Published November 1, 1980

Arrangement of Subunits in Microtubules with 14 Profilaments

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ABSTRACT The structure of 14-protofilament microtubules reassembled from dogfish shark brain tubulin was analyzed by high resolution electron microscopy and optical diffraction. The simultaneous imaging of the protofilaments from near and far sides of these tubules produces a moiré pattern with a period of ~96 nm. Optical diffraction patterns show that the 5-nm spots that arise from the protofilaments for the two sides of the tubule are not coincident but lie off the equator by a distance of 1/192 nm^{-1}. These data provide evidence that in reassembled microtubules containing 14 protofilaments, the protofilaments are tilted 1.5° with respect to the long axis of the tubule, giving a left-handed superhelix with a pitch of 2.7 μm. The hypothesis is that the tilt of the protofilaments occurs to accommodate the 14th protofilament. It is determined that when the 14th protofilament is incorporated, the 3-start helix is maintained, but the pitch angle changes from 10.5° to 11.2°, the angle between protofilaments measured from the center of the microtubule changes by 2°, and the dimer lattice is discontinuous. These observations show that the tubulin molecule is sufficiently flexible to accommodate slight distortions at the lateral bonding sites and that the lateral bonding regions of the α and β monomers are sufficiently similar to allow either α-α and β-β subunit pairing or α-β subunit pairing.

The number of protofilaments in a microtubule assembled from temperature-cycled tubulin (17) can differ from the number of protofilaments in the native microtubule (22). Several studies have shown that the predominant population of reassembled microtubules contain 14 protofilaments (9, 17). The arrangement of subunits in the native microtubule that contains 13 protofilaments is known to be a 3-start left-handed helix with a helical pitch angle of 10.5° (2, 3, 7, 8, 14). The change in this arrangement of subunits when the 14th protofilament is incorporated into the tubule is not known. Our observation that negative contrast electron microscope images of reassembled shark brain tubules display a moiré pattern and that these tubules contain 14 protofilaments prompted a study of the helical surface lattice of the tubulin subunits (12).

The results show that when the 14th protofilament is incorporated into reassembled tubules, it causes the tubule to twist into a shallow superhelix. The 3-start helical family is retained but the pitch angle of the helix changes by 0.7° to accommodate the additional protofilament. The retention of the 3-start helix requires that the dimer lattice be discontinuous. Therefore, the reassembled microtubule with 14 protofilaments contains elements of both the A-tubule and the B-tubule dimer lattice.

These findings are consistent with the recently reported data of McEwen and Edelstein (15). These authors used electron microscopy, in conjunction with computer analysis based on Fourier transforms, to obtain evidence in favor of a 3-start monomer helix and a discontinuous dimer lattice which combines elements of both the A- and B-type lattices.

MATERIALS AND METHODS

Dogfish brain tubulin was prepared by the temperature-dependent assembly-disassembly procedure of Shelanski et al. (18) as previously described (13). The polymerization solution contained 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.60, 1.0 mM EGTA, 0.5 mM $MgCl_2$, and 1.0 mM GTP. Glycerol (25%) was added to the crude extract to enhance polymerization because very little polymerization occurred without it. Because polymerization proceeds without glycerol in subsequent polymerization steps, it was not used for assembly after the first step of the purification process.

SDS-polyacrylamide gel electrophoresis was performed according to the procedures of Bryan (5) and Stephens (20). 5% gels, 12 cm in length, were run in 25 mM Tris-glycine buffer, pH 8.3, containing 0.1% SDS. The gels were stained with Coomassie Brilliant Blue and scanned at 560 nm with a Zeiss PM 10 spectrophotometer (Carl Zeiss, Inc., New York).

The electron microscope grids used in this study were coated with only a carbon film by a procedure described by Linck and Amos (14). 400-mesh grids were coated first with a plastic film made from 0.25% Formvar and then a thin film of carbon. The Formvar film was removed by floating the Formvar-carbon-coated grids over dichloroethane, which dissolves the Formvar, leaving only a carbon film over the grid holes. Such carbon-coated grids gave much improved resolution by the negative-staining procedure. To stain for microtubules, a drop of the solution containing tubules was placed on the carbon-coated grid for 20 s, after which the grid was rinsed with four to five drops of buffer and stained with several drops of 1% uranyl acetate. Excess stain was removed by blotting the

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edge of the grid with filter paper. Stained grids were observed as soon as possible in a JEOL-100S electron microscope (EM). The tannic acid staining procedure described by Tilney et al. (22) was used to establish the number of protofilaments in these tubules.

