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# Adenosine Triphosphate-Induced Sliding of Tubules in Trypsin-Treated Flagella of Sea-Urchin Sperm

**(motility/microtubule/cilia/sliding filament model/axonemes)**

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**ABSTRACT** **Axonemes isolated from the sperm of the sea urchin, *Tripneustes gratilla*, were briefly digested with trypsin. The digested axonemes retained their typical structure of a cylinder of nine doublet-tubules surrounding a pair of single tubules. The digestion modified the axonemes so that the subsequent addition of 0.1 mM ATP caused them to disintegrate actively into individual tubules and groups. The nucleotide specificity and divalent-cation requirements of this disintegration reaction paralleled those of flagellar motility, suggesting that the underlying mechanisms were closely related. Observations by dark-field microscopy showed that the disintegration resulted from active sliding between groups of the outer doublet-tubules, together with a tendency for the partially disintegrated axoneme to coil into a helix. Our evidence supports the hypothesis that the propagated bending waves of live-sperm tails are the result of ATP-induced shearing forces between outer tubules which, when resisted by the native structure, lead to localized sliding and generate an active bending moment.**

Although much is known about the detailed fine structure of the flagellar axoneme (1–5), there is relatively little evidence concerning the functional role of this structure in the mechanism of flagellar motility. In this paper, we report preliminary observations on the active disintegration produced when ATP is added to axonemes whose structure has been modified by brief digestion with trypsin. By studying the movements of the flagellar tubules during this disintegration, we have been able to obtain information about the nature of the mechanical forces induced by ATP. Our results strongly support the "sliding filament" model of flagellar bending (6, 7).

Axonemes were isolated from sperm of the sea urchin, *Tripneustes gratilla*, by extraction and differential centrifugation in a solution containing 1% (w/v) Triton X-100, 0.1 M KCl, 5 mM $MgSO_4$, 1 mM ATP, 1 mM dithiothreitol, 0.5 mM EDTA, and 10 mM Tris-phosphate buffer, pH 7.0, (Gibbons and Fronk, manuscript in preparation). The resultant preparations contained about equal numbers of intact axonemes (50 $\mu$m long) and of shorter fragments formed by shearing. The centriole was visible, by dark-field microscopy, as a brighter granule at one end of many axonemes. A substantial proportion of the axonemes are capable of normal motility if diluted into reactivating solution at pH 8.0 (see above).

The suspension of axonemes was centrifuged and resuspended at a concentration of about 0.6 mg of protein/ml in Tris–Mg solution (2.5 mM $MgSO_4$, 0.1 mM dithiothreitol, 30 mM Tris·HCl, pH 7.8), at room temperature. Sufficient trypsin was then added to give a trypsin to axonemal protein ratio of about 1 to 1500. The course of the digestion was monitored by measurement of the turbidity of the suspension at 350 nm as a function of time. In routine preparations, further digestion was stopped by the addition of an excess of soybean trypsin-inhibitor after the turbidity had decreased to about 80% of its initial value. This suspension of digested axonemes could be stored at 0°C for up to 2 days, and constituted the starting material for most of our experiments. In some cases, the trypsin and inhibitor were removed by centrifugation of the digested axonemes and resuspension in fresh Tris–Mg solution.

Preliminary electron-microscopic examination of the digested preparations showed that the cylindrical structure of nine outer doublet tubules surrounding the two central-tubules remained largely intact in most axonemes (Fig. 1). Comparison with undigested preparations indicated that only slight structural changes resulted from digestion. The most apparent change was the disruption of the radial spokes near the point where they normally connect to the sheath surrounding the central tubules. Disruption of the nexin links that connect adjacent doublets (3, 5) was not directly apparent in the micrographs, but was revealed upon dialysis of digested axonemes against low concentrations of EDTA. The dynein arms on the doublets (3) appeared to be relatively resistant to the digestion. More detailed descriptions of the effects of digestion on the fine structure of the axoneme will be published elsewhere.

