Different Structural States of a Microtubule Cross-linking Molecule, Captured by Quick-freezing Motile Axostyles in Protozoa

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Abstract. Freeze-etch preparation of the laminated bundles of microtubules in motile axostyles demonstrates that the cross-bridges populating individual layers or laminae are structurally similar to the dynein arms of cilia and flagellae. Also, like dynein, they are extracted by high salt and undergo a change in tilt upon removal of endogenous ATP (while the axostyle as a whole straightens and becomes stiff). On the other hand, the bridges running between adjacent microtubule laminae in the axostyle turn out to be much more delicate and wispy in appearance, and display no similarity to dynein arms. Thus we propose that the internal or "intra-laminar" cross-bridges are the active force-generating ATPases in this system, and that they generate overall bends or changes in the helical pitch of the axostyle by altering the longitudinal and lateral register of microtubules in each lamina individually; e.g., by "warping" each lamina and creating longitudinal shear forces within it. The cross-links between adjacent laminae, on the other hand, would then simply be force-transmitting elements that serve to translate the shearing forces generated within individual laminae into overall helical shape changes. (This hypothesis differs from the views of earlier workers who considered a more active role for the latter cross-links, postulating that they cause an active sliding between adjacent layers that somehow leads to axostyle movement.) Also described here are physical connections between adjacent intra-laminar cross-bridges, structurally analogous to the overlapping components of the outer dynein arms of cilia and flagella. As with dynein, these may represent a mechanism for propagating local changes from cross-bridge to cross-bridge down the axostyle, as occurs during the passage of bends down the length of the organelle.

THE vigorous undulation of the axostyle in certain primitive protozoa is a fascinating example of microtubule-based motility. Previous observers have demonstrated that the axostyle is composed of thousands of parallel microtubules arranged in a characteristic laminar pattern in which each lamina is composed of several dozen microtubules spaced evenly apart, and several dozen of these laminae are stacked on top of each other to form a relatively thick ribbon (15–17, 28, 34). The undulation is presumed to be an active process involving conformational changes in the cross-links amongst the microtubules. This somehow generates bends perpendicular to the long axis of the whole ribbon (3, 8, 17, 24–26, 29, 34), and because the axostyle is permanently twisted along its length, it also involves a change in helical pitch. Additionally, the bends propagate from one end of the axostyle to the other, causing the organelle to rotate inside the cell as well as to undulate.

Isolated axostyles can regenerate this movement in vitro when exposed to ATP in an appropriate ionic environment (2, 29, 40). The challenge is thus to explain how molecular ATPases that presumably reside in the cross-bridges between microtubules can affect the helical pitch of the overall lattice. This is generally thought to involve some sort of sliding of microtubules relative to each other (2, 17, 26, 29, 40), analogous to the dynein-generated sliding of microtubules in cilia and flagellae (33); but the geometrical complexity of the axostyle has thwarted efforts to specify exactly which cross-bridges are involved and what sort of sliding occurs.

A previous report by Woodrum and Linck (40) provided the first deep-etch images of axostyle cross-bridges. These turn out to look sufficiently similar to dynein cross-bridges in deep-etched ciliary and flagellar axonemes (11–14) to suggest a functional homology. Woodrum and Linck did not describe any changes in cross-bridge structure in the presence vs. absence of ATP, but because such changes have been seen in axonemal dynein cross-bridges (11, 39), further deep-etch study of axostyles seemed warranted.

The present study provides additional deep-etch images of the various cross-bridges that exist in the axostyle, as they appear in vivo as well as in vitro in the presence vs. absence of ATP, and demonstrates that a change in cross-bridge conformation accompanies the straightening that occurs when axostyles are removed from inside the cell and depleted of ATP (i.e., when they enter a "rigor" condition). This change involves only the cross-bridges that link microtubules together laterally within each lamina. These cross-bridges assume a more upright or perpendicular configuration in vitro, some 20–40° different from the inclined configuration they

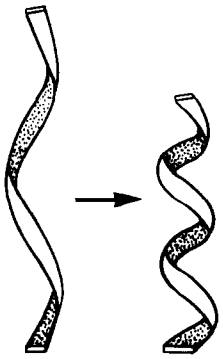


Figure 1. Isolated axostyles exposed to Mg-ATP display this increase in helical pitch by phase-contrast microscopy. A few axostyles display rhythmic conversions back and forth between these two extremes in ATP. The more typical undulation of the organelle in situ involves the propagation of short segments of the more tightly coiled configuration, on the right, down the length of an axostyle that is otherwise in the configuration on the left.

display in more helical regions in vivo. In interpreting these new data, we discuss how such microscopic changes in cross-bridge conformation, if they were actively controlled by ATP hydrolysis, could generate macroscopic changes in the helical twist of the whole organelle.

Materials and Methods

The two "classic" axostyle-bearing protozoa were studied together; *Pyrsonympha* that reside in the guts of termites (*Reticulotermes*, supplied by Carolina Biological Supply Co., Burlington, NC) and several related species of *Saccinobaculus* that reside in the guts of wood-eating roaches (*Cryptocercus*, kindly provided by Dr. Hope Ritter [31], Department of Zoology, University of Georgia, and Cristine Nalepa [30], Department of Entomology, North Carolina State University). In both cases, living organisms were quick-frozen in as close to their natural state as possible by dissecting out an insect's gut, expressing its contents onto the sample stage of our freezing machine (the "Cryopress" manufactured by Med-Vac, Inc., St. Louis, MO), and immediately dropping the sample onto the machine's liquid helium-cooled copper block. The protozoa thus remained in their natural environment of gut contents and were frozen within 30 s of dissection, so they should not have been damaged by atmospheric oxidation (cf. reference 32). In other experiments, the contents of an insect's gut were extruded into a "suspension solution" whose composition was compiled from previous recipes (25, 32, 38, 41) and found empirically to maintain protozoan viability for ≥12 h; this was composed of 60 mM K^+, 2 mM Na^+, 3 mM Mg^{++}, 1 mM Ca^{++}, 20 mM Cl^-, 20 mM acetate$^-$, 10 mM PO_4^-, and 10 mM HCO_3^-. To prevent oxidation in this case, 2 mM fresh dithiothreitol (DTT) was added to the above solution just before use, and thereafter it was kept under mineral oil. Each fresh batch of gut contents was allowed to stand in this solution for 5 min to permit settling of larger protozoa and wood chips, which were discarded. The *Pyrsonympha* and *Saccinobaculus* species under study were then pelleted out of the 1-*g* supernatant at 300 *g* and briefly exposed to either of two "rupturing solutions" to obtain their axostyles, one solution being 0.5% Nonidet P-40 (NP-40) in 70 mM KCl, 30 mM Hepes buffer, pH 7.2, 5 mM $MgCl_2$, 3 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The second rupturing solution was simply hypotonic buffer (5 mM Na-Pipes, pH 7.0). Within 60 s, both of these solutions ruptured most of the larger axostyle-bearing protozoa but left

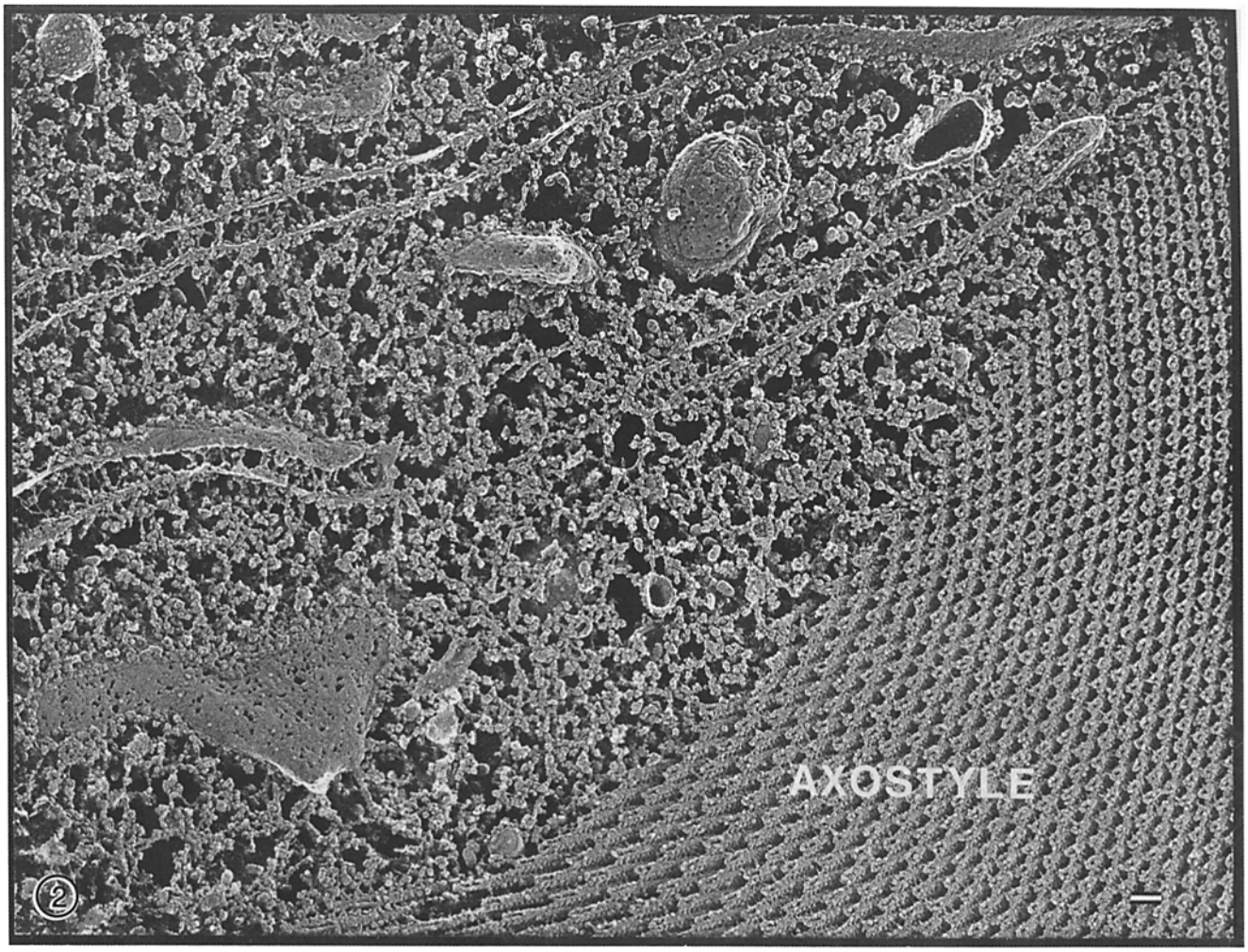


Figure 2. Deep-etch appearance of the axostyle (*lower right corner*) and adjacent cytoplasm in a *Pyrsonympha* that was extruded from the hindgut of a termite directly into glutaraldehyde fixative. Note that the granular components of the cytoplasm do not penetrate the axostyle, which thus can be examined in a "clean" form in situ. Bar, 0.1 μm.

smaller flagellates intact. Thereupon, axostyles were collected by centrifugation at 3,000 *g* for 3 min, resuspended in the first solution without NP-40, and then exposed to 1 mM Mg-ATP. (Their behavior upon such ATP exposure will be described in Results.) Finally, the extracted and reactivated axostyles were quick-frozen as above, without intervening chemical fixation.