For optical diffraction studies, images of microtubules were transferred from the original EM film to photographic glass plates. Segments of microtubules were selected and prepared for diffraction by masking out unwanted areas of the plate. The diffraction analysis was made with an optical diffractometer (10, 11) in the laboratory of R. W. Linck, Harvard Medical School, Boston, Massachusetts. The optical transforms were recorded on photographic film, using a Hasselblad 2 × 2 single-lens reflex camera. Optical filtering was performed by the method of Klug and DeRosier (11).

RESULTS

Tubulin Assembly Without Accessory Proteins

Dogfish brain tubulin preparations contain little or no accessory protein after purification by two cycles of the temperature-dependent assembly-disassembly procedure of Shelanski et al. (18). Such twice-cycled tubulin preparations (C_2S tubulin) analyzed for purity by SDS-polyacrylamide gel electrophoresis (13) show α- and β-tubulin bands but no protein bands in the high and intermediate molecular weight ranges corresponding to microtubule-associated proteins (1, 4, 6, 16, 19, 23). A typical gel profile is shown in Fig. 1. C_2S tubulin preparations, which contained no detectable amounts of accessory proteins, were warmed to induce polymerization and the reconstituted microtubules were negatively stained for use in the study that follows.

Structure of Reconstituted Microtubules

Dogfish brain microtubules negatively stained and examined in the electron microscope display a twist of shallow pitch. The twist can be seen by sighting along the axis of the tubule or by tracing along the length of a protofilament at the edge of the tubule (Fig. 2). One sees that the protofilaments lie at a slight angle to the longitudinal axis of the tubule.

In addition to the twist, a moiré pattern is seen along the axis of the tubule. The pattern consists of a set of two alternating bands or striations that repeat at regular intervals along the tubule. The striation pattern represents regions along the tubule where the protofilaments appear alternately distinct and indistinct. The striation containing obscure protofilaments appears as though the protofilaments at the back of the tubule project exactly between those at the front. When this happens, the grooves between protofilaments are not discernible and the path of individual protofilaments and the tubulin subunits are difficult to identify. The adjacent striation, containing distinct protofilaments, appears as though the protofilaments on the front of the tubule are exactly superimposed on protofilaments at the back. In the latter stripe, the globular substructure of the protofilaments is very evident and individual tubulin monomers are seen. The two bands of the moiré pattern alternate along the axis of the tubule (Fig. 3). Each band is ~48 nm long, inclined at an angle of ~40°, and repeats at ~96-nm intervals along the length of the tubule.

The presence of the moiré pattern is supporting evidence that the protofilaments lie at an angle to the long axis of the tubule. The period of the pattern is a measure of the degree of

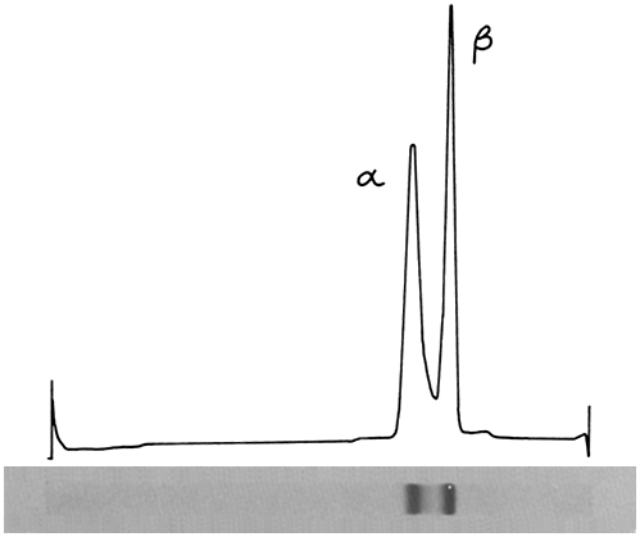

FIGURE 1 SDS-polyacrylamide gel of dogfish brain tubulin after two cycles of purification (C_2S tubulin). A photograph of the gel is shown below the densitometer tracing. α- and β-tubulin bands are present but no accessory protein bands.