Although the trypsin digestion did not itself destroy the basic structure of the axoneme, it modified it in such a way that the structure became highly sensitive to ATP. Addition of a low concentration of ATP to the digested preparation caused a rapid disintegration of the axonemes into individual doublet-tubules and small groups (Fig. 2). The dynein arms and a portion of the spoke were still present on most of the separated doublets. The central tubules usually remained together as pairs, but they did not appear to be associated with the groups of doublets.

The specificity of the reaction responsible for this axonemal disintegration was examined by the use of the decrease in turbidity of the suspension, measured at 350 nm, as an assay of disintegration. With concentrations of ATP between 50 $\mu$M and 1 mM, complete disintegration of the axonemes occurred within 30 sec, and the decrease in turbidity amounted to about 50%. With lower concentrations of ATP, the decrease was smaller and slower to develop, amounting to about 25% after 3 min with 10 $\mu$M ATP. The reaction was highly

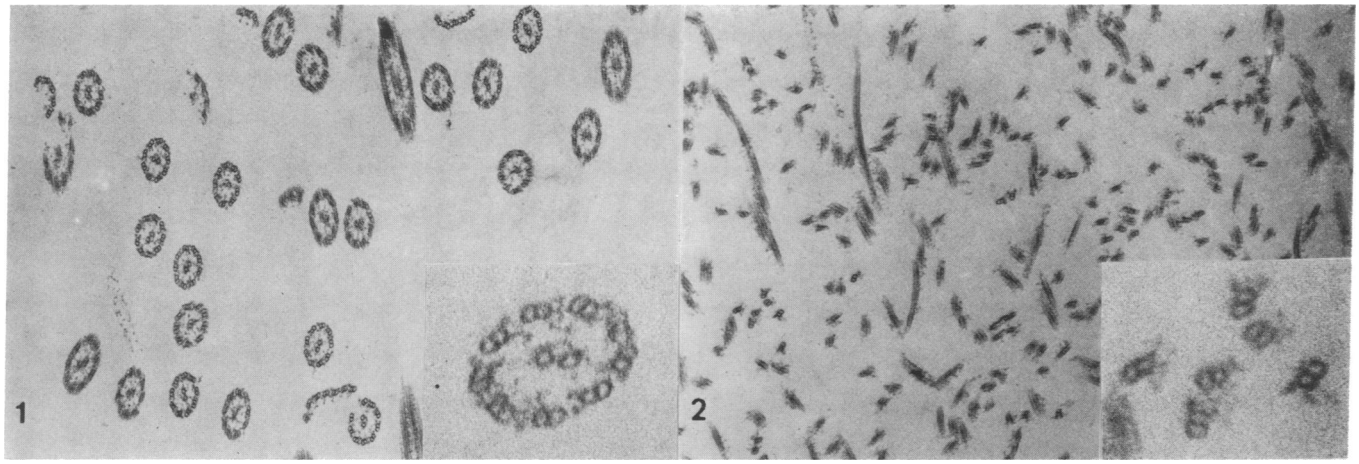



FIG. 1. Electron micrograph of trypsin-treated axonemes. The samples were fixed with glutaraldehyde and post-fixed with osmium tetroxide. After samples were embedded in epoxy resin, thin sections were cut, and then stained with uranyl acetate and lead citrate. ×27,000; insets ×109,000.

FIG. 2. The same preparation ot trypsin-treated axonemes as in Fig. 1, but 0.5 mM ATP was added 3 min before fixation.

specific for ATP; other nucleotides, including GTP, CTP, and ITP, gave only a 3–5% decrease in turbidity, even when added at a concentration of 1 mM.

