Published October , 1984

Alternate Patterns of Doublet Microtubule Sliding in ATP-disintegrated Macrocilia of the Ctenophore *Beroë*

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ABSTRACT We have used the unique properties of macrocilia from the lips of the ctenophore *Beroë* to test whether the ciliary beat cycle is caused by sequential activation of doublet sliding on opposite sides of the axoneme (Satir, P., 1982, *Soc. Exp. Biol. Symp.*, 35: 179–201; Sugino, K., and Y. Naitoh, 1982, *Nature (Lond.)*, 295: 609–611; Wais-Steider, J., and P. Satir, 1979, *J. Supramol. Struct.*, 11:339–347). Macrocilia contain several hundred axonemes linked into rows by lamellae between doublets 3 and 8. These connections provide morphological markers for numbering the doublet microtubules in thin sections. Demembranated, detached macrocilia undergo ATP-induced sliding disintegration by extrusion of thick fragments and finer fibers from the proximal end. Disintegration can easily be followed with low-magnification brightfield or phase-contrast optics. Sliding occurs with or without added elastase, and is reversibly inhibited by vanadate. Thin sections through 16 ATP-disintegrated macrocilia showed two mutually exclusive patterns of doublet extrusion with equal frequency. Doublets 9, 1, and 2 or doublets 5, 6, and 7 were usually extruded, but not both groups. We conclude that both subsets of doublets slide by their own active arms, and that the two extrusion patterns represent alternate activation and inactivation of doublet sliding on opposite halves of the axoneme. These findings provide the first direct experimental support for a switching mechanism regulating microtubule sliding in cilia.

Current work shows that ciliary and flagellar motility is caused by active sliding between outer doublet microtubules, powered by dynein ATPase cross-bridges, coupled with controls that regulate and resist sliding to convert it into local, propagated bending (6, 8, 17). In trypsin-treated axonemes all nine doublet microtubules (except for doublets 5 and 6 in certain cases) actively slide in a single direction (16). If a constant polarity of dynein force generation holds for intact cilia, then arms on opposite sides of the axoneme would act antagonistically with respect to creating bends (3); therefore, not all doublets can be actively sliding with equal force at the same time. This has led to the view that a planar beat cycle is caused by alternate activation of doublet sliding on opposite sides of the axoneme (17, 18, 22).

So far, no direct experimental evidence has been obtained for this "switch-point" hypothesis, i.e., for the sequential activation of dynein arms on alternate halves of the axoneme during the ciliary beat cycle. In this report we take advantage of the unique properties of macrocilia (9) from the lips of the ctenophore *Beroë* to attack this problem. Using the technique of ATP-induced sliding disintegration developed by Summers and Gibbons (19), we demonstrate alternate patterns of doublet microtubule sliding around the axoneme for the first time.

MATERIALS AND METHODS

Ctenophores: Medium to large specimens of *Beroë cucumis* were collected by Mr. Anthony Moss from Cape Cod Bay. *Beroë* in excellent condition were also sent from Friday Harbor Laboratory by Dr. Claudia Mills. Animals were maintained in fresh-running seawater in the laboratory.

Electron Microscopy of Intact Macrocilia: *Beroë* were placed in 7.0% $MgCl_2$:seawater (1:1) to relax body muscles before dissection and fixation. Macrocilia are found in a dense band around the inner margin of the lips of *Beroë*. Lips were excised and fixed in 2.5% glutaraldehyde, 1% paraformaldehyde, 0.15 M NaCl, 0.01 M $CaCl_2$, 0.2 M Na cacodylate, pH 7.8, for 2 h at 4°C. Lips were stored in buffer (0.3 M NaCl, 0.2 M Na cacodylate, pH 7.8) overnight at 4°C, then cut into smaller pieces and postfixed in 1% osmium tetroxide, 0.37 M NaCl, 0.1 M Na cacodylate, pH 7.8, for 30 min at room temperature. After rinsing in distilled water, lip pieces were placed in 0.5% uranyl acetate in veronal-acetate buffer for 6 h at room temperature. Dehydration in acetone was followed by embedding in Araldite. Thin sections were stained with uranyl and lead salts, and examined in a Zeiss 10CA electron microscope at 80 kV.

ATP Reactivation and Sliding Disintegration: Solutions were modified from those devised previously for reactivation and sliding disintegra-

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 OCTOBER 1984 1364–1371

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tion of comb plate cilia (21). Small pieces of lips were placed in 0.5 ml of extraction solution (ES),[1] consisting of 0.05% Triton-X 100, 10% glycerol, 150 mM KCl or K acetate, 5 mM $MgCl_2$, 2 mM EGTA, 30 mM PIPES, pH 6.9, for 8–10 min at room temp. Pieces were then transferred into two drops of reactivation solution (RS) or wash solution (WS) on a microscope slide, and macerated vigorously to detach macrocilia from the epithelium before adding a coverslip. Reactivation solution consisted of 2 mM ATP, 1 mM dithiothreitol, 150 mM KCl or K acetate, 5 mM $MgCl_2$, 2 mM EGTA, 30 mM PIPES, pH 6.9. WS was identical to RS, but lacked ATP. Elastase (20 μg/ml), as originally introduced by Brokaw (2), was routinely added to RS for sliding disintegration experiments. Sodium vanadate (Mallinckrodt, Inc., St. Louis, MO) was used to inhibit reactivation and sliding; norepinephrine (Sigma Chemical Co., St. Louis, MO) was perfused under the coverslip to reverse vanadate inhibition.

