MICROTUBULES: EVIDENCE FOR 13 PROTOFILAMENTS

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ABSTRACT

When microtubules are fixed in glutaraldehyde in the presence of tannic acid and thin sections cut, the subunit structure of the microtubule is readily observed without the need of image reinforcement. Seven types of microtubules were analyzed: those in the heliozoan axoneme, the mitotic apparatus, the contractile axostyle, repolymerized microtubules derived from the chick brain, the central pair in flagella, and the A tubules of flagella and the basal body. In all cases microtubules were composed of 13 equally spaced protofilaments. The B tubules in flagella and the basal body appear to be composed of 11 subunits. The connections of the B to the A and the C to the B are described. A model of a microtubule is presented.

INTRODUCTION

Many reports have been published which attempt to describe the substructure of cytoplasmic microtubules and of the microtubules which make up the axoneme of cilia and flagella. In the negatively stained image, microtubules appear to be composed of linear strands of subunits, the so-called "protofilaments" (see André and Thiéry, 1963; Gall, 1966; Pease, 1963; Grimstone and Klug, 1966; Behnke and Zelander, 1966; Kiefer et al., 1966; Warner and Satir, 1973; and many others). Because of the superposition of subunits in microtubules dried on a grid, accurate and reproducible counts of the number of protofilaments cannot be made. Likewise, image reinforcement techniques (Markham rotation, Markham et al., 1963) applied to transverse sections of microtubules are notoriously difficult to interpret if the number of subunits is not known beforehand. Thus infor-mation obtained from this technique should be viewed with extreme caution (see Friedman, 1970; Agrawal et al., 1965). For example, numbers of subunits as diverse as 8, 9, 10, 11, 12, and 13 have been proposed (Burton, 1966; Ross, 1968; Silver and McKinstry, 1967; Fuge, 1968; Bertolini et al., 1970; MacDonald and Kitching, 1967; to list just a few references). The exact number cannot be resolved by X-ray diffraction (Cohen et al., 1971), and judgments based on geometric arrangements of microtubules (Tilney, 1971) must await clear demonstration of bridges.

We have found in the literature only three instances in which the number of subunits making up the wall of a microtubule appears certain. One of these is a transverse section of the flagellum of a spermatozoon of the black scavenger fly, *Sepsis* (Phillips, 1966), in which the number of subunits

of one of the central microtubules in the flagellum can be counted directly on the micrograph without "reinforcement." A second case can be seen in transverse sections of microtubules near the walls of certain plant cells; these microtubules appear negatively stained(Ledbetter and Porter, 1964). Porter makes reference to the microtubules in these plant cells in the discussion following his review (1966) and suggests that they "are nonfunctional microtubules preserved in tannin-related materials." A third case has recently been demonstrated by Mizuhira and Futaesaku (1971) and Futaesaku, Mizuhira, and Nakamura (1972). These investigators obtained some elegant pictures of cilia and of a microtubule in the spermatid by fixing cells in the presence of tannic acid. Presumably, the idea of adding tannic acid to the fixative came from the "negative stained" image of microtubules from the plant cells published by Ledbetter and Porter (1964). Penetration of the tannic acid into the cilium was achieved by the addition of a detergent. In all these instances the microtubules, in transverse section, definitely have 13 subunits.

Recent biochemical studies have demonstrated that the basic subunit of a microtubule, irrespective of the source, appears to be a heterodimer of tubulin (Bryan and Wilson, 1971; Feit et al., 1971). There is considerable controversy over the number of different tubulins present (see Witman et al., 1972), but as Feit et al. (1971) suggest, there may be as few as four. However, there are striking differences in the relative stability of microtubules (see Behnke and Forer, 1967; Tilney and Gibbins, 1968), and in the number and arrangement of bridges which connect them, as, for example, the dynein, spoke, and nexin connections attached to the ciliary outer doublet. It is, therefore, of considerable interest to determine if there really are variations in subunit number from tubules of different sources as might appear to be the case from library research or if all microtubules, universally, are made up of the same number of subunits.

In this brief report we will describe seven cases in which the subunit number of microtubules can be readily determined by the addition of tannic acid to the fixing solution as described by Mizuhira and Futaesaku (1971). We have improved the visibility of the subunits by using tubule systems which have been isolated from the cell. We have found that the walls of all the microtubules examined are composed of 13 protofilaments. We

are further able to determine accurately how the B tubule is attached to the A in the outer doublets of cilia and flagella and in centrioles. Further reports will describe to what subunits of the A tubule in flagella the dynein arms and the spokes attach and to what subunits in the axostyle the bridges attach. It is our hope that the precise determination of the arrangement of bridges to microtubules will be of use in our ultimate understanding of the morphogenesis of tubule clusters and their associated motility.

MATERIALS AND METHODS

Tannic acid was obtained from Merck and Company, Inc., Rahway, N. J. Tannic acid goes into solution upon heating, but when cooled a brown precipitate forms. Solutions of 8%, 4%, or 2% were made up in buffer, heated, and after cooling, centrifuged to clear. The resultant fluid is a clear, brownish liquid. The tannic acid was dissolved in phosphate buffer and, just before fixation, glutaraldehyde was added.

Echinosphaerium

Echinosphaerium was obtained from Carolina Biological Supply Company, Burlington, N. C., and cultured in the laboratory. Organisms were fixed in 2% glutaraldehyde to which 8% tannic acid, 0.05 M phosphate buffer, 0.015 M $CaCl_2$, and 1% digitonin were added. Fixation was carried out for 1 h. The cells were rinsed in buffer, postfixed in 1% OsO_4 in buffer at pH 6.8, dehydrated rapidly in acetone, and embedded in Araldite.

Isolated Mitotic Apparatus

The mitotic apparatus was isolated from *Strongylocentrotus purpuratus* eggs by treatment with 12% hexylene glycol in phosphate buffer at pH 6.3 (Kane, 1962). 2% glutaraldehyde containing 0.05 M phosphate buffer and 8% tannic acid at pH 6.3 was added to the isolated spindles. After fixation they were washed in buffer and postfixed in 1% OsO_4 in phosphate buffer at pH 6.3 and processed as above.