Subsequent preparation of deep-etch replicas and three-dimensional (3-D) electron microscopy was performed as described in previous reports (11, 18–21). All stereo micrographs were taken at 68,000× initially, then studied at 10× further magnification with a Wild APT-1 stereo map reader. Data were transferred directly from the 3-D viewing field of this instrument to the digitizing tablet of a Zeiss MOP-3 stereology calculator, or to graph paper for drawings such as Fig. 22 below, via a standard Wild camera lucida attachment.

Results

Phase-contrast Microscopy of Living Organisms vs. Reactivated Axostyles

The vigorous undulation typical of axostyles in living *Pyrsonympha* and *Saccinobaculus* is rarely observed in isolated axostyles after they are prepared according to the bulk methods used here. Successful reactivation has generally been obtained only when detergent extraction and ATP exposure are performed in rapid succession directly on the microscope stage, without washing away soluble cellular contents or subjecting axostyles to centrifugation (2, 29, 40). In fact, the only axostyles in our preparations that show rhythmic, propagated activation upon exposure to ATP are ones that attach to the glass slide during viewing; these happen to retain their nucleus and other cytoplasmic components. Most axostyles in our hands simply convert upon ATP exposure from extended, shallow coils to shorter more obvious spirals (Fig. 1) and thereafter remain stationary. This was apparently also the experience of Woodrum and Linck (40) who prepared axostyles by techniques very similar to ours. Rarely, a few unusually large *Saccinobaculus* axostyles display dramatic coiling–uncoiling movements that occur simultaneously along their entire length. Such overall changes in helical pitch between the two states shown in Fig. 1 were also seen by Mooseker and Tilney in a few of the axostyles in their reactivation studies (29). Witnessing this unusual "clockspring" movement in the present study inspired a mechanistic interpretation, based on a general theory of helices, that will be offered in the Discussion.

Electron Microscopy of Whole Cells

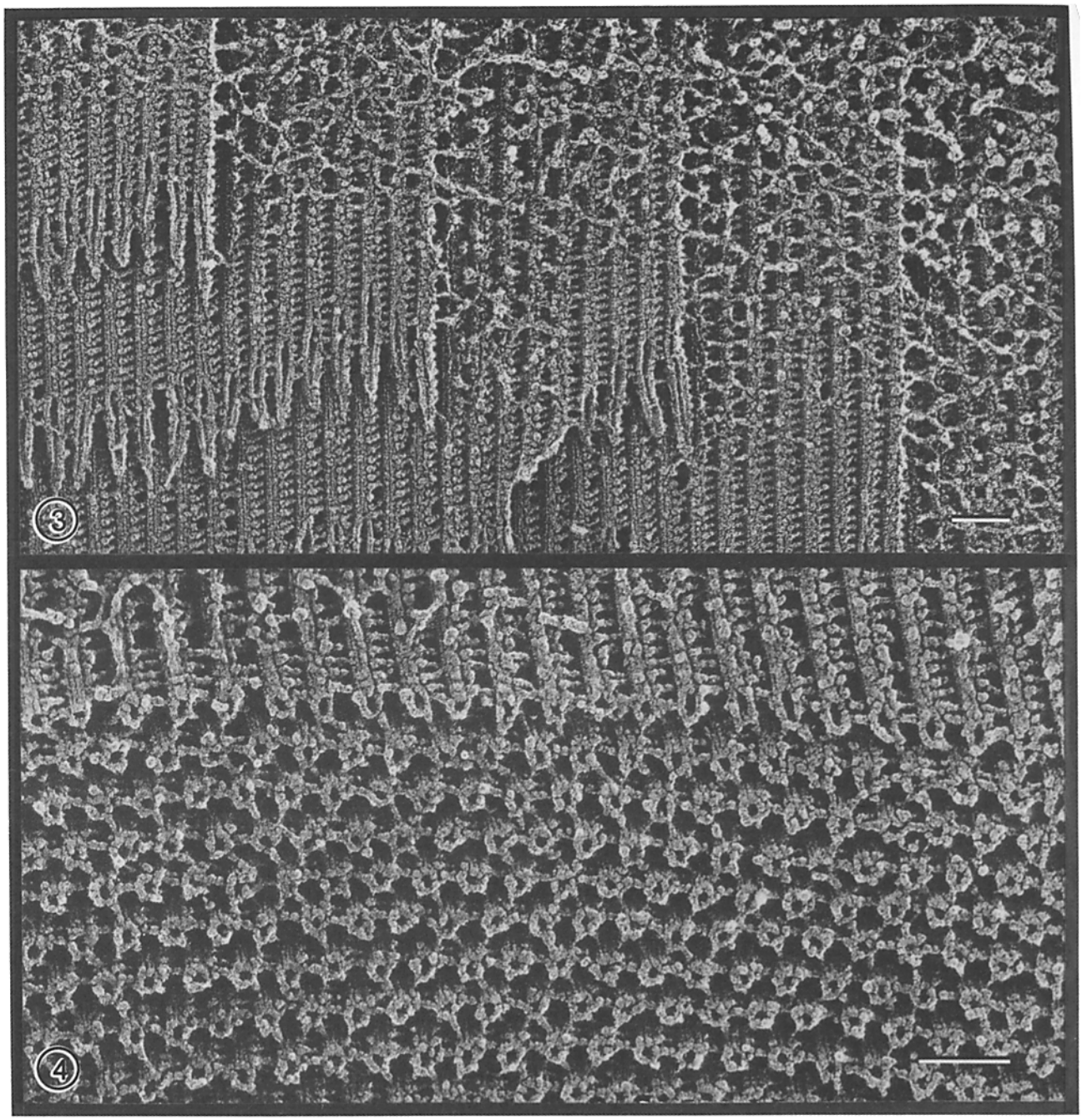
In previous studies of other cell types, deep-etch imaging of motile organelles in situ has been thwarted by the inevitable presence of uniformly dispersed, non-etchable granular material in the cytoplasm. This we have called "granola," for lack of a better term (21, 23). We believe this "granola" represents the freeze-dried counterparts of what were formerly the soluble contents of the cells' cytoplasm. The concentration of soluble proteins in most cells is thought to be ~10% wt/vol. Likewise, this "granola" is so concentrated in most cells that organized protein lattices such as muscle fibrils or ciliary axonemes are almost totally obscured (11, 20). To study such motile lattices by deep etching, it has been necessary to interject detergent extraction or osmotic rupture of the cells before quick-freezing, to wash out the obscuring material. Thus it was surprising to find that axostyles, on the other hand, always look "clean" in situ (Fig. 2). The cytoplasm of these protozoa looks like "granola", as usual, but apparently this material does not penetrate into the axostyle proper. (Either the microtubules are too closely packed or the granola is too internally cohesive.) In any case, it becomes possible to observe this motile organelle, in situ, in a cell that was living up to the moment of freezing.

One advantage this offers is that we can determine whether any changes occur when the organelle is removed from the cell and manipulated in vitro. On this point deep-etching agrees with previous thin-section studies, which have all concluded that isolation does not alter the basic architecture of the axostyle (2, 4, 8, 28, 29, 40). Readily apparent in vitro as well as in vivo is the bundling of microtubules into a series of sheets or "laminae," in which each lamina is composed of a planar, parallel array of tightly cross-linked microtubules (Fig. 3) and laminae are stacked on top of each other to form a thick, close packed bundle (Fig. 4). Also visible in vivo as well as in vitro are the two different sets of microtubule appendages that maintain this arrangement: those within each sheet (henceforth called "intra-laminar cross-bridges") and those between adjacent sheets of microtubules (henceforth called "inter-laminar cross-links"). Both sets of appendage are visible in cross-fractures such as Fig. 4, but are individually much more apparent in longitudinal fractures such as Fig. 3, which will be described in some detail next. Since one or both of these sets of cross-links are thought to be the critical force-generators in the axostyle, space will be taken here to review what is currently known about the geometry of movement vis-à-vis these structures, and what is known about their ultrastructural organization.

Past Work on the Geometry of Microtubule Cross-bridges vis-à-vis Overall Axostyle Movements

Unfortunately, any attempt to analyze the local structural changes that underly bend formation in axostyles is complicated by the fact that there is currently no accepted description of the geometry of the bend. Two diametrically opposed views are held (Fig. 5), one claiming that *Saccinobaculus* axostyles bend in a plane perpendicular to the broad axis of their laminae (Fig. 5 *b*), and the other claiming that *Pyrsonympha* axostyles bend in a plane parallel to the broad axis of their laminae (Fig. 5 *d*). This discrepancy would not be suspected from light microscopy alone; it shows that in both organisms, bends occur in a plane perpendicular to the broad axis of the axostyle as a whole (8, 17, 25, 34, 40). However, the electron microscopy has shown that the microtubule laminae run across the narrow axis of *Pyrsonympha* axostyles (15, 16, 25, 34), but across the broad axis of *Saccinobaculus* axostyles (28, 29, 40). The bend imagined for *Saccinobaculus* (Fig. 5 *b*) would require slippage of one lamina over another longitudinally and thus would boil down to active "rowing" movements by inter-laminar cross-links, as most former authors have entertained. The bending in *Pyrsonympha* would require longitudinal displacements of adjacent microtubules within one lamina, which would boil down to active "rowing" movements by the intra-laminar cross-bridges. Thus, differing interpretations of the geometry of the bends in the two organisms leaves ambiguity about which set of cross-bridges ought to be involved.