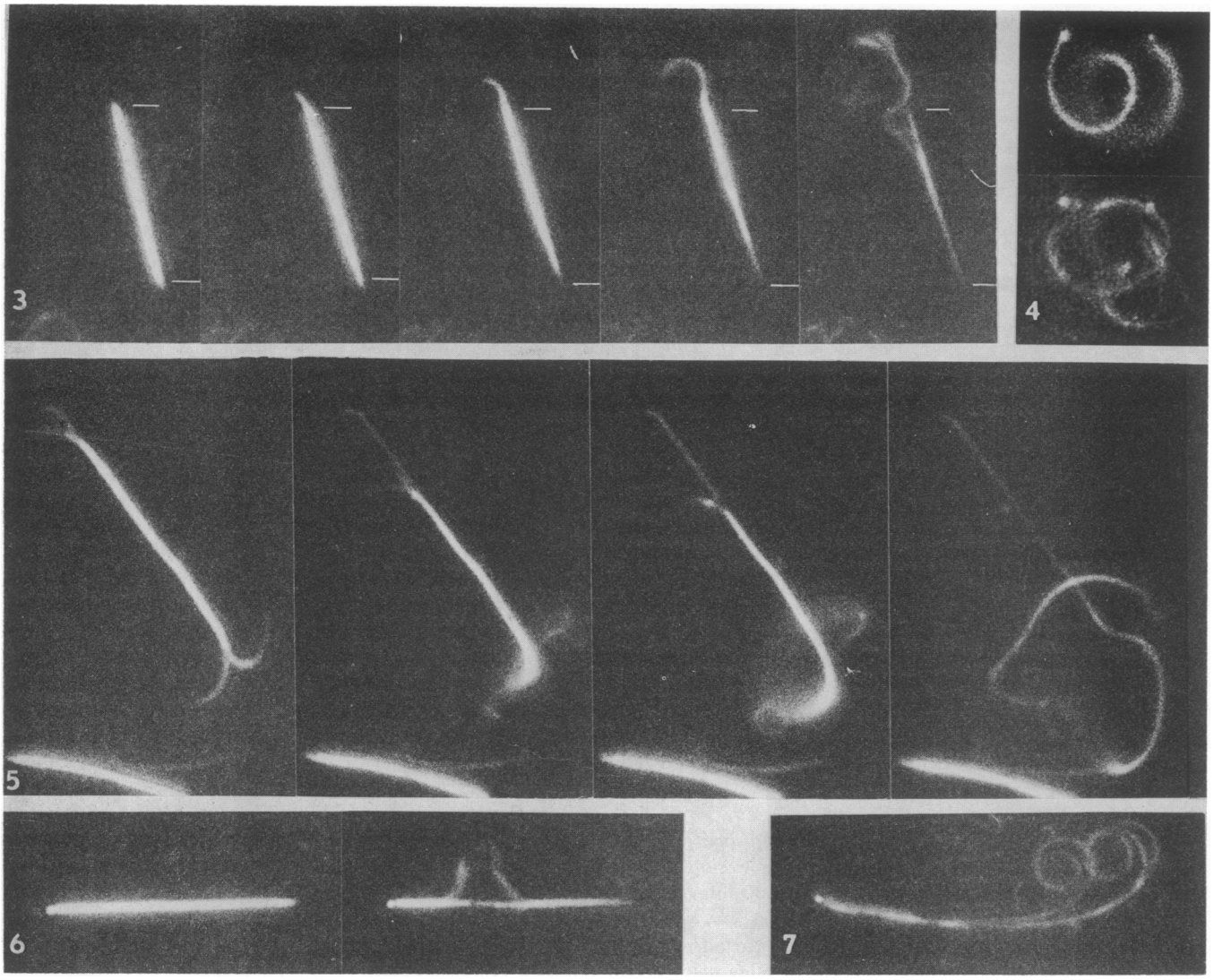
In addition to ATP, the presence of a divalent cation was required for the reaction to occur. When the digested axonemes were suspended in 0.1 M KCl, 0.5 mM EDTA, 30 mM Tris buffer, pH 7.8, the addition of 0.1 mM ATP gave no change in turbidity; subsequent addition of 2.5 mM $MgSO_4$ to the suspension gave a 40% decrease. $MnSO_4$ was about as effective as $MgSO_4$ in activating the response. Activation with 2.5 mM $CaCl_2$ gave a 27% decrease, but the response was slower than with $MgSO_4$ and took about 3 min to develop.

The manner in which the digested axonemes disintegrated was studied in more detail by dark-field microscopy. Under these conditions, structures probably as small as individual doublet-tubules were visible. When the doublets were in groups, they could not be resolved individually, but the approximate number in the group could be estimated from the relative intensity of its image. Photographs were taken at a magnification of ×210, with a 5–10 sec exposure. Because of Brownian movement, only structures attached to the coverglass could be photographed.

