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ASSEMBLY OF MICROTUBULES FROM PREFORMED, RING-SHAPED PROTOFILAMENTS AND 6-S TUBULIN

Harold P. Erickson

Department of Anatomy, Duke University Medical Center, Durham, North Carolina 27710

Tubulin obtained from disassembly of microtubules at 0°C exists in two forms: 6-S tubulin and a larger, curved or ring-shaped filament. These two forms have been separated chromatographically and their roles in assembly examined. The purified rings reassemble to microtubules with high efficiency by uncoiling and straightening out, to be incorporated directly as protofilaments in the microtubule wall, and are thus identified as preformed protofilaments. Purified 6-S tubulin has not been observed to reassemble into microtubules by itself but will contribute to assembly when mixed with rings. Addition of glycerol at 0°C induces the 6-S tubulin to form rings, and the treated fraction will then reassemble to microtubules. Electron microscope observations indicate that assembly begins with the formation and growth of an incomplete microtubule wall. This wall grows wider by the addition of new protofilaments until the intact, circular microtubule, with 13 protofilaments, is formed. It is suggested here that growth of this wall from individual 6-S tubulin subunits may be energetically unfavorable. The direct incorporation of preformed protofilaments may be much more favorable, in which case the rings would be required for this initial stage of assembly.

INTRODUCTION

Mircotubules are labile structures that assemble and disassemble in a variety of cellular processes. It has recently been demonstrated that assembly and disassembly of microtubules can be achieved in vitro from a preparation of brain tissue homogenized in an appropriate buffer (1). The in vitro assembly has been characterized in terms of chemical requirements and sensitivities (1, 2, 3, 3a), and these show many similarities to the conditions required for the assembly and stability of microtubules in vivo. In addition, the microtubules formed in vitro are morphologically identical to microtubules from a variety of natural sources (4).

When prepared under conditions of disassembly, at 0°C or in high Ca^{++} concentra-

tion, negatively stained electron-microscope specimens showed ringlike structures, which disappeared during the reassembly of microtubules (2). Centrifugation experiments indicated that these rings were essential for microtubule assembly, and it was suggested that they might be a preformed circular nucleation site for the growth of the cylindrical microtubules (2). However, observation of the early stages of assembly by electron microscopy indicated that assembly began with the formation of an incomplete microtubule wall (4). This sheet appeared to curve as it grew laterally until the edges met and sealed to form the intact, cylindrical microtubule. Although the mechanism of this growth was not known, it appeared to be a self-assembly process with no need for a circular nucleation site.

The experiments reported here are based on a chromatographic separation of rings and 6-S tubulin (5) and a determination of the activity of each fraction in assembly. The rings were found to be more active in assembly, with 6-S tubulin contributing under particular conditions. A model has been developed to explain the results in terms of the formation and growth of an incomplete wall as the initial stage of assembly. A similar chromatographic separation of rings and 6-S tubulin has been demonstrated independently by Sander and Kirschner (6) and by Kirschner et al. (7, 7a). Their interpretation of the role of rings in assembly is similar to that presented here.

Before the discussion of assembly, a brief summary of the structure of microtubules will be presented.

The Structure of Microtubules

Electron microscopy of embedded and sectioned specimens shows microtubules to be hollow cylinders of 250 Å outside diameter, 50 Å wall thickness, and up to many micrometers long. A finer substructure of the wall is generally not seen in sectioned material but is easily revealed in negatively stained specimens. The most obvious feature is the division of the wall into longitudinal filaments, termed protofilaments. Special fixation and staining techniques have recently been used to visualize the protofilament structure in sectioned material, and a survey of microtubules from a wide variety of sources has shown 13 protofilaments in every microtubule examined (8). Each protofilament appears in negatively stained specimens to be composed of a string of globular subunits. Analysis of the structure by optical diffraction techniques (9) showed that the microtubule wall could be represented as an array of subunits on a skewed 40 × 50 Å lattice.

The lattice parameters have been specified more precisely, and finer detail of the structure of individual subunits has been visualized in a recent study of reassembled neuronal microtubules (4). The lattice is seen in Fig. 1, which shows a computer reconstruction of a microtubule wall unrolled and flattened. The subunits are aligned in protofilaments, and adjacent protofilaments are staggered slightly so that the subunits form an obliquely horizontal line, which is a helix in the intact microtubule. Since this oblique line is continuous with the third subunit up after completing one circuit of 13 protofilaments, the lattice may be described as a 13-filament, three-start helix. A somewhat different lattice structure was proposed on the basis of x-ray diffraction analysis (10), but

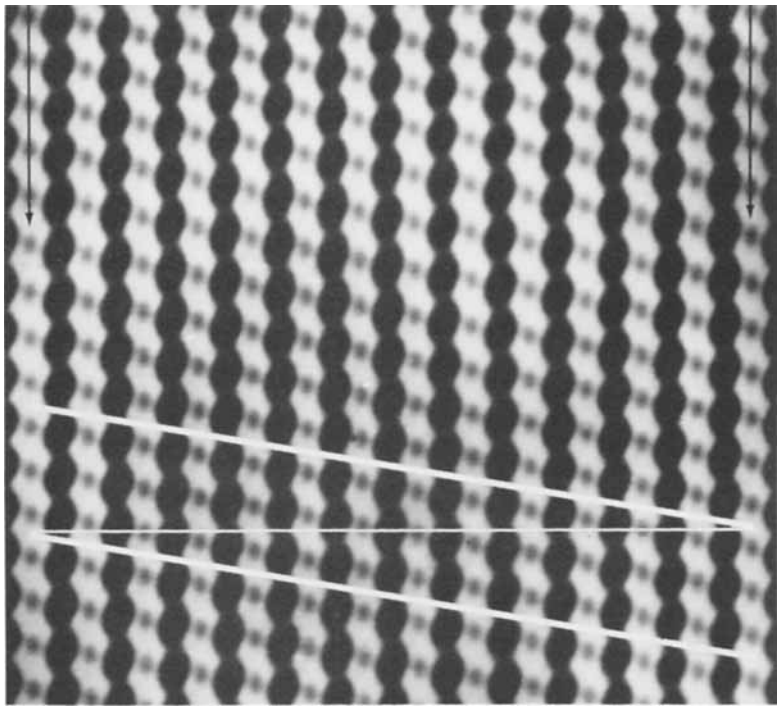

Fig. 1. Computer reconstructed image of the microtubule wall to 25 Å resolution, showing the arrangement of subunits on the surface lattice. Protein is white and stain is black. The apparent small size of the subunits and the large spaces (stain) between them is an artifact, since the absolute size of the subunits is not reliably represented in this type of reconstruction. The sheet may be rolled up to make a cylinder, superimposing the first and fourteenth protofilaments (arrows), without bending or distorting the lattice. The oblique line indicates one basic helix of the three-start helical lattice.

a more recent interpretation (10a) indicates that these data are actually consistent with the lattice seen by electron microscopy.

The structure of the individual subunits has been determined from these reconstructed images from which nonperiodic noise has been filtered. The subunit structure is shown in Fig. 1 and at higher magnification in Fig. 2. The images are a 2-D projection of the flattened microtubule wall and contain data to a resolution of 25 Å. Each of the 40 × 50 Å subunits is seen to be split symmetrically into two lobes. Each subunit has two prominent connections (one for each lobe) to the subunits above and below it within a protofilament, and there is a single connection between subunits in adjacent protofilaments.

Although the morphological division into 40 × 50 Å subunits is obvious in these images, the identification of tubulin molecules is less clear. It is now known that microtubules are composed of two slightly different tubulins, designated α and β (11, 12), each of which has a molecular weight (MW) of 55,000. The stable 6-S tubulin that has been isolated and characterized (13) is a dimer (probably an α-β heterodimer) of 110,000 MW.

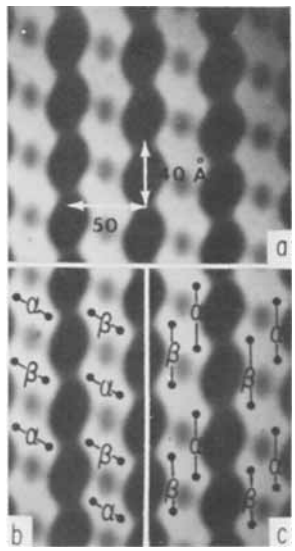

Fig. 2. A detail of the microtubule wall at higher magnification. (a) Each of the 40 × 51 Å morphological subunits, which correspond in size to 55,000-dalton tubulin monomers, appears to have a twofold axis of symmetry which divides it into two identical lobes. There are two longitudinal contacts between subunits within a protofilament and a single lateral contact between subunits on the basic helix. (b) and (c) show two possibilities for the identification and arrangement of the α and β tubulins. The most obvious identification is that shown in (b), where each of the morphological subunits is labeled as either an α or β tubulin monomer. The alternating arrangement of the two types indicated is the only one consistent with the 13 filament, three-start helix. Alternatively, the α and β monomers might be arranged longitudinally, spanning two 40 Å subunits. One such arrangment is shown in (c). Reprinted from Ref. 4.

From the division of the 40 × 50 Å subunits into two lobes as seen in the reconstructed images, it would be tempting to identify each of these subunits as a 6-S tubulin dimer. However, the volume of 40 × 50 × 50 Å (assuming a 50 Å wall thickness) is barely sufficient to contain a 55,000 MW protein molecule and is almost certainly too small for the 110,000 MW dimer (14). Thus, it would seem that each of the morphological subunits may be identified as either an α- or β-tubulin monomer. For the 13-filament, three-start helical surface lattice there is only one arrangement of the two types of subunits that preserves helical symmetry. This is shown in Fig. 2b, where the subunits are seen to alternate both along each protofilament and around the basic helix. Another possible identification is that indicated in Fig. 2c, in which the α and β monomers are elongated to span two of the 40 Å morphological subunits. At present, these identifications are purely speculative, since no information has been obtained to actually locate the two types of tubulin. In fact, the images of brain microtubules and of purified flagellar microtubules examined in this study have consistently failed to show any structural difference in the subunits. However, such a structural difference between alternate 40 Å subunits has been observed in images of flagellar microtubules by other investigators (9). Three-D reconstruction has recently shown that this is due to a radial staggering of alternate subunits, which may be related to the arrangement of heterodimers in the microtubule wall (15).

METHODS

Preparation of Tubulin

The buffer used routinely for chromatography and for reassembly studies was 0.05

M PIPES [piperazine-N, N′-bis (2-ethanesulfonic acid)], 1 mM EGTA [ethyleneglycol-bis-(β-amino-ethyl ether) N, N′-tetra-acetate], 0.5 mM $MgSO_4$, and 0.5 mM GTP (guanosine triphosphate), pH 6.5. This is similar to that recommended previously (2), but with half the concentration of PIPES and GTP.

Fresh hog brain (100 g) was obtained within one hour of slaughter, mixed with 150 ml of buffer (which usually contained no GTP), and homogenized in the cold with a teflon-glass homogenizer. The homogenate was centrifuged for 30 min at 65,000 g and 2°C and the supernatant mixed with cold glycerol (to 25%) (16). GTP was added to a concentration of 1 mM, and the mixture was incubated at 35°C for 30–60 min to allow polymerization of microtubules. These were collected by centrifugation for 35 min at 115,000 g and 30°C and resuspended in 10–20 ml of buffer. This suspension was put on ice for an hour to depolymerize the microtubules and centrifuged for 20 min at 65,000 g and 2°C. The supernatant was sometimes used directly for chromatography and was sometimes mixed with glycerol (to 50%) and stored at −20°C. The stored tubulin was collected by another step of assembly and disassembly as described by Shelanski et al. (16). The stored tubulin retained considerable activity for several days, but storage for several weeks, as originally described (16), was not successful.

Spectrophotometric Determination of Tubulin Concentration

Tubulin preparations and purified fractions were routinely assayed by spectrophotometry. An extinction coefficient $E^{1\ mg/ml}_{280\ nm}$ of 0.72 has been reported for purified 6-S tubulin (7) and has been assumed in this work for all statements of protein concentration. Analysis on SDS gels showed that a number of high-molecular-weight components constituted one-fourth or more of the material in the ring fractions, and spectrophotometry showed optical density at 260 nm equal to that at 280 nm, indicating possible nucleic-acid contamination. In stating the tubulin concentration of the ring fractions, an extinction coefficient of 1.0 has been assumed to account approximately for these contaminants.

Chromatography and Analysis

In a typical experiment (Fig. 3a), a column of Sepharose 6B (registered trade mark, Pharmacia Fine Chemicals, Uppsala, Sweden), 25 mm in diameter × 390 mm high, was equilibrated with buffer at 4°C. Five ml of tubulin preparation, 3–5 mg/ml, was applied to the column and eluted at a rate of 15 ml/hr. Five-ml fractions were collected, and a preliminary assay of protein was obtained by measuring the $O.D._{280}$ of a 1/10 dilution of each fraction. Electron microscope specimens were prepared, and assembly experiments performed as quickly as possible, usually within 15 hr after beginning the chromatography. In later experiments, a shorter (250 mm) column and faster elution (60 ml/hr) were used to reduce this time to 3 hr.

The fractions were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis.

