

# ELECTRON MICROSCOPE STUDIES OF pH EFFECTS ON ASSEMBLY OF TUBULIN FREE OF ASSOCIATED PROTEINS

## Delineation of Substructure by Tannic Acid Staining

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### ABSTRACT

Bovine brain tubulin, purified by phosphocellulose chromatography (PC), was assembled in the presence of 10% dimethyl sulfoxide (DMSO), and the reaction was monitored turbidimetrically. Samples were fixed in glutaraldehyde-tannic acid after completion of polymerization, as indicated by no further change in absorbance, and then sectioned and studied by electron microscopy, with special attention being given to the arrangement of protofilaments in the walls of formed elements. Samples of PC-tubulin were polymerized in buffer having various pH values from 6.0 to 7.7. At the lower pH values, only branched and flattened ribbons of protofilaments are formed. At intermediate values, the ribbons are unbranched, narrower, and more curved in cross section; complete microtubules are also seen. At the higher pH values, the predominate formed elements are complete microtubules. Most of the complete microtubules examined in this study had 14 wall protofilaments. The effect of pH on tubulin assembly was shown not to be an effect of DMSO. The dimers of associated protofilaments in ribbons and microtubules are conceptually viewed as having trapezoidal profiles in cross section, and, as additional dimers are added, the "C"-shaped ribbon closes to form a tube. The tilt angle of the lateral surfaces of the "trapezoidal" dimers will determine the number of wall protofilaments in the microtubules. At low pH, it is theorized that the trapezoidal profile of the dimer is shifted to a more rectangular configuration such that flat ribbons are formed by the lateral association of dimers. Also, variously shaped ribbon structures are formed at intermediate pH values, including "S"- and "W"-shaped structures, and elements shaped like a figure "6," all representing ribbons viewed in cross section. By visualizing the trapezoidal dimer in three-dimensions, and by arbitrarily indexing its six binding surfaces, it is possible to discuss interdimer binding in terms of preferred and possible binding interactions.

KEY WORDS tubulin · microtubular  
elements · pH effects · substructure · tannic  
acid

The self-assembly of tubulin into microtubules

and the possible factors that may modulate such assembly have been given much consideration in the past few years. Proteins that copurify with tubulin facilitate assembly of the dimer into complete microtubules in vitro (4, 12, 18, 24). Puri-

fied tubulin lacking "microtubule-associated proteins," or MAPs, is unable to polymerize at protein concentrations routinely used for assembly studies (1-5 mg/ml), although addition of isolated MAPs induces tubulin to assemble into microtubules (8, 18, 24). It has been reported that MAP-free tubulin can be induced to polymerize in the presence of high magnesium and glycerol concentrations (15), and we have recently demonstrated that, in assembly medium containing 10% dimethyl sulfoxide (DMSO), highly purified tubulin obtained with phosphocellulose chromatography (PC-tubulin) rapidly polymerizes into microtubular elements (10,11).

The assembly of microtubules from tubulin containing MAPs has been studied by a number of workers. At low temperature before polymerization, much of this tubulin is in the form of ringlike structures (3, 5, 14); upon raising the temperature, these structures appear to be converted into ribbons of parallel protofilaments, and there is evidence that the ribbons then close to form complete microtubules (9, 13). Recently, Bryan (6) studied microtubule elongation by using a polyanion (polyadenylic acid) to retard tubulin assembly in a controlled manner, and he concluded that the rings are not obligatory intermediates in microtubules but are probably cold-stable aggregates of tubulin.

The research described in the present paper is directed toward studying the assembly of MAP-free tubulin in 10% DMSO solutions. Although MAP-free tubulin assembles in the absence of DMSO, the protein concentration necessary for assembly is lower in the presence of this solvent (10, 11). Most of the observations were made with sectioned material fixed in glutaraldehyde-tannic acid (7, 17, 23) for the following reasons: (a) microtubule wall protofilaments can be visualized and counted, and (b) it is possible to obtain more three-dimensional information on the structure of formed elements than by negative staining, which has been the method used by others in studying assembly of tubulin into microtubules.

In previous studies we observed an abundance of variously shaped ribbons of protofilaments in samples of PC-tubulin polymerized with 10-20% DMSO (10, 11). The present study shows that by adjusting the pH of assembly one can preferentially induce formation of branched ribbons of protofilaments or complete microtubules; by studying the protofilamentous structures formed between pH 6.0 and 7.7, it is possible to predict certain preferred and possible binding interactions

between tubulin dimers composing the protofilaments.

## MATERIALS AND METHODS

A modification of the method of Shelanski et al. (22) was used to isolate tubulin from the brains of freshly slaughtered beef cattle, as previously described (10, 11). Highly purified tubulin devoid of contaminating accessory proteins was obtained from tubulin by phosphocellulose chromatography and then polymerized in assembly buffer containing 10% DMSO (10, 11). The buffer systems generally used for assembly of tubulin were: (a) 20 mM 2-[*N*-morpholino]ethane sulfonic acid (MES), 0.1 M NaCl, 1 mM ethyleneglycol bis[ $\beta$ -aminoethyl ether]*N,N'*-tetraacetic acid (EGTA), 0.5 mM MgCl<sub>2</sub>, 0.5 mM guanosine 5'-triphosphate (GTP), at pH 6.5 (this buffer system is hereafter designated MES-NaCl) and (b) 0.1 M piperazine-*N,N'*-bis[2-ethane sulfonic acid] (PIPES), 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, 0.5 mM GTP, at pH 6.9 (hereafter designated PIPES buffer). Changes in the composition and pH of these assembly buffers will be set forth in the text as appropriate.

Polymerization reactions were monitored at 37°C by recording turbidity (absorbance) changes at 350 nm in a Gilford 2000 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) or Hitachi 100 spectrophotometer. Protein concentrations usually ranged from 1 to 2 mg/ml. To fix elements formed during or upon completion of assembly for electron microscope examination, 5  $\mu$ l of 25 or 50% aqueous unbuffered glutaraldehyde was added to 0.5-ml samples of assembly buffer containing polymerizing tubulin, and the solutions were quickly mixed. For tannic acid staining (7, 23), 1.5 ml of 8% tannic acid (Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.) in 0.05 M phosphate buffer (pH 6.8) was added to the sample 5 min after glutaraldehyde was added.

Our choice of fixation methods was based on several preliminary studies. Concentrated glutaraldehyde was used to stop assembly reactions and fix formed elements in order to avoid excessive dilution of the assembly medium. It was considered possible that, depending on concentration, glutaraldehyde might cross-link ribbons of protofilaments to form more complex structures of an artifactitious nature. In studies with fixative concentrations of glutaraldehyde from 25  $\times$  10<sup>-2</sup>% to 25  $\times$  10<sup>-5</sup>%, no differences were noted between *in vitro* polymerized structures as studied in tannic acid-stained, sectioned material and in negatively stained material. It was found desirable to fix assembled microtubular elements before centrifugation in order to avoid the possibility that centrifugal forces might induce packing and even binding between formed elements which did not exist before their sedimentation (also, staining with tannic acid is better achieved before rather than after sedimentation).