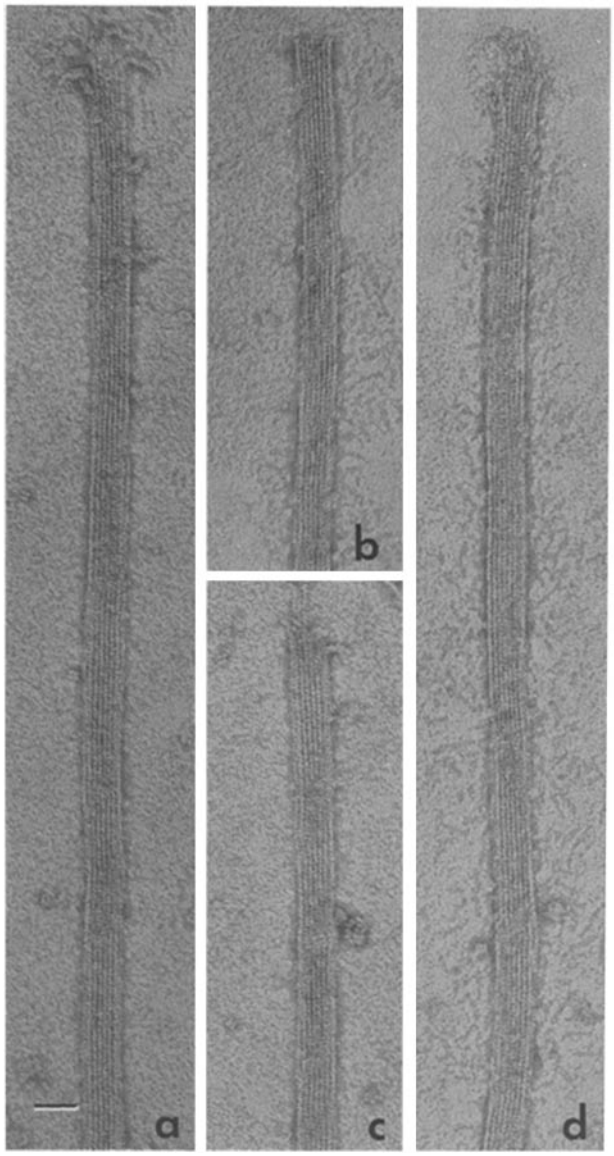

FIGURE 2 Electron micrographs of negatively stained dogfish brain microtubules. The tubules contain a shallow twist with pitch between 1 and 3 μm. The twist can be seen by sighting along the axis of the tubules or by following the path of protofilaments at the edge of the tubules. The tubules are flattened and measure 34–36 nm in width. Six to seven protofilaments can be counted across the width of the tubule. Bar, 50 nm. *a–d*, × 125,000.

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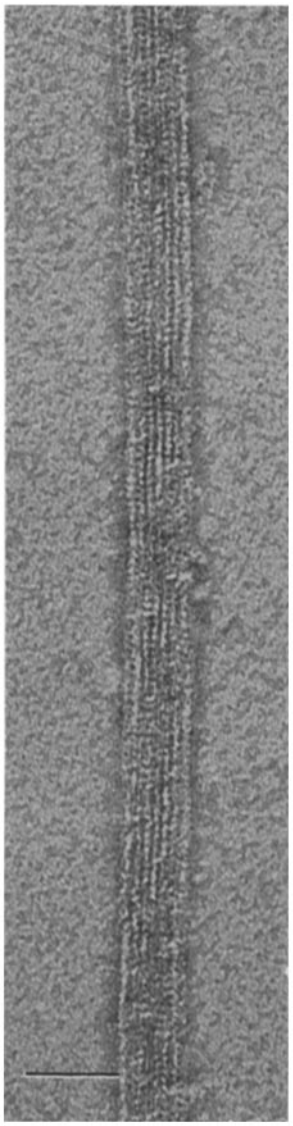

FIGURE 3 Electron micrograph of a tubule that shows several periods of the 96-nm axial repeat. A periodic banding pattern can be seen in the wall of this tubule (sight along the axis of the tubule). The pattern appears as helical bands or striations containing protofilaments that appear alternately distinct and indistinct at regular intervals along the tubule. Bar, 50 nm. × 250,000.