A suspension of the digested axonemes in Tris–Mg solution was placed in a trough 0.2 mm deep on a microscope slide and covered with a coverglass, except for a small opening at one end. A small volume of 0.5 mM ATP was placed into the opening, and evaporation and diffusion then drew the ATP slowly across the suspension. The time of disintegration of individual axonemes varied from a few seconds to several minutes, depending on such factors as the steepness of the ATP gradient and its rate of progression.

The overall disintegration of the axoneme appeared to result partly from active sliding movements between groups of tubules, and partly from the tendency for the partially disintegrated structure to coil into a helix. The sliding movements could be seen most clearly in short- to medium-length fragments of axoneme that initially were stuck to the coverglass along much of their length. In Fig. 3, the process begins with the protrusion of a group of tubules from the upper end of the axoneme. This protrusion gradually extends further and begins to coil around onto itself. Simultaneously with the growth of the protrusion, a region of reduced intensity extends upward from the lower end of axoneme, as a result of the smaller number of tubules remaining in this region. The lower end of the structure remains in a constant position on the coverglass. In the fourth micrograph of the series, two distinct steps of intensity are visible in the lower part of the structure, indicating the presence of two groups of tubules moving upward at different speeds. At the end of the disintegration, the protruded groups of tubules are partly outside the focal plane, but they appear to be at least as long as the initial axoneme and to have undergone further separation. One small group of the tubules has remained behind in the position of the original axoneme. The disintegration of a second, somewhat longer, axonemal fragment is shown in Fig. 5. In this case a large group of tubules is sliding toward the bottom-right of the micrograph. Although the free, forward end of the sliding group soon coils around out of the plane of focus, the progress of the group can be followed from the movement of its rear end as it moves downward, leaving behind it a stationary group of a few tubules attached to the coverglass. In the last micrograph of the series, the forward end of the sliding group has coiled completely around and come back into the focal plane, while the rear end has moved a total distance of about 25 $\mu$m, about equal to the length of the original axoneme. Further disintegration of the group into a tangle of mainly individual doublet-tubules occurred subsequently. No further movement was observed in the separated tubules.

In both of the above examples, the sliding groups of tubules left behind them a small stationary group of tubules attached to the coverglass in the same position as the original axoneme. It seems reasonable to suppose that these stationary tubules are the ones by which the axoneme had been attached to the coverglass, and that they formed the fixed surface relative to which the active sliding of the other tubules occurred. Where two groups of sliding tubules were observed in a given axoneme, they were most often moving in the same direction along the axoneme, with different speeds (Fig. 3). However, we have occasionally observed two groups of tubules moving in opposite directions relative to a third stationary group. The total length produced by sliding is sometimes greater than five times that of the original axonemal fragment.




FIGS. 3–7. Dark-field light micrographs of trypsin-treated axonemes reacting to ATP. The successive micrographs in each series were taken at intervals of 10–30 sec. The *white lines* in Fig. 3 indicate the initial position of each end of this fragment. The centriole is visible in the *upper-left* corner of Fig. 4, and at the *left end* of the axonemes in Figs. 6 and 7. For further details see *text*. ×1730.

The disintegration of full-length axonemes and of fragments derived from the centriolar end is modified by the apparent presence of a block to sliding in the region of the centriole. With full-length axonemes that are attached to the coverglass, disintegration usually begins with the opening of a split about midway along their length. The group of tubules constituting one side of the split gradually billows out to form a large loop, while the group on the other side remains in about the original position on the coverglass (Fig. 6). As the size of the initial billow increases, the end of a moving group of tubules can be seen sliding up along the axoneme from the distal end toward the split, suggesting that the billow is a consequence of the force generated by active sliding in the region distal to the split. Further disintegration occurs subsequently, and the final result is a tangle of tubules, which is often still held together at the centriolar end. Short fragments of axoneme derived from the centriolar end are relatively resistant to disintegration, and they can often be found intact when full-length axonemes and distal fragments have disintegrated.

The disintegration of axonemes that are floating free in solution is difficult to study in detail, because they rotate irregularly through the focal plane as disintegration proceeds. In most cases, free axonemes first bend near the middle, and then coil into helices of rather uniform pitch (Fig. 4). The helices subsequently break down into balls of tangled tubules. No motion is seen once the separation into individual doublets is complete.

As mentioned above, partially disintegrated axonemes show a marked tendency to coil. This is illustrated by the helices formed in free-floating axonemes and by the coiling of the groups of tubules protruded from axonemes attached to the coverglass. Another expression of this tendency is seen in some axonemes, where disintegration occurs by means of one or more groups of tubules peeling back along the length of the axoneme, coiling up as they do so (Fig. 7). On occasion, we have observed what appear to be individual doublet-tubules peeling back in this way. In many axonemes coiling or peeling occurred simultaneously with sliding, giving complex patterns of disintegration. The relative predominance

of sliding or coiling appeared to differ from one preparation to another, and it may be affected by the exact degree of digestion.

## DISCUSSION

Our observations indicate that the principal factors responsible for the disintegration of the digested axonemes are the active sliding movements induced by ATP between groups of tubules, and the tendency for the partially disrupted structure to coil up. Although such other factors as length, attachment to the coverglass, presence or absence of the centriole, and exact degree of digestion exert a modifying influence on the detailed manner in which particular axonemes disintegrate, they are of less general significance.

The sliding movements that occur between groups of tubules indicate the presence of an active force induced by ATP, and are of particular interest because they seem likely to be closely related to the mechanism underlying normal motility. The force that tends to cause sliding might result either from the interaction of the outer doublets with the central sheath and tubules, or from the interaction of each outer doublet with its neighboring doublet. Interaction with sheath and tubules seems less probable, because the interaction would presumably have to occur through the spokes, and these structures appear to be the ones most damaged by the trypsin digestion. Moreover, it would be difficult to explain how the final length after sliding can be greater than three times the initial length. The second possibility, that the shearing forces derive from the interactions between adjacent doublets, appears more probable. In this case the interaction would occur through the dynein arms, and these appear to be relatively resistant to digestion. The close similarity between the specificity requirements of dynein ATPase (8) and those of the disintegration reaction provides support for believing that the dynein arms play an active mechanochemical role in inducing sliding.

We have no direct evidence concerning the detailed mechanism by which the dynein arms might induce sliding. One possibility would be for the binding and hydrolysis of ATP to cause a cyclic change in the angle of the arms, which, coordinated with the repeated making and breaking of their attachment to successive binding sites along the length of the B-tubule, would result in the arms "walking" one tubule along the other. Such a mechanism would be analogous to that thought to occur in muscle, where cyclic movements of the bridges on the myosin filaments cause sliding relative to the actin filaments (9).

If the interaction between tubules occurs through the arms, this implies that the arms on one doublet are capable of binding chemically to specific sites on the B-tubule of the adjacent doublet, and thus forming crossbridges between the two doublets. The gap of 5–10 nm usually observed in electron micrographs between the arms and the adjacent B-tubule could be a real separation and perhaps form part of the mechanism for coordination of the interactions between tubules (see below), although the possibility of this gap being an artefact resulting from the failure of glutaraldehyde fixation to preserve the interaction between the arm and B-tubule cannot be neglected.

Since the sliding movements between groups of tubules frequently continue for almost their whole length, a distance of 25 $\mu$m or more, the polarity of the structures generating the sliding must maintain a constant direction along the length of each doublet. Considerations of symmetry incline us to think that the direction of this polarity is probably the same on all nine doublets, but our observations on the patterns of sliding are not yet adequate to determine whether or not this is the case.

The coiling of partly disintegrated axonemes may be partly an indirect effect of the ATP-induced forces between tubules, but it seems unlikely that this is a complete explanation for this mechanism fails to account for the coiling of individual doublet-tubules. Further work is needed to elucidate the factors involved in this coiling. The coiling of the flagellar tubules is reminiscent of that of bacterial flagella (10), although the resemblance may be no more than coincidental.

The conditions necessary for obtaining the disintegration response closely match those necessary for obtaining propagated bending waves in undigested axonemes. In both cases, there is a high degree of specificity for ATP; other nucleotides are essentially inactive. The concentration of ATP that gives half-maximal disintegration (as judged by the decrease in turbidity) is about the same (10 $\mu$M) as the minimum concentration necessary for motility in undigested axonemes. The divalent-cation requirements are also similar, and can be satisfied by $Mg^{++}$ or $Mn^{++}$ or, less effectively, by $Ca^{++}$ (ref. 11; Gibbons, B. H., manuscript in preparation). This similarity in conditions suggests that the active process underlying the ATP-induced disintegration of the trypsin-treated axonemes is closely related to that which produces bending waves in normal flagella.

Two general types of model have been proposed to explain the mechanism of flagellar bending. In the "local contraction" model, the bending of a short segment of flagellum is a direct result of the active contraction of longitudinal elements on one side of the flagellum within that segment (12–14), while in the "sliding filament" model, bending occurs as a result of active sliding between noncontractile longitudinal elements (1, 6, 7). The sliding filament model has been favored in recent theoretical work because it provides a better explanation for the fact that the propagation velocity of flagellar bending waves is constant, and does not change with the varying local viscous—resistive—moment they encounter (15–17). Brokaw has recently shown that the relationship between sliding and bending in such a model provides a possible mechanism for the generation of propagated bending waves (7).

Our demonstration that ATP causes active sliding between tubules in trypsin-treated flagella, together with Satir's earlier finding that the length of ciliary tubules remains constant during bending (6), provides strong experimental support for the general validity of the sliding filament model.

On this basis, the propagated bending waves of normal flagella can reasonably be explained as the result of ATP-induced shearing forces between adjacent doublet-tubules, which, when opposed by the elastic resistance of the native structure, lead to localized sliding and generate an active bending moment (7). We postulate that the trypsin treatment of the axonemes damages those structural components that are responsible for coordinating the shearing forces and for providing the elastic resistance that normally restrains sliding, with the result that unlimited sliding can occur, leading to complete disintegration of the axoneme. Since the spokes and the nexin links appear to be the components most susceptible to damage by trypsin, we would suggest that they may form part of the coordinating and resistive structures. The possi-

bility that the trypsin modifies significantly the properties of the dynein itself must also be considered.

The coordination necessary to produce propagated bending waves implies that the active process that generates the forces between tubules must be sensitive to some local property of the wave, such as its curvature or the amount of shear in the structure. There is no direct evidence as to how active forces generated by the dynein arms, which are arranged in a basically helical pattern in the axoneme, could be coordinated to produce a planar bending wave. However, since the direction of bending is correlated with the plane of the central tubules (18, 19), it seems possible that the central tubules and sheath, together with the spokes that normally connect them to the outer doublets, are involved in the coordination process. The gap of 5–10 nm usually observed between the arms and the adjacent B-tubule could also form part of the coordination mechanism by preventing effective interaction of the arms with their corresponding sites on the B-tubule until the gap was closed by the distortions resulting from curvature or shear. Our evidence tentatively suggests that the active process can generate a force in only one direction between a given pair of tubules, but it is not easy to see how such a unidirectional force could give rise to almost symmetrical, planar waves, and the possibility of a reverse force being generated under appropriate conditions has to be considered. The differences reported in the structure and chemical properties of the two rows of dynein arms on each doublet (2, 20) suggest that the production of forces between tubules is complex. The largest bend angles normally observed in flagella are about 3 radians (ref. 21; Gibbons, I. R. manuscript in preparation), and bends of this magnitude indicate that sliding can amount to about 400 nm between tubules on opposite sides of the axoneme, with at least 100 nm between adjacent doublets, if the tubules are inextensible. This much sliding is considerably greater than can be accommodated by the spokes and nexin links, unless they have considerable ability to stretch or unless their links to the tubules are broken and remade. The further possibility that the tubules themselves undergo a significant amount of elastic compression and extension during extreme bending must also be considered. Although our observations provide strong support for the general basis of the "sliding filament" model, it seems likely that further evidence regarding the coordination and polarity of the active forces between tubules will be necessary before it will be possible to present a reasonably detailed hypothesis relating this theoretical model to the actual structure of the axoneme.

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