Material was fixed with glutaraldehyde-tannic acid (GTA) for at least 1 h at room temperature and sedi-

mented at 40,000 g for 30 min; the supernate fluid was poured off and 1% osmium tetroxide in 0.05 M phosphate buffer was added to the centrifuge tube, after which the sedimented material was carefully teased from the inside of the tube into suspension. After secondary fixation for 60 min, the sample was rapidly dehydrated in an acetone series and embedded in Araldite. Sections were obtained with a diamond knife, collected on 400-mesh uncoated specimen screens, and stained in methanolic uranyl acetate followed by lead citrate as previously described (7).

Material was examined with a Phillips 300 electron microscope, and micrographs were obtained at initial magnifications of up to 88,000 diameters.

## RESULTS

To study the effects of the pH of the assembly buffer on tubulin polymerization, samples of PC-tubulin were polymerized with 10% DMSO under numerous buffer conditions with a range of pH values from 6.0 to 7.7. Counts were made of ribbons and complete microtubules in electron micrographs of sectioned material that had been fixed in GTA. The results are shown in Table I.

With use of 20 mM or 0.1 M MES buffer at pH 6.0–6.2, only ribbons are seen (Figs. 1, 2, and 3). At pH 6.0, such structures generally appear in small, well-defined groups (Fig. 1); between the groups, some amorphous material can be seen, as well as a few flattened ribbons. The appearance of the groups of ribbons is reminiscent of that of a branching (dendritic) salt crystal. Upon closer inspection of such groups, highly branched ribbons of protofilaments are seen, the protofilaments having a center-to-center spacing of about 45 Å (Figs. 2 and 3). The ribbons are generally flattened and may form broad sheets composed of as many as 100 protofilaments. In a branched structure, the axes of the protofilaments of interconnected ribbons always appear to parallel one another. Individual protofilaments or small groups of protofilaments may be adherent to the ribbons (Figs. 2 and 3). Single adherent protofilaments appear flattened in the plane of the ribbon, and most often they lie directly over the junction between two adjacent protofilaments of the ribbon. In branched ribbons, a single protofilament may provide the center or branch point for up to four ribbons (Fig. 3), and the "treelike" appearance of many groups suggests that growth may have started at the base of a central sheet which formed the "trunk" of the tree; ribbons branch off from one another at various angles, and no consistent branching pat-

tern is observed. At the edges of many of the ribbons extending to the free margin of a group of branched elements, the protofilament structure is lost, and the ribbon trails off into amorphous material (Figs. 2 and 3), and this appears not to be due to the plane of the section.

At pH 6.5 in 0.1 M PIPES-DMSO buffer, the predominate elements formed by PC-tubulin are ribbons, although these are not so elaborately branched as at lower pH values—also, about 35% of the structures formed are complete microtubules, some of which may be associated with ribbons (Fig. 4). The ribbons formed are not so wide as those formed at pH 6.0–6.2, and they are much more curved in cross section; many ribbons are attached to one another in various configurations, and "C"- and "S"-shaped configurations are also seen. In complete microtubules where protofilaments can be counted, 14 subunits are most often seen.

When PC-tubulin is polymerized in 0.1 M PIPES-DMSO buffer at pH 6.9, many more complete microtubules are seen than at pH 6.5. In the first of three samples examined in this set of experiments, 47% of the formed elements were complete microtubules. In this sample run, however, there was reason to believe that the pH of the assembly medium may have been lower at completion of polymerization than at the beginning. Subsequently, two additional samples were examined on different occasions, the samples showing 74% and 78% complete microtubules. In numerous other samples polymerized under these conditions, it was generally observed that over half the formed elements were complete microtubules, although exact counts were not obtained. The number of protofilaments in such microtubules varies, but most microtubules have 14 (Figs. 5, 6, 8, and 10). The ribbons in these samples are relatively narrow and highly curved in cross section, and many are attached to one another (Figs. 8–10). In addition to having other more complex configurations, ribbons may appear in the shape of a "C" (Figs. 8 and 9), an "S" (Fig. 7), a "W" (Fig. 9), or a figure "6" (Fig. 10). Often, as in samples polymerized at pH 6.5, PC-tubulin polymerized at pH 6.9 forms complete microtubules with attached ribbons (Fig. 10). Fig. 6 shows a microtubule with two extra protofilaments adherent to the inner surface of its wall. If the concentration of PIPES buffer is raised to 0.4 M, at pH 6.9 very few complete microtubules are formed; rather, groups of narrow but curved

TABLE I  
Effects of pH on Assembly of Purified Tubulin in the Presence of 10% DMSO

pH and buffer system	Ribbons and incomplete microtubules	Complete microtubules	Complete (microtubules/total) %	Refers to Figure
pH 6.0, 20 mM MES-0.1 M NaCl	Groups of rebranched ribbons	None	—	1
pH 6.1, 20 mM MES-0.1 M NaCl	Groups of rebranched ribbons	1	—	
pH 6.2, 0.1 M MES (X2)*	Groups of rebranched ribbons	None	—	2, 3
pH 6.5, 20 mM MES-0.1 M NaCl	116	73	38	
pH 6.5, 0.1 M PIPES (X2)*	60 78	31 49	34 48	4
pH 6.9, 0.1 M PIPES (X3)‡	112 47 45	99 139 159	47 74 78	5-10
pH 6.9, 0.4 M PIPES	Groups of ribbons curved in x-section	1	Estimated less than 2	11
pH 7.1, 0.1 M PIPES				
Early§	45	59	56	12
Mid	34	33	49	
Late	54	104	66	13
pH 7.5, 0.1 M PIPES	13	80	86	
pH 7.7, 0.1 M PIPES	26	127	83	14

Tubulin samples were polymerized to completion (no further increase in absorbance) and fixed as described in Materials and Methods. Two or more embedded pieces from each sedimented sample were examined; if the formed elements differed in some way between the two specimens, additional pieces of that sample were sectioned and examined until a representative set of micrographs was obtained. All samples were coded and then processed and examined in a blind manner. In micrographs taken at magnifications of 33,000–54,000 diameters, ribbons and microtubules were counted only if they were transversely sectioned such that at least some of their protofilaments could be clearly seen. Microtubules with incomplete walls were counted as ribbons, and if doubt existed as to whether a microtubule was complete, e.g., whether one protofilament was missing, the microtubule was not counted at all. A complete microtubule with a ribbon or ribbons adherent to its surface was counted as one microtubule and one ribbon. Attached ribbons, e.g., overlapping or attached at right angles, were counted as one. Formed elements were studied in numerous other samples polymerized under the above conditions, but comparative counts were obtained only in this series of studies dealing with effects of pH on formation of ribbons and microtubules.