Repolymerized Chick Brain Microtubules

Brains from 14-day old chick embryos were homogenized in 0.1 M 2-(N-morpholino)ethane sulfonic acid buffer, 1 mM $MgCl_2$, 1 mM EGTA at pH 6.5. They were centrifuged at 100,000 g for 60 min and the supernate was isolated and warmed to 37°C for 20 min (Weisenberg, 1972). The microtubules were pelletted at 27,000 g for 20 min and the pellet was fixed with 2% glutaraldehyde which contained 8% tannic acid and phosphate buffer at pH 6.5. The pellet was processed as was *Echinosphaerium*.
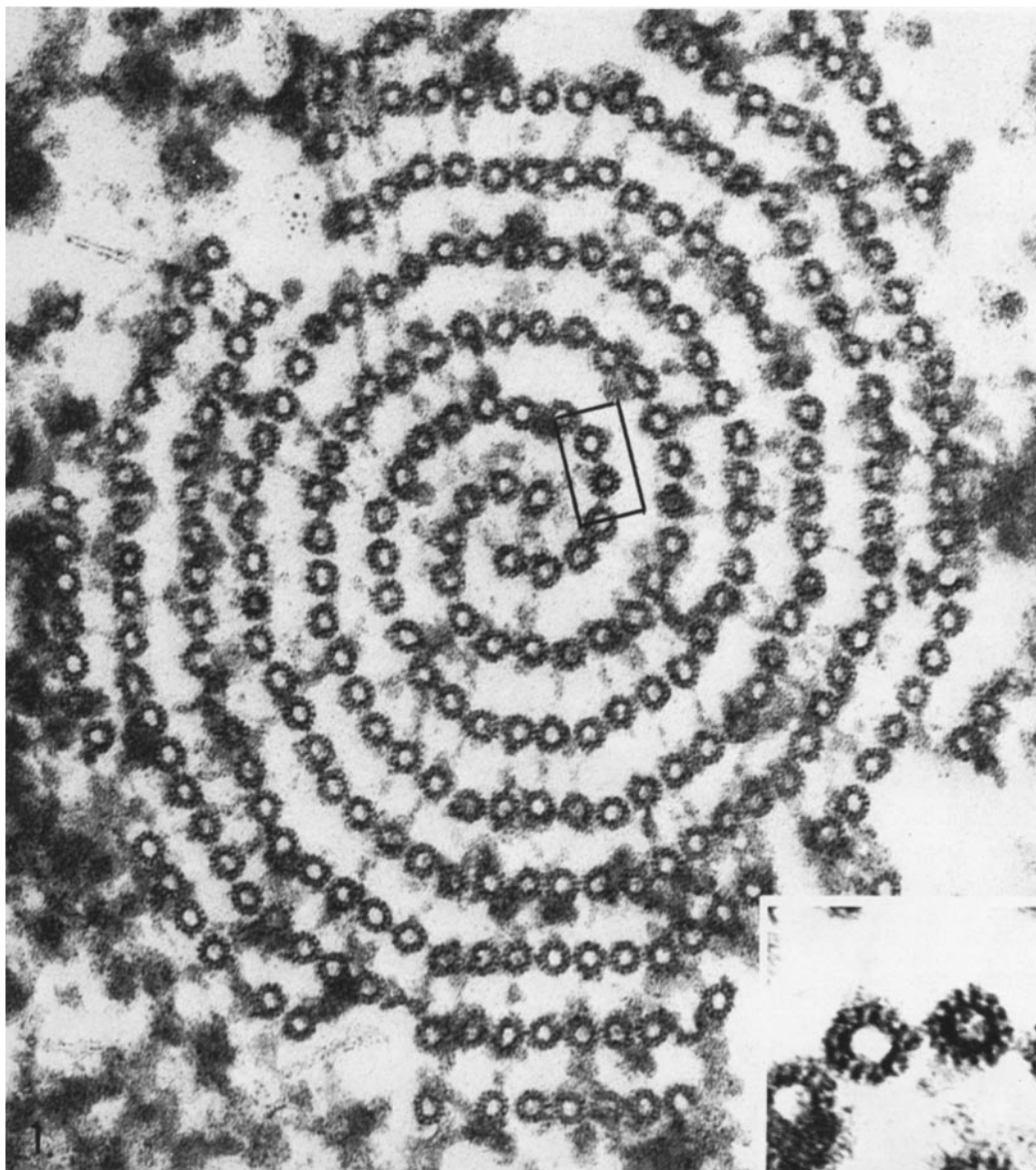
FIGURE 1   Transverse section through an axoneme of *Echinosphaerium* fixed in the presence of 8% tannic acid. The substructure of a number of the microtubules in this axoneme can be seen. × 118,000. *Insert.* Two of the microtubules are enlarged photographically. × 480,000.

Isolated Axostyles

Axostyles were isolated from the protozoa in the hind gut of the wood-eating roach, *Cryptocercus*, as described by Mooseker and Tilney (1973). They were fixed in 2% glutaraldehyde containing 4% tannic acid at pH 6.8. They were processed in the same way as the isolated mitotic apparatus.

Isolated Flagellar Axonemes

Axonemes from *Lytechinus pictus* sperm were obtained following the procedure of Stephens (1970). They were fixed in 2% glutaraldehyde which contained 2%, 4%, or 8% tannic acid at pH 7.0–7.5, and processed as the isolated mitotic apparatus.
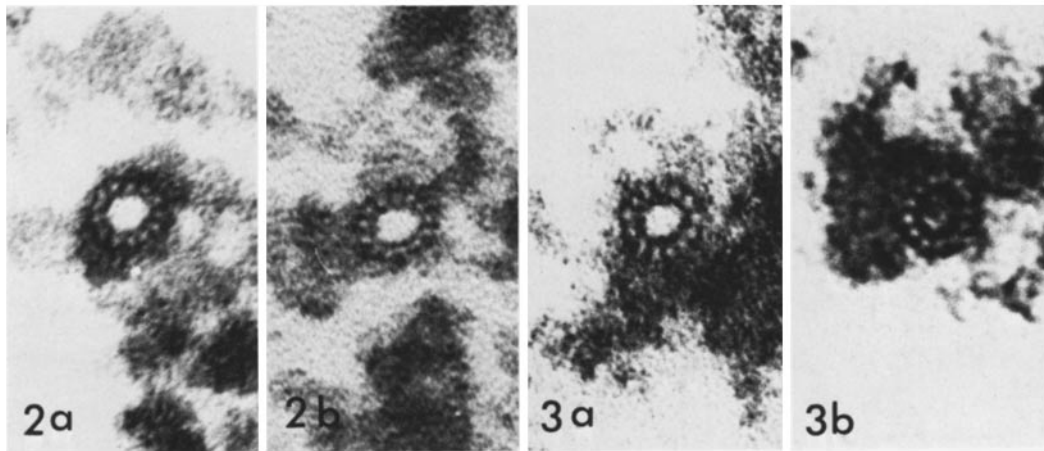
In other experiments isolated axonemes were fixed

FIGURE 2 Transverse section through microtubules from the isolated mitotic apparatus. 8% tannic acid. × 450,000.

FIGURE 3 Transverse section through repolymerized chick brain microtubules. 8% tannic acid. × 450,000.

in 2% glutaraldehyde in phosphate buffer at pH 7.5 for 30 min, then treated with glutaraldehyde containing tannic acid. After 30 min more they were washed, postfixed, dehydrated, and embedded as mentioned above. Isolated axonemes were also pretreated with tannic acid in buffer at pH 7.5 for 30 min. They were then fixed for 30 min in glutaraldehyde containing tannic acid and processed as above.

Isolated Sperm Heads

To investigate centriole ultrastructure, we fixed sperm heads in 2% glutaraldehyde containing 8% tannic acid and phosphate buffer. They were processed as above.

Electron Microscope Techniques

Thin sections were cut with a diamond knife on a Sorvall Porter-Blum MT-2 ultramicrotome, collected on uncoated grids, and either viewed directly or stained with uranyl acetate and lead citrate and then viewed with the Philips 200 electron microscope.

RESULTS

Echinosphaerium

Although full reports have not appeared, there are two papers which mention that the microtubules in the axoneme of Echinosphaerium are composed of 12 subunits (MacDonald and Kitching, 1967; Roth et al., 1970). This information, at least in the former case, was obtained by using Markham rotations. Since the axoneme has

12-fold pseudosymmetry (see Fig. 1), this in itself might predispose the viewer to assume that the microtubules are composed of 12 subunits, particularly so since earlier work demonstrated that much of the axonemal pattern was derived from bridges of two sizes and the substructure of the microtubule (Tilney and Byers, 1969). In fact, the microtubules in Echinosphaerium would appear to be the one instance in which there "ought to be" 12 subunits.

An enlargement of several of the microtubules in Fig. 1 (see insert of Fig. 1) demonstrates, however, that there are 13 equally spaced subunits making up the wall of each microtubule. The dense material surrounding the inner diameter of the wall and the dark spokes are because of tannic acid. The subunits, by contrast, appear light. Other microtubules from this micrograph and from others are invariably composed of 13 subunits.

Isolated Mitotic Apparatus

In transverse section the walls of both the chromosomal and astral microtubules contain 13 subunits (Fig. 2).

Repolymerized Microtubules from Chick Brain

Transverse sections through the microtubules (Fig. 3) show that the walls of these microtubules are composed of 13 subunits.
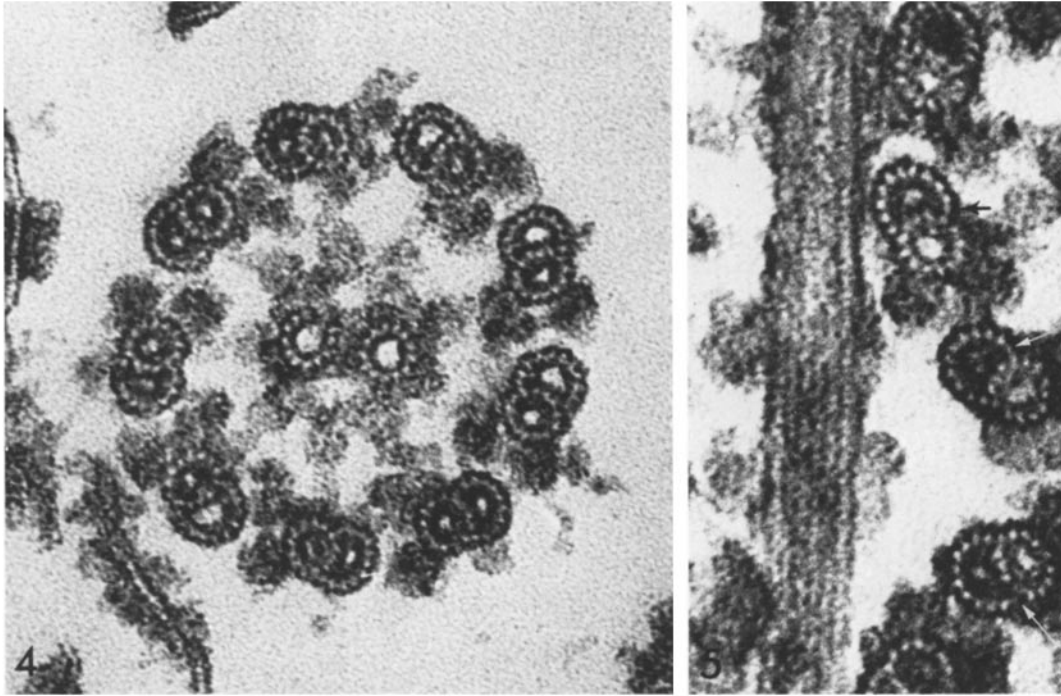
FIGURE 4 Transverse section through the isolated flagellar axoneme of *Lytechinus*. 4% tannic acid. × 330,000.

FIGURE 5 Section through a pellet of flagellar axonemes. The outer doublet, cut in longitudinal section, clearly displays the protofilament structure. Two transverse sections of portions of axonemes are seen to the right of the micrograph. The outer sides of these doublets are towards the left; their inner sides to the right. The number of subunits making up the A and the B can be counted easily and the connection of the B to the A clearly demonstrated. The arrows point towards the 11th subunit of the B. 8% tannic acid. × 360,000.

Isolated Axoneme of Sperm and Hypermastigophoran Protozoan Flagella

As expected, both the central pair of microtubules in the axoneme and the A subfiber of the outer doublet contain 13 subunits in transverse section (Figs. 4, 5, and 8). The B subfiber appears as a "c" attached to the A. Excluding the subunits in the A, the B contributes 11 subunits, though frequently the 11th subunit is smaller and less obvious than the other subunits in the B (see arrows in Fig. 5). Sometimes, in fact, it is missing altogether (see Fig. 7 of the centriole). The connection between the B and the A is from subunit to subunit, not from subunit to groove as has been suggested by others (Ringo, 1967; Warner and Satir, 1973) (see Fig. 6). The B tubule invariably appears to be bent inwards in such a way that it is
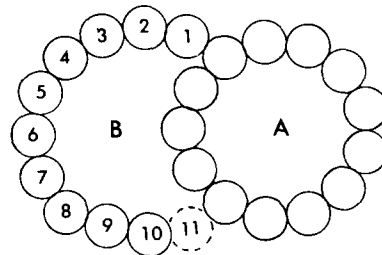

FIGURE 6 Drawing of an outer doublet. The A tubule (*A*) is composed of 13 subunits, the B (*B*) of 11 subunits. The inside of the cilium would be beneath this doublet, the outside above it.

slightly flattened. Thus the first subunit of the B, in some micrographs, appears to lie nearly in the groove between two subunits of the A (see Figs. 5 and 6). The partition between the A and the B is composed of 5 subunits with the B fiber attached

to only the two outer subunits (Fig. 6). When the isolated axoneme is allowed to warm up to room temperature after isolation, the outer connection of the B to the A is broken first. The subunits of the B begin to become solubilized at this region and are gradually lost, apparently subunit by subunit, until the innermost connection between the B and the A is reached.

Since an identical subunit morphology is present in the outer doublets of echinoderm sperm flagella and flagella from protozoa, we conclude that this pattern is universal. Thus earlier reports on the number of subunits making up the B fiber and on the precise connection of the B to the A (Ringo, 1967; Hausmann and Hinssen, 1972; Warner and Satir, 1973) are most likely invalid.

In longitudinal section the protofilaments can be easily identified (Fig. 5).

Basal Body of Lytechinus

As seen in Fig. 7, the A tubule contains 13 subunits, the B fragment 10 and a space, and the C fragment 10 and a space. As in the case of the axoneme the connections of the B subunits to the A or the C subunits to the B appear to be subunit to subunit, not a subunit of B to a groove in the

A etc. The partition between the A and the B and the B and the C involves five subunits.

Prefixation or Pretreatment with Tannic Acid

In order to be sure that the tannic acid was not itself producing an artifact, we prefixed the isolated axonemes in glutaraldehyde for 30 min, then treated them with tannic acid and glutaraldehyde. They appeared indistinguishable from those fixed in the presence of tannic acid, although usually the intensity of the staining was not as great and thus the subunits tended to be more difficult to observe. In another preparation we pretreated the isolated axoneme with tannic acid in buffer for 30 min, then fixed the axoneme in glutaraldehyde and tannic acid (Fig. 8). No difference could be found from those fixed in the presence of tannic acid. Thus tannic acid, by itself, appears not to affect the structure.

Isolated Axostyles

Transverse sections through the axostyle reveal that all the microtubules are composed of 13 subunits (Fig. 9).
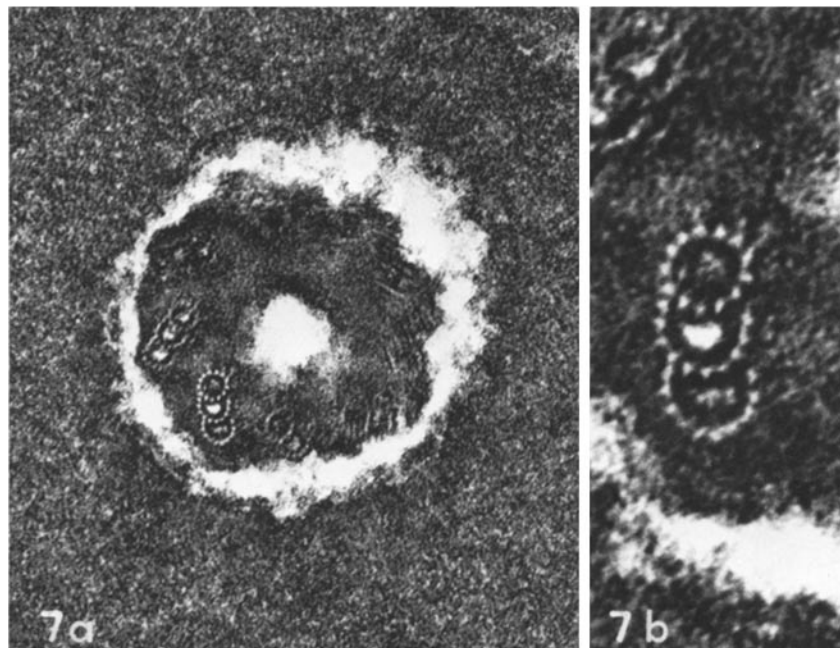