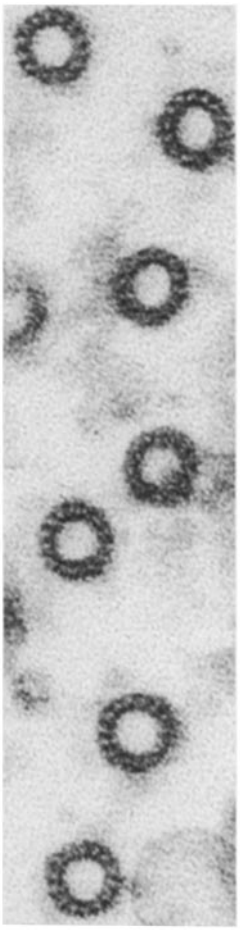

FIGURE 4 Electron micrograph of a thin section of a pellet of microtubules stained with tannic acid. The protofilaments making up the wall of the tubules can be seen. 95% of the tubules in a typical section contain 14 protofilaments. The protofilaments do not appear with equal clarity around the entire circumference of the tubule. This occurs because the protofilaments are inclined at a shallow angle to the long axis of the tubule. Therefore, when the plane of section is perpendicular to the protofilaments on one side of the tubule, it is at an oblique angle to the protofilaments on the opposite side.

tilt of the protofilaments. The moiré pattern occurs because the protofilaments from both sides of the microtubule are imaged simultaneously. The tilt of the protofilaments, such that they no longer lie parallel to the longitudinal axis but follow a helical path along the tubule, is defined in this paper as the superhelix of the microtubule.

The superhelix is thought to arise because these tubules contain one more than the usual number of protofilaments found in native microtubules. Tannic acid staining of reassembled shark brain tubules shows that ~95% of them contain 14 protofilaments (Fig. 4). The other 5% of the microtubules contain either 13 or 15 protofilaments in roughly equal numbers. The hypothesis is that the twist in microtubules with 14 protofilaments occurs to accommodate the extra protofilament. It is not known whether a twist is present in reassembled microtubules with 15 protofilaments. The assumption is that in vitro assembled tubules with 13 protofilaments, like flagella A-tubules, do not contain a twist. Optical diffraction was used to determine the changes that take place in the arrangement of subunits when the 14th protofilament is incorporated into the reconstituted microtubule.

Optical Diffraction of Microtubules

A microtubule that is twisted into a superhelix should give rise to a diffraction pattern that differs in a predictable way from the normal microtubule. In a 13-protofilament microtubule, the protofilaments from the near and far sides are parallel to the cylinder axis and the equatorial diffraction spots that arise from the protofilaments are coincident. However, if the protofilaments have a helical orientation with respect to the cylinder axis, as appears to be the case for 14-protofilament tubules, the diffraction spots that arise from the protofilaments should appear off the equator (11).

The pitch angle of the superhelix that is computed based on measurements from the diffraction patterns should agree with the pitch angle determined from measurements of the period of the moiré pattern. Specifically, for a moiré repeat of 96 nm, the equatorial spots should be split into two spots at a spacing

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of 1/192 nm^{-1} above and below the equator. This corresponds to a slight twist or superhelix of the protofilaments, at an angle of $\tan^{-1}(5/192) = 1.5°$ from the microtubule axis.

The diffraction pattern obtained from the tubule shown in Fig. 3 is shown in Fig. 5. The diffraction pattern shows two orders of the 5-nm equatorial spots and has four spots on the 4-nm layer line. These aspects of the pattern are typical of a 13-protofilament microtubule, but the equatorial spots arising from the protofilaments for the two sides of the 14-protofilament tubule are not coincident but are separated and lie above and below the equator. They are separated from each other by a distance of 1/96 nm^{-1} and from the equator by 1/192 nm^{-1} as predicted by the moiré pattern.

The off-equatorial spots for the two sides do not image at precisely the same distance from the origin. This difference in position occurs because one side of the tubule partially collapses, squeezing the filaments on that side together. Erickson (7) has shown that the near side or the side away from the carbon film is the one that flattens and shrinks. On this basis the off-equatorial reflections that arise from the near side and the ones that arise from the far side of the tubule can be established. Based on this determination and on the assumption that the basic monomer helix in the reassembled tubule is left-handed, as is the case in the native microtubule that contains 13 protofilaments, it is possible to establish the handedness of the superhelix. The diffraction pattern shows that the equatorial reflections for a given side lie in the same quadrant of the diffraction pattern as the 4-nm spots for the monomer helix of that side. This is proof that the superhelix and the monomer helix are of the same hand, i.e., left-handed.

In summary, the diffraction patterns show that in reassembled shark brain tubules containing 14 protofilaments, the protofilaments are tilted 1.5° with respect to the long axis of the tubule, giving a left-handed superhelix with a pitch of 2.7 μm.

DISCUSSION

The electron microscope images and the optical diffraction data are consistent with the interpretation that microtubules with 14 protofilaments are slightly twisted into a superhelix of shallow pitch. The hypothesis is that the tilt of the protofilaments occurs to accommodate the 14th protofilament. These data provide a means to establish the changes that take place in the subunit lattice when the 14th protofilament is incorporated into the tubule. There are three parameters that may change when the 14th protofilament is incorporated into the tubule: (*a*) the number of starts in the monomer helical family; (*b*) the pitch angle of the monomer helix; and (*c*) the tilt of the protofilaments. Changes in these three parameters do not require changes in the spacing of the subunits along the protofilaments or the spacing between the protofilament but they do require changes in the lateral bonding between subunits.

If one assumes, as a first approximation, that the pitch angle of the monomer helix in the 14-protofilament tubule is the same (10.5°) as the pitch angle for the monomer helix in the 13-protofilament tubule, one can calculate the tilt of the protofilaments and the corresponding moiré pattern required to accommodate the 14th protofilament. The calculated tilt of the protofilaments and the period of the moiré patterns are shown in Table I for the 2-, 3-, and 4-start helices. These three helices are chosen because they require the smallest departure from the lattice parameters of the 13-protofilament tubule. It is seen that neither the 2-, 3-, or 4-start helix generates a moiré pattern with a period of 96 nm, the period of the observed moiré pattern. Therefore, one can conclude that the native subunit helix pitch angle of 10.5° must be distorted slightly in the 14-protofilament tubule. This is reasonable because the lateral bonds that define this helix are relatively weak and may be easily distorted.

If one fixes the value of the protofilament tilt at 1.5° (the

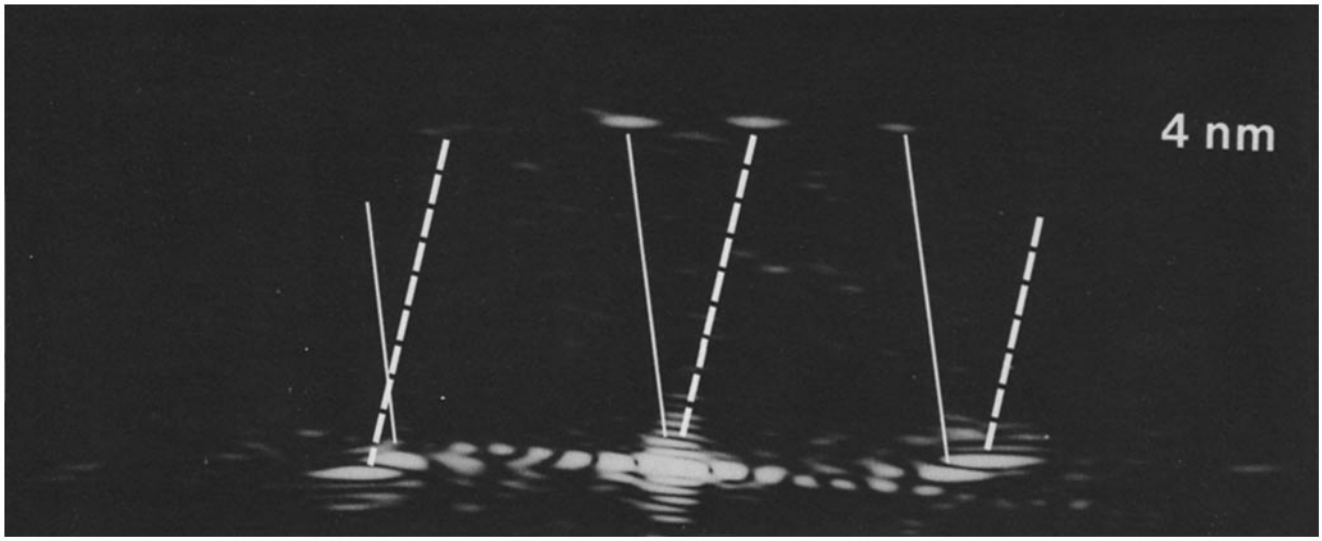

FIGURE 5 Optical diffraction pattern of the microtubule shown in Fig. 3. Only one half of the pattern is shown. Four spots are seen on the 4-nm layer line, two from the near and two from the far side of the tubule. These spots arise from the subunits aligned along the protofilaments. Two pairs of bright off-equatorial reflections arising from the protofilaments are seen on either side of the center of the pattern. One member of each pair of spots arises from the near and the other from the far side of the tubule. Each of the equatorial spots is displaced 1/192 nm^{-1} above or below the equator. The equatorial reflection arising from the near side (connected to the 4-nm layer line reflection by a dotted line) and the far side (connected by solid line) of the tubule can be distinguished because the spots from the near side occur at approximately 1/4.5 nm^{-1}, whereas the reflections from the far side occur at 1/5 nm^{-1} from the center. Note that the 4- and 5-nm spots for a given side occur in the same quadrant of the diffraction pattern. The unit cell is arbitrarily drawn in the form of the B-type lattice.

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observed protofilament tilt), one can calculate the corresponding pitch angles for the 2-, 3-, and 4-start helices. The calculated values plus the handedness of each superhelix are shown in Table II. It is seen that only the 3-start helix gives both a left-handed superhelix and a pitch angle for the basic subunit helix that is reasonably close to the 10.5° pitch of the normal, 13-protofilament microtubule. By this process of elimination, it is possible to conclude that the 3-start helical family is maintained in the 14-protofilament tubule but the pitch angle changes from 10.5° to 11.2°. This model is diagramed in Fig. 6.

These data support the conclusion that the angle of the lateral bonds between subunits in the reassembled tubules with 14 protofilaments is distorted slightly from the lateral bond angle in native 13-protofilament tubules. In addition, it should be noted that the angle between protofilaments measured from the center of the microtubule must change from 27.69° (360°/13) to 25.71° (360°/14) when the 14th protofilament is incorporated. This 2° distortion represents a somewhat larger angular distortion than the distortion of the helix angle. Apparently, the tubulin molecule is sufficiently flexible to accommodate these distortions of the lateral bonding site.

The dimer lattice in reassembled microtubules is not known. The lattice could be either like the dimer lattice in the A-subfiber (21) or the B-subfiber (15) of flagella outer doublet microtubules. Amos and Klug (2) showed that whereas in the A-tubule the dimers in adjacent protofilaments are in a half-stagger arrangement (along the 5-start helix), the dimers in adjacent protofilaments of the B-tubule are lined up obliquely at a shallow angle (along the 3-start helix). Because the A-tubule is a complete cylinder and because the B-tubule dimer lattice will not allow for the formation of a 13-protofilament tubule with a symmetric dimer lattice, it is argued that cytoplasmic singlet microtubules must have the same dimer lattice as flagella A-tubules. The model of Amos and Klug (2) shows that for a 13-protofilament tubule, an odd-start (e.g., 3-start) monomer helix is required to generate a symmetric dimer lattice if the dimers are arranged in the A-tubule lattice. However, an even-start monomer helix is required to generate a symmetric dimer lattice if the dimers are in the B-tubule lattice. But for a 14-protofilament tubule, an even-start monomer helix is required to generate a symmetric dimer pattern for both the A- and B-tubule dimer lattice patterns. Consequently, the 3-start monomer helix in 14-protofilament tubules is a surprising finding because it requires the dimer lattice to be discontinuous. For example, in a 14-protofilament tubule that forms from a ribbon with dimers in the A-tubule lattice (α-β lateral pairing), the pair of protofilaments forming the seam will have the B-tubule dimer lattice (α-α and β-β lateral pairing) and vice versa.

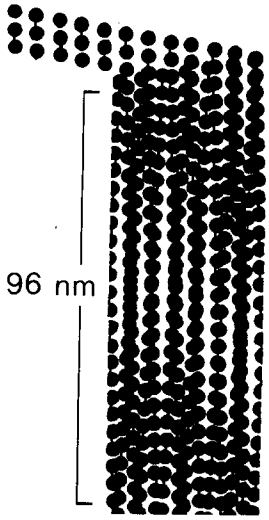

FIGURE 6 Model of the 14-protofilament microtubule. The protofilaments are tilted 1.5° to the tubule axis giving rise to a left-handed superhelix with a pitch of 2.7 μm. Superposition of near and far sides of the tubule gives rise to a 96-nm moiré pattern. A 3-start helix is the basic subunit helix with a pitch angle of 11.2°. One turn of the 3-start helix is unwound at one end of the tubule to illustrate the pitch angle. In this model, the dimers are arranged in the A-type lattice.

TABLE I

Tilt of the Protofilaments and Corresponding Period of Moiré Pattern

Helical family	Pitch angle of the subunit helix	Period of the moiré pattern	Tilt angle of protofilaments	Hand of the superhelix
		nm		
2-start	10.5°	35.6	4.02°	Left
3-start	10.5°	188.5	0.76°	Left
4-start	10.5°	57.3	2.50°	Right

The table shows the tilt of the protofilaments and the corresponding period of the expected moiré pattern for the 2-, 3-, and 4-start helical families when the pitch angle of the helical family is 10.5° and the number of protofilaments is 14.

TABLE II

Pitch Angle of Subunit Helix and Hand of the Superhelix

Helical family	Pitch angle of the subunit helix	Period of the moiré pattern	Tilt angle of protofilaments	Hand of the superhelix
2-start	5.0°	96 nm	1.5°	Left
3-start*	11.2°	96 nm	1.5°	Left
3-start	8.3°	96 nm	1.5°	Right
4-start	11.4°	96 nm	1.5°	Right

The table shows the pitch angle of the basic subunit helix and the hand of the superhelix when the number of protofilaments is 14, the tilt of the protofilaments is 1.5°, and the corresponding moiré pattern is 96 nm.
* The only model of the microtubule that fits the data.

This observation suggests that the lateral bonding regions of the α and β monomers are sufficiently similar to allow either α-α and β-β subunit pairing or α-β subunit pairing. Therefore, the reconstituted, 14-protofilament, brain microtubule that retains the 3-start monomer helix must contain elements of both the A-tubule and the B-tubule dimer lattice.

The determination that the monomer helix in reassembled microtubules with 14 protofilaments is a 3-start helix agrees with the data of McEwen and Edelstein (15). These authors used computer analysis based on Fourier transforms and helical diffraction theory, to determine n, the Bessel order that corresponds to the number of starts in the helical lattice. The tilt of the protofilaments reported here further defines the subunit arrangement and has permitted a determination of the pitch angle of the 3-start helix. The relative amounts of A- and B-type dimer lattices in the reassembled tubule remains a matter of conjecture (15), because reflections in optical transforms corresponding to the dimer repeat are not seen.

I am indebted to Drs. R. E. Stephens and R. W. Linck for their encouragement and stimulating discussions. I thank Dr. Linck for the use of his optical diffractometer, and Lascelles Lyn-Cook for able technical assistance.

I wish to recognize the contribution made to this paper by one of

the reviewers appointed by the Journal of Cell Biology, Dr. Harold P. Erickson of the Department of Anatomy, Duke University, who provided me with new insight into the data.

This research was supported in part by fellowships from the Macy Foundation and the Steps Toward Independence Program, Marine Biological Laboratory, Woods Hole, Massachusetts, and by National Institutes of Health grants GM24446 and GM 28107.

Received for publication 9 July 1980, and in revised form 8 August 1980.

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