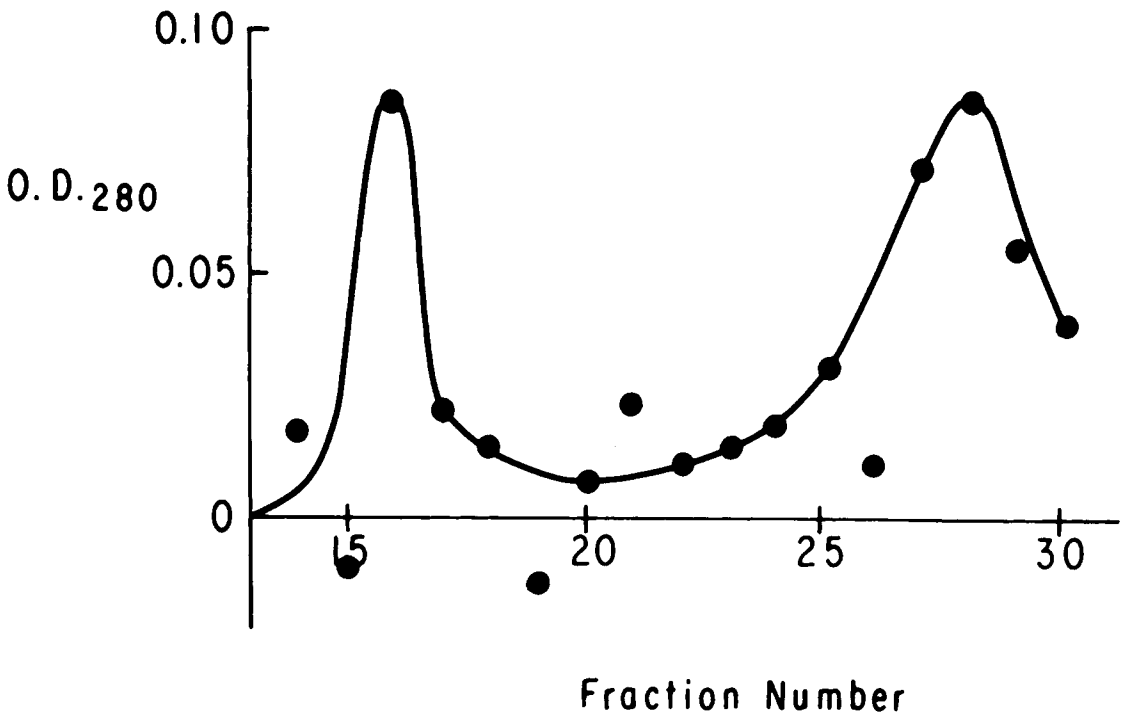

Fig. 3a. Chromatography of tubulin at 0°C on Sepharose 6-B. Optical density assay of fractions diluted 1/10 before spectrophotometry. Fraction #16 is the peak "ring" fraction, and #27 and #28 are the peak "6-S" fractions. Chromatography was run overnight on a 25 X 390 mm column.

Samples were mixed with SDS (3%) and β-mercaptoethanol (1%) and heated in boiling water for 1 min. They were then run on 7.5% acrylamide gels as described by Fairbanks et al. (17) and stained with Coomassie Blue.

Electron Microscopy

Negatively stained specimens were prepared by placing a drop of the sample on a grid coated with a thin carbon film. This was drained with a filter paper and the surface was washed with several drops of 2% uranyl acetate and then drained and dried. A reproducible spread of microtubules and rings was obtained which gave a rough measure of their concentrations. Speciments of 0°C preparations were made in the cold room and stained with cold uranyl acetate since good images of rings were seldom obtained from specimens stained at room temperature. Specimens of microtubules at 35°C were prepared and stained with uranyl acetate at room temperature. Micrographs were taken on a Philips EM-301 electron microscope at magnifications of 45,000 X to 70,000 X. The microscopy and printing conventions were such that the image is a view of the specimen

through the carbon film, which corresponds to a view from the outside of the microtubule for the images of the wall and lattice (4).

RESULTS AND DISCUSSION

Incomplete Microtubule Walls – A Precursor in Assembly

One of the most direct methods for following the assembly of microtubules has been to prepare negatively stained specimens at different times after raising the temperature from 0° to 35°C. In such a series, assembly normally appears to be complete in about 10 min, as specimens show a constant number of long intact microtubules after this time. At earlier times there are shorter microtubules, and in many circumstances, short incomplete segments of the microtubule wall are found. These appear as flattened sheets with prominent protofilament structure, and the arrangement of the subunits on the surface lattice may be seen directly. In most of the images presented here, the sheets appear to be flattened in the stain, but in solution they probably are curved to the same degree as the wall of an intact microtubule (4). The smallest sheets, which are found at the earliest times of assembly, have only a few protofilaments, while at later times sheets with 9–12 protofilaments are more common. Sheets with the full complement of 13 protofilaments are rarely seen, presumably because the curvature of the sheet is such that the edges meet, and the subunits on the adjacent edges are in correct alignment to contact and attach. Thus, the edges may seal, forming an intact cylinder, and growth continue by elongation.

The occurrence of these incomplete walls is somewhat variable. The concentration of tubulin in the assembly mixture seems to be important, with a low concentration being most favorable for seeing the incomplete walls. At a protein concentration greater than 1 mg/ml they are only rarely observed, even in the first minute of assembly, since short intact microtubules apparently form very quickly. The most reproducible and convincing specimens of these sheets have been obtained from assembly in purified "ring" fractions, diluted to 0.1 mg/ml. In these preparations, the rate of assembly appears to be reduced, and the progression from small sheets to larger sheets to intact microtubules is clearly demonstrated over a 5–10-min period after raising the temperature. In addition, the direct incorporation of rings into the growing microtubule wall is seen, as described below.

Perhaps the most striking manifestation of the incomplete walls occurs in assembly in the presence of glycerol (16). Here, the sheets may be several micrometers long and persist even after long periods of assembly. They frequently have 13 protofilaments, sometimes more, and microtubules which appear to be intact along part of their length will be split open at other places. The glycerol may somehow interfere with the lateral growth of the microtubule wall – i.e., the addition of new protofilaments and the joining of the edges of the sheet to form the intact cylinder.

Dissasembled Microtubules – Chromatographic Separation of Rings and 6-S Tubulin

In order to understand the mechanism of assembly of microtubules at 35°C, it is

first necessary to determine the state of the tubulin when the microtubules are disassembled at 0°C. Early observations of Borisy and Olmsted (2) showed the presence of ring-shaped structures in negatively stained specimens of disassembled microtubules, and centrifugation experiments indicated that these rings might be essential for microtubule assembly.

In the investigation of assembly intermediates reported here, the presence of the rings in specimens of disassembled microtubules was confirmed in purified preparations of tubulin. In order to investigate the nature of these rings and their role in the assembly process, attempts were begun to separate the rings and to determine the state of the tubulin at 0°C.

The first attempt, which failed, involved sucrose-gradient centrifugation, in which a sample of disassembled microtubules at 2°C was sedimented overnight in a 10–30% sucrose gradient at 40,000 rpm in the SW-41 rotor (Spinco Division, Beckman Instruments). Ninety percent of the tubulin was recovered in a single peak traveling with a sedimentation coefficient of 5–6 S. Surprisingly, however, negatively stained specimens prepared in the cold from the 6-S peak fractions showed the presence of rings. Since the rings should have a sedimentation coefficient of 30–36 S (7, 3a, 7a, 17a), they must have been dissociated to 6-S tubulin during the centrifugation and reformed afterwards. It was felt that the high pressure during the centrifugation may have caused the dissociation. Microtubules in vivo are known to be sensitive to pressures around 3,000 lb/in^2 (18), which were reached in these centrifuge runs at a distance of 1 cm from the top of the gradient. This hypothesis has been confirmed by Borisy and coworkers (3a) in a series of experiments demonstrating changes in the relative amounts of 6-S tubulin and rings at different pressures.

A successful separation of rings from 6-S tubulin was obtained by chromatography on Sepharose 6-B. Tubulin prepared by disassembly of microtubules at 0°C was separated into two peaks, as shown in Fig. 3a. The first peak contained 20–50% of the applied protein and appeared in the excluded volume of the column. This should correspond to a molecular weight greater than 4×10^6 for globular proteins (19) and would be expected to contain only very large molecules and aggregates. The second peak, containing 50–80% of the protein, traveled in the included volume, with a $K_{av} = 0.5$, corresponding to a molecular weight of 100,000 daltons for a globular protein (19). This second peak thus corresponds in size to the 6-S tubulin dimer and is referred to as the "6-S" peak. The first peak is referred to as the "ring" peak on the basis of identification by electron microscopy discussed below.

The analysis of fractions by SDS gel electrophoresis (Fig. 3b) showed that most of the tubulin was in the "6-S" peak (fractions 25–30), a smaller amount in the "ring" peak (fraction 16), and a small residual amount spread in the fractions between the two peaks. The high-molecular-weight bands, which are normally seen in preparations of tubulin purified by assembly and centrifugation of microtubules (4), are concentrated in the first peak, with a slight trailing through later fractions. It is not yet known if this high-molecular-weight material is involved in microtubule assembly or if it is a contaminant which aggregates with the microtubules when they are assembled and centrifuged at 35°C. Negatively stained specimens prepared in the cold showed that the first peak contained a

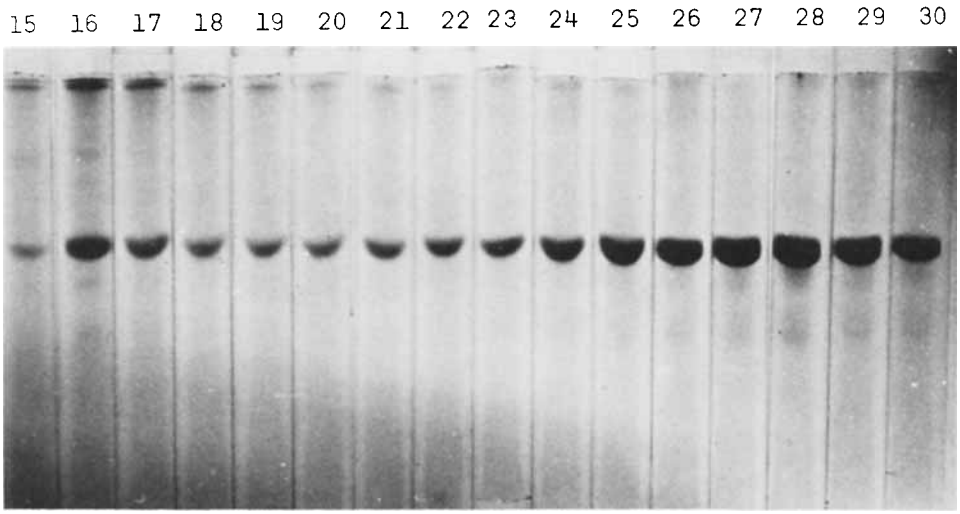

Fig. 3b. Chromatography of tubulin at 0°C on Sepharose 6-B. Analysis of fractions by electrophoresis on SDS polyacrylamide gels. The prominent band near the center is the tubulin. A number of high-molecular-weight bands are seen near the tops of the gels in fractions #16 and #17.

high concentration of rings (Fig. 4) like those found in unfractioned preparations of dissociated microtubules. Specimens prepared from the "6-S" peak usually showed a large number of particles 50–100 Å in size. The shape of individual particles appeared to be variable, probably because of differences in orientation, and has not been investigated further.

Structure of the Rings

The micrograph of a specimen of purified rings presented in Fig. 4 shows a variety of structures typical of these specimens. All of them seem to be based on a common structure – namely, a single continuous filament 50 Å thick which is uniformly curved to form an arc of a circle 420 Å in outside diameter. The filament at a is only long enough to form half a circle, while that at b forms almost a complete circle. In the two structures marked c and d the ring appears complete, but they probably do not consist of a single filament whose ends are joined to make a simple circle. Rather, the impression is gained from looking at a large number of these structures that the filament is longer than the circumference of the circle, with the extra length overlapping and coiling on top of the first loop. The somewhat greater density of the right-hand segment of c and the left-hand segment of d would correspond to this overlap. In the structure at e, a large upper loop of the coil seems to have slipped off the lower loop and become flattened inside. A slippage may occur at the arrow, but the continuity of the strand is not obvious, and the appearance is perhaps more that of two concentric rings. A clearer picture of the