FIGURE 7 Transverse section through the basal body of a flagellum of *Lytechinus* sperm. (a) × 150,000. (b) × 470,000.
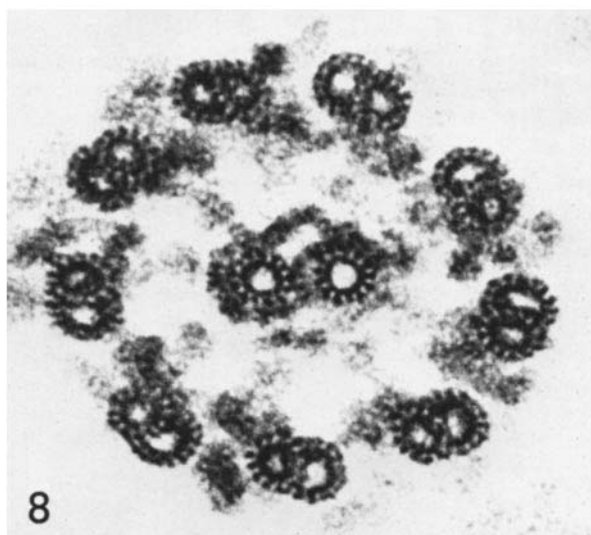
FIGURE 8   Transverse section through the flagellum of *Lytechinus* sperm which had been pretreated in 4% tannic acid in buffer for 30 min before fixation with glutaraldehyde containing tannic acid. Thus tannic acid by itself has no effect on the axoneme. × 300,000.
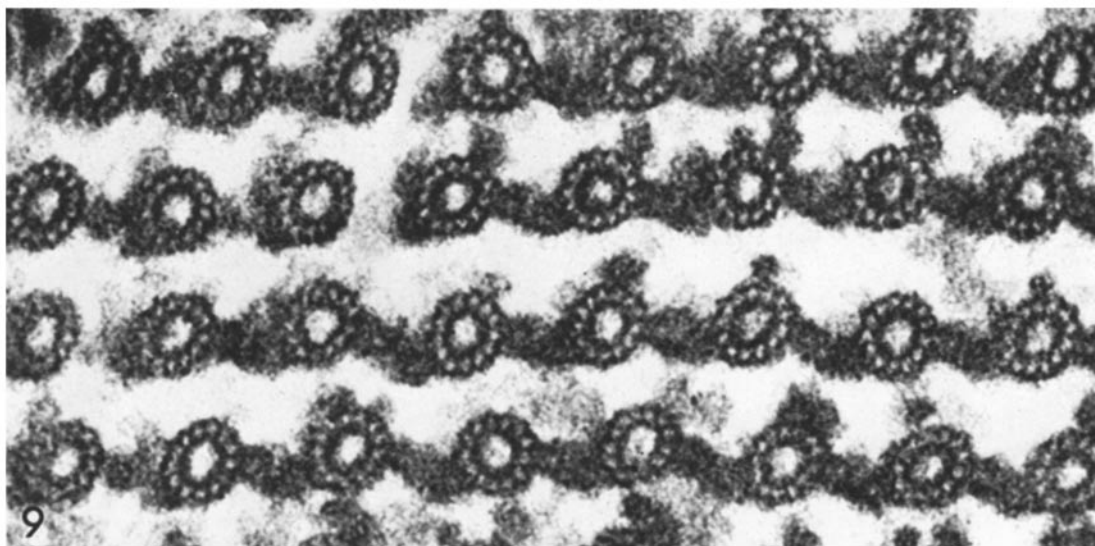

FIGURE 9   Transverse section through a portion of an isolated axostyle. × 450,000.

DISCUSSION

We have reported on the substructure of seven different types of microtubules from protozoa, echinoderms, and birds. We illustrated four types of cytoplasmic microtubules, namely those in the heliozoan axoneme, in the mitotic apparatus, in chick brain homogenates and in the contractile axostyle, and three types of microtubules in

flagella and the associated basal body, namely the A tubules and the central pair. In all cases, microtubules are composed of 13 equally spaced subunits.

In the longitudinal section the wall of the microtubule is made up of protofilaments similar to what has been described by negative staining (see Introduction for references) indicating that the

protofilament structure is not an artifact induced by drying. The fact that the microtubule is composed of linearly arranged protofilaments is essential for the applicability of tannic acid staining. As can be seen by the micrographs, the tannic acid surrounds the subunits and is cross-linked in place by the glutaraldehyde producing, in effect, a "negative stained" image. Thus the groove between adjacent protofilaments is filled with tannic acid, leaving the subunits, by contrast, unstained. When thin sections are cut and the microtubules examined exactly normal to their axes, the grooves show up very clearly as there is a column of electron-dense material running through the width of the section. Clearly, if the protofilaments did not run parallel or nearly parallel to the long axis of the microtubule, observation of the grooves would be impossible. It is not surprising, therefore, that the section has to be cut exactly normal to the long axis of the microtubule in order to see the subunits; a small angle of tilt completely eliminates their visibility. Consistent with the above

explanation is the observation that the subunits can be readily seen in the thinnest sections if 8% tannic acid is added to the fixative, yet if 1% tannic acid is used, considerably thicker sections must be cut in order to clearly distinguish the subunits.