\* Two different samples polymerized on separate occasions.

‡ Three different samples polymerized on separate occasions.

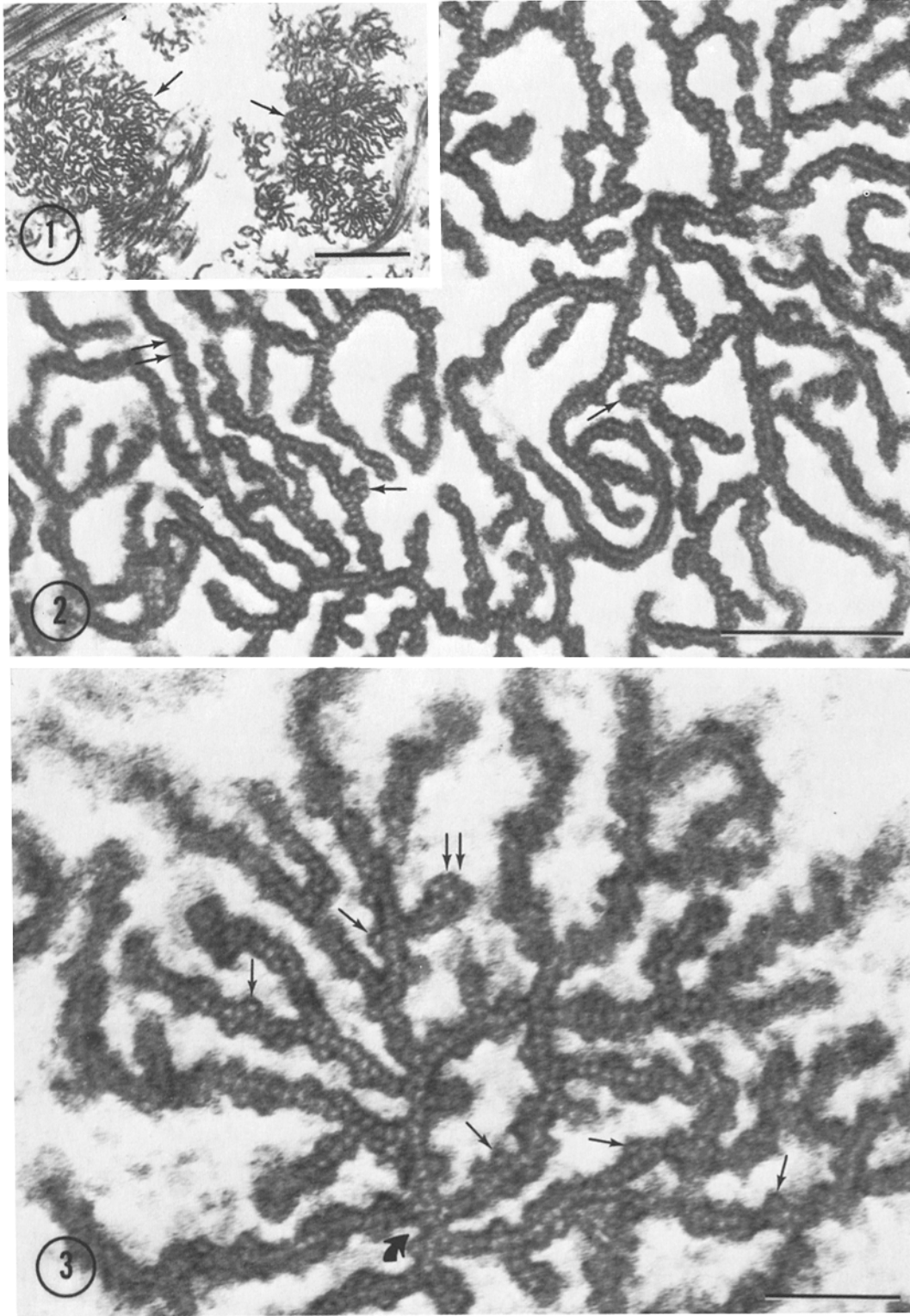
§ Refers to examination of sample at beginning, midway, and at completion of polymerization as determined by absorbance.

ribbons are seen which often are adherent to one another to form complex configurations (Fig. 11).

At pH 7.1, PC-tubulin polymerized in 0.1 M PIPES-DMSO buffer shows about as many complete microtubules as at pH 6.9, but the ribbon structures tend to be less complex. Samples taken at different time-points during polymerization showed that early in assembly the percentage of ribbons and microtubules is about the same while at completion of assembly 66% of the formed elements are complete microtubules. Early in polymerization, numerous "C"- and "S"-shaped ribbons are seen, along with abundant aggregate

material (Fig. 12); upon completion of polymerization, numerous microtubules show ribbons attached to their surfaces, and there is much less aggregate material in the background (Fig. 13). If PC-tubulin is polymerized in 0.1 M PIPES-DMSO at pH 7.5 or 7.7, the number of ribbons is greatly reduced, and complete microtubules comprise 80–90% of all formed elements. Most of the ribbons present in this material are "C" shaped (Fig. 14). Counts of wall protofilaments in microtubules formed at high pH show that in most cases 14 are present.

Data presented in Table I show that elevated



pH enhances formation of microtubules rather than ribbons in this system. To determine whether the phenomenon could be reversed, samples of the PC-tubulin were polymerized with DMSO at low pH, then dialyzed against buffer-DMSO at (a) the same pH or (b) at a higher pH. Dialysis was performed at 37°C for 1 h, and upon completion of dialysis the final pH of the sample was measured before fixation. If tubulin is polymerized in 0.1 M MES-DMSO at pH 6.2, then dialyzed for 1 h against the same buffer at pH 6.2, only ribbons are observed in tannic acid-stained and sectioned material. If dialysis is performed against 0.1 M PIPES-DMSO buffer at pH 7.7, a pH of 6.9 was attained in the sample, and 46% of the formed elements were complete microtubules. In another experiment, tubulin was polymerized in 0.1 M PIPES-DMSO buffer at pH 6.5, then dialyzed against buffer at pH 6.5, and 27% of the formed elements were complete microtubules. In the sample prepolymerized in the same manner but dialyzed against buffer at pH 7.5, a pH of 7.2 was attained within the sac, and examination showed that 33% of the formed elements were microtubules. Although reversibility studies were not pursued, the evidence suggests that by raising the pH of the buffer medium preformed ribbons are replaced by complete microtubules. Whether this occurs by direct conversion or by disassembly and reassembly is not known.