Reactivation and disintegration of free macrocilia were observed with Zeiss phase-contrast optics using 16 × or 25 × objectives, and filmed with a Locam 16-mm cine camera (Redlake Labs, Santa Clara, CA) on Kodak Plus-X Reversal or Negative film at 12 or 25 frames s^{-1}.

Electron Microscopy of Sliding Disintegration: Microscope slide preparations of extracted macrocilia in RS were prepared as above, and disintegration was monitored by phase-contrast microscopy. After extrusion of fibers had begun in many macrocilia, glutaraldehyde-paraformaldehyde fixative (see above) was immediately perfused under the coverslip, and the contents flushed into a 1.5-ml microcentrifuge tube. Macrocilia were pelleted and allowed to remain in fixative for 2–3 h. Pellets were washed twice in buffer and postfixed in osmium tetroxide as above. Pellets were then dehydrated in acetone, embedded in Araldite, and thin-sectioned and stained for electron microscopy as above. Patterns of doublet extrusion in individual macrocilia (Table I) were usually determined from sections through a single level, although occasionally sections at different levels of the same macrocilium were available (Fig. 5). 13 of the 16 macrocilia analyzed in Table I were fixed from the same microscope slide preparation.

RESULTS

Structure and Motility of Macrocilia

A macrocilium consists of several hundred ciliary axonemes cross-linked to one another and surrounded by a common membrane (9) (Fig. 1). Macrocilia beat in a planar ciliary pattern at right angles to the rows of bridged axonemes (Fig. 1). The effective stroke is directed toward doublet 1 (three doublet compartment of the axoneme); reversal of beat direction has never been observed. Macrocilia rarely show continuous activity in normal seawater; typically, they beat discontinuously with separate effective and recovery strokes, resulting in a unique pattern of activity called "split-cycle" coordination (20).

Reactivation of Beating

Maceration of ES-treated lip tissue detaches many macrocilia from the epithelium. The macrocilia break off evenly at the base (where membranes of individual axonemes have not yet fused and cross-connections between adjacent shafts are lacking) so that the proximal end appears flat, in contrast to the tapered distal end (Figs. 2 and 3).

In the absence of ATP (WS with or without elastase), detached macrocilia were never observed to beat. In RS, detached macrocilia occasionally quivered rapidly or beat continuously with large amplitude, distally propagated bends. Time delays between effective and recovery bends, characteristic of split-cycle motility in vivo (20), have so far not been observed under our conditions of reactivation. Reactivated beating of detached macrocilia lasted for several minutes, then gradually slowed down and stopped. The presence or absence of elastase in RS had no noticeable effect on the form or extent of reactivated beating.

[1] *Abbreviations used in this paper*: ES, extraction solution; RS, reactivation solution; WS, wash solution.

TABLE I

Patterns and Frequencies of Outer Doublet Extrusion in Macrocilia

Macrocilium	Outer Doublets 9	1	2	3	4	5	6	7	8	No. of axonemes	Outer membrane
A*	–	–	–							15	–
	–									29	
						–	–	–		1	
B	–	–	–							13	–
C	–	–	–							11	–
D	–	–	–							11	–
E	–	–	–							5	–
		–	–							1	
F	–	–	–							14	–
		–	–							1	
	–	–	–	–						1	
						–	–	–		1	
G	–	–	–	–						5	–
H	–	–	–	–						10	+
		–	–	–						2	
I†						–	–	–		46	+
J						–	–	–		9	–
K						–	–	–		5	–
L						–	–	–		3	–
M						–	–	–		4	–
	–	–	–	–						1	
N					–	–	–			5	+
					–	–				2	
						–	–			1	
O					–	–	–			19	–
	–	–	–						–	1	
P						–	–	–		3	–
					–	–	–			3	
	–	–	–	–						1	

* Shown in Fig. 4.
† Shown in Fig. 5a.

ATP-induced Sliding Disintegration

Sliding disintegration of detached macrocilia started after reactivated movements ceased, or, as happened more commonly, without prior reactivation of bending. ATP-induced disruption occurred without elastase, but was more reproducible in the presence of the enzyme.

The large size of macrocilia, and their characteristic method of disintegration, allowed the disintegration process to be observed and filmed under low magnification phase-contrast optics (Figs. 2 and 3).

ATP-induced sliding disintegration often begins by the sliding out of a thick fragment from the flat proximal end of the macrocilium (Fig. 2). Sometimes thin fibers are first extruded from the proximal end (Fig. 3). Of 24 macrocilia filmed from the start of disintegration, 23 showed extrusion of elements from the proximal end only. The fragments progressively slide apart into thinner and thinner elements (Fig. 3), often forming long-curved or looped structures that extend out of the field of view. During the later stages of disintegration it is common to see two parts of a fragment slide apart in opposite directions if the parts are similar in thickness and neither one is attached to the slide or restrained by adjacent cilia. Eventually, only a tangled mass of fine filaments remains (Fig. 3).

No sliding disintegration occurred in WS with or without elastase, although lateral fraying of the macrocilia was com-

Published October , 1984

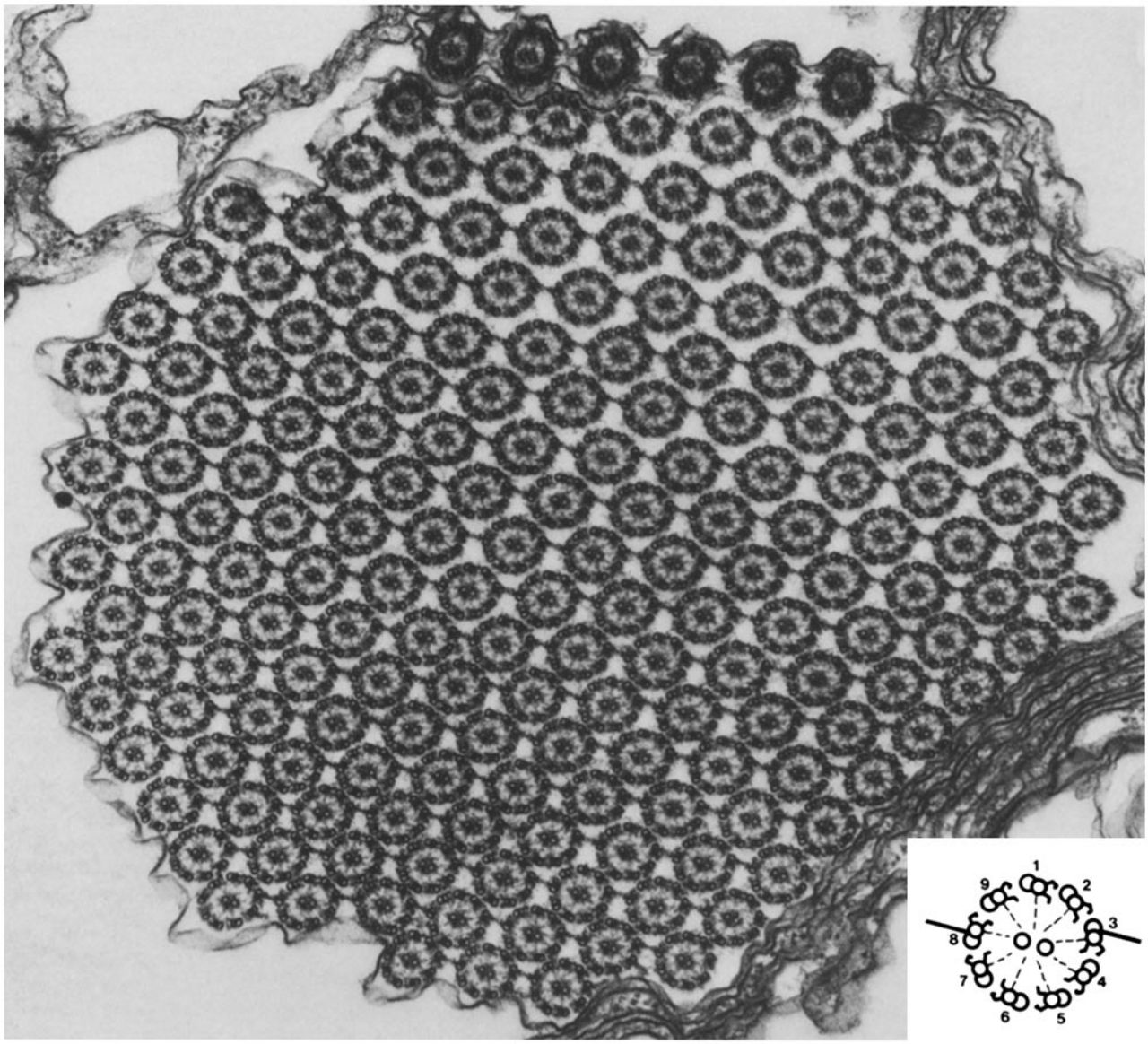

FIGURE 1 Transverse thin section just above the base of an intact macrocilium from a living *Beroë*. 200–250 hexagonally-packed axonemes are surrounded by a common outer membrane. The axonemes are linked into horizontal rows or sheets by compartmenting lamellae (reference 1) between doublet 3 of one axoneme and doublet 8 of the next, dividing the axoneme into two unequal compartments, one containing three doublets (9, 1, and 2) and the other containing four doublets (4–7). These connections provide markers for numbering the nine outer doublet microtubules (*inset*), and identifying which ones have been extruded during ATP-induced disintegration (cf. Figs. 4 and 5). Less regular connections extend between axonemes of adjacent rows. The effective stroke is directed towards the top of the figure (doublet 1), and the recovery stroke is towards doublets 5 and 6. Macrocilia in Figs. 1, 4, and 5 are viewed from base-to-tip. At the base individual axonemes have separate membranes (not shown), which fuse distally as seen in the upper row. × 49,700.

mon in the presence of the enzyme, particularly after 5–10 min.

Reversible Inhibition of Reactivation and Sliding Disintegration by Vanadate

At low concentrations, vanadate in the +5 oxidation state is a potent inhibitor of dynein ATPase activity and reactivated beating of cilia and flagella (7, 10, 23). This inhibition is reversed by norepinephrine, which reduces vanadate to the inactive +4 oxidation state. Recently, vanadate has also been shown to inhibit ATP-induced sliding disintegration of sperm flagellar axonemes (11, 14, 15), although its reversibility was not reported in these cases.

50 μM vanadate in RS with elastase completely inhibited reactivated beating and sliding disintegration of macrocilia. Perfusion of 50 mM norepinephrine induced beating almost immediately, followed by extensive sliding disintegration (Fig. 3). To our knowledge, this is the first documentation for the reversibility of vanadate inhibition of microtubule sliding.

Electron Microscopy of Disintegration

Cross sections through macrocilia fixed in the early stages of sliding disintegration showed the complete absence of a surrounding membrane in most, but not all cases (Table I; Figs. 4 and 5). The axonemes usually remained linked into sheets by the compartmenting lamellae, but adjacent rows

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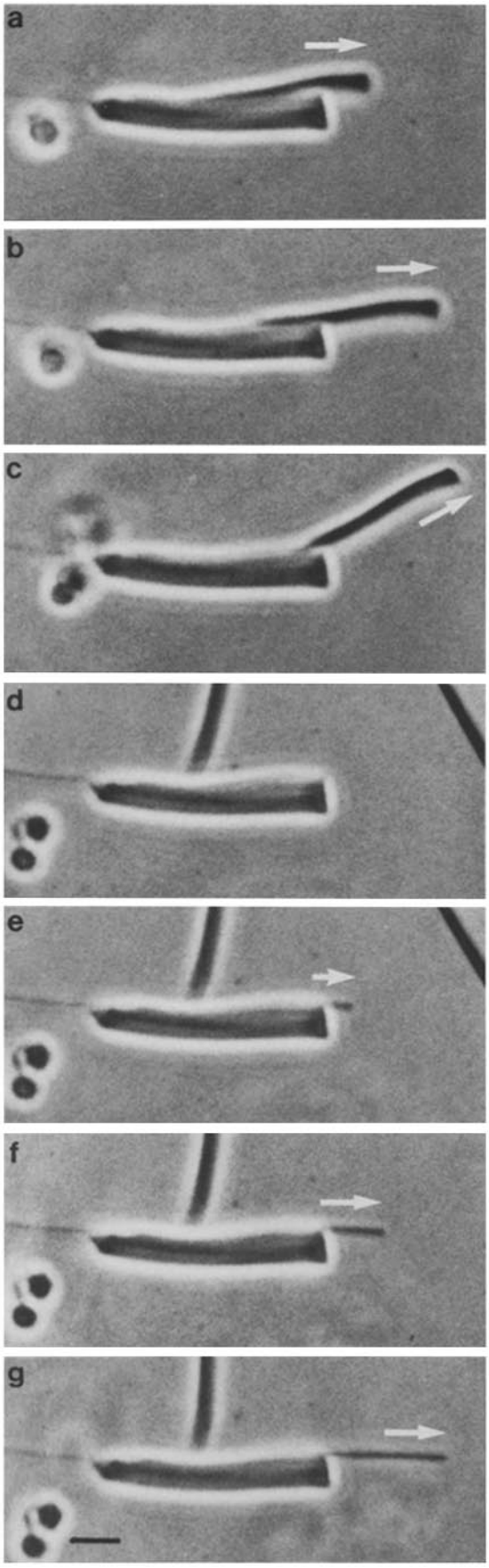

FIGURE 2 Cine prints showing ATP-sliding disintegration of a detached macrocilium. The broad flat proximal end (to right) is clearly distinguishable from the tapered distal tip (*left*). A thick fragment first slides out from the base (arrows, *a–c*), and is displaced to a perpendicular position along the upper side of the main piece (*d–g*). Later, a second fiber is also extruded from the proximal end (arrows, *e–g*). It is not known whether the thin filament at the tip slid distally, or whether it remained attached while the main piece moved to the right. Time elapsed from print *a*: *b*, 2 s; *c*, 7.7 s; *d* 24 s; *e*, 25 s; *f*, 26 s; *g*, 28.5 s. Bar, 10 μm.

were often separated from one another (Figs. 4 and 5). In some sections only a few isolated rows were present, apparently the result of having been extruded from the main part of the macrocilium. The morphology of the various axonemal structural components in ATP-disintegrated, elastase-treated macrocilia does not appear significantly different from that of controls in WS without enzyme, except that the radial spokes are sometimes fainter and less distinct in the former preparation.

Specific doublet microtubules were regularly missing from a number of axonemes in ATP-disintegrated macrocilia (Table I, Figs. 4 and 5). In contrast, doublets were never observed to be missing in control macrocilia fixed in the absence of ATP (WS with elastase). This indicates that the missing doublets in RS preparations disappeared by sliding extrusion.

Two mutually exclusive patterns of doublet extrusion were observed. Individual macrocilia displayed one or the other of the patterns, but rarely both, with equal frequency (Table I). Of 16 macrocilia analyzed, most were from the same slide preparation, and thus experienced identical conditions of extraction and reactivation. In eight of the macrocilia, doublets 9, 1, and 2 were extruded from many axonemes (macrocilia *A–H*, Table I). In one example, doublet 9 alone was extruded more frequently than subset 9, 1, and 2 (Fig. 4). In the other 8 macrocilia (*I–P*, Table I), three adjacent doublets in the opposite compartment of the axoneme were extruded. In five of these macrocilia doublets 5, 6, and 7 were missing almost exclusively; in two macrocilia doublets 4, 5, and 6 were extruded, and in the remaining case both groups of three doublets were missing with equal frequency. Cross sections at different levels through the same macrocilium showed that the doublets (5, 6, and 7) missing from 46 axonemes at one level (Fig. 5 *a*) were present, but sometimes displaced slightly, in 31 of these axonemes at a more distant level (Fig. 5 *b*). The latter section evidently represents a more proximal level showing doublets in the act of sliding but not yet completely extruded.

The central pair microtubules were almost never extruded, nor were doublets 3 and 8 usually lost.

DISCUSSION

Although dynein-driven microtubule sliding has been shown to be the basis for ciliary and flagellar movement (6, 17), little is known about the control systems responsible for the bidirectional bending cycle of cilia (effective and recovery strokes) and flagella (principal and reverse bends).

To investigate this problem, we applied the ATP-induced sliding disintegration method (19) to ctenophore macrocilia. Macrocilia offer several technical advantages for studying control of microtubule sliding: (*a*) the hundreds of axonemes within a single organelle act synchronously, yielding a high signal-to-noise ratio for analyzing doublet extrusion patterns; (*b*) the single outer membrane is easily disrupted, allowing ready access of reagents to all the component axonemes; (*c*) the connections between doublets 3 and 8 of adjacent axonemes provide clear markers for identifying which doublets

Published October , 1984

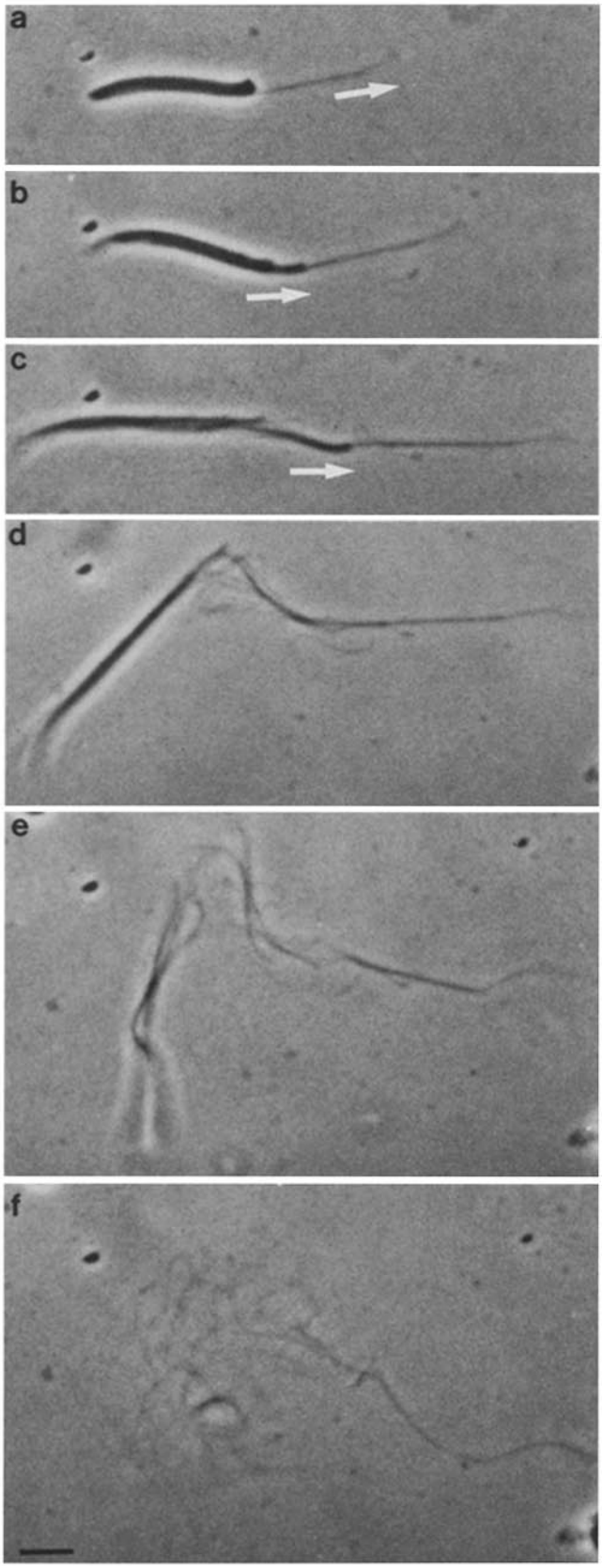

FIGURE 3 Cine prints of the reversal of vanadate inhibition of sliding disintegration by norepinephrine. Reactivation and disintegration of this macrocilium were initially inhibited by 50 μM vanadate in RS with elastase (see text). Norepinephrine in RS was then perfused into the preparation, resulting in extrusion of a single thin fiber from the flat proximal end (*right*, arrow in *a*). Perfusion of additional norepinephrine (*b*) caused further sliding out of fibers sequentially from the base (arrows, *b* and *c*), followed by complete disintegration into a tangled mass of fine filaments (*d–f*). Time elapsed from print *a*: *b*, 10.7 s; *c*, 12.3 s; *d*, 13.1 s; *e*, 14.4 s; *f*, 18.8 s. Bar, 10 μm.

have been extruded. In addition, the large diameter and unique structure of macrocilia allow the process of disintegration to be observed by low-magnification phase-contrast optics, in contrast to sliding disintegration of single axonemes which requires high-resolution darkfield microscopy (19).

The large-scale sequential disintegration of macrocilia can readily be explained on the basis of conventional inter-doublet sliding if the connections between adjacent rows of axonemes are taken into account. Extrusion of either doublet subset (9, 1, and 2 or 5, 6, and 7) from a number of axonemes in a single row would be expected to passively carry along neighboring rows linked to the sliding doublets by inter-row bridges, much like a geological fault. The sensitivity to low concentrations of vanadate, and the reversal of inhibition by norepinephrine, provides further evidence that disintegration of macrocilia is due to the mechanochemical cycle of dynein arms.

Disintegration of macrocilia occurs by extrusion of elements from the proximal end of the organelle. Since electron microscopy of such preparations showed doublet subsets 9, 1, and 2 or 5, 6, and 7 (4–6) missing with about equal frequency, both subsets of tubules must be extruded proximally. Sale and Satir's (16) demonstration of a single polarity of active sliding in *Tetrahymena* cilia implies that doublets that slide towards the base do so by their own active dynein arms, whereas doublets that move toward the tip are pushed passively by the activity of arms on the adjacent doublet. Applying this unidirectionality of active sliding to macrocilia, then both subsets of doublets must slide by their own active arms, since both are displaced towards the base.

Our results may thus be taken to demonstrate, for the first time, selective activation of doublet sliding on opposite sides of the axoneme. An alternative explanation, however, is that the two extrusion patterns may not reflect anisotropy of active sliding, but may result from differential sensitivity to proteolysis of radial spoke/nexin link restraints on the two halves of the axoneme. This possibility seems unlikely since (*a*) both patterns of doublet extrusion were found with about equal frequency in macrocilia subjected to identical conditions of ATP and elastase treatment prior to fixation, i.e., from the same slide preparation; (*b*) if the resistive structures in one compartment of the axoneme were more sensitive to protease digestion than those in the opposite half, then doublets on the more sensitive side should be missing (limited digestion), or both sets of doublets should be extruded (further digestion), but not one or the other set as actually observed. In this regard, the unilateral pattern of extrusion of doublets 4–7 found in rodent sperm (12–14) may be due to greater sensitivity of nexin/spoke structures to proteases in the doublet 4–7 half of these flagella.

We therefore conclude that the two patterns of doublet extrusion in macrocilia represent alternate activation and inactivation of dynein arms in the two compartments of the axoneme. This provides the first direct experimental support for a switching mechanism regulating microtubule sliding in cilia, and argues against other possible control mechanisms involving the resistive elements (4), bidirectional dynein

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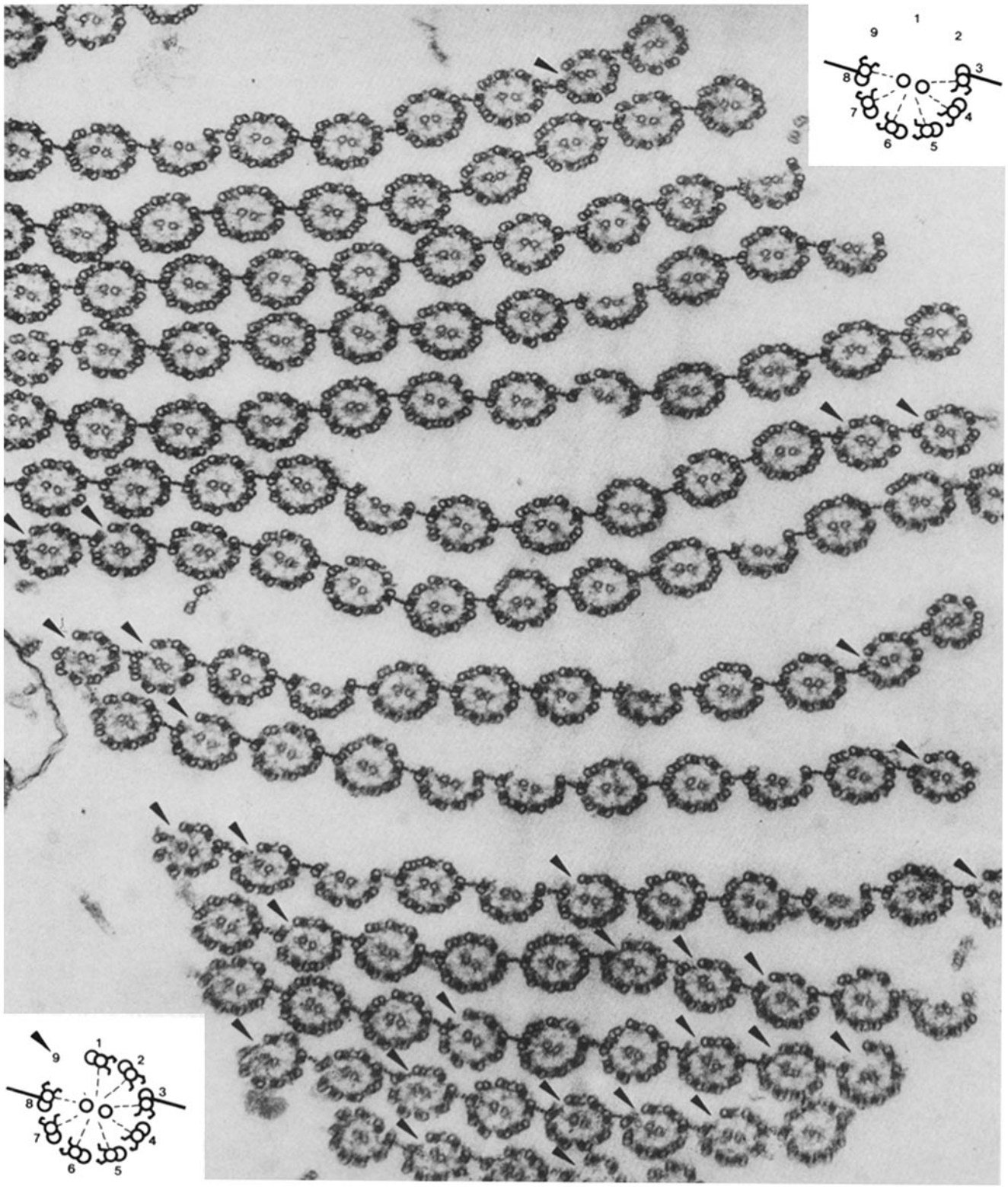
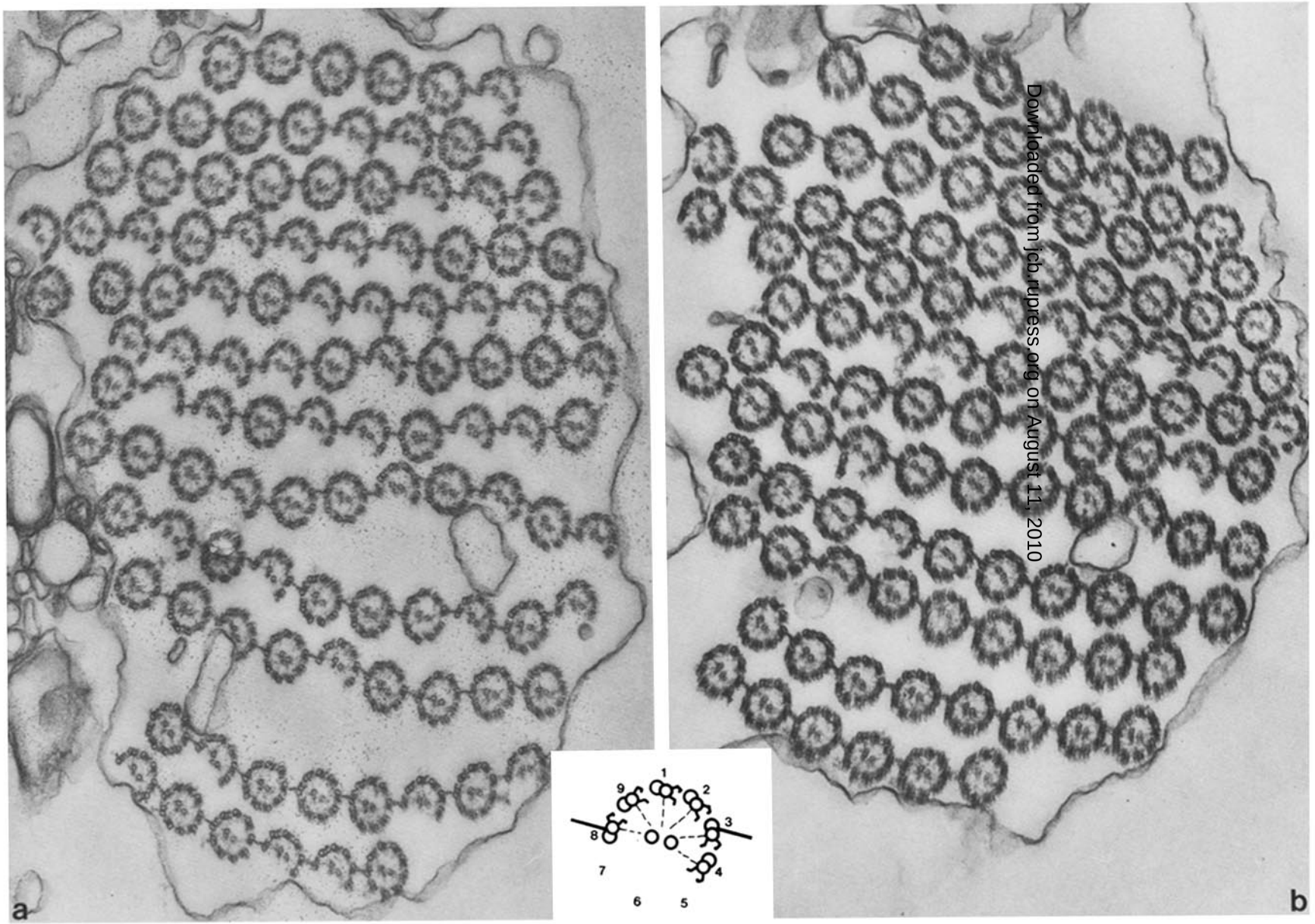


FIGURE 4 Transverse section through an ATP-disintegrated macrocilium (macrocilium A in Table I), showing extrusion of doublets 9, 1, and 2 from 15 axonemes (*inset, upper right*). In addition, doublet 9 alone has been extruded from 29 other axonemes (arrowheads, *inset lower left*), although in many cases its space has been filled in by neighboring doublets. Only one axoneme is missing doublets 5, 6, and 7 (fifth row from top). The structure of the various axonemal components is similar to that of intact macrocilia, but some rows of axonemes are separated. Same orientation as in Fig. 1. × 81,200.

power strokes (6), or direction-dependent dynein force generation (8).

Since the effective stroke of macrocilia is toward doublet 1, then according to the switch-point hypothesis of Satir and coworkers (17, 22), one would expect doublet subset 6–9 to slide actively during this phase of the beat cycle, and doublets 1–4 to be activated in the recovery bend. However, we observed that doublet subsets 9, 1, and 2 or 5, 6, and 7 (4–6) were



FIGURES 5 Transverse sections at different levels through the same ATP-disintegrated macrocilium (macrocilium I in Table I) showing extrusion of doublets 5, 6, and 7 from many axonemes (*inset*). In a, 46 axonemes are missing this doublet subset. In *b*, judged to be at a more proximal level, doublets 5, 6, and 7 are now present in 31 of the axonemes that are lacking these doublets in *a*, although they are occasionally displaced slightly from the ring of doublets. Same orientation as in Figs. 1 and 4. × 50,200.

actually extruded. The question arises as to the reason for these patterns of tubule extrusion, and whether they can account for the plane of bending.

It is not surprising that doublets 3 and 8 were rarely observed to slide, since these doublets are firmly attached to the compartmenting lamellae that link adjacent axonemes into rows, as in comb plate cilia (1). Similarly, doublets 3 and 8 in mammalian sperm are attached to the inward extensions of the fibrous sheath (5), and are likewise not extruded during disintegration of rodent sperm axonemes (12, 13).

We have assumed that the proximal extrusion of doublet subsets 9, 1, and 2 or 5, 6, and 7 (4–6) represents activation of sliding of these specific doublets. This criterion for identifying which doublets are actively sliding may not be completely valid. For example, not all the doublets within a given subset may have active dynein arms (i.e., the middle doublet may be bridged to the adjacent doublet by inactive arms, and carried along passively by its active neighbor). Alternatively, doublets in addition to those actually extruded may also have active arms, but be unable to slide themselves or to push out adjacent doublets. Does the inability of doublets 3 and 8 to slide out have an effect on the resultant pattern of doublet extrusion? It is also unclear whether the dynein arms of doublets 3 and 8 can interact with their neighbors, although cases of extrusion of doublet 3 (Table I) suggest that this doublet can actively slide.

Until these various questions are resolved, we cannot be certain that the two patterns of doublet extrusion we observe are functionally related to the beat cycle of macrocilia. However, the fact that the two patterns involve opposite sides of the axoneme, are mutually exclusive in a given macrocilium, and occur with similar frequencies, leads us to believe that this is the case, and that the two sliding patterns may represent the effective and recovery strokes of the planar beat cycle of macrocilia. Force diagrams show that if each pattern indeed represents activation of dynein arms solely on the doublets extruded (i.e., 9, 1, and 2, or 5, 6, and 7 [4–6]), a net downward force would be produced in either compartment of the axoneme, resulting in a bend towards the side opposite that of the actively sliding doublet subset. Thus, activation of doublets 5, 6, and 7 would cause bending in the effective stroke direction, while active sliding of doublets 9, 1, and 2 would lead to bending in the recovery direction. Although this pattern of alternate sliding may not be as "efficient" for producing bends as activation of doublet subsets 6–9 and 1–4, the important point is that we have shown that anisotropy of active sliding does occur. It remains to be seen whether the control of microtubule sliding in macrocilia represents a special case, or whether the switch-point hypothesis must be modified to fit our findings.

We are very grateful to Claudia Mills for kindly sending us *Beroë* from Friday Harbor, Washington. We also appreciate the skill and patience of Dorothy Hahn in continually processing and reprocessing these words.

This work was supported by National Institutes of Health grant GM 27903 and National Science Foundation grant 83-14317.

Received for publication 10 May 1984, and in revised form 25 June 1984.

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