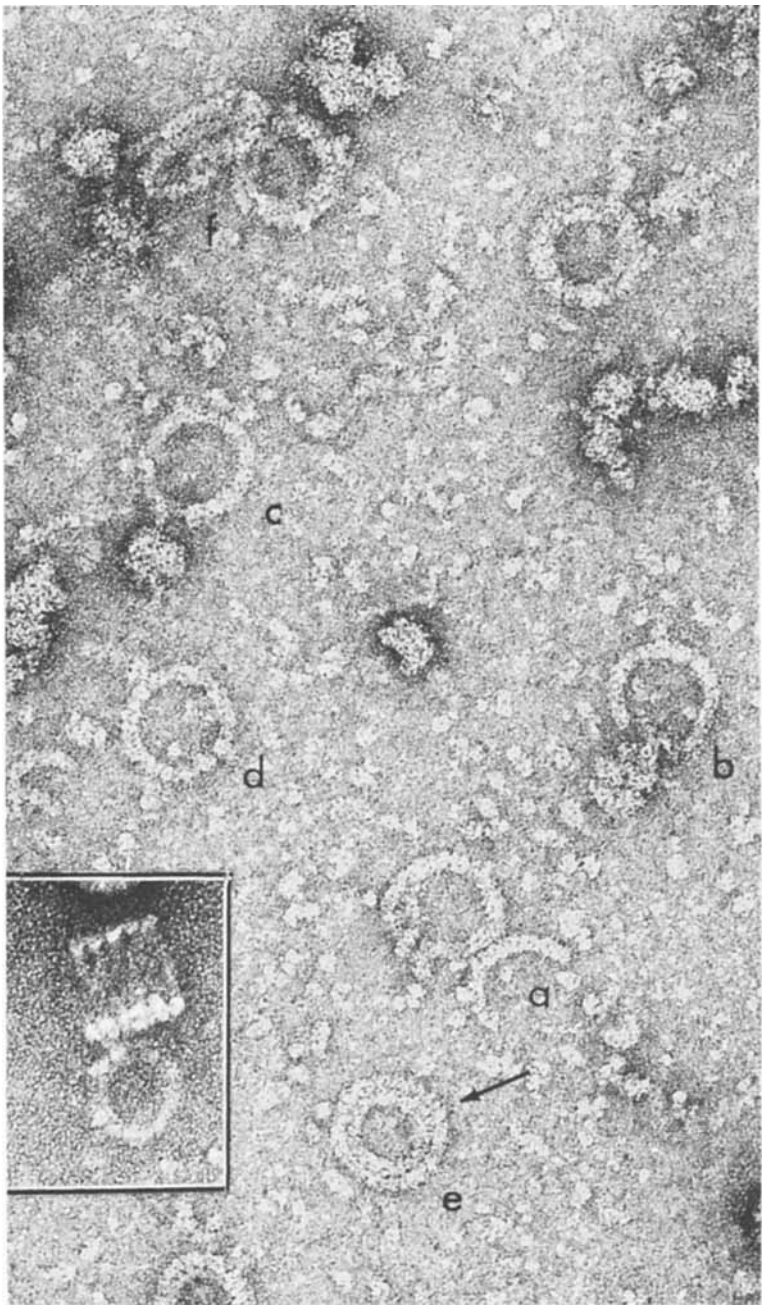

Fig. 4. Negatively stained specimen prepared from the "ring" fraction. The identification and discussion of the structures is given in the text. Magnification, 350,000 ×.

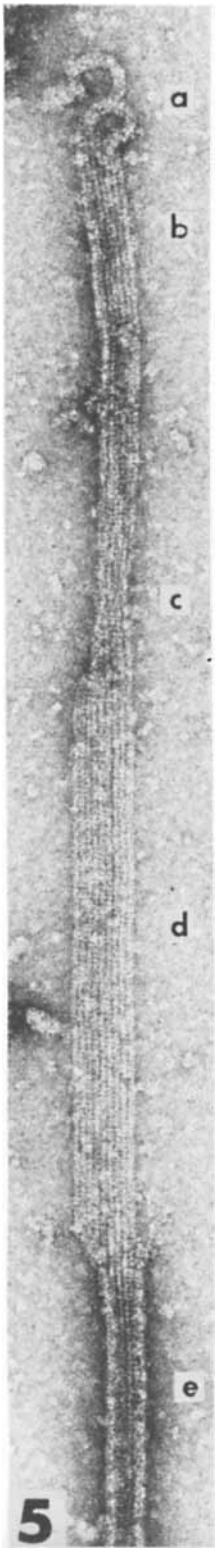

Fig. 5. Stages of microtubule assembly seen on a single structure. (a) rings at the growing tip; (b–d) incomplete, flattened microtubule wall; (e) intact microbutule. Discussion is given in the section on assembly from rings. Magnification, 175,000 ×.

coiled structure may be seen at f, which appears to be an oblique view of a coil. An even more striking image is that in the inset, which appears to be an unusually long coil lying on its side. Although the images presented here can generally be interpreted as single continuous filaments in a coil, similar micrographs obtained by Kirschner et al. (7) indicate that these coils can be transformed into concentric closed rings, especially after prolonged incubation in the cold.

The 50 Å thickness of the ring suggests that it might be related to a protofilament, which adopts a curved configuration when freed from the constraints of bonding to adjacent filaments in the microtuble wall. Observations of microtubules during disassembly by Kirschner et al. (7) and during assembly (reported below) strongly support this identification. The filament that constitutes the ring is apparently a segment of protofilament, which remains intact and coils when the microtubule disassembles and is reincorporated directly upon reassembly.

Assembly of Microtubules from Rings – Direct Incorporation as Protofilaments

Assembly experiments with the purified "ring" fraction showed that the tubulin in the ring aggregates was very active in assembly and could in fact account for a large part of the total assembly in the unfractionated tubulin preparation. Efficiency of assembly was determined by centrifuging the reassembled microtubules at 25,000 rpm for 30 min at 30°C. The supernatant and resuspended pellet were examined by spectrophotometry and SDS chromatography to determine the amount of tubulin reassembled. In undiluted "ring" fractions in which the tubulin concentration was 1 mg/ml, more than 75% of the tubulin was recovered in the microtuble pellet. Assembly could be obtained (although the efficiency was reduced to 25–50%) even after dilution to below 0.1 mg/ml, at which unfractionated tubulin would give no assembly.

The identification of the rings as curved protofilaments was confirmed, and their role in assembly indicated, by electron microscopy of negatively stained specimens prepared during assembly. As discussed above, the progression from small segments of the microtuble wall to the intact microtubule was seen most clearly in specimens prepared from dilute (0.1 mg/ml) "ring" fractions. In addition, images were frequently obtained from these preparations that showed rings actually attached to the growing microtubule wall. Figures 5 and 6 show several examples, in some of which a protofilament in the wall is seen to be continuous with the curved filament that constitutes the ring. Sometimes two or more protofilaments are continuous with a cluster of rings. It is from a study of these and similar images that the conclusion was formed that the ring is a curved, intact protofilament which uncoils and straightens out as it is incorporated into the growing microtubule wall.

Normally, the rings are found only on one end of the sheet, frequently in clusters of three or more, but cases with rings at both ends definitely occur. Rings are occasionally seen attached to the side of a sheet, continuous with a protofilament at the free edge (Fig. 6a, c). They are also found attached to the end of intact microtubules, and it seems likely that the curled structures noted by Borisy and Olmsted (2) are such rings or clusters. It is suggested here that the rings are found at the site of growth of the sheet or

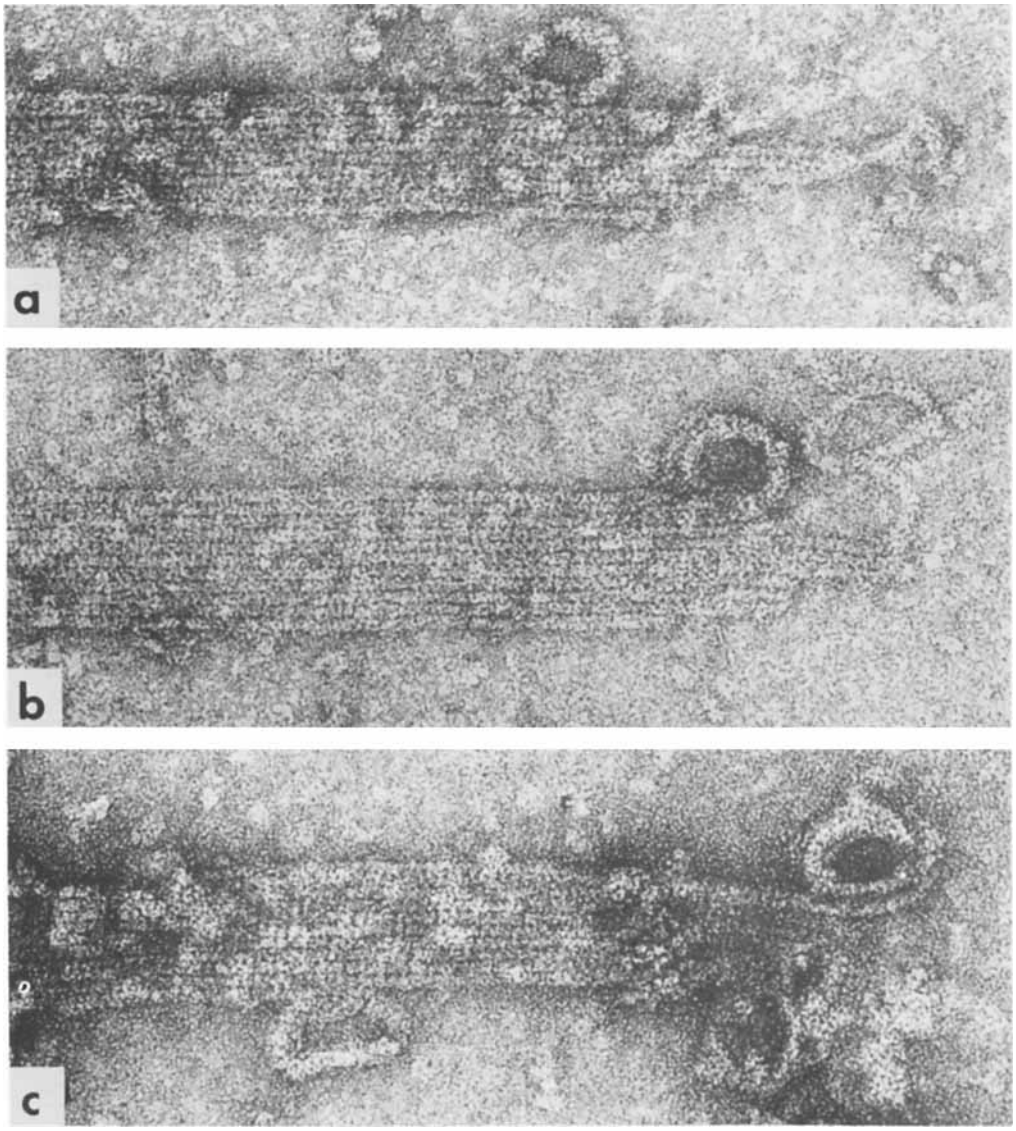

Fig. 6. Incomplete microtubule walls showing the attachment of rings and the continuity of rings with protofilaments. Specimens were prepared from a purified "ring" fraction, diluted to 0.1 mg/ml, 1.5–4 min after raising the temperature to 35° C. Magnification, 350,000 X.

microtubule, rather than at a site of initiation. The occurence of rings mainly at one end is consistent with the predominantly unidirectional growth of microtubules in dilute solutions of tubulin (20, 21, 22). The rarity of attachment to the side is consistent with the slower addition of new filaments relative to longitudinal growth in these dilute solutions of tubulin. The images presented here are all from specimens prepared during the first few minutes of assembly in dilute purified "ring" fractions. Similar pictures of rings and their attachment to sheets and microtubules during reassembly have been obtained from unfractionated tubulin preparations. They have also been found during reassembly from both "ring" fractions and "6-S" fractions after addition of glycerol.

It should be noted that although the rings usually appear in the images to be flattened in the plane of the growing sheet or tangential to the microtubule wall, they are sometimes seen to project perpendicular to the wall, toward the outside of the microtubule. The normal curvature is probably in this perpendicular direction, with the flattening occurring on specimen preparation. The negatively stained specimens of purified rings would then be a view of the protofilament perpendicular to that seen in the flattened microtubule wall.

In an independent investigation of the nature of the rings, Kirschner et al. (7) have examined specimens of microtubules in the process of disassembly. Images were obtained in which protofilaments from fraying microtubules could be seen coiling up to form rings, and the rings were thus identified as arising directly from protofilaments during disassembly. The experiments reported here confirm the identification of the rings as coiled protofilaments, and more importantly, they show that the rings are incorporated directly as protofilaments in the assembly of the microtubule wall.

Figure 5 shows a single growing microtubule in which all the stages of assembly, from rings to incomplete wall to intact microtubule, may be seen. At the top (a), two rings are associated with the sheet, the lower one being continuous with the protofilaments of the incomplete microtubule wall. The wall seems to have seven protofilaments at this point (b). It then twists (c) and becomes wider, having nine protofilaments at d. At e, the wall has apparently reached its full complement of 13 protofilaments and has curled and sealed to form a normal intact microtubule.

Contribution of 6-S Tubulin to Assembly

In the chromatography experiments most of the tubulin was recovered in the "6-S" fraction, but this material was never observed to produce microtubules when the fraction was simply warmed to 35°C. However, when some of the "ring" fraction was added to the "6-S" fraction, the 6-S tubulin could be demonstrated to contribute to assembly, provided the chromatography was done rapidly so that assembly experiments could be performed 3–5 hr after starting the chromatography.

In a typical experiment the tubulin concentration in both the "ring" and "6-S" fractions was 0.7–1.5 mg/ml. Equal aliquots of the "ring" fraction were added to different amounts of 6-S tubulin and to plain buffer as a control, to give a dilution of the rings of 1/2 to 1/10. The mixtures were incubated at 35°C for 15 min, and negatively stained specimens were prepared. A semiquantitative assay of the extent of assembly was

obtained by noting the density of microtubules in these specimens. For samples in which rings were diluted into buffer the density of assembled microtubules decreased roughly in proportion to the dilution. In each case, however, the sample diluted into 6-S tubulin showed a significantly greater (two to five times) density of microtubules than the control diluted into buffer. Both an increase in average length and in total number of microtubules may be responsible for the increased density, but the analysis has not been sufficiently quantitative in these preliminary experiments to distinguish these two.

The assembly has also been assayed by spectrophotometry and SDS gel electrophoresis of the pellets and supernatants after centrifugation. Preliminary results confirm the electron microscope observations that 6-S tubulin contributes to assembly. In one experiment approximately 40% of the 6-S tubulin was lost to the microtubule pellet when the "ring" fraction (1 mg/ml) was diluted 1/2 to 1/5 into 6-S tubulin (1.5 mg/ml). When smaller amounts of "ring" fraction were added (1/10 to 1/30 dilution) more of the 6-S tubulin remained in the supernatant, indicating that the contribution of 6-S tubulin to assembly was limited by the concentration of rings. This may imply that the assembly is more complicated than a simple nucleation by rings. Alternatively, it may be due to rapid inactivation of the 6-S tubulin at 35°C, competing against a slower rate of initiation of assembly at the greater dilutions of rings. A quantitative assay of assembly with different proportions of rings and 6-S tubulin is in progress.

The Effect of Glycerol – Rings from 6-S Tubulin

Since glycerol had been shown to enhance microtubule assembly (16), its effect was tested on the separate fractions from chromatography. In all cases, cold glycerol was added to the fractions to a concentration of 4–6 M before raising the temperature to 35°C. With the "ring" fraction there was no effect on the extent of assembly: 75% of the tubulin centrifuged into the microtubule pellet both with and without glycerol. The mode of assembly was changed somewhat in that specimens from the glycerol mixture showed predominantly opened or incomplete microtubule walls, rather than intact microtubules. This has been noted in previous studies with unfractionated tubulin (16, 4).

When glycerol was added to the 6-S tubulin, microtubule assembly was obtained from the previously inactive fraction. Since the glycerol thus seemed to induce the 6-S tubulin to form microtubules without any requirement for added rings, it was important to determine whether the treatment induced the formation of rings at 0°C. Specimens prepared in the cold at different times after addition of glycerol showed that rings were indeed formed. Specimens prepared after 2 min showed rings and many partial rings (curved filaments forming 1/4–3/4 of the circumference of a normal ring, Fig. 4a and b). After 10 min, the number of rings had increased and the number of partial rings had decreased somewhat. There was little further change over 2 hr at 0°C. Microtubule assembly at 35°C appeared to be the same (about 10% pelleted) regardless of the time the sample was previously kept with glycerol at 0°C. Thus, the primary effect of the glycerol may be to induce the 6-S tubulin at 0°C to form rings which are more stable and more active in assembly and which appear to be necessary for 6-S tubulin to contribute to assembly.

Stability and Interconversion of 6-S Tubulin and Rings

The chromatographically purified "ring" fraction is relatively stable at 0°C and retains competence for reassembly for several days. The 6-S tubulin appears to be much more labile. Its competence to contribute to assembly (after the addition of rings) was lost about 10 hr after chromatography was begun. The effect of glycerol in inducing rings and subsequent microtubule assembly from 6-S tubulin could usually be obtained even after overnight chromatography, but was lost after 15–20 hr.

The chromatographic separation of rings and 6-S tubulin demonstrated in these experiments indicates that there is no significant interconversion of the two forms during the time of chromatography, either 3 or 12 hr. This seems at first to contradict evidence based on sedimentation velocity analysis of unfractionated tubulin (7, 3a) that 6-S tubulin rapidly establishes an equilibrium with 20-S to 36-S aggregates at 0°C. The apparent stability of the rings and 6-S tubulin in the chromatographic separation may be considered separately.

The rings isolated by chromatography do correspond in size to the 36-S aggregates (7), but they are obviously quite stable and break down to 6-S tubulin only after a period of days. It seems, therefore, that there may be a transition from the labile aggregates formed in equilibrium with 6-S tubulin to the stable rings isolated by chromatography. The aggregates are probably all closely related to the stable ring form, but the nature of the transition or stabilization is not known. Any labile aggregates could break down completely to 6-S tubulin as they are diluted during the chromatographic separation.

The isolation of a fraction of 6-S tubulin free from any rings or other aggregates may be due to the relatively low concentration of active tubulin in these fractions. In a typical experiment the maximum protein concentration in the eluted "6-S" fraction is about 1–1.5 mg/ml, of which less than half can contribute to assembly. Borisy and coworkers (3a) have observed less than 5% aggregates at a tubulin concentration of 1 mg/ml, so the concentrations obtained in the chromatography experiments may be below a threshold for ring or aggregate formation. The 6-S tubulin in these fractions is still competent to form rings when glycerol is added. The effect of the glycerol in these experiments may be simply to shift the equilibrium toward aggregation, causing the formation of rings at a lower tubulin concentration. Mention should also be made of a number of "unsuccessful experiments" in which the leading "6-S" fractions gave microtubule assembly at 35°C without any addition. In every such case, electron microscopy showed that these leading fractions of the "6-S" peak were contaminated with a small number of rings and partial rings. These results were obtained in several cases when a larger than normal amount of tubulin was applied to the column; perhaps they reflect equilibrium conditions at higher tubulin concentrations.

A Tentative Model for Assembly: Initial Wall Formation and Elongation

In the experiments presented here, 6-S tubulin has been demonstrated to contribute to assembly, but only after assembly has been initiated by addition of rings. In other investigations, 6-S tubulin obtained as a supernatant from a high-speed centrifuga-

tion was found to assemble onto fragments of microtubules added as seeds or nucleation centers (20, 21, 22). Thus it seems that 6-S tubulin can contribute to growth of existing microtubules by elongation but may be incapable of supporting an initial stage of assembly. It is suggested here that this stage may be the formation and growth of the incomplete microtubule wall and more specifically, that the unaggregated 6-S tubulin may be incapable of attaching to the side of the wall to start the formation of a new protofilament. In this case, the incorporation of the preformed protofilaments from the ring aggregates may be the only mechanism by which the wall can grow laterally and would be essential for the formation of the intact helical lattice.

A simple rationale for this suggestion may be given in terms of the bonding patterns available to the subunits in the growing microtubule. It was pointed out in the discussion of structure that there are two types of bonds between subunits: longitudinal bonds within a protofilament and lateral bonds between adjacent protofilaments. The longitudinal bonds are probably the stronger of the two, since it is these that are maintained, or are reformed from 6-S tubulin, when the microtubule is disrupted at 0°C. The basis of the argument is that there must always be one point at the growing end of an intact helical lattice at which a newly attached subunit could form both a longitudinal and a lateral bond. In a multiple-start helical lattice, one would expect several such points. Attachment of subunits would be much more favorable at sites where two bonds could be formed than at sites where only a single longitudinal or lateral bond could be formed. Under the conditions of assembly obtained in these experiments, 6-S tubulin may attach only to the more favorable sites. In this case, it would not be competent to generate the initial assembly and growth of the incomplete microtubule wall. Before the wall closes to make the intact microtubule, sites with two bonds available would be filled in quickly, leaving a sheet with smooth edges. Further growth of this sheet would be impossible, since subunits attaching to the edge could form only a single bond. Circular growth would be especially unfavorable, since only weaker lateral bonds would be formed. Thus, the 6-S tubulin could contribute to elongation of intact microtubules, attaching to the sites with two available bonds, under conditions where it could not produce assembly of an unclosed segment of the microtubule wall.

The initial segment of intact, cylindrical microtubule must then be produced by some mechanism other than assembly of individual subunits. It is suggested here that assembly of the incomplete microtubule wall from rings, as described above, may provide such a mechanism. In this preformed protofilament the longitudinal bonds are already formed, and when it attaches to the growing wall a large number of lateral bonds could be formed, perhaps in a cooperative manner. This would be much more favorable energetically than the formation of a single bond in the attachment of an individual subunit. Thus, the preformed, ring-shaped protofilaments would be more active in all stages of assembly and would be essential for the initial assembly and growth of the microtubule wall. The steps in assembly proposed in this model are indicated schematically in Fig. 7.

This model of assembly is, of course, rather speculative at present, but it provides a rationale based on simple principles of self-assembly for understanding the assembly experiments with 6-S tubulin and rings. The key point of this model is the suggestion

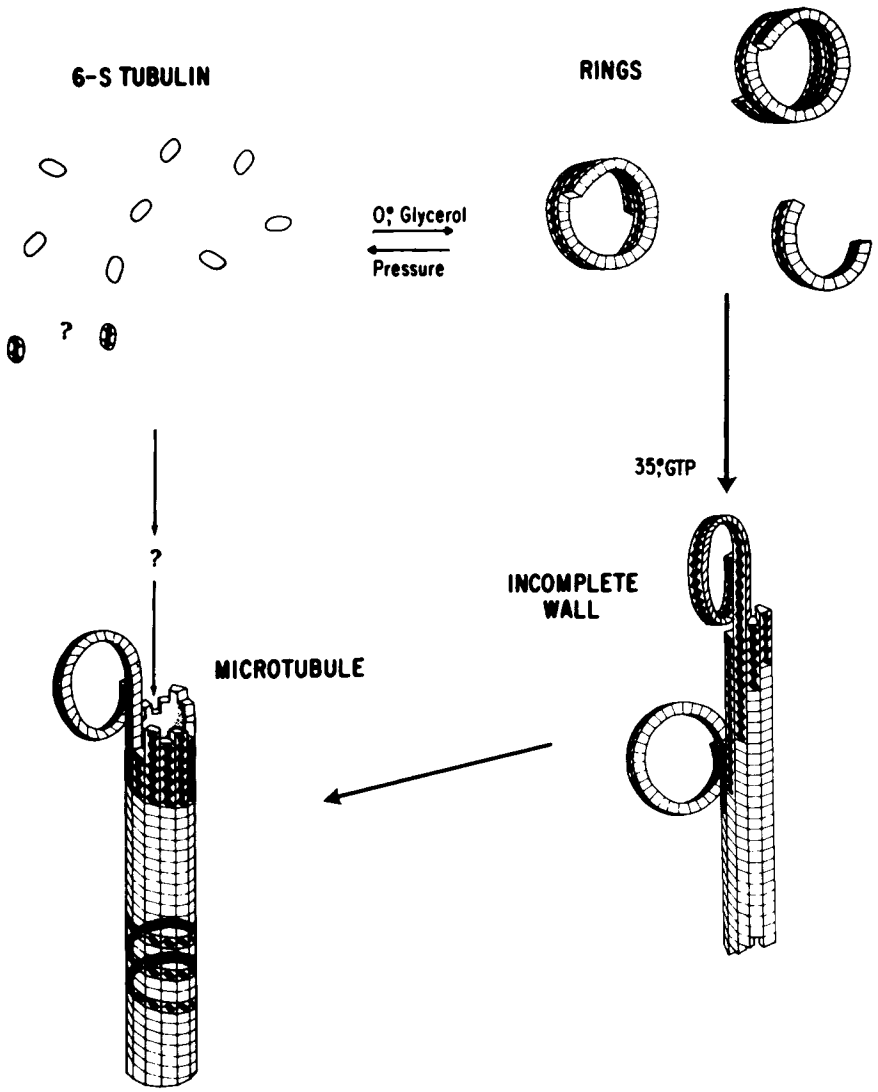

Fig. 7. Schematic summary of microtubule assembly in the in vitro system. The model presented here is speculative in several respects, some of which are indicated by question marks. The most important features for which experimental evidence is strongest are the formation of rings from 6-S tubulin and the direct incorporation of rings into the growing incomplete wall and intact microtubule. Very little is known about the structure of 6-S tubulin, and the details of its incorporation and bonding are quite speculative.

that assembly begins with the incomplete microtubule wall and that the preformed protofilaments provide a mechanism for the energetically unfavorable circular growth. Both rings and 6-S tubulin may then participate in elongation. Quantitative measurements of assembly with different proportions of rings and 6-S tubulin, under different conditions of initiation and assembly, will be important in confirming the proposal and in refining details. It should be emphasized that details of the attachment and uncoiling of rings are obscure at present, and even less is known about the structure and incorporation of 6-S tubulin. Present thinking would suggest that the 6-S tubulin is an α-β dimer, consisting of two of the 40 × 50 Å morphological subunits connected by a longitudinal bond. Such a structure is consistent with the assembly mechanism proposed here, but to specify these details in the model seems premature until the identification of the dimer is better established.

The model of assembly presented by Kirschner et al. (7) on the basis of their independent investigation agrees with the identification of rings as intact protofilaments. The role of 6-S tubulin is somewhat different in their model in that they suggest that 6-S tubulin is chemically and functionally different from the tubulin subunits in the rings. Kirschner and Williams (7a) have presented evidence that the differences are retained when the tubulin is assembled into microtubules and subsequently released during disassembly. The suggestion that the differences in the two states are retained when the subunits are incorporated in the microtubule lattice is disturbing since the subunits originated as identical polypeptide chains and occupy equivalent positions in the helical lattice. This would be a significant departure from the principals of construction of regular structures (23). It would be particularly surprising, for example, to find that 6-S tubulin and "ring" tubulin (their X and Y state molecules) are differentially involved in lateral and longitudinal bonding, as they suggest. This would produce a microtubule that is mechanically and chemically less stable than one with identical lateral and longitudinal bonds for all subunits. The difference between 6-S tubulin and that isolated in the stable "ring" fraction by chromatography is an important question that requires further consideration.

The most obvious alternative to the model of assembly in which an incomplete wall is a precursor to the intact microtubule would be initiation of assembly by a preformed circular organizing center, or nucleation site. Such initiation could obviate the need for lateral growth of the microtubule wall, but there are conceptual difficulties in constructing a circular nucleation site. Of particular importance is the need to initiate three turns of the three-start helix (or five turns if α-β dimers constitute the basic subunit) and propagate them until a full turn of the lattice is completed. It is difficult to imagine anything other than a segment of intact microtubule serving this role. The model presented here, in which the microtubule segment is formed by addition of protofilaments to an incomplete wall, is conceptually straightforward and consistent with data presently available on in vitro assembly.

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