The 11th subunit of the B (Fig. 6) needs further clarification. This subunit appears either smaller or less dense than the others in the B or is completely absent. The possibility exists that this subunit may be biochemically different from the 10 others in the B and the 13 in the A; if this is true, the amount of tubulin for this subunit would be very small (1/23) relative to the amount of tubulin present in the other subunits of the outer doublets. Why the 11th subunit is sometimes absent is not evident because when the B tubule breaks down, as by heating, it starts from its outer connection to the A, *not* from the inner connection.

In conclusion, the number of subunits and their arrangement as protofilaments appear universal
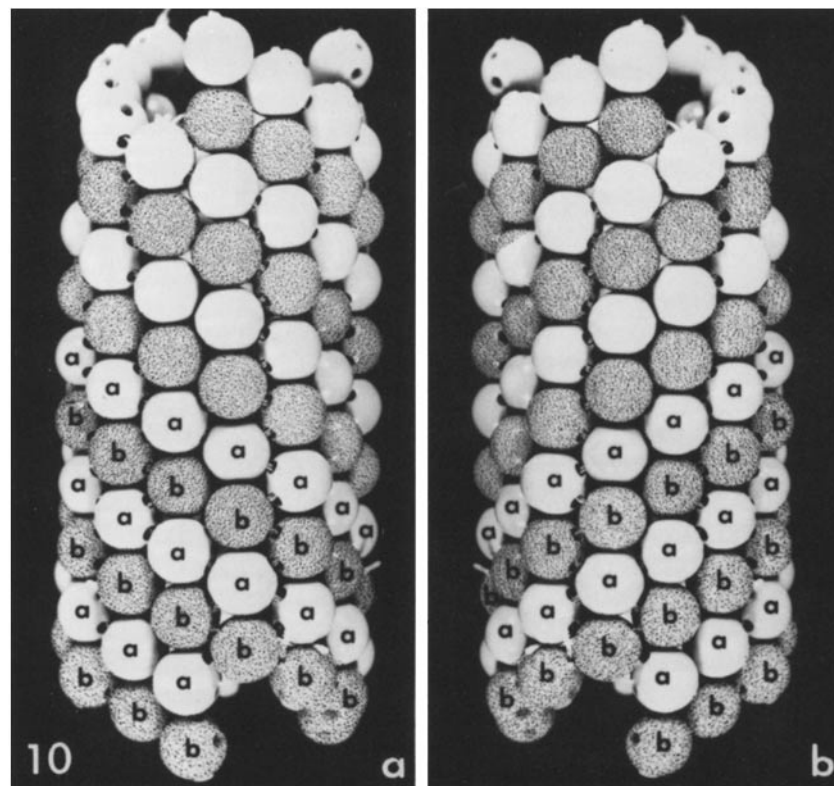

FIGURE 10 Possible models of a microtubule which could account for the protofilament structure described in this paper. Model *b* is probably the correct model for *Echinosphaerium*.

both as to phylogeny and as to location, whether in a cilium or in cytoplasmic microtubules. Thus differences in the relative stability of microtubules (Behnke and Forer, 1967; Tilney and Gibbins, 1968) and in the precise determination of bridges connecting microtubules must be accounted for in other ways than by varying subunit numbers. In addition, since all the microtubules have an odd number of subunits, since the basic unit appears to be a heterodimer (Bryan and Wilson, 1971; Feit et al., 1971), and since the units are half staggered (Cohen et al., 1971), there can be only a limited number (two) of possibilities as to how the subunits can be arranged: that is, each protofilament is composed of a succession of repeating heterodimers (Fig. 10). If we now establish the chirality of a tubule, then only one model will suffice. In *Echinosphaerium* the axoneme is a chiral structure. From unpublished observations we know that the chirality of the axoneme is counterclockwise when viewed from the base to its tip. (There are a few exceptions to this statement, less than 1% of the thousands of axonemes observed; these are best interpreted as artifacts caused by fixation.) We presume that this handedness is related to the substructure of the microtubule. Thus model B in Fig. 10 is probably the only correct model for a microtubule. A further feature of this model is that the tubules have an intrinsic polarity, a feature which is necessary for a number of models of microtubule-associated motility.

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