Biochemical studies of the self-assembly of PC-tubulin indicate that the presence of DMSO raises the optimal pH value for assembly of complete microtubules (10). To determine that ribbon

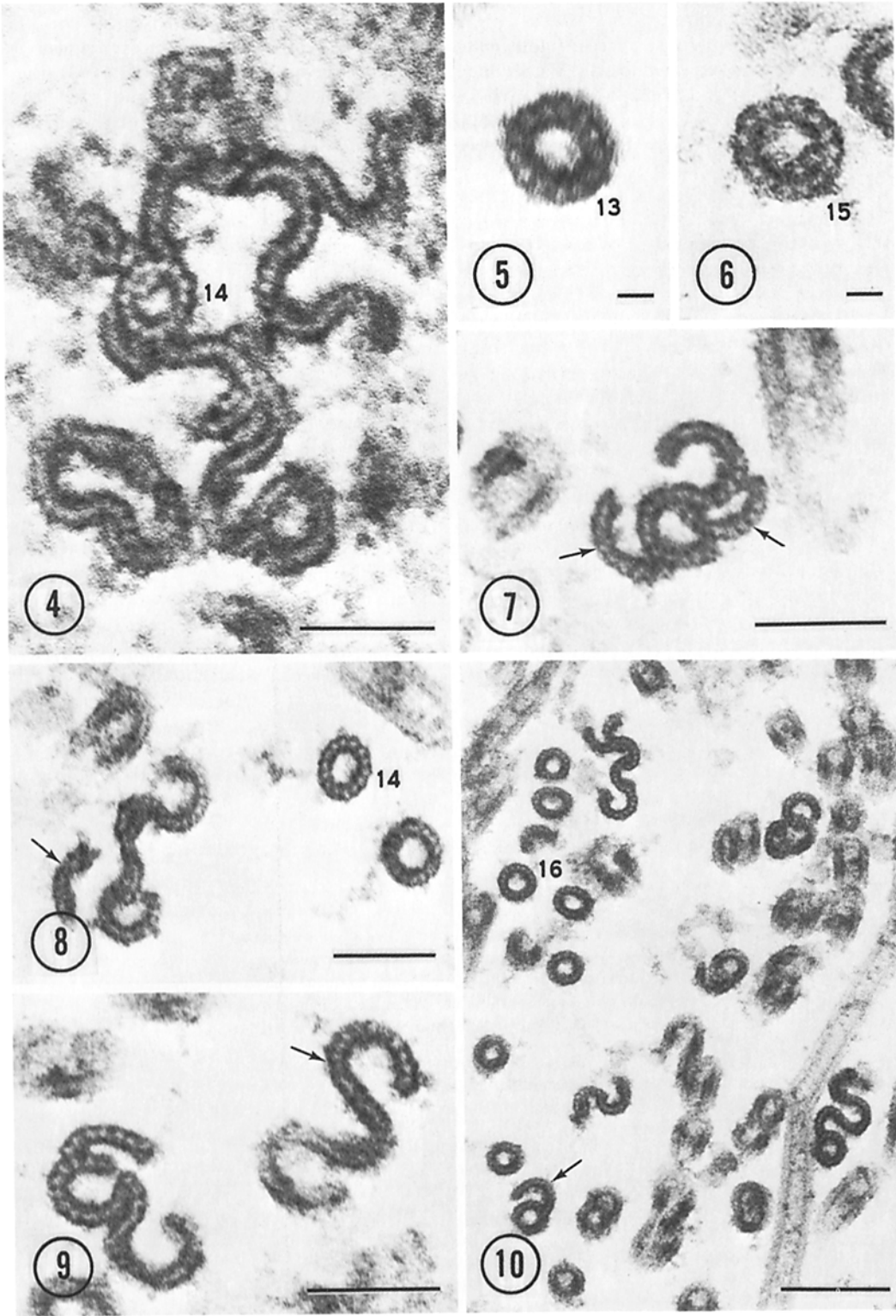
formation is not solely due to the presence of DMSO, PC-tubulin was polymerized to completion in 0.1 M PIPES buffer containing 4 M glycerol, the buffer being adjusted to pH 6.43 for one sample and to pH 7.25 for the other sample. Formed elements were fixed in GTA, sedimented, and ultimately sectioned for examination in the electron microscope. At the lower pH value, ribbons were abundant and complete microtubules represented only 15% of the formed elements ( $n = 164$ ). At the higher pH value, however, 92% of the formed elements were complete microtubules ( $n = 184$ ), although ribbons were attached to some of the microtubules (Fig. 15*a-e*) to give rise to configurations often seen when PC-tubulin is polymerized in the presence of DMSO. Most of the microtubules formed in both samples showed 14 wall protofilaments. The microtubule-ribbon structures formed by PC-tubulin assembled at pH 7.25 in the presence of 4 M glycerol were unusually well-stained, and micrographs from this material are used to illustrate the frequently observed configurations encountered throughout this study. In other words, the protofilament configurations shown in Fig. 15*a-e* are often seen after polymerization of PC-tubulin under buffer conditions that induce formation of both ribbons and microtubules. The figures illustrate some of the various ways in which ribbons and microtubules can bind with one another to form complex structures. Fig. 15*a* shows an 8-protofilament ribbon attached to a 13-protofilament microtubule to form a figure "6;" note that the proximal or attached end of the ribbon involves a ribbon protofilament which binds to the

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**FIGURE 1** Low-magnification view of groups (arrows) of branched ribbons of protofilaments polymerized from PC-tubulin in 20 mM MES-DMSO at pH 6.0. Polymerization was carried out to completion, i.e., until no further increase in absorbance occurred. The groups generally occur as separate and distinct units and give the overall appearance of having grown as would crystals. GTA;  $\times 28,000$ . Bar, 0.5  $\mu\text{m}$ .

**FIGURE 2** Branched ribbons from PC-tubulin polymerized to completion in 0.1 M MES-DMSO at pH 6.2. Note the flatness of the wide ribbons, e.g., the ribbon shown by the double arrow. Two small masses of protofilaments are shown by the small arrows. GTA;  $\times 276,000$ . Bar, 0.1  $\mu\text{m}$ .

**FIGURE 3** High-magnification view of a branched structure formed by polymerization to completion of chromatographed tubulin in 0.1 M PIPES-DMSO at pH 6.2. Note the distinct protofilaments in the flattened ribbons. The large arrow indicates a region with four branch points from a single protofilament. The small arrows indicate some of the single protofilaments adherent to the surfaces of ribbons, and the double arrows show a knoblike structure formed by a small group of protofilaments at the edge of a ribbon. Note the way in which the ribbons near the edge of the group (top) trail off into amorphous material. Center-to-center spacing between adjacent protofilaments of this structure is about 45 Å. GTA;  $\times 415,000$ . Bar, 0.05  $\mu\text{m}$ .



wall of the microtubule at the junction of two wall protofilaments. Fig. 15*b* shows a "piggy-back" configuration formed by a 7-protofilament, "C"-shaped ribbon attached to a 14-protofilament microtubule. One end of the "C"-shaped ribbon is attached at the junction of two microtubule protofilaments, while the other end of the ribbon appears to be attached to the outer surface of a single protofilament of the microtubule wall. Fig. 15*c* depicts an "earmuff" configuration in which two microtubules are bridged by a 6-7-protofilament ribbon. An overlapping between adjacent ribbons is seen in Fig. 15*d*, where "C"-shaped ribbons adherent to two microtubules overlap and appear to be bound together by two protofilaments (star). If the concave surface of the ribbons is considered the inner surface, then the two terminal protofilaments of each of the ribbons are linked by their inner aspects. Fig. 15*e* shows a configuration that suggests interlocking rings formed by two microtubules; in this case, various interpretations of protofilament relationships are possible, but one can best begin by viewing the microtubules as "C"-shaped elements that share in common a complex wall of several protofilaments.

## DISCUSSION

Our results demonstrate that changes in the hydrogen ion concentration in the pH region from 6 to 7.7 exert a great influence on the type of formed element produced by the self-assembly of MAP-free tubulin. This effect is seen whether 10% DMSO or 4 M glycerol is used to stimulate

polymerization. By examining negatively stained material, Matsumura and Hayashi (16) found that bovine brain tubulin containing MAPs forms ribbons of protofilaments when assembled at pH 5.8-6.2. Further, ribbons formed at pH 5.8-6.0 were wider than those at pH 6.0-6.2. Above pH 6.2, microtubules were produced. Thus, the assembly of tubulin into broad ribbons is not a result of the presence of DMSO or glycerol. The ribbon structures observed in our studies were formed over a broader range of pH values than in the studies of Matsumura and Hayashi (16) with unchromatographed tubulin, which may be due to the solvents used in our system or the absence of MAPs.

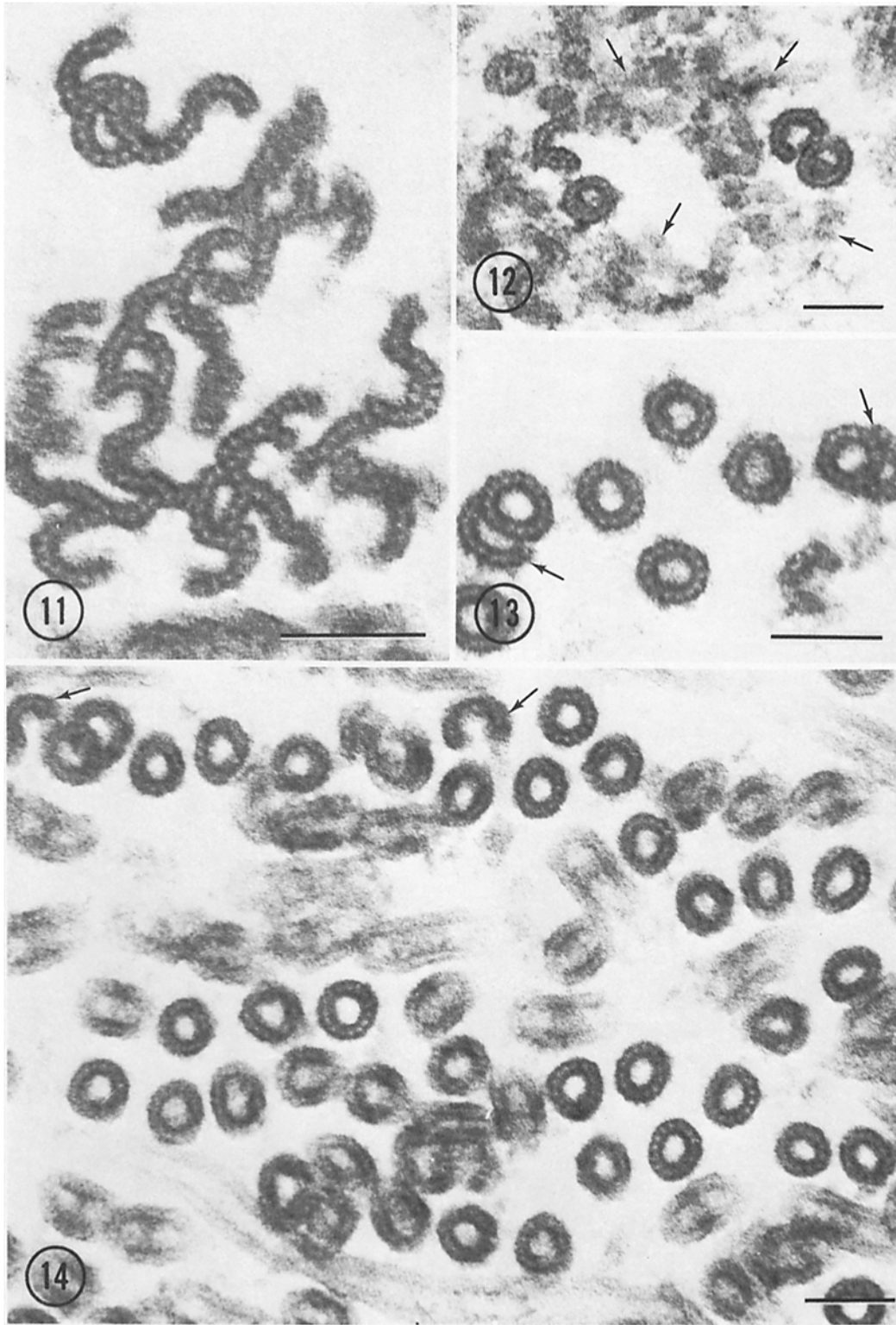
Before considering the various binding interactions of PC-tubulin dimers that provide for variously shaped ribbon structures, mention should be made of the biased or "unidirectional" manner in which microtubules elongate (1, 2, 9, 19, 21, 25, as well as our own unpublished observations of assembly of tubulin onto microtubule fragments coated with cationized ferritin). This property must reflect a tendency toward unidirectional addition of dimers to a growing protofilament, and in fact most protein polymers appear to have polarity (20). In branched ribbons of protofilaments seen when PC-tubulin is polymerized in 10% DMSO at low pH, all the protofilaments are oriented in the same direction, indicating polarity within the system (Fig. 16*B*). Thus, if we arbitrarily designate one end of an elongate dimer as the "anterior" (A) and the other the "posterior" (P) end, a preferred binding between dimers would involve the A and P ends, and unidirec-

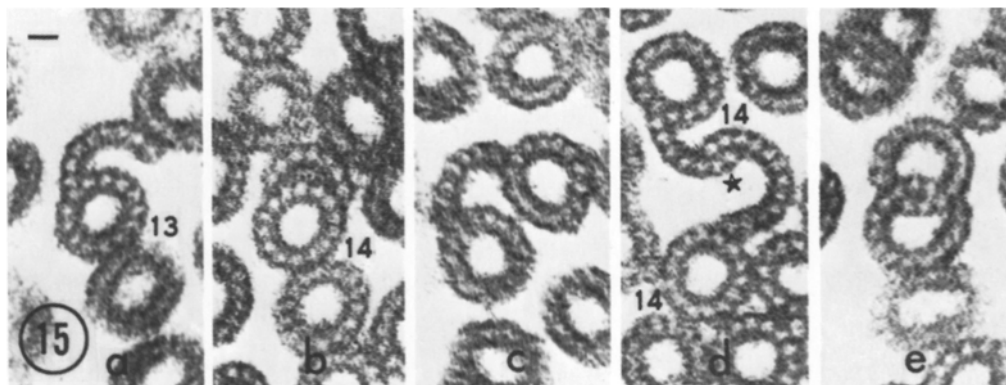
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**FIGURE 4** Typical formed elements seen in polymerization of PC-tubulin polymerized to completion in 0.1 M PIPES-DMSO at pH 6.5. At this pH, about 35% of the formed structures are microtubules, with which ribbons may be adherent, as shown here. Note that ribbons formed at this pH are more curved in cross section than those seen at lower pH values. The microtubule shown has 14 protofilaments, with two protofilaments adherent to its inner wall. GTA;  $\times$  415,000. Bar, 0.05  $\mu$ m.

**FIGURES 5-10** Structures formed by PC-tubulin polymerized to completion in 0.1 M PIPES-DMSO at pH 6.9. At this pH, numerous complete microtubules are formed, although narrow ribbons in the form of "C-," figure "6-," and "W"-shaped elements are also present. The number of wall protofilaments can be determined in some of the microtubules shown (see number beside microtubule), and such numbers are variable, with 14 the predominant number. Two extra protofilaments are seen on the inner wall of the microtubule in Fig. 6. In Fig. 7, two "C"-shaped elements (arrows) are adherent to an "S"-shaped ribbon. Fig. 8 shows a short "C"-shaped ribbon (arrow) which is only slightly curved, and a "W"-shaped ribbon is shown in Fig. 9 (arrow). Various shaped ribbons are seen in Fig. 10, including one shaped as a reversed figure "6" (arrow). GTA; Fig. 5,  $\times$  567,000; Fig. 6,  $\times$  527,000; Fig. 7,  $\times$  400,000; Fig. 8,  $\times$  313,000; Fig. 9,  $\times$  400,000; and Fig. 10,  $\times$  168,000. Fig. 10: Bar, 0.1  $\mu$ m. Figs. 7-9: Bars, 0.05  $\mu$ m. Figs 5 and 6: Bars, 0.01  $\mu$ m.







**FIGURE 15** (a-e) Micrographs illustrating various configurations resulting from ribbon-ribbon and ribbon-microtubule binding of protofilaments. In this sample, PC-tubulin was polymerized to completion in 0.1 M PIPES without DMSO at pH 7.25, and containing 4 M glycerol. Although over 90% of the formed elements were complete microtubules, configurations such as those shown were also present; under the same conditions at pH 6.4, only 15% of the formed elements were complete microtubules. The structures shown were frequently seen in many of the studies where conditions of polymerization, e.g., pH, selected for ribbon formation. See text for details. GTA;  $\times$  415,000. Bar, 0.01  $\mu\text{m}$ .

tional growth of a protofilament requires dimer addition only at one or the other of the free ends. The A-P axis of the dimer, or protofilament, can be visualized as providing polarity, and the  $\alpha$ - and  $\beta$ -subunits of the dimer are likely candidates to provide for longitudinal polarity.

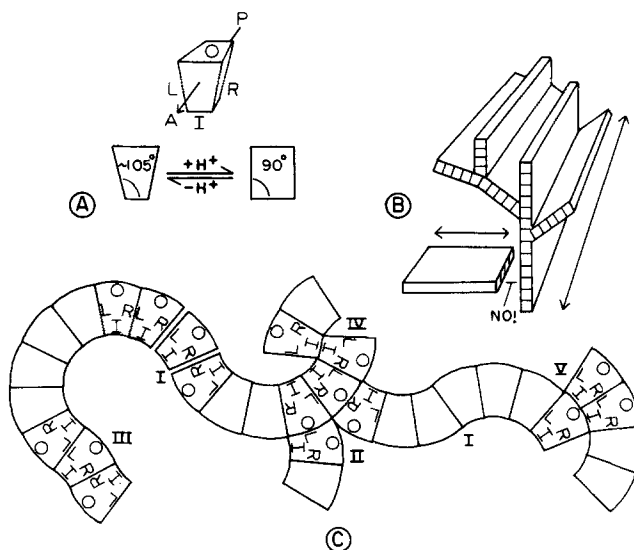
To account for "C"-shaped ribbons of protofilaments, and any ribbon that is curved in cross section, it is useful to perceive the tubulin dimer as having a trapezoidal profile when seen in end-view, as shown at the top of Fig. 16 A. The A-P axis is designated in the diagram, as well as left (L) and right (R), and inner (I) and outer (O) surfaces. In the trapezoidal configuration, the

lateral surfaces are tilted with respect to a horizontal plane through the dimer. The tilt angle of these lateral surfaces will determine the number of protofilaments required to close a microtubule resulting from the side-to-side association of dimers, and the number of protofilaments will be reflected in the diameter of the tubule. If the lateral surfaces of the dimers are tilted at an angle of about  $105^\circ$ , as shown in Fig. 16 A, 13 of them will be required to complete the wall of a microtubule. At lower pH values, we theorize that the conformation of the dimer is altered such that the tilt angle approximates  $90^\circ$ , and the side-to-side association of such dimers would give rise

**FIGURE 11** Formed elements from PC-tubulin polymerized to completion in 0.4 M PIPES-DMSO at pH 6.9. At this buffer concentration, only an occasional microtubule is seen; the many ribbons in such samples are curved in cross section and are frequently attached to one another to form various complex configurations. GTA;  $\times$  439,000. Bar, 0.05  $\mu\text{m}$ .

**FIGURES 12-13** Formed elements seen early and late in the polymerization of PC-tubulin in 0.1 M PIPES-DMSO at pH 7.1. Fig. 12 is from a sample taken at the beginning of polymerization. Much aggregate material is present (arrows), although a few ribbons (often as "S"-shaped elements) and complete microtubules are seen. Upon completion of polymerization (Fig. 13), little aggregate material and many microtubules are present, although a few ribbon structures are also seen, often adherent to the complete microtubules (arrows). Microtubules oriented such that protofilaments can be counted generally show 14 wall subunits. GTA; Fig. 12,  $\times$  244,000; Fig. 13,  $\times$  345,000. Bars, 0.05  $\mu\text{m}$ .

**FIGURE 14** Complete microtubules are predominant in PC-tubulin polymerized to completion in 0.1 M PIPES-DMSO at pH 7.7. A few ribbons are present, most of which are "C" shaped (arrows). Most of the microtubules have 14 wall protofilaments. GTA;  $\times$  276,000. Bar, 0.05  $\mu\text{m}$ .



**FIGURE 16 (A-C)** Effect of pH on tubulin dimers and protofilaments. These diagrams summarize the apparent effects of pH on the relationships between protofilaments formed from purified tubulin. The evidence favors a tubulin dimer having the symmetry shown at the top in Fig. 16 A, where in three-dimensional view the dimer is visualized as having an anterior (*A*) to posterior (*P*) polarity, an inner (*I*) and an outer (*O*) surface, and left (*L*) and right (*R*) lateral binding sites. The dimer is visualized as having a trapezoidal shape when seen in cross section as it would appear as one subunit of a protofilament in the wall of a microtubule. The angle of the lateral surfaces (~105°) is such that side-by-side arrangement of 13 dimers forms a tubule about 250 Å in diameter. In the lower part of Fig. 16 A, the end of a dimer is viewed as a trapezoid which is modified by assembly buffer at low pH such that the angle of its lateral walls is now 90°. At low pH, the side-to-side association of these dimers, or protofilaments constructed of these dimers, forms flat ribbons or sheets. Fig. 16 B is a diagrammatic representation of a group of branched ribbons, similar to those formed by PC-tubulin at low pH. The arrow at the right indicates the protofilament axes in the branched structure; at the bottom left is a portion of a ribbon with the protofilaments oriented with their long axes perpendicular to those of the branched structure. The diagram emphasizes that in branched structures all the protofilament axes parallel one another, and attached ribbons are never seen with protofilament axes perpendicular to the axes of other interconnected ribbons in the group. Fig. 16 C shows the various ways in which dimers bind together to give rise to the complex ribbon structures formed when PC-tubulin is polymerized at suboptimal pH. Normally, to form "C"-shaped elements which then add dimers (protofilaments) to become complete microtubules, the model requires the association of surfaces *L* and *R*, this being the preferred configuration. The Roman numerals indicate the possible deviations from this arrangement as described in the text. The numerals reflect the estimated frequency of observation of a particular kind of arrangement, with arrangement *I* being the most frequently observed and arrangement *IV* being rarely seen. Configuration *I* would provide for "S"-shaped structures while configuration *III* would provide for a "W"-shaped ribbon. According to the model, the degree of curvature of the ribbons is influenced by the pH of the assembly buffer. See the text for a more complete description of Fig. 16 C.

to a flat ribbon. This conversion is viewed as passing through intermediate stages, and dimers having lateral surfaces tilted at angles intermediate to the extremes shown would be formed over a range of pH values; in this manner, dimers would associate to form ribbons having greater or lesser curvature. Since ribbon and microtubule formation involves a two-dimensional polymerization,

the preferred binding interactions between dimers may be designated A to P and L to R.

The protofilamentous structures formed by PC-tubulin at various pH values provide clues to the way in which dimers normally bind to one another, without having to consider the involvement of MAPs in the process. Certainly, most of the complex structures observed would not be formed

in the cytoplasm of a normal cell, but they are of interest in that they provide information on the possible ways in which dimers can bind with one another. Curved ribbons of protofilaments, or "C"-shaped structures in cross section, are without question intermediate elements in microtubule assembly of both PC-tubulin and MAP-containing tubulin. In this study, we demonstrate various ribbon structures into which PC-tubulin can self-assemble; such ribbons occur individually and in association with one another and with complete microtubules. Fig. 16C illustrates the kinds of binding between protofilaments of the various ribbon structures observed in this study; in this diagram, the protofilaments are seen in cross section, and it is assumed that the dimer has a trapezoidal profile, as discussed above, even though at a given pH tubulin may exist in a variety of conformational states having different trapezoidal and rectangular profiles (Fig. 16A). The Roman numerals I-V in Fig. 16C designate the five aberrant ways in which dimers were observed to be associated with one another, and the ranking is in order of their estimated frequency, i.e., type I is the most commonly observed, while type V is rarely observed. Although diagrammed in Fig. 16C as having trapezoidal profiles, the illustration should be viewed as though the dimers involved in the aberrant binding interactions (I-V) have undergone some sort of pH-induced conformational change. Types I-V are representative of possible binding interactions between adjacent dimers; it should be borne in mind that the preferred binding between dimer surfaces is A to P, which is highly conserved even in branched ribbon structures, and between L and R surfaces. At the left end of the diagram a portion of a forming microtubule is shown with eight dimers laterally oriented in the preferred manner, with their O-surfaces outward and the I-surfaces directed toward the prospective lumen of the microtubule.

As shown in Fig. 16C, a frequently observed (Type I) binding involves insertion of a trapezoidal dimer such that its inner or narrower surface now faces outward instead of toward the lumen of the prospective tube, and with addition of like-oriented dimers to this "upside-down" dimer the result is an "S"-shaped ribbon. This involves an R to  $\bar{R}$  or L to  $\bar{L}$  kind of association, and it does not alter the A-P polarity of the ribbon. Another frequently observed kind of binding is that designated Type II, which involves addition of the

lateral surface (L or R) of a dimer to the outer (O) surface of another. This would give rise to one ribbon adherent to and growing perpendicular to another, with their protofilaments oriented in the same direction. If this were an L to O kind of binding, the two ribbons would have the same polarity, but an R to O binding would mean that the two ribbons would have opposite A-P polarity and would grow in opposite directions if we assume unidirectional growth. The Type III kind of binding is a sometimes-seen derivation of Type I, where only one dimer in a laterally associated series is inserted "upside-down," giving rise to a "W"-shaped ribbon in cross section. Another possible but rare kind of binding involves the association of dimers by their inner surfaces (I to I, as designated Type IV); it is obvious in this case that, as other dimers were added to their R and L surfaces, curvature of the forming ribbons would interfere with continued lateral growth. One other rare kind of binding is that involving the inner (I) surface of a dimer adherent to the outer (O) surface of another (Type V). This kind of binding would provide for three-dimensional growth, which is rarely seen even in flat ribbons at low pH. As mentioned above, the most unlikely, if not normally impossible, kind of binding is that which would involve adherence of the A or P surface of one dimer to any but the A or P surface of another. If binding between A or P and lateral or inner and outer surfaces of dimers could occur, branched ribbons formed at low pH would *not* have all their protofilaments oriented in the same direction (see Fig. 16B, which stresses that a ribbon with its protofilaments oriented in a given direction cannot form in association with a ribbon having protofilaments oriented perpendicular to that direction). It is the preferred end-to-end, side-to-side association of dimers which gives rise to the orderly two-dimensional walls of microtubules.

Microtubules with fourteen wall protofilaments were seen with remarkable frequency in material examined in the present study, as well as in other of our studies (10). This is not an effect of DMSO, nor is it due to the complete absence of MAPs, since the assembly of unchromatographed tubulin in the absence of DMSO predominately produces microtubules with 14 protofilaments. On the basis of the model of a tubulin dimer described above, where the subunit is trapezoidal in profile, only a slight change in the angle of the lateral surfaces would provide for the formation of microtubules

with 14 rather than the normal 13 wall protofilaments.

The tubulin preparations used in this work do not contain other proteins detectable by SDS-gel electrophoresis, nor do they contain rings, spirals, or other aggregate forms of tubulin and associated proteins. By using such a "clean" system, details of microtubule initiation and elongation can be studied to greatest advantage. Previous studies on the mechanism of tubulin assembly have used preparations which contained existing protofilaments in the form of ring and spiral structures. Such preparations can provide but limited information about the formation of initiating centers before elongation occurs. We are currently using the MAP-free system to investigate the early stages of the assembly process in order to determine the structure of the initiation centers.

This investigation was supported by U. S. Public Health Service grants AI 06448 (P. R. Burton) and NS 11360 (R. H. Himes), by grants from the Biomedical Science Support grant (PR-07037 to R. H. Himes) and the University General Research Fund (P. R. Burton), and was supported also by the Mid-America Cancer Center Program. The authors express their appreciation to Drs. William L. Dentler and George B. Pierson for their critical reading of the manuscript.

Received for publication 19 May 1977, and in revised form 3 October 1977.

Note Added in Proof: Microtubules having 14 wall protofilaments were observed with remarkable frequency in this study, an observation which is in keeping with evidence set forth in a recent paper of ours which deals with alterations in number of protofilaments in microtubules assembled *in vitro* (Pierson, G. B., P. R. Burton, R. H. Himes. 1978. *J. Cell Biol.* **76**:223-228).

## REFERENCES

1. ALLEN, C., and G. G. BORISY. 1974. Structural polarity and directional growth of microtubules of *Chlamydomonas flagella*. *J. Mol. Biol.* **90**:381-402.
2. BINDER, L. I., W. L. DENTLER, and J. L. ROSENBAUM. 1975. Assembly of chick brain tubulin onto flagellar axonemes of *Chlamydomonas* and sea urchin sperm. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1122-1126.
3. BORISY, G. G., K. A. JOHNSON, and J. M. MARCUM. 1976. Self assembly and site-initiated assembly of microtubules. In *Cell Motility*. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. Vol. 3. 1093-1108.
4. BORISY, G. G., J. M. MARCUM, J. B. OLMSTED, D. B. MURPHY, and K. A. JOHNSON. 1975. Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly *in vitro*. *Ann. N. Y. Acad. Sci.* **253**:107-132.
5. BORISY, G. G., and J. B. OLMSTED. 1972. Nucleated assembly of microtubules in porcine brain extracts. *Science (Wash. D. C.)*. **177**:1196-1197.
6. BRYAN, J. 1976. A quantitative analysis of microtubule elongation. *J. Cell Biol.* **71**:749-767.
7. BURTON, P. R., R. E. HINKLEY, and G. B. PIERSON. 1975. Tannic acid-stained microtubules with 12, 13, and 15 protofilaments. *J. Cell Biol.* **65**:227-233.
8. DENTLER, W. L., S. GRANETT, and J. L. ROSENBAUM. 1975. Ultrastructural localization of the high molecular weight proteins associated with *in vitro*-assembled brain microtubules. *J. Cell Biol.* **65**:237-241.
9. DENTLER, W. L., S. GRANETT, G. B. WITMAN, and J. L. ROSENBAUM. 1974. Directionality of brain microtubule assembly *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1710-1714.
10. HIMES, R. H., P. R. BURTON, and J. M. GAITO. 1977. The dimethylsulfoxide-induced self-assembly of tubulin lacking associated proteins. *J. Biol. Chem.* **252**:6222-6228.
11. HIMES, R. H., P. R. BURTON, R. N. KERSEY, and G. B. PIERSON. 1976. Brain tubulin polymerization in the absence of "microtubule-associated proteins." *Proc. Natl. Acad. Sci. U. S. A.* **73**:4397-4399.
12. KEATES, R. A. B., and R. H. HALL. 1975. Tubulin requires an accessory protein for self assembly into microtubules. *Nature (Lond.)*. **257**:418-420.
13. KIRSCHNER, M. W., L. S. HONIG, and R. C. WILLIAMS. 1975. Quantitative electron microscopy of microtubule assembly *in vitro*. *J. Mol. Biol.* **99**:263-276.
14. KIRSCHNER, M. W., R. C. WILLIAMS, M. WEINGARTEN, and J. C. GERHART. 1974. Microtubules from mammalian brain: some properties of their depolymerization products and a proposed mechanism of assembly and disassembly. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1159-1163.
15. LEE, J. C., and S. N. TIMASHEFF. 1975. The reconstitution of microtubules from purified calf brain tubulin. *Biochemistry*. **14**:5183-5187.
16. MATSUMURA, F., and M. HAYASHI. 1976. Polymorphism of tubulin assembly. *In vitro* formation of sheet, twisted ribbon and microtubule. *Biochem. Biophys. Acta.* **453**:162-175.
17. MIZUHIRA, V., and Y. FUTAESAKU. 1971. On the new approach of tannic acid and digitonine to the biological fixatives. 29th Annual Proceedings of the Electron Microscope Society of America, Boston, Mass. Claitor's Publishing Division. Baton Rouge, La. 494-495.
18. MURPHY, D. B., and G. G. BORISY. 1975. Associ-

- ation of high-molecular-weight proteins with microtubules and their role in microtubule assembly *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2696-2700.
19. OLMSTED, J. B., J. M. MARCUM, K. A. JOHNSON, C. ALLEN, and G. G. BORISY. 1974. Microtubule assembly: some possible regulatory mechanisms. *J. Supramol. Struct.* **2**:429-450.
  20. OOSAWA, F., and S. ASAKURA. 1975. Thermodynamics of the polymerization of protein. Academic Press, Inc., New York. 204 pp.
  21. ROSENBAUM, J. L., L. I. BINDER, S. GRANETT, W. L. DENTLER, W. SNELL, R. SLOBODA, and L. HAIMO. 1975. Directionality and rate of assembly of chick brain tubulin onto pieces of neurotubules, flagellar axonemes, and basal bodies. *Ann. N. Y. Acad. Sci.* **253**:147-177.
  22. SHELANSKI, M. L., F. GASKIN, and C. R. CANTOR. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **70**:765-768.
  23. TILNEY, L. G., J. BRYAN, D. J. BUSH, K. FUJIIWARA, M. S. MOOSEKER, D. B. MURPHY, and D. H. SNYDER. 1973. Microtubules: evidence for 13 protofilaments. *J. Cell Biol.* **59**:267-275.
  24. WEINGARTEN, M., A. H. LOCKWOOD, S. Y. HWO, and M. W. KIRSCHNER. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1858-1862.
  25. WITMAN, G. B. 1975. The site of *in vivo* assembly of flagellar microtubules. *Ann. N. Y. Acad. Sci.* **253**:178-191.