incorporation of X-state molecules acting as strong lateral-bonding agents. (An attractive aspect of this scheme is that the spirals and rings would serve as reservoirs of preformed protofilament segments, about 230 nm long, thus facilitating rapid

microtubule assembly.) Although a preparation of repolymerized microtubules is found to contain about 25% 6S protein, the relative amount of X-state and Y-state molecules in this nonincorporated fraction is unknown. Fig. 4 is a schematic representation of our proposed mechanism of disassembly and assembly of microtubules.

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