Electron microscopic studies have not resolved this ambiguity, to date. Varying interpretations of the organization and relative abundance of the two sets of cross-bridge have



Figures 3 and 4. (*Fig. 3*) Surface view of the lateral edge of an isolated *Saccinobaculus* axostyle (in Mg-ATP before freezing), illustrating that the microtubules forming stacks of laminae run strictly parallel to each other and to the edges of the laminae, as was generally thought before one recent claim to the contrary (40). Bar, 0.1 μm. (*Fig. 4*) A cross-fracture through an axostyle extracted from *Saccinobaculus* by NP-40 detergent treatment and then exposed to ATP before aldehyde fixation and quick-freezing, showing adequate preservation of its overall architecture after these manipulations. It remains a thick helical ribbon composed of sheets or laminae of microtubules (here oriented horizontally). Microtubules in adjacent laminae are packed in hexagonal register in this area, but in general have no fixed position from layer to layer. Bar, 0.1 μm.

been offered (see Fig. 6 and its legend for elaboration), but no concensus has been reached. One source of the discrepancies here may be differences in sample preparation; namely, thin sectioning accentuates the relative abundance of interlaminar cross-links while deep-etching reveals very few of them. Thin sectioning is of course subject to image-overlap problems; for example, a typical 100-nm thick cross section of an axostyle will superimpose six or seven intra-laminar cross-bridges (since, as we shall see below, they are spaced every 16 nm along the microtubules). Hence, if the interlaminar cross-links were even 5% as abundant, they would end up appearing in thin sections at ~30% the frequency of the former. In fact, careful review of previously published thin section electron micrographs suggested to us that interlaminar cross-links have always looked frail and insubstantial. Moreover, they often allow laminae to separate or dislo-

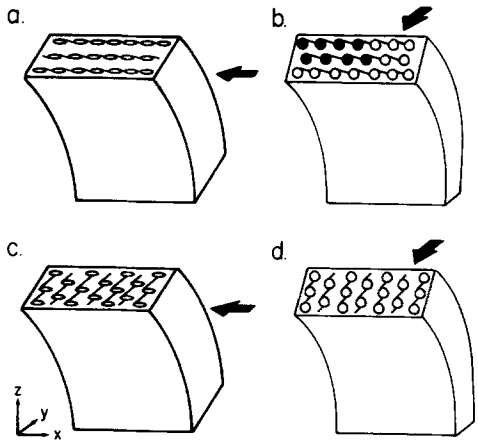


Figure 5. Comparison of the two purely orthogonal bending moments possible in *Saccinobaculus* axostyles (*a* and *b*) vs. *Pyrsonympha* axostyles (*c* and *d*). The two on the left show bends parallel to the broad axis (or "width") of the axostyle; the two on the right show bends perpendicular to their broad axis. Light microscopy has revealed that only the two on the right actually occur. Note, however, that the bend that occurs in *Saccinobaculus* (that shown in *b*) would be mechanically equivalent to the *Pyrsonympha* bend shown in *c*, relative to the cross-links that form the laminae of microtubules (shown here simply as diagonal lines). However, according to current interpretations (24, 25), the bend that actually occurs in *Pyrsonympha* is that shown in *d*. This complication is discussed in Results. (The blackened microtubule profiles illustrate the area enlarged in Fig. 6.)

cate from each other, even in healthy in vivo axostyles. On the other hand, intra-laminar cross-bridges have always looked stout and immutable, and obviously hold adjacent microtubules in near perfect register. The former set seems too frail and the latter set too robust to be the prime generator of axostyle movement. Thus past electron microscopy of the cross-bridges has also left ambiguity about which set is involved in axostyle movement.

With these unresolved issues in mind, we determined to examine axostyles more closely with freeze-etching, which offers certain technical advantages. First, platinum replicas provide *en face* views with such high contrast that individual cross-bridges can be seen; and second, these images are totally free from image-overlap problems because they represent true surface views of structures supported by ice that has been fractured in various planes.

Appearance of Intra-laminar Cross-bridges in Longitudinal Fractures

The most commonly observed fracture of the axostyle is one that travels along its length in a plane roughly parallel to the broad axis of the laminae. Here, deep-etching exposes *en face* views of laminae from either an outer or inner vantage point relative to the central axis of the axostyle. Views of the outer surfaces of laminae (Fig. 7) display the intra-laminar cross-bridges most distinctly, since these are located slightly above an imaginary axis joining the centers of the microtubules. (Laminae look generally convex in such views because the axostyle is usually cupped across its width as well as helically wrapped along its length.) As was noted in thin section studies, these intra-laminar cross-bridges are abundant and are regularly spaced every 16 nm along the microtubules (four times the microtubule's 3-start period [1]). Previ-

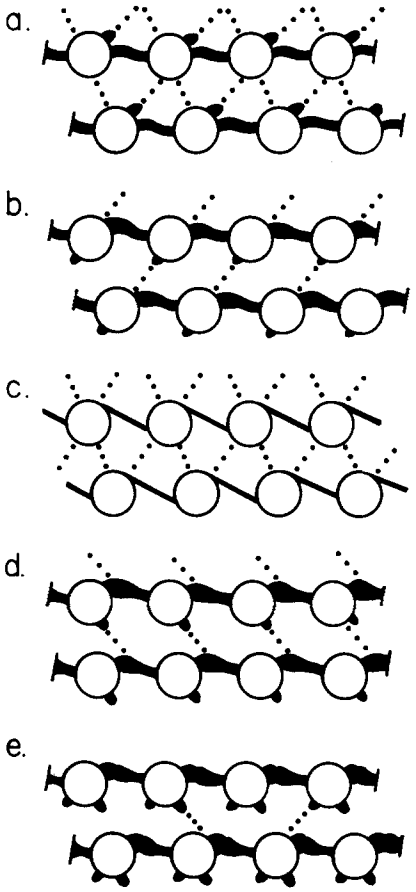


Figure 6. Changing views of the organization and deployment of microtubule cross-bridges in the axostyle, redrawn from earlier thin section studies in order to achieve a consistent vantage point, with rows of microtubules (here termed "laminae") oriented horizontally. The view in *a* (from reference 28) stressed the relative prominence of small "dog ears" that appeared to be separate from the lateral "intra-row links," as they were called in that study; deep-etching indicates that these "dog-ears" are in fact the inner domains of the intra-laminar cross-bridge "heads". The view in *b* (our interpretation of the thin sections of tannic acid–stained axostyles in references 29 and 37) revealed additional small projections on the bottoms of microtubules. These were interpreted to be the origins of inter-laminar bridges (dotted in this drawing). The view in *c* (from reference 4) was the author's interpretation of the relative abundance and hexagonal deployment of "inter-row bridges" seen in freeze-fractures of glycerinated axostyles; however, in our opinion the relative abundance of these structures was exaggerated by the form of freeze-etching used in this study. The view in *d* is our redrawing of the conclusions of the deep-etch study of reference 40, showing that this technique actually reveals very few inter-laminar cross-bridges and accentuates the relative prominence and abundance of the cross-bridges within laminae. The drawing in *e* is our current view of the organization and relative abundance of these two sets of axostyle cross-bridges. It also shows that deep-etching clearly displays both sets of granules that were seen before on the bottoms of the microtubules. Either of these sets could be the origins of inter-laminar cross-bridges, but the paucity of dotted connections between the laminae indicates how rare we think these actually are.

ous deep-etch views of these cross-bridges by Woodrum and Linck (40) further illustrated that each bridge is differentiated into a globular "head" (~9-nm diameter) and a thinner, elongated "stalk" (4 nm wide and 14 nm long). By monitoring

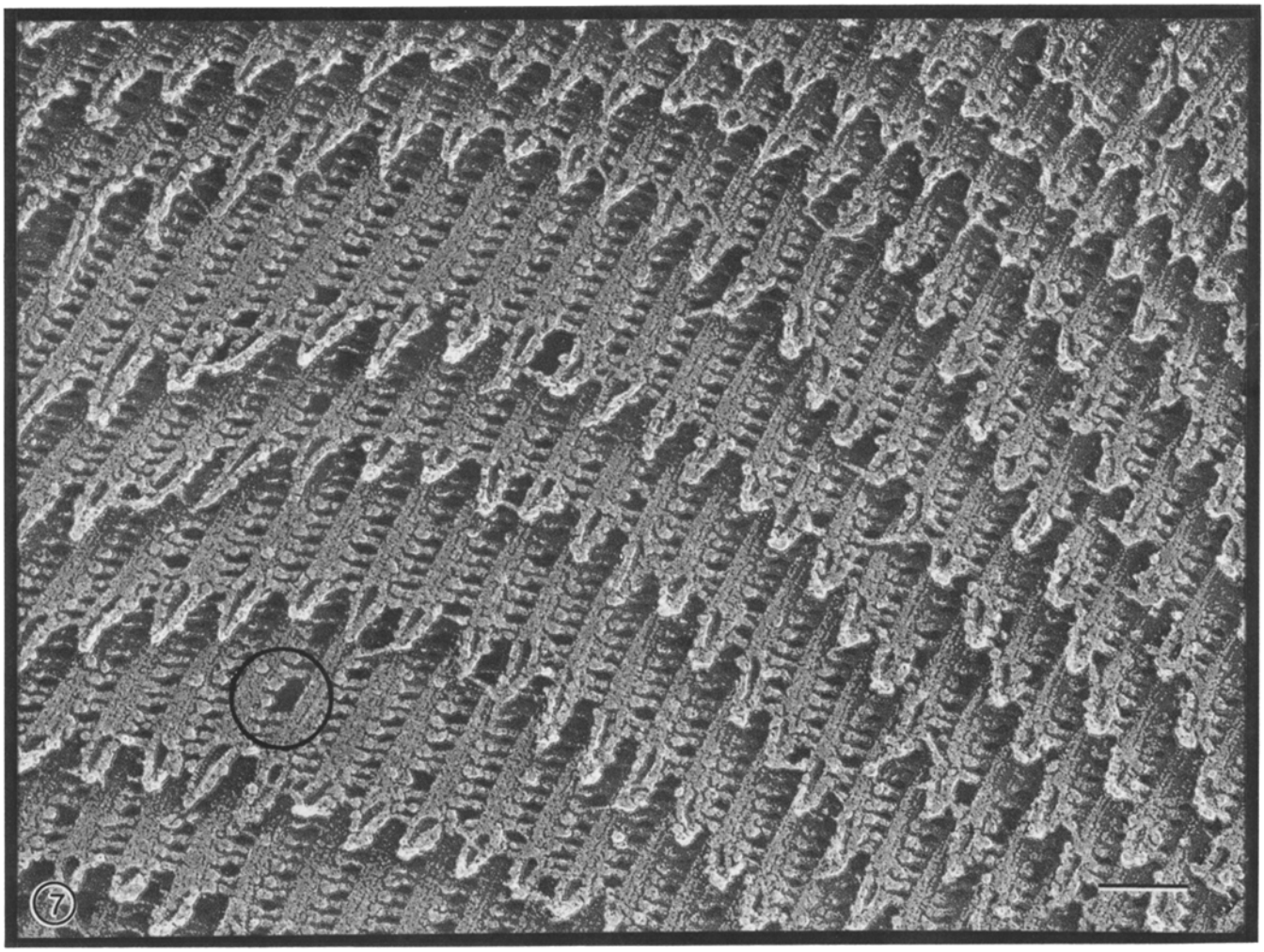


Figure 7. Oblique fracture through a *Saccinobaculus* axostyle isolated by detergent extraction and depleted of ATP (e.g., placed in "rigor") before quick-freezing. From this outer vantage point, laminae are curved convexly and intra-laminar cross-bridges are readily visible. The circle denotes two examples of a few cross-bridges in this field that are lacking their stalks, discussed further in Results (see also Fig. 21). Bar, 0.1 μm.

the orientation of our replicas and comparing these features with other landmarks on the microtubules (Fig. 8), we can show that when the anterior end of the axostyle is up and it is viewed from the outside (i.e., it looks convex), the globular "head" of each cross-bridge rests on the leftward member of each pair of microtubules and its stalk extends to the right. In addition, optimal replicas of *Pyrsonympha* axostyles demonstrate that the globular "heads" can be further resolved into two relatively distinct domains (Fig. 9). These sit roughly side-by-side in the gap between microtubules, so that only the one domain of the head rests on the microtubule to the left, while the elongated "stalk" emerges from the other domain.

Fig. 10 illustrates two additional features of the intra-laminar cross-bridges. First, an axis through the center of the two head-domains is usually slightly tilted, relative to a perpendicular between the microtubules. (This point bears on the description of changes in cross-bridge inclination presented below.) Second, the outer domain of the head (i.e., the portion that does not sit directly upon a microtubule) often appears to be linked to the next cross-bridge along. That is, this domain is teardrop-shaped, with a tapering extension that brings it into contact with the cross-bridge one up from it. A few such cross-bridge "links" are pointed out in Fig. 10 (they will be seen even more clearly in the rigor axostyle in Fig. 13; see below). The overall intra-laminar cross-bridge geometry determined from these observations is diagrammed in Fig. 11.

Variations in Cross-bridge Inclination

The exceptional clarity of intra-laminar cross-bridges in deep-etch replicas has allowed us to recognize that their stalks assume distinctly different inclinations under different conditions. Sometimes they tilt downward from left to right (using the convention of orienting the axostyle with anterior end upwards and viewing from the convex outer surface). This is seen, for example, in the three rightward rows of Fig. 9. Since this stalk inclination is opposite to the upward cant of the two head-domains, the cross-bridges in this case appear slightly check-shaped. In other axostyles, stalks can be found with the opposite inclination, upward to the right

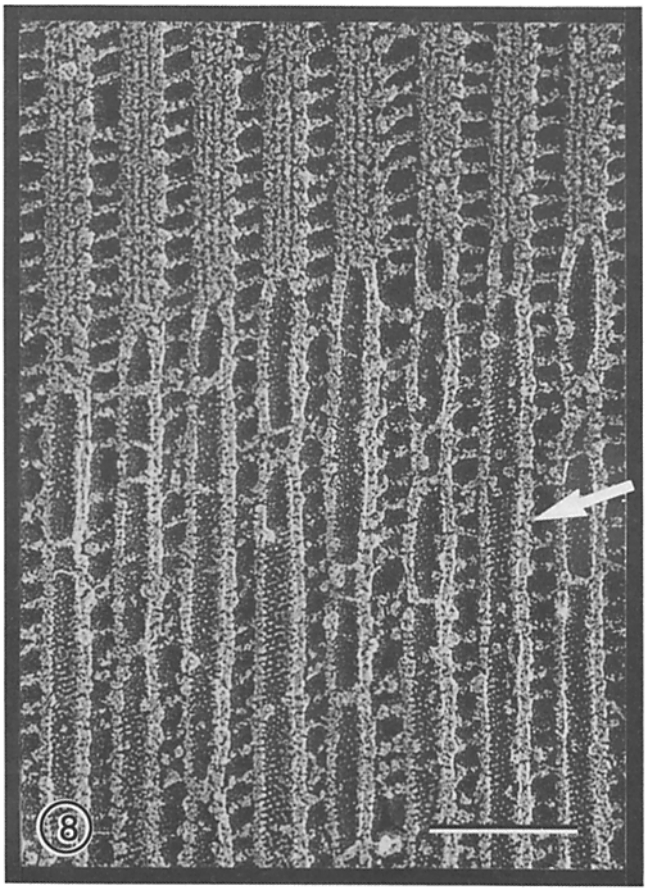


Figure 8. An isolated *Saccinobaculus* axostyle (in rigor) in which the microtubules are split open in the lower portion of the field to reveal their internal "ribbing" (sight along the arrow inclined in parallel with the ribs). We know from previous deep-etch work (19, 21) that these ribs represent the microtubule's three-start genetic helices, which repeat at 4 nm and are left-handed (1); thus they should slope up to the right when seen on the inside wall of the microtubule. These internal standards permit unambiguous determination of the actual spacing of the intra-laminar cross-bridges (4 × 4 nm) and of their proper orientation in our replicas (head to the left, stalk to the right when the axostyle apex is off the top of the picture and the lamina is viewed from its outer convex surface). Bar, 0.1 μm.

(Figs. 7 and 8). Since this is in the same direction as the cant in the head-domains, it eliminates their checked shape and makes the cross-bridges look straighter.

One interesting aspect of the observed differences in stalk inclination from axostyle to axostyle is that they are nearly always of sufficient magnitude, and sufficiently uniform across the width of each lamina, to bring all of the laminae's cross-bridges into oblique "register" (Figs. 12 and 13). This curious phenomenon is manifest in two ways. First, the heads of intra-laminar cross-bridges within any single lamina usually remain in almost perfect transverse registration (i.e., perpendicular to the long axis of the microtubules), regardless of how tilted the cross-bridges are. Second, the stalks of tilted cross-bridges generally point toward the next cross-bridge up (or down) in the adjacent row, or towards the second or third cross-bridge up or down. (This second point would of course be a necessary concomitant of the maintenance of transverse head-registration, if cross-bridge origins and insertions were fixed.) Such is the case, for example, in the drawing of Fig. 14, in which adjacent microtubules were displaced by 32 nm relative to each other, without detaching the cross-bridges. Both before and after this displacement, heads remain in transverse (horizontal) registration because the displacement was an integral of their natural repeat (2 × 16 nm). Moreover, cross-bridges continue to point at the same neighboring cross-bridge in the next row as they had pointed at before, which is drawn as either one 16-nm "step" up or down from the horizontal. What results is formation of oblique planes of cross-bridge registration. (This is also due to certain features of axostyle geometry explained in the legend to Fig. 14.)

In fact, the observed range of cross-bridge tilts suggests that 1, 2, or rarely 3 such 16-nm "steps" of microtubule displacement can occur in the axostyle. Angles of registration at ~18° and ~33°, either up or down, are most commonly observed (Figs. 3 and 7–10), while angles of ~44° are seen occasionally (Fig. 15). Since adjacent rows of cross-bridges are separated by 50 nm (transversely) and cross-bridges are spaced 16 nm apart (longitudinally), one step should create a tilt of TAN 16/50 = 17.7°, 2 steps a tilt of TAN 2× 16/50 = 32.6°, and 3 steps a tilt of TAN 3× 16/50 = 44°. Though we did not measure large numbers of cross-bridges individually to confirm that their mean angles matched these predictions, it was readily apparent (simply from looking diagonally across all our negatives, as suggested in the legend to Fig. 15) that one or another of these three patterns of registration was always present.

Indeed, the images strongly suggest that we are witnessing "quantal" differences in microtubule registration amongst different axostyles, reflecting perhaps the existence of several different stable states in which all the shearing forces within an axostyle are distributed amongst the cross-bridges uniformly. Rarely do we observe sudden shifts in cross-bridge inclination within any one axostyle, whether between adjacent laminae or within one lamina, although Fig. 9 illustrates an exception. Foreshortening this field in a plane perpendicular to the microtubules illustrates an abrupt shift from one step down (−18°) to two steps up (+33°). The interface where this occurs is enlarged in Fig. 9 *b*. Note that cross-bridges in the upwardly tilted (left-most) row look identical to those throughout Fig. 13. Such an abrupt, internal variation in cross-bridge angle is extremely rare; we have seen it only in a few isolated axostyles that were collected by centrifugation, and suspect that under these conditions a relatively rigid axostyle may have been subjected to artifactual shear.

The important question raised by the observations so far is whether such differences in cross-bridge inclination are passive manifestations of different degrees of shear within different axostyles, or whether they are actively generated changes in conformation that create the different shearing forces. In either case, it would be helpful to correlate the different internal cross-bridge conformations with overall axostyle shapes. Unfortunately, this has been hard to do, simply because freeze-fracture rarely provides enough exposure of an axostyle to determine the latter parameter; e.g., its overall degree of "bending" (longitudinal helicity) or "cupping" (transverse curvature). Nevertheless, an obvious trend in our observations bears mentioning. Downward cross-

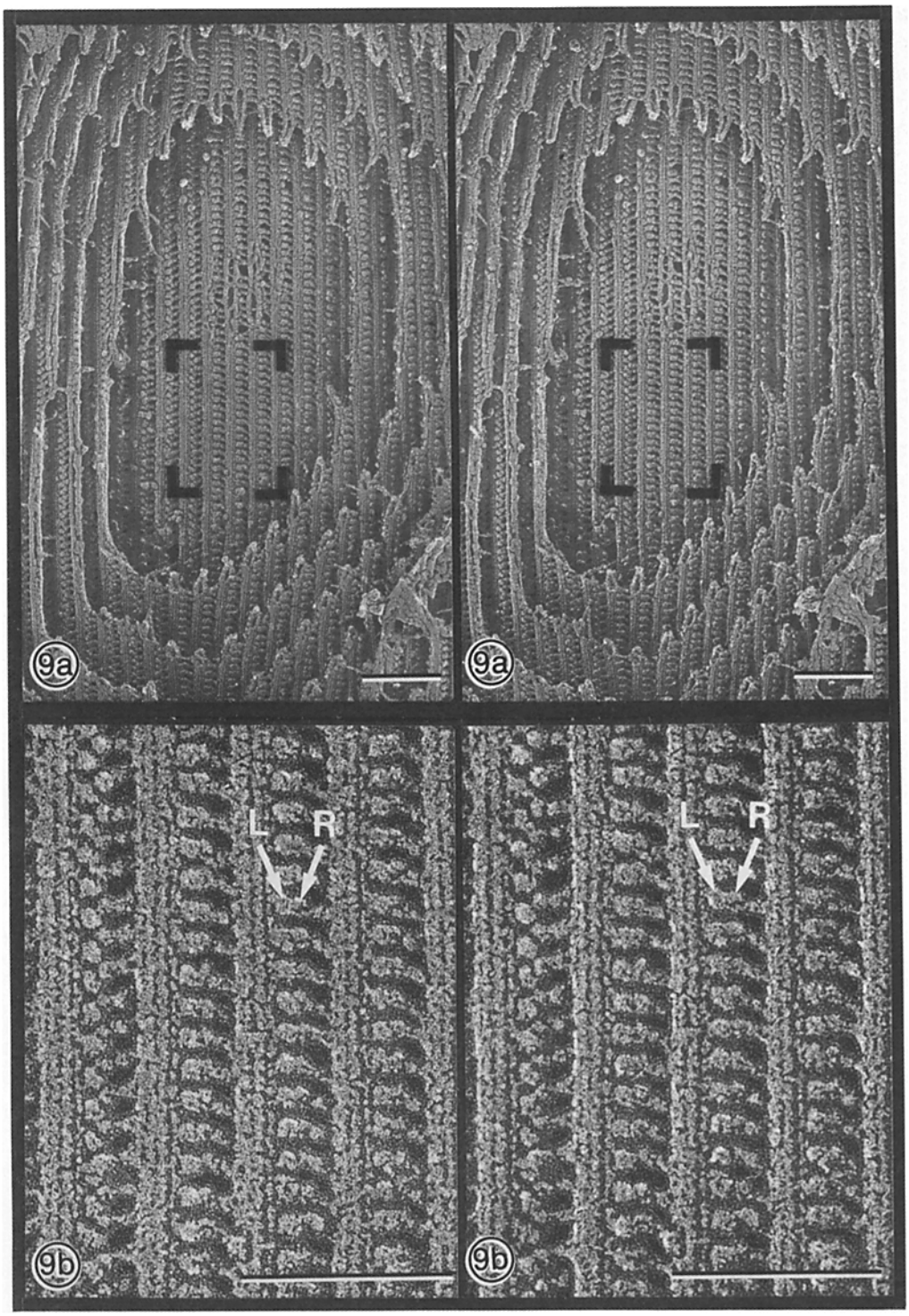


Figure 9. En face stereo views of intra-laminar cross-bridges in a *Pyrsonympha* axostyle isolated with detergents and exposed to Mg-ATP before quick-freezing. The high power view below (taken from the bracketed area above) includes four rows of cross-bridges, the three on the right displaying a "partitioning" of each head into two domains (labeled *L* and *R* on one head). (The row of cross-bridges on the left display a different "extended" morphology described in detail in Fig. 11.) Bars, 0.1 μm.

bridge tilts are found more commonly in situ (Figs. 2 and 12) and in ATP-reactivated axostyles (Figs. 3, 9, 5, 16, and 17), axostyles that would be expected to contain a greater proportion of bends or to be more helically coiled throughout. On the other hand, upward cross-bridge tilts are found more commonly in isolated axostyles frozen in the absence of ATP (Figs. 7, 8, 10, and 13). These nonmotile, "rigor" axostyles are typically longer and straighter, but narrower and more crescent-shaped transversely than are ATP-reactivated ones. This suggests a correlation: cross-bridges down in bent axostyles vs. cross-bridges up in straight axostyles. However, a complete review of all our observations (Table I) indicates that, to date, this correlation is no more than a trend. Apparent exceptions are common, though some of them can be explained. Upwardly canted bridges in whole cells or in ATP-reactivated samples (at least 25% of the total in Table I) could come from relatively straight regions between bends, or from axostyles that were inactive at the moment of freezing.

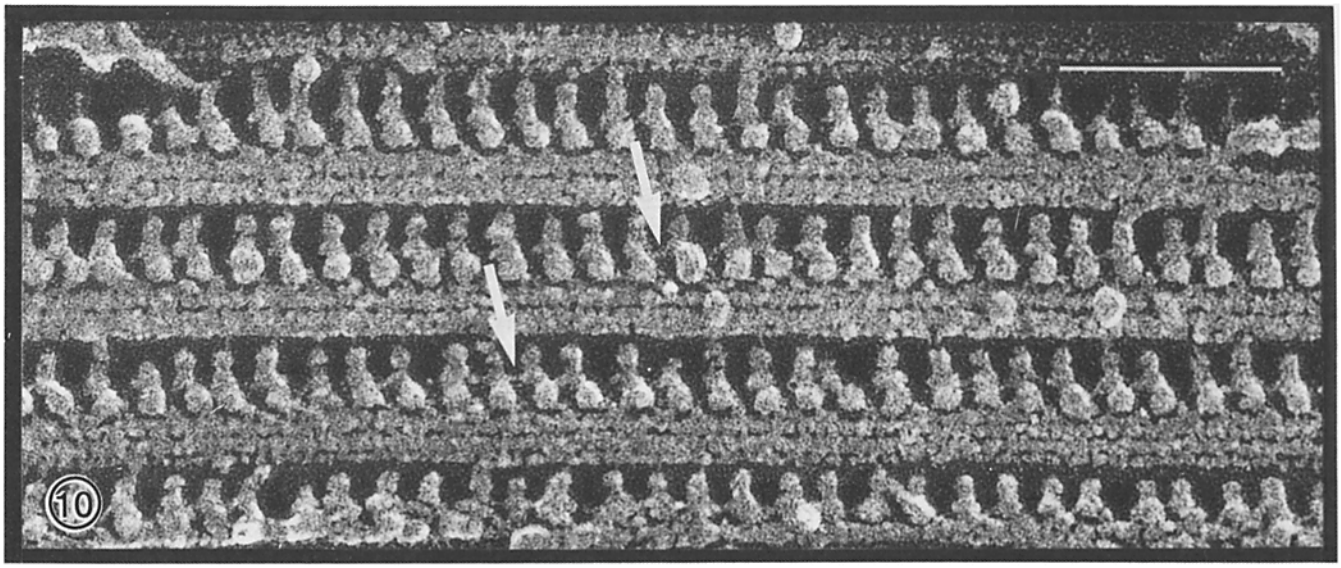


Figure 10. Our best replica (in terms of its subtlety of platinum shadowing) of intra-laminar cross-bridges in an isolated *Pyrsonympha* axostyle in rigor (oriented 90° counterclockwise relative to Figs. 8 and 9, so the apex of the axostyle is to the left), showing most clearly the partitioning of their "heads" into two domains, as well as the longitudinal "linkers" between their heads (a few of which are pointed out by the arrows). Bar, 0.1 μm.

On the other hand, downwardly tilted bridges in rigor axostyles (found in roughly one-third of them) could come from bent regions that became "frozen in" during ATP depletion, as are observed occasionally. In any case, the data in hand are consistent with the possibility that a conversion of cross-bridges from tilted up to tilted down could actively generate microtubule sliding within individual laminae. How this internal shear could lead to overall changes in the axostyle will be considered in the Discussion.

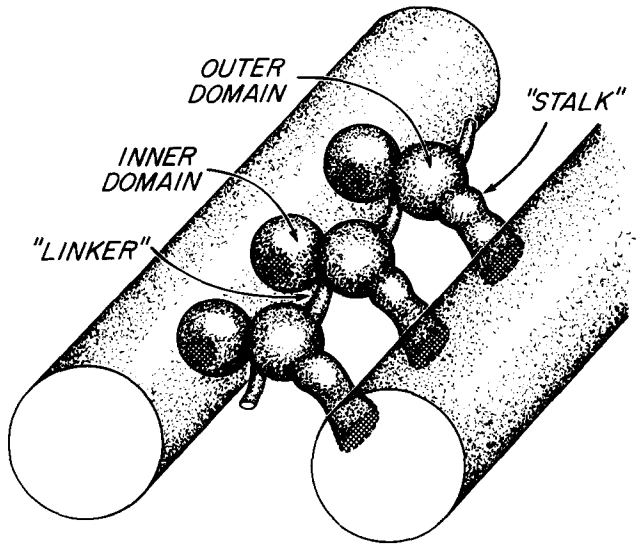


Figure 11. Diagram of our conclusion on the deep-etch appearance of intra-laminar cross-bridges, illustrating (*a*) the partitioning of their "heads" into two domains, inner and outer; (*b*) the diagonal "links" between adjacent cross-bridges; and (*c*) the relative positioning of the "stalks" that reach across to the adjacent microtubule.

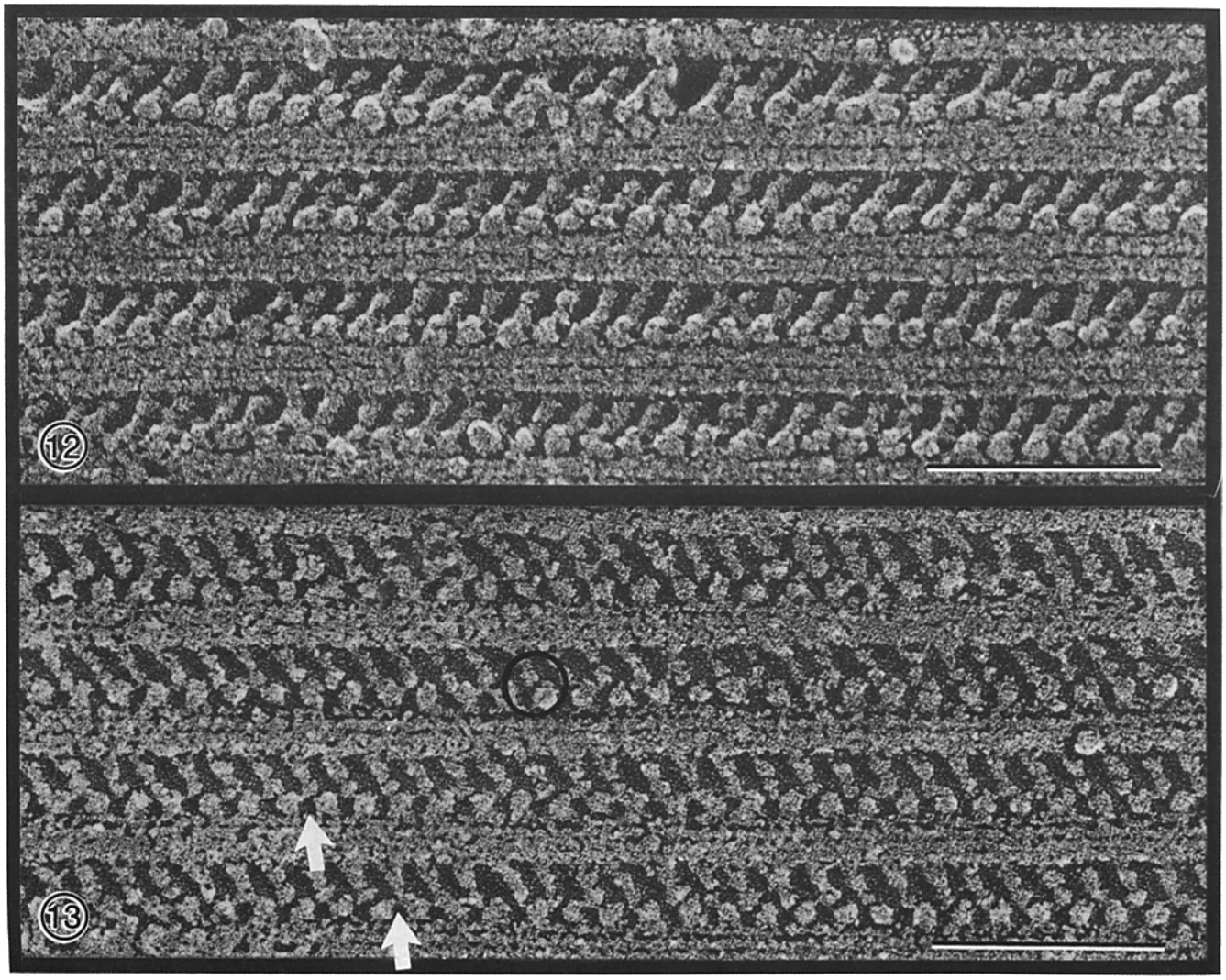
Table I. Cross-bridge Inclinations Tabulated

	No. of replicas	No. of axostyles	Intra-laminar cross-bridge inclination			
			Tilted down	Tilted up	Mixed	Indeterminant
P whole	15	35	14	4	3	14
P rigor	11	23	5	9	7	2
P ATP	13	27	2	17	1	7
S whole	9	20	9	6	1	4
S rigor	10	22	8	4	3	7
S ATP	12	18	6	10	2	0

Summary of our total number of observations of different axostyles in freeze-etch replicas of *Pyrsonympha* (P) and *Saccinobaculus* (S). The first two columns indicate the total number of replicas examined and the total number of axostyles found in them. The final four columns indicate the predominant intra-laminar cross-bridge orientation in each of the observed axostyles. Note that downward tilts are generally more abundant in whole organisms that are wiggling up to the moment of fixation or freezing, while upward tilts are relatively more abundant in extracted axostyles, regardless of whether they are placed in rigor by ATP depletion or provided with 5 mM Mg-ATP in the moments before freezing.

Structures Seen on the Undersides of the Laminae

En face views of the undersides of microtubule laminae (Figs. 16 and 17) reveal the same crystalline arrays of intra-laminar cross-bridges; however, from this vantage point, these cross-bridges appear less prominent because they are below the microtubules and hence partially lost in the shadows of the metal replica. Also, only the outermost domains of their dimeric "heads" can be seen, because their inner domains are underneath the microtubules. The terminal portions of their "stalks" look broader and more distinct in this view, on the other hand, indicating that the stalks are slewed inward in the radial direction relative to an axis joining microtubule centers. This geometry was indicated in Fig. 11, above.



Figures 12 and 13. (*Fig. 12*) An in situ axostyle in a *Saccinobaculus* that was wiggling until the moment of quick-freezing, showing the basal tilt of intra-laminar cross-bridges typical of the in situ organelle. Bar, 0.1 μm. (*Fig. 13*) An isolated *Pyrsonympha* axostyle that was allowed to go into rigor (via ATP depletion), showing an upward inclination of its intra-laminar cross-bridges that is so steep that the two portions of their heads appear slightly separated (one such bridge is circled). Note that the diagonal "links" between adjacent cross-bridges are much more visible when the heads are this extended (*arrows*). Bar, 0.1 μm.

Saccinobaculus axostyles viewed from beneath display two further sets of microtubule appendages that have not been explicitly described before. One consists of barely visible 7-nm "granules" that are extremely abundant and regularly deployed (16 nm center-to-center in regions of closest packing) along one edge of the underside of each microtubule (Figs. 16 and 17, small arrows). (Being not much wider than a microtubule protofilament, this set of granules is often very hard to see unless one sights down along the length of the microtubules to foreshorten the image.) The second set consists of larger (~10 nm) "granules" that are less abundant, but partially occupy another set of 16 nm-spaced sites on the microtubules (cf. ref. 27). These are located on the opposite edge of the microtubule from the small granules, immediately beneath the heads of the intra-laminar cross-bridges (Figs. 16 and 17, large arrows). Fig. 6 included these granules when comparing our results to previous reports. In retrospect, the small granules were particularly prominent in the thin section images of tannic acid stained axostyles of Tilney et al. (37), while the large granules were particularly apparent in Woodrum and Linck's thin section images (Fig. 4 *c* of reference 40). The latter authors concluded, in agreement with earlier interpretations (28), that one or both of these sets of appendages might be the more visible parts of cross-bridges that extend between rows of microtubules. As described next, the present deep-etch views of such inter-laminar cross-bridges do not support this conclusion. Moreover, neither of these two types of granule are found in *Pyrsonympha* axostyles. In the following description, however, bear in mind the possibility that the granules in question could represent detached inter-laminar cross-bridges, and that we have failed so far to maintain the physiological conditions needed to preserve their attachment.

Inter-laminar Cross-links in Edge-views of Laminae

The second major plane of fracture along the length of the axostyle, which occurs much less often, is in a plane or-

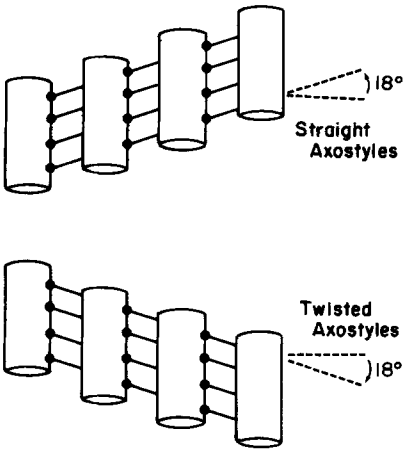


Figure 14. Diagram showing how 16-nm "quantal steps" of microtubule displacement lead to oblique registration of cross-bridges in adjacent rows. This occurs because stalks do not point directly at a cross-bridge in the adjacent row, but rather at the space immediately between its closest neighbors. Stated another way, the head-binding sites and stalk-binding sites on opposite sides on any single microtubule are exactly out of phase (i.e., 8 nm apart). This half-stepping combines with the fact that the cross-bridges are about half as long as the center-to-center spacing between microtubules (a natural consequence of the fact that the microtubule diameter is also about half of this spacing) to create the registration. (This is best understood by raising the journal page and citing down along the dotted lines.)

thogonal to that described above: namely, perpendicular to the broad axis of the laminae. Here, deep-etching exposes edge-on views of the laminae, in which intra-laminar cross-bridges are transected (Fig. 18). Such edge-views are valuable because they reveal most distinctly the links that extend between laminae. Of course, such inter-laminar cross-links have to exist for the axostyle to hold together. A key question, however, is whether they play a primary role in motility, as most previous investigators have assumed by analogizing them to ciliary dynein (2, 26, 28, 29). Actually, the deep-etch images obtained in this study, like those obtained by Woodrum and Linck (40), show that the inter-laminar connections in question are relatively few in number, thin and wispy in appearance, and variable in deployment and inclination (Fig. 18); hence they do not look at all like the substantial dynein side-arms that generate microtubule sliding in cilia and flagella (11–14, 39). Fig. 18 is in fact from an axostyle frozen in situ (a different part of Fig. 2), where there should be no suspicion that certain cross-bridges were extracted during sample preparation. Nevertheless, the inter-laminar links are <5% as abundant as the stout intra-laminar cross-bridges described above. These links course randomly across the gaps between laminae, which are much more variable in width than the spaces between microtubules within each lamina, hence the links are of widely variable length and inclination. They look rather like the bridges we find in nonmotile sheets of microtubules, such as those found beneath the plasma membrane of trypanosomes (Fig. 19; see also reference 35). They also look like the nondescript "wisps" that span microtubules and neurofilaments in nerve axons (22) and the cross-links between microtubules and pigment granules in chromatophores (23).

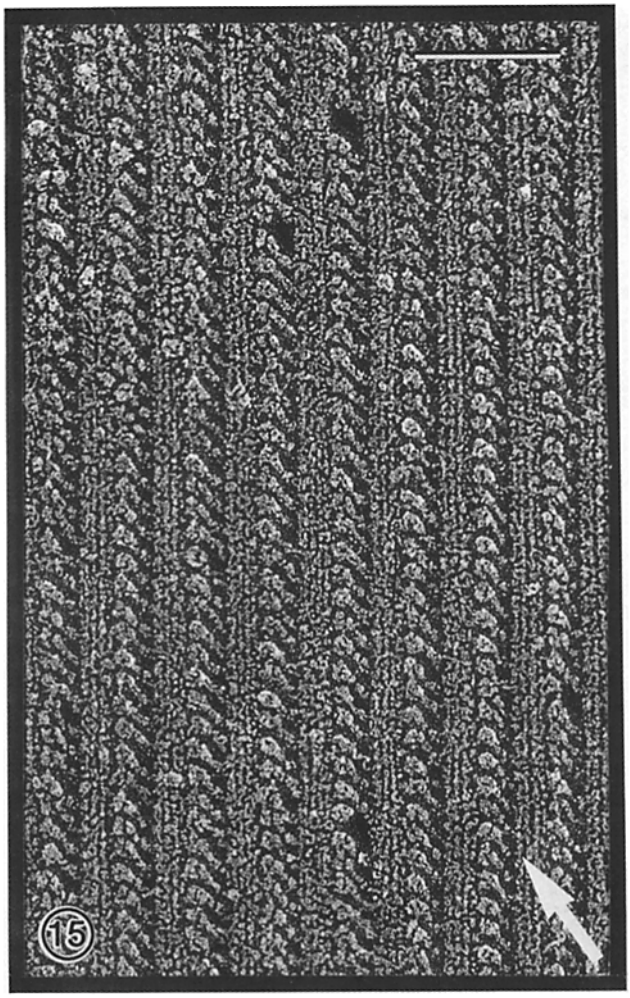
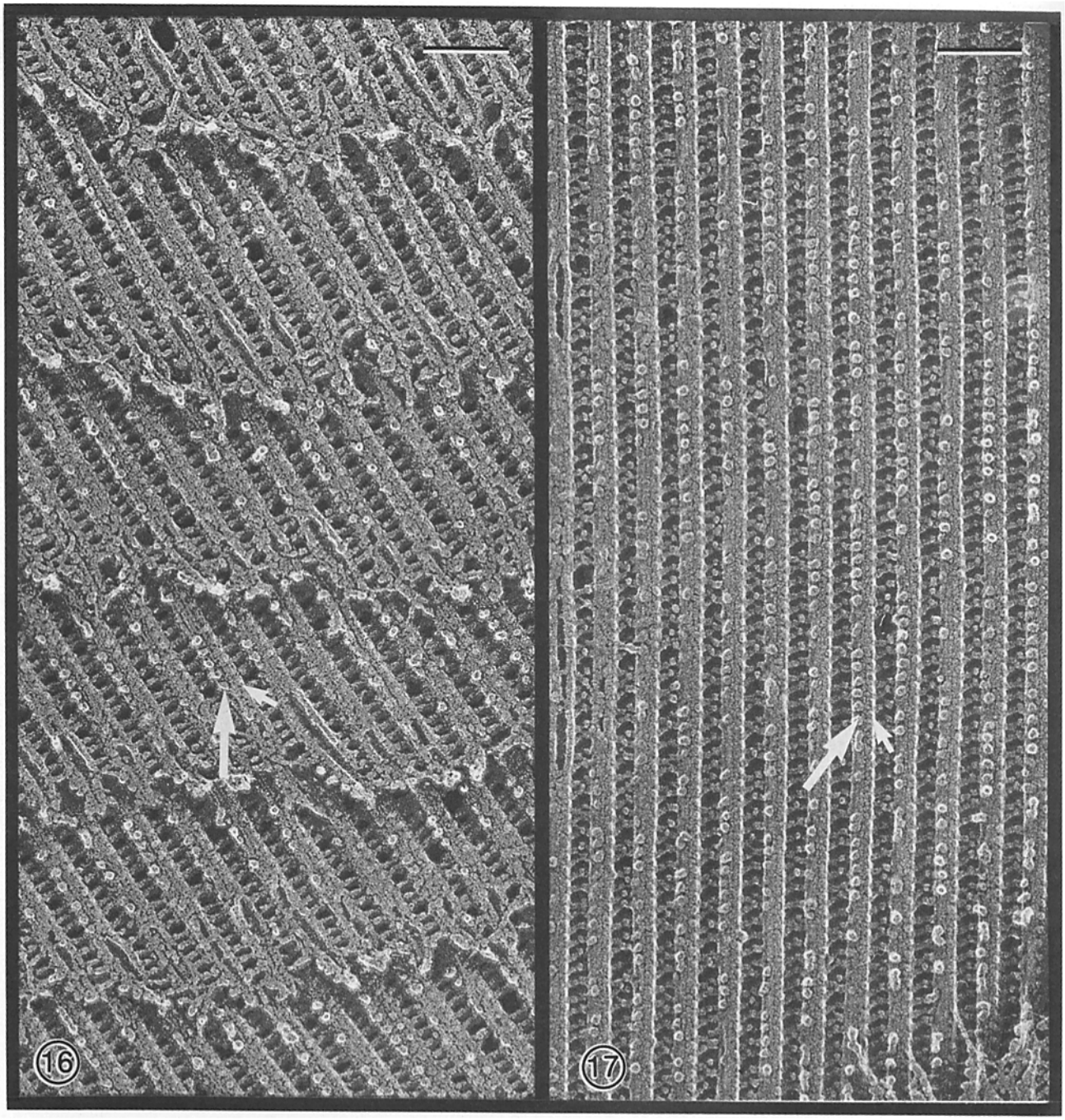


Figure 15. An exceptional *Saccinobaculus* axostyle exposed to ATP before quick-freezing, whose cross-bridges were tilted more severely than usual, thus creating the "three-step" downward registration described in the text. This produces 44° diagonals that can be seen when the image is foreshortened along the arrow. Bar, 0.1 μm.

Comparison of Cross-bridges in Cross-fractured Views

Fractures perpendicular to the long axis of the axostyle provide images analogous to transverse thin sections, but are somewhat less informative because the cross-bridges are more deformed by fracturing than by thin sectioning. Even so, their preservation is usually adequate to confirm the above-mentioned differences between the two sets of cross-bridges (Fig. 20). Those within laminae are ubiquitous while those between laminae occur between <10% of the subjacent microtubules, regardless of whether the axostyles are viewed in situ (Fig. 2) or after removal from the cell (Fig. 20). Underscoring our impression that links between laminae are relatively rare and physically insubstantial is the common finding that upon removal from the cell, adjacent laminae often peel apart from each other (2, 17, 29, 40). Earlier observers also noted that adjacent laminae are not uniformly spaced in fixed whole cells (3, 15, 16, 34), and that axostyles can even split completely apart in living cells, with both halves continuing to function (25).



Figures 16 and 17. (*Fig. 16*) Oblique fracture through a *Saccinobaculus* axostyle (bathed in Mg-ATP before freezing) which yielded *en face* views of the concave undersides of several laminae, opposite to the side on which the intra-laminar cross-bridges occur (such as Fig. 7). Portions of the underlying cross-bridges can be seen in the gaps between microtubules. Also visible are large and small granules dotting the lateral edges of the microtubules (large and small arrows). Bar, 0.1 μm. (*Fig. 17*) Purely *en face* view of the underside of a lamina in a rigor *Saccinobaculus* axostyle, illustrating particularly clearly the two sets of large and small granules that decorate the inner edges of the microtubules (*large and small arrows*). The distribution of these granules suggests random occupation of regularly repeating sites (cf. reference 27), sites that are in register with the 16-nm repeat of the underlying intra-laminar cross-bridges, portions of which can be seen in the gaps between microtubules. Bar, 0.1 μm.

At a grosser level, the cross-fractures obtained here display overall features of the axostyle lattice that substantiate previous thin section images; namely, by demonstrating that they are also found in unfixed samples. These features include, most importantly, the distinct curvature of each lamina, which creates the overall "cupped" appearance of the transected axostyle (Figs. 2, 7). McIntosh et al. (26, 28) were the first to note that this transverse curvature diminishes where longitudinal (helical) bending increases, an observation that required careful serial thin sectioning and thus is not accessible to confirmation by random freeze-fracturing. Freeze-fractures do show, however, that the degree of trans-

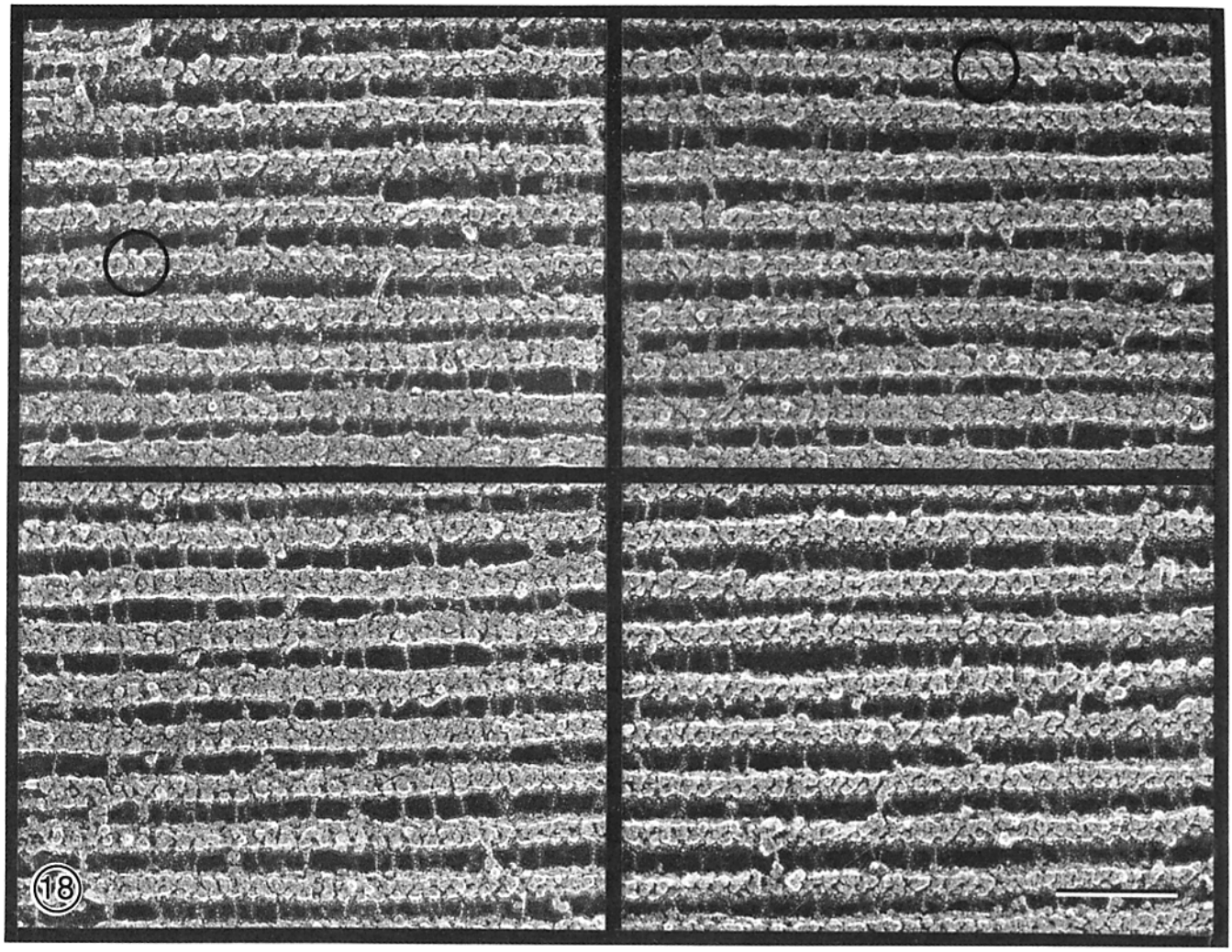


Figure 18. Four different edge-on views of an axostyle in situ, from a *Pyrsonympha* that was aldehyde fixed while still wiggling, in order to reduce the likelihood of any cross-bridge breakage or extraction. The axostyle runs horizontally here, so adjacent laminae are stacked on top of each other. Foreshortened views of fractured intra-laminar cross-bridges look like "corn kernels" in the plane of each lamina (a few are circled). Inter-laminar cross-links run more-or-less vertically between the stacks; these are extremely thin, amounting to only one platinum grain in width (2–3 nm), and occur in much lower frequency than the intra-laminar cross-bridges shown heretofore. Bar, 0.1 μm.

verse curvature varies from one axostyle to the next and varies even within each axostyle, tending to be more extreme at its lateral edges (not shown). In addition, transverse freeze-fractures confirm that although the microtubules in adjacent laminae run parallel to each other, the stacking of adjacent laminae varies continuously, as would be expected from their variable radius of curvature. Thus the overall packing of microtubules is sometimes orthogonal (Fig. 20), sometimes hexagonal (Fig. 4), and sometimes intermediate (Fig. 2). Unlike the strict order within laminae, there appears to be no fixed position of microtubules in one lamina relative to those in the next. The cross-links between laminae must thus be flexible enough to adapt to these changes in packing, or must be capable of "jumping" from one binding site to another.

Cross-bridge Extraction

As earlier studies uncovered the structural analogies between axostyles and ciliary axonemes, efforts were made to see if dynein-like molecules could be extracted from axostyles by the sorts of protocols that remove dynein from axonemes (9, 10). Two earlier reports claimed some success with the typical high salt extraction (2, 29). Thin sections in Bloodgood's report (2) showed that extracted axostyles fall apart into individual laminae, indicating that cross-bridges between laminae were broken or gone. Since these cross-bridges are now shown to be unlike dynein in morphology, a key question is the response of the dynein-like intra-laminar cross-bridges to high salt exposure. Fig. 21, *C* and *D*, provides *en face* views of laminae from *Saccinobaculus* and *Pyrsonympha*, respectively, separated by high salt extraction. These demonstrate that a considerable proportion of the intra-laminar cross-bridges are also removed by such treatment. Thin sections of single laminae embedded in plastic apparently did not provide adequate contrast to demonstrate this (2), yet it is readily apparent in the present high-contrast surface replicas. We conclude that high salt extraction can remove a major proportion of the intra-laminar cross-bridges as well

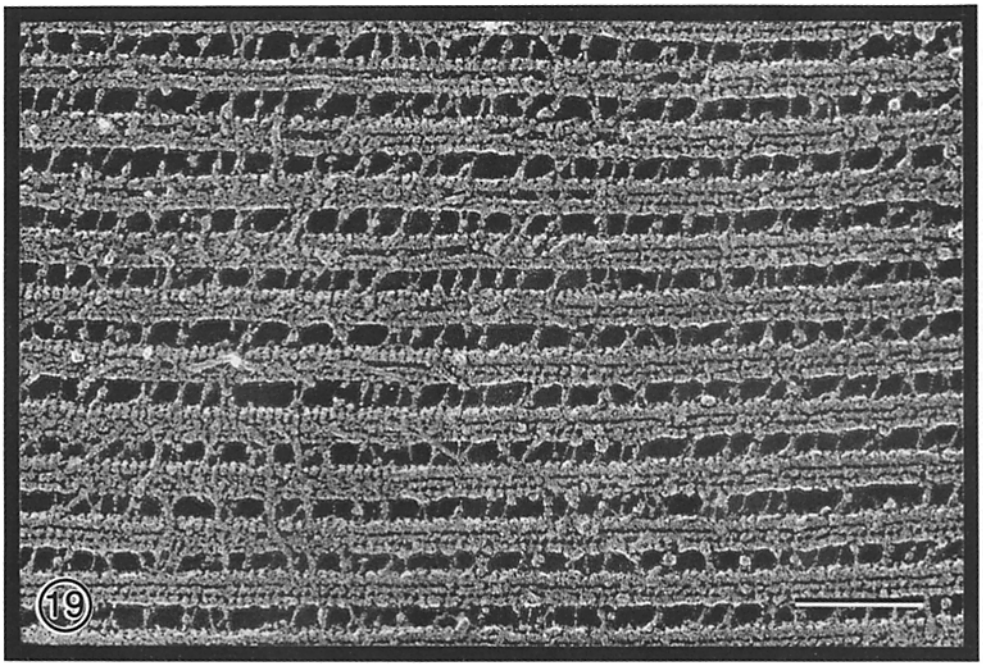


Figure 19. Sub-pellicular array of microtubules from a trypanosome protozoan extracted with detergents to remove the overlying plasma membrane. Delicate cross-bridges hold the microtubules in permanent parallel alignment; these are similar in thickness and nondescript appearance to the inter-laminar cross-links of axostyles in Fig. 18. This array is considered to be nonmotile. Bar, 0.1 μm.

as the inter-laminar cross-bridges; apparently, however, this does not show up as internal "fraying" of laminae because their internal cross-bridges are so abundant to start with. Thus the extracted dynein-like ATPase (2, 29) could just as well reside in either type of cross-bridge (assuming it is not simply extracted from the flagellar axonemes that inevitably contaminate such preparations).

A further observation, made serendipitously, may contribute toward localizing the ATPases in axostyles. While exploring methods to obtain axostyles from protozoa by hypotonic shock, in an effort to avoid exposing them to detergents, we found that solutions of very low ionic strength containing low divalent cations (for example, 5 mM Pipes buffer, pH 7.0 with Mg^{++} <0.3 mM) also extract intra-laminar cross-bridges, but without separating adjacent laminae. This may be related to Gibbon's early success at removing ciliary dynein by exposing axonemes to low ionic strength, divalent cation-free solutions (9), a procedure that was used before the advent of high salt extraction of dynein (10). Images of axostyles partially extracted by this protocol are particularly

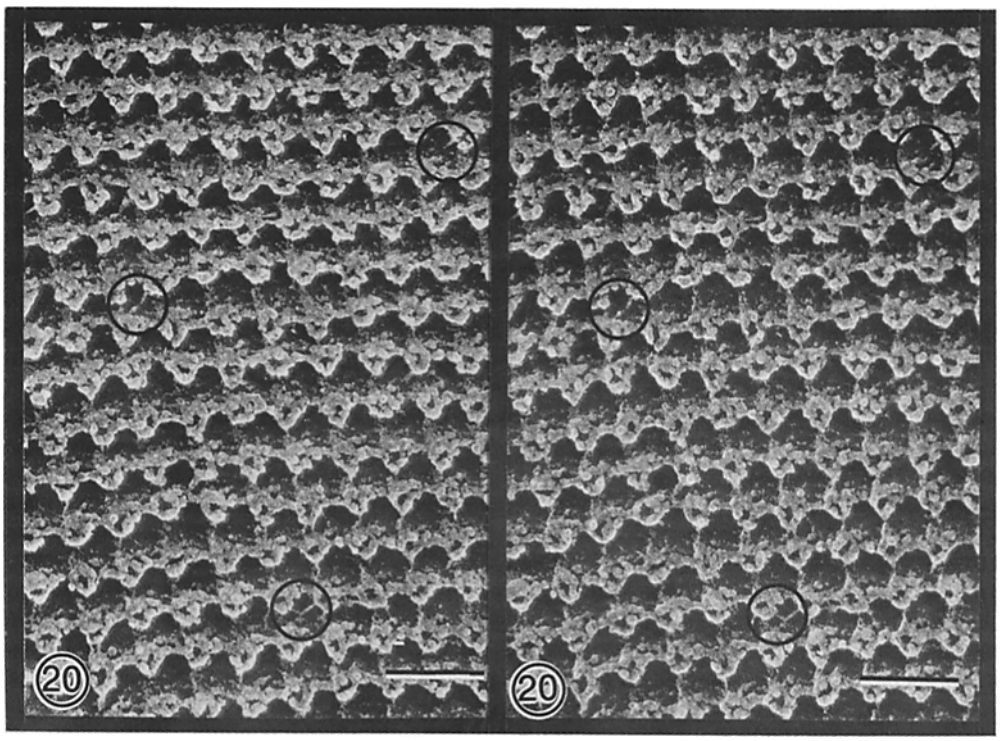


Figure 20. Stereo view of a cross-fractured *Pyrsonympha* axostyle, after in vitro treatment with ATP and aldehyde fixation (this protocol being chosen to increase the likelihood of finding inter-laminar cross-links and to "toughen" them against distortion during fracturing and etching). In comparison with the ubiquitous intra-laminar cross-bridges, which here run horizontally, inter-laminar cross-links are few and far between. A few are circled; others can be found, but in a small proportion to the former. Bars, 0.1 μm.

informative (Fig. 21, *A* and *B*). They illustrate that in some regions, the stalks of the intra-laminar cross-bridges are extracted selectively; that is, they leave their heads behind, still attached to the microtubules of origin (Fig. 21 *B*). In other regions, cross-bridges appear to be extracted wholly but apparently at random (Fig. 21 *A*). Such intra-laminar changes are found throughout axostyles that are exposed to low salt for as little as 0.5–2 min at 4°C, at which point they still appear to be held together normally, so their inter-laminar cross-links ought to be relatively intact. Clearly, the thesis proposed in this report would be strengthened by showing that such low salt treatment extracts a dynein-like ATPase from the axostyle. We are currently attempting to collect enough organisms to do such biochemistry.

Discussion

The relative fraility and variability of the cross-links between microtubule laminae in the axostyle was once considered to be evidence that they were dynein-like (26, 28, 29, 34), but with the realization that dynein cross-bridges are stout, multi-faceted structures (9–12), this analogy no longer holds. Indeed, deep-etching shows that intra-laminar cross-bridges in the axostyle look much more like dynein arms. This, plus our demonstration that intra-laminar cross-bridges assume different tilts under different conditions (Fig. 22) and also fall out under extraction protocols used to remove dynein from cilia, would suggest that they, instead, are the dynein analogs. If so, they would be expected to generate longitudinal displacements of adjacent microtubules within laminae. This would produce the type of bend imagined for *Pyrsonympha* axostyles (diagrammed in Fig. 5 *d*), but whether it would produce enough of a bend seems doubtful. For example, a change in cross-bridge angle of ±18° would displace adjacent microtubules by only 32 nm (Fig. 23). Projected into the total width of a lamina 100 microtubules wide, this would produce a shape change roughly like that shown in Fig. 23 *a*. If constrained at one end, as in cilia, this would generate only a very shallow curvature in the plane of the lamina (Fig. 23 *b*). The amount of curvature actually observed in *Pyrsonympha* axostyles would require that cross-bridges detach, rebind downstream, and repeat this change in angle several times. Microtubule displacements in cilia and flagella, for example, exceed ~100 nm in maximally curved areas (33). This is thought to require at least four dynein outer arm cycles of attachment and detachment, since these arms are spaced 24 nm apart (11). However, we should stress that there is no evidence for cross-bridge cycling in axostyles. At least, we have never observed a phase of detachment of the intra-laminar bridges, nor has anyone ever obtained even indirect evidence that any of the bridges normally detach. Such evidence has of course been obtained for cilia and flagella (36); since they actively slide apart if they are exposed to proteases and then reactivated with ATP. No such "telescoping" has ever been observed in reactivated axostyles, however.

Barring cross-bridge "cycling," the only other way to increase curvature in the plane of the axostyle laminae would be to shorten or contract the microtubules on one side relative to those on the other. This of course would alter the spacing of the intra-laminar cross-bridges. Indeed, McIntosh obtained indirect evidence, from optical diffraction of thin sections, for a reduction in spacing of these cross-bridges on the insides of bends in three *Saccinobaculus* axostyles (26). Unfortunately, in the present study we have never observed such changes in spacing, even though we can scan across individual laminae and view their cross-bridges directly. We have seen only the changes in tilt and resultant patterns of oblique registration, described above. We suspect that optical diffraction may have picked up some of these differences. (Looking diagonally down a row of obliquely registered cross-bridges would give the impression that they were closer together, due to the foreshortening introduced via the cosine rule.) Moreover, in the years since McIntosh's study, no other evidence for microtubule contraction in any other system has come to light. Thus it is hard to imagine that it plays a role in axostyle movement.

On the other hand, we can envisage a way in which concerted changes in the conformation of cross-bridges, even if they didn't cycle, could generate major bends. If we assume that conformational changes in the cross-bridges create shearing forces between adjacent microtubules and that these changes tend to occur uniformly across the breadth of a lamina and for some limited distance up and down it, then the involved area would attempt to shear until it looked like a parallelogram (Fig. 23 *a*). If we further assume that regions above and below the involved area resist actual displacement of microtubules, then it seems likely that the shearing forces would be relieved by altering the helical twist of the involved area. Simple planar bending would not occur, because unlike the situation in cilia and flagella where sliding of microtubules is permitted at the top of the axoneme (Fig. 23 *b*), here we imagine that sliding would be resisted at both ends by the intra-laminar cross-bridges that were not involved in the change. Instead of bending, the involved area would change its helical pitch (Fig. 23 *c*). At least this is the only way to insert a long parallelogram between two flat, horizontal surfaces; it has to be twisted into a helix in order for its ends to become horizontal and parallel to each other. Another instance in which such local shear forces are converted into helical shape changes in a whole organelle—the actin bundle of limulus sperm—has been analyzed in detail by De Rosier, Tilney, and colleagues (5–7). Their papers may throw further light on the process envