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THE SUBSTRUCTURE OF CILIARY MICROTUBULES

F. D. WARNER* AND P. SATIR
Department of Physiology-Anatomy, University of California, Berkeley, California, 94720, U.S.A.

SUMMARY

Gill cilia from the freshwater mussel *Elliptio complanatus* were examined after negative staining with phosphotungstic acid. The doublet and central pair microtubules typically collapse on the grid supporting film so that the protofilaments making up the wall of each tubule spread into a visible array. Thirteen protofilaments can be seen to form both the central pair microtubules and subfibre A of each doublet. Subfibre B of the doublet consists of 10 protofilaments.

As viewed in both normal and thiourea-treated cross-sectioned cilia, subfibre A appears as a complete microtubule with a circular profile and 26 nm diameter. Subfibre B is an incomplete, elliptically profiled tubule that shares, as a common wall or partition, 4 or 5 protofilaments of the A subfibre. The organization of the 3·5-nm thick protofilaments within the microtubules and their relationship to the various doublet appendages is discussed.

INTRODUCTION

Inasmuch as microtubules are the main structural component of several important cell organelles, they have elicited interest and controversy concerning both their structure and biochemistry. Early studies of negatively contrasted microtubules from eukaryotic flagella (André & Thiéry, 1963; Pease, 1963) demonstrated that the wall of individual tubules was made up of a number of linearly arrayed 'protofilaments'. Because of the tendency of microtubules to collapse upon themselves when negatively stained, the number of protofilaments most often observed in single tubules was 4–6. Occasionally as many as 10 filaments were observed at the end of a broken and frayed tubule. The protofilaments were generally observed lying both straight and parallel to one another in both intact and partially collapsed microtubules, suggesting that the stronger intermolecular bonding was in the linear dimension and that a weaker, co-lateral bonding held adjacent filaments into the wall lattice. Each protofilament had a diameter of about 4 nm and a repeating 4-nm period occurred along its axis (Grimstone & Klug, 1966). The 4-nm repeat is thought to represent the globular and monomeric form of microtubule protein of molecular weight $\sim$ 60000 (Shelanski & Taylor, 1968).

Subsequent observations of sectioned flagella have placed the number of protofilaments per microtubule nearer 12–14 for both central tubules and doublet subfibre A, and 8–10 filaments for subfibre B (for review, see Arnott & Smith, 1969). Moreover, Phillips (1966) has shown that in the doublet microtubules, subfibre A is a

* Present address: Department of Biology, Biological Research Laboratories, Syracuse University, Syracuse, New York, 13210, U.S.A.

complete tubule, while subfibre B is an incomplete, C-shaped tubule that shares, according to Ringo (1967), 3 or 4 filaments of the adjacent A subfibre.

Recent biochemical studies of doublet microtubules of *Chlamydomonas* flagella (Witman, 1970; Olmsted, Witman, Carlson & Rosenbaum, 1971) have convincingly shown that each doublet consists of a minimum of 2 proteins or tubulins, both of which are present in each subfibre of the doublet. In addition, certain cytoplasmic microtubules may also consist of 2 tubulins (Bibring & Baxandall, 1971; Bryan & Wilson, 1971; Olmsted *et al.* 1971). Because of this protein heterogeneity in single microtubules, determination of the precise organization of the tubule wall subunits assumes some significance; particularly in view of the seemingly requisite, periodic attachment sites for the various appendages occurring along the wall of both the A subfibres and the central pair microtubules (Hopkins, 1970; Warner, 1970, 1972; Satir, 1972).

In this study we present unequivocal evidence, based on direct observation, that clarifies the number of protofilaments making up the 3 kinds of ciliary microtubules. Furthermore, we present evidence concerning the organization of the protofilaments in the doublet microtubules based on observations of thiourea-treated, 'negatively stained' cilia.

MATERIALS AND METHODS

Gill cilia of the freshwater mussel *Elliptio complanatus* were utilized in this study. No distinction is made between the 4 kinds of cilia that occur along the gill epithelium since, with regard to axoneme structure, all are morphologically indistinguishable, although each can be identified by other criteria.

For negatively stained preparations, gill cilia were mechanically separated from the epithelium into 10 mM HEPES buffer at pH 7·4. A drop of the cilia-containing suspension was placed on a coated grid and the buffer diluted away with distilled water. A drop of 1 % phosphotungstic acid (PTA) at pH 5·0 or 7·4 was then added to the grid, excess fluid was removed, and the preparation allowed to air dry. Total staining time was 1–2 min at room temperature.

For thin-section microscopy, gill tissue was excised and fixed at 4 °C for 1 h in 2 % glutaraldehyde adjusted to pH 7·4 with 0·05 M sodium cacodylate buffer. The tissue was postfixed for 30 min in 1 % OsO_4 in the same buffer and embedded in Epon 812. Thin sections were stained for 15 min in 5 % aqueous uranyl acetate followed by 2 min in Reynolds' lead citrate. 'Negatively stained' thin-sections (Fig. 10) were prepared by treating the gill tissue with 0·5 M thiourea for 15 min prior to fixation in 1 % OsO_4. Thiourea is generally used as an inhibitor of ciliary motility (Brokaw, 1964). Thin sections were stained as above.

All electron micrographs were calibrated to less than 5 % magnification error using carbon-grating replicas.

OBSERVATIONS AND DISCUSSION

Elliptio gill cilia negatively contrasted with PTA are typically observed minus their membrane and with the axoneme splayed apart on the grid supporting film in such a way that the 9 doublet and 2 central microtubules are visible. The doublet tubules generally appear reasonably intact, while subfibre A shows little evidence of its wall subunits (Fig. 2). Subfibre B and the central microtubules are often partially splayed open but, since the tubules tend to collapse upon themselves, no more than 5 or 6

protofilaments are generally visible (Fig. 2). Subfibre A is always distinguishable from subfibre B by its tendency to remain intact or by the presence of the attached arms and radial links that are sometimes visible.

Occasionally, and most often in the distal region of the cilium, the degradative action of the PTA results in the complete collapse of the axoneme microtubules and the spreading of all of their protofilaments into a visible array. Figs. 3–5 show different doublet tubules in this condition and in each, the protofilaments making up the subfibres can be accurately counted. Fig. 4 shows 13 protofilaments in subfibre A, while in the lower region of the micrograph subfibre B is incompletely spread and only 7 filaments are visible. Proceeding distally, however, subfibre B becomes completely spread and 10 protofilaments can be counted. The distance separating the 2 subfibres is sufficient to preclude any confusion between A and B. In addition, both subfibres were traced back to intact regions where their identification was obvious. Fig. 4 shows subfibre A comprises 13 protofilaments, although again, only 7 are visible in subfibre B. Similarly, Fig. 5 shows 10 protofilaments in subfibre B, while subfibre A is intact. Figs. 6 and 7 show 2 completely spread central microtubules and in each it is clear that 13 protofilaments make up the tubule wall.

The protofilaments have an average diameter of about 3·5 nm and a minimum centre-to-centre spacing of about 4·5 nm in all of the spread microtubules observed. In regions where microtubules have broken, the protofilaments appear to curl and form a fountain-like array (Fig. 8) and some broken segments form nearly complete circles on the supporting film. Curling of the protofilaments suggests that they are under tension along the linear axis.

In summary, we have observed numerous cilia where the number of protofilaments forming their microtubules can be counted. Thirteen protofilaments make up each of the A subfibres and central pair microtubules. Similarly, we have observed a maximum of 10 protofilaments in the B subfibre. Since electron-microscopic (Behnke & Forer, 1967; Phillips, 1966) and X-ray diffraction studies (Cohen, Harrison & Stephens, 1971) have set the number of protofilaments at 12 or 13 for single flagellar microtubules, it seems likely that 13 is the true number in typical cilia and flagella. It can be strictly stated, however, that we have seen only the minimum number of protofilaments possible in *Elliptio* gill cilia. Exceptions do occur, as in the accessory microtubules of cricket sperm flagella, where 16 protofilaments appear to make up the tubule wall (Kaye, 1970). In addition, variation in protofilament number may occur among the various kinds of cytoplasmic microtubules, although in several instances, 12 or 13 filaments have been reported (Behnke & Zelander, 1966; Kiefer, Sakai, Solaris & Mazia, 1966; Ledbetter & Porter, 1964).

Protofilament organization within doublet microtubules

Knowledge of the number of protofilaments making up the ciliary doublet microtubules, along with detailed measurements taken from both normal and thiourea-treated, cross-sectioned cilia (Figs. 9, 10), enabled us to construct a model of protofilament organization as it appears in the doublet microtubules. This model is shown diagrammatically in Fig. 1, along with the pertinent measurements.

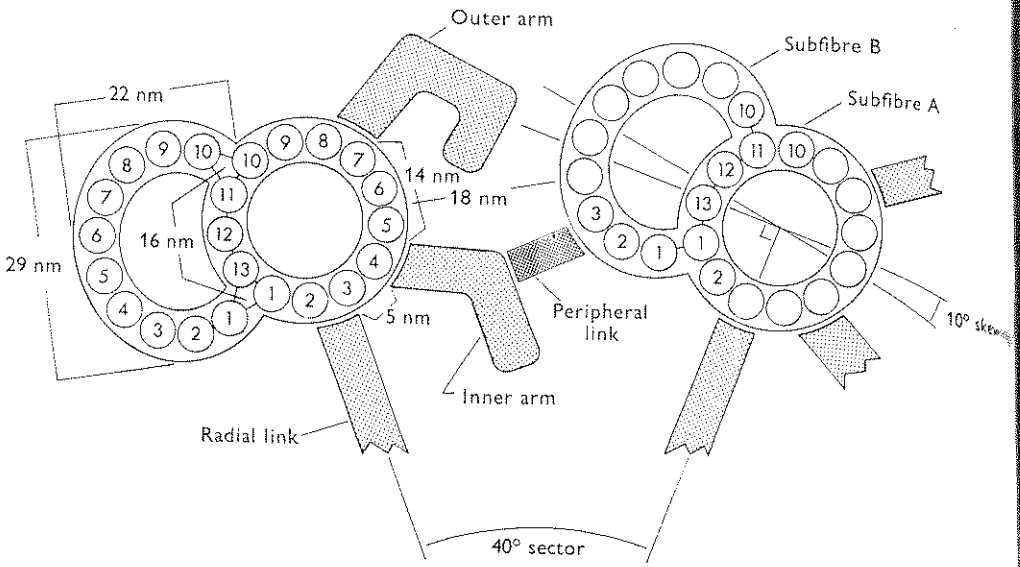

Fig. 1. Schematic interpretation of the protofilament organization in the doublet microtubules of *Elliptio* gill cilia, as viewed from base-to-tip. The axoneme's 9-fold radial symmetry is divided into nine 40° sectors delineated by the plane of the radial links. Two possible A to B subfibre junction patterns are shown in the doublets. Distinction between the two involves only a rotational shift of about 2·5 nm in subfibre A. Measurements not indicated on the diagram are given in the text. Drawn approximately to scale.

Subfibre A of the doublet typically has a circular outline or profile when viewed in cross-section but occasionally may appear laterally compressed. Subfibre B is decidedly out-of-round and is foreshortened to the form of an approximate 60° ellipse (Fig. 9). This is particularly clear in the thiourea-treated cilia (Fig. 10) where, even though the axoneme symmetry has been disrupted, the dimensions and form of the doublet subfibres remain as they appear in untreated cilia. Each doublet lies in a plane that is 8–10° to the tangent of the axoneme radius as drawn through the centre of subfibre A; which is about 80 nm from the axoneme axis (Figs. 1, 9). It is this 8–10° doublet skewing and the dynein arm attachment to subfibre A that impart the enantiomorphic asymmetry to the axoneme profile and allow specific doublet numbering (Afzelius, 1959); although it should be noted that enantiomorphism is also an intrinsic property of the individual doublet subfibres, independent of attached arms or links (Satir, 1972). Consequently, knowing the number of protofilaments in the subfibres enables us to number each filament reliably (Fig. 1) and the number assignment remains the same regardless of the direction in which the axoneme is viewed. As used in Fig. 1, protofilament numbering in subfibre A is counterclockwise and in subfibre B it is clockwise when the axoneme is viewed from base-to-tip. A tip-to-base view would reverse the numbering ascension in each subfibre.

In *Elliptio* gill cilia, subfibre A has an outside diameter of about 26 nm and a wall thickness of 6–6·5 nm. The latter is considerably greater than the 3·5-nm protofilament diameter observed in negatively stained preparations, but this may be accounted

for by the deposition of the electron-opaque stain on the tubule surfaces. This seems particularly evident in the thiourea-treated cilia (Fig. 10), where the protofilaments stand out in negative relief in the tubule wall. The filaments have an approximate diameter of 3·5 nm and a centre-to-centre spacing of about 4·5 nm. The tubule wall thickness (positive staining) shows considerable variation, apparently dependent upon the amount of stain that has accumulated on the protofilament surface. Support for this interpretation also comes from observations of insect sperm flagella, where the microtubule wall thickness may reach 8·5 nm yet the protofilaments are still approximately 4 nm in diameter when negatively stained (Warner, 1970). Moreover, in sectioned flagella and some cytoplasmic microtubules, the 4-nm protofilaments often appear in negative relief (Kaye, 1970; Ledbetter & Porter, 1964; Phillips, 1966; Ringo, 1967), as in our thiourea-treated cilia. This suggests that, for unknown reasons, the electron-opaque stain sometimes does not penetrate the protofilament but rather either stains material around the filament or simply accumulates on the filament surface. This interpretation may account for the great diversity of tubule wall thicknesses that have been reported (*ca.* 5–8·5 nm), whereas a fairly uniform protofilament diameter of 3·5–4·5 nm has been consistently observed in negatively stained microtubules.

As shown by Phillips (1966) and supported by morphogenetic evidence (Warner, 1971), subfibre B is an incomplete, C-shaped microtubule that shares as a midwall perhaps 3–5 protofilaments of the A subfibre (Ringo, 1967). The A subfibre is always seen as a complete microtubule after subfibre B termination near the ciliary tip (Satir, 1968). The shared or partition protofilaments can be isolated by Sarkosyl solubilization of the doublet tubules, and in *Chlamydomonas* flagella they appear as intact groups of 3 protofilaments (Witman, 1970). Since it is apparent that a minimum of 10 protofilaments make up subfibre B in *Elliptio*, according to the filament numbering sequence used in Fig. 1, and morphological criteria given below, the B subfibre can share as many protofilaments as numbers 10, 11, 12, 13, and 1 of subfibre A (notation $A_{10,11,12,13,1}$). It is not clear, however, if filaments $B_{1,10}$ bond individually to filaments $A_{1,11}$ respectively, or if $B_{1,10}$ each bonds to 2 protofilaments ($A_{13,1}$ and $A_{10,11}$). Both patterns of junction are shown in Fig. 1 and in either pattern, the distance separating $B_{1,10}$ remains the same. All of the doublets that are clear in Figs. 9 and 10 have a $B_{1,10}$ separation in the range of 16–19 nm. These measurements are particularly reliable on the thiourea-treated cilia, where the position of the protofilament axis can be predicted. Consequently, our observations favour a 4 or 5 protofilament partition model and indeed, 3 protofilaments can be seen to lie completely free of the $B_{1,10}$ junctional position indicated in Fig. 10. It is also conceivable, based on our measurements of the $B_{1,10}$ separation, that $B_{1,10}$ each bonds to $A_{1,10}$, respectively, rather than the shared bonding pattern shown in Fig. 1 (left). However, the solution to this bonding pattern (1:1 or 1:2) must await a more refined method of viewing the joining of the A and B subfibres.

In Fig. 1 left (shared bonding) one could say that subfibres A and B are equivalent, i.e. composed of 10 protofilaments and sharing numbers 11, 12 and 13, in accord with Ringo's (1967) idea that subfibre B should be considered as, at least in principle, a

13-protofilament microtubule. Our own morphological observations on *Chlamydomonas* flagella indicate that the geometrical and dimensional parameters are quite similar to those in *Elliptio*. Therefore our model might suggest how 3 protofilaments can be isolated as the partition filaments in *Chlamydomonas* (Witman, 1970), although it remains to be seen whether these protofilaments are numbers 11, 12 and 13 as the model predicts.

In addition to the junctional pattern between the A and B subfibres, the relative positions of the radial link and the dynein arms on subfibre A can be predicted. The radial link lies in the sector plane (40°) and thus abuts $A_{2,3}$ or A_3, depending on the A–B junctional pattern used (Fig. 1). Similarly, although with less accuracy, the dynein arms project from either $A_{7,8}$ or A_8 (outer arm) and A_4 or $A_{4,5}$ (inner arm). In the latter case, the inner arm appears to make contact, via the peripheral link (Allen, 1967; Kiefer, 1970; Warner, 1972) with $B_{2,3}$. Since so little is known about either the linkages or arms, very little can be said of their respective attachment sites except that, given their specific longitudinal periodicities (Hopkins, 1970; Warner, 1970), their attachment must be very specific both with respect to the individual protofilament involved and the longitudinal binding site along that protofilament. Such specificity suggests that protein heterogeneity within the microtubule, and possibly within the protofilament, is to be expected.

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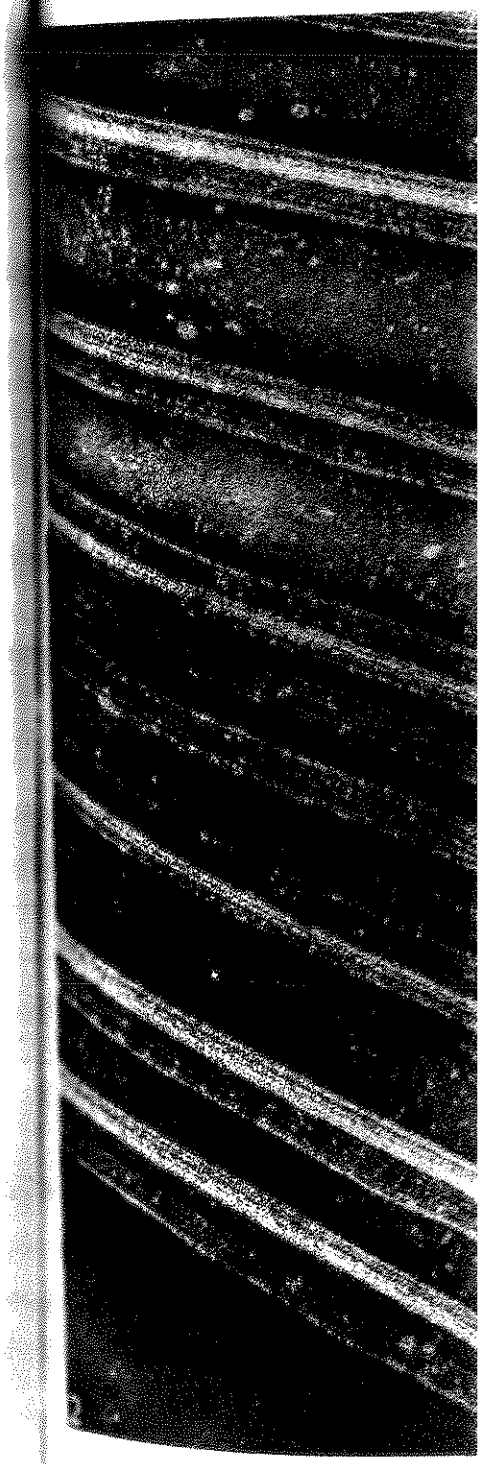

Fig. 2. PTA negatively stained cilium showing 7 doublet and 2 central (*cm*) microtubules. Subfibre A (*A*) of each doublet can be distinguished from subfibre B (*B*) by its tendency to remain intact. Both the B subfibres and the central tubules are partially collapsed, revealing their wall protofilaments. × 190 000.

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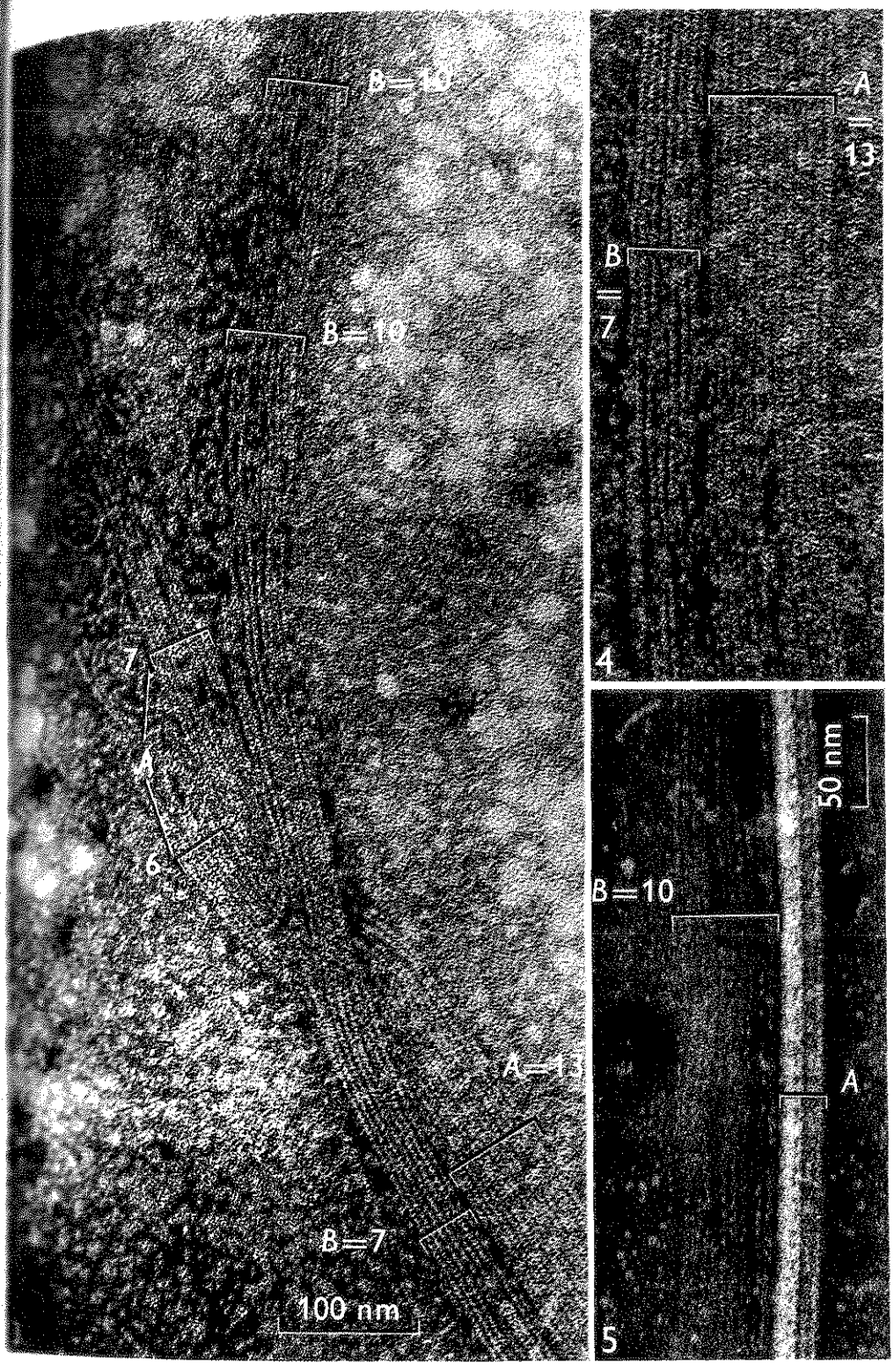
Fig. 3. A completely spread doublet which clearly reveals the number of protofilaments making up both subfibres. In the lower region of the micrograph, subfibre A consists of 13 protofilaments while only 7 are visible in subfibre B. Proceeding distally, subfibre B becomes completely spread and 10 filaments are visible. × 200000.

Fig. 4. A spread doublet showing 13 protofilaments in subfibre A and only 7 in subfibre B. × 260000.

Fig. 5. A single doublet showing an intact A subfibre and 10 protofilaments in subfibre B. × 260000.



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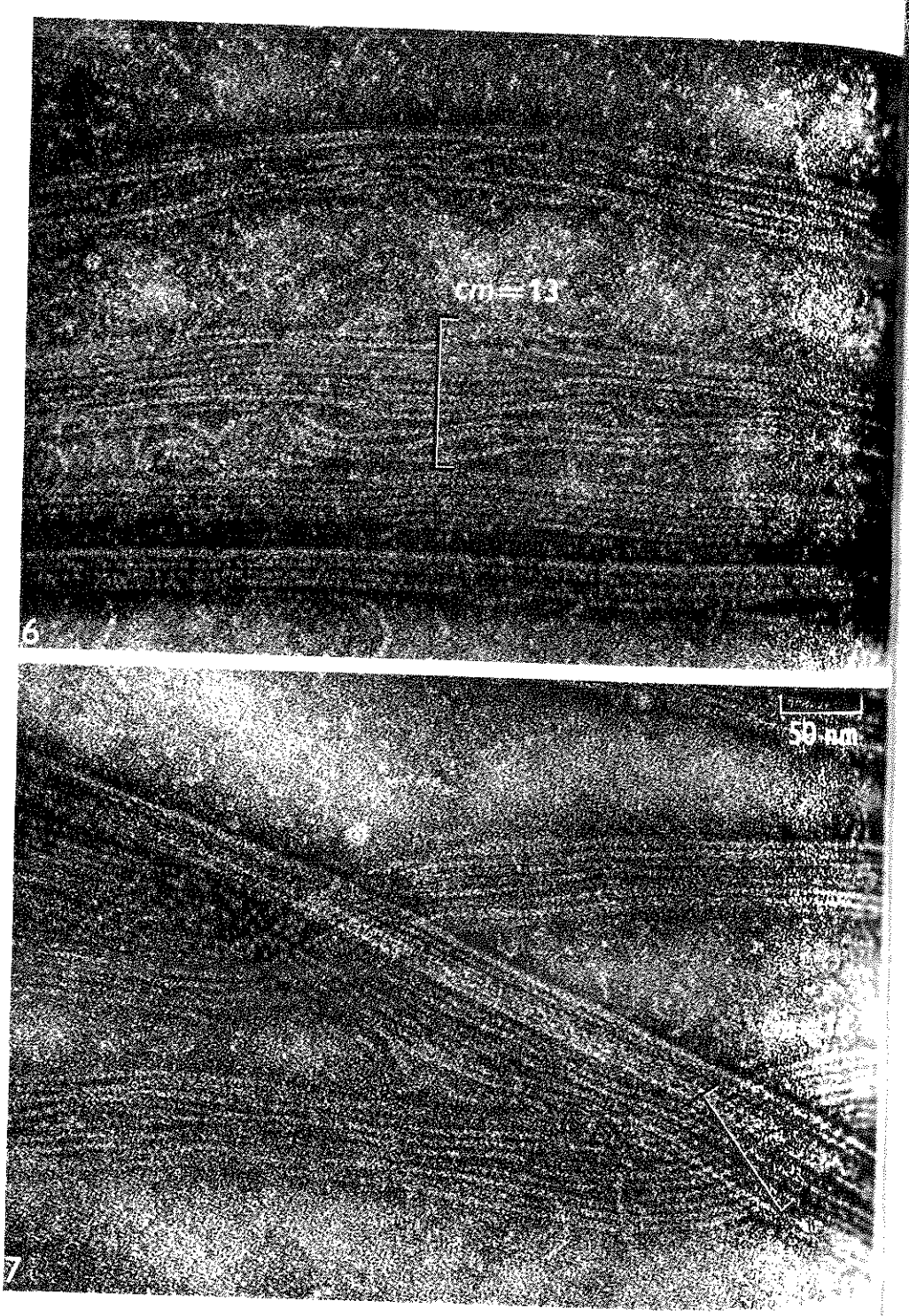

Figs. 6, 7. Negatively stained central pair microtubules (*cm*) that have spread so th
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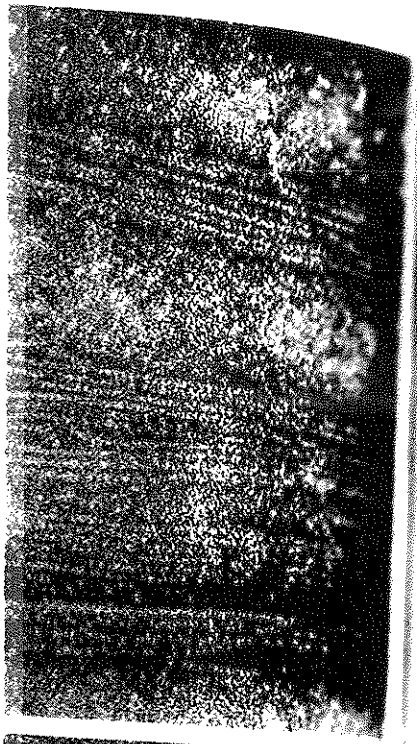

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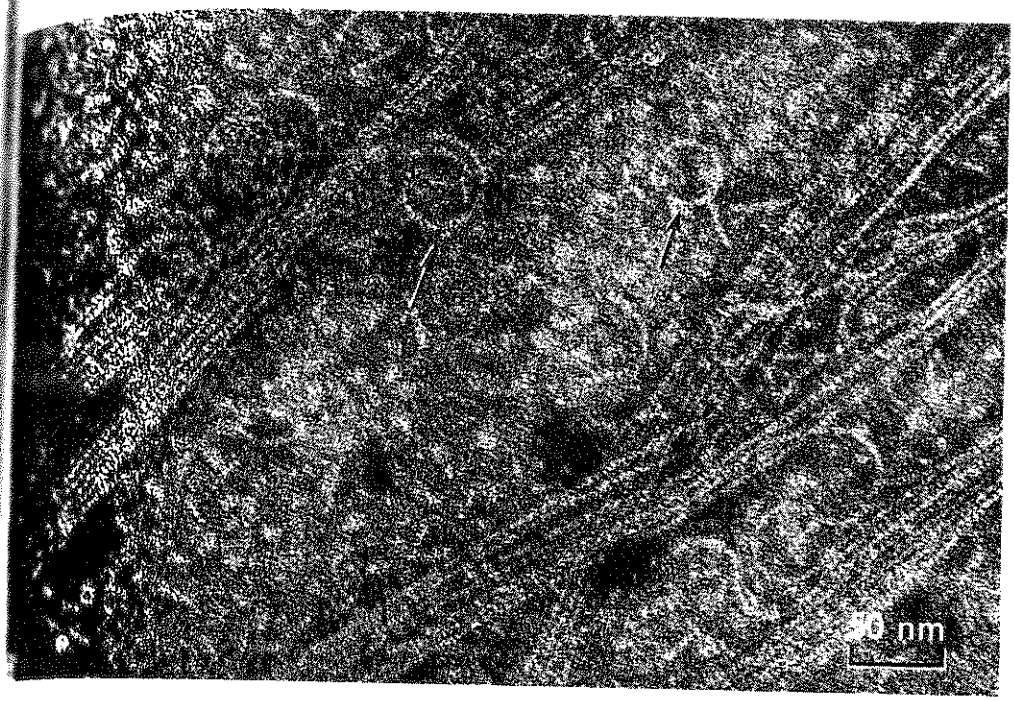

. 8. Negatively stained doublet microtubules that have broken on the supporting
. The disrupted protofilaments curl into a fountain-like array and some broken
ments form nearly complete circles (arrows). × 190000.

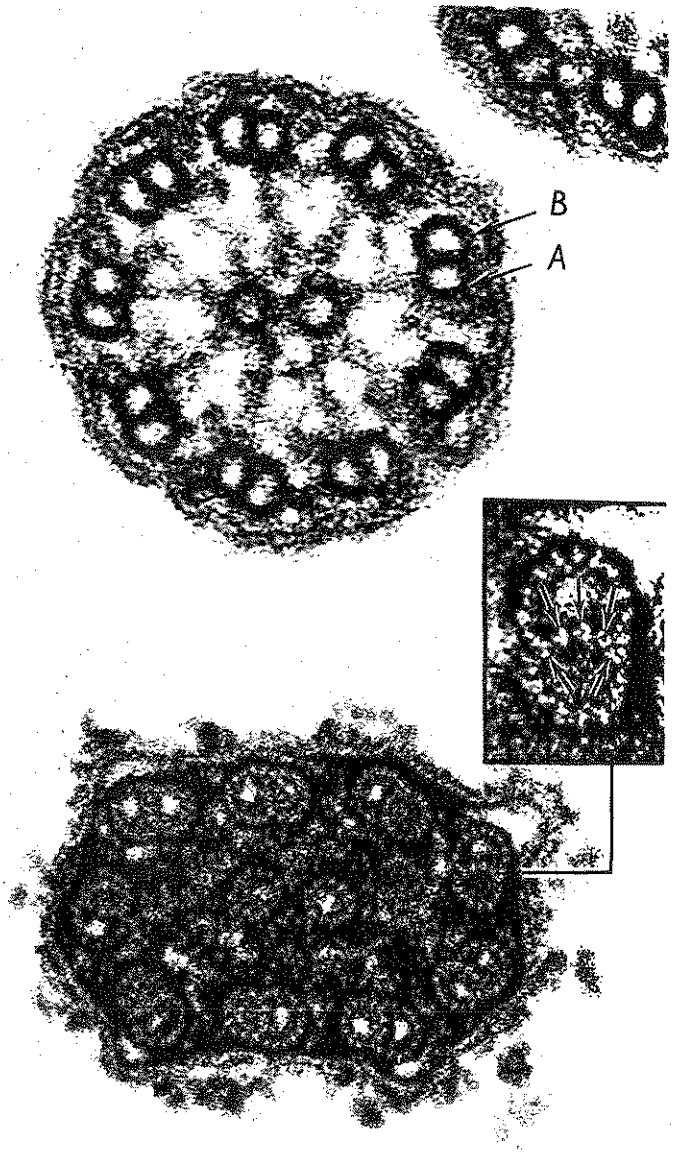

Fig. 9. Cross-sectioned lateral-frontal cilium viewed from asbe-to-tip. D number 1 lies in the 12 o'clock position and numbering proceeds clockwise. ×

Fig. 10. Cross-sectioned lateral cilium that has been treated with thiourea p fixation. The protofilaments of the tubule wall stand out in negative relief. The from cilium base-to-tip. × 290 000. The inset is an enlargement of the indicated d with subfibre B at the top. Three protofilaments appear to lie free (3 upper arro the A–B subfibre junction (2 lower arrows). The calibration scale is 1 mm = 1 × 620 000.

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MICROFILAMENT STREAMING: INH WITH CYTOCHAL

M. O. BRADLEY
*Department of Biological Scien U.S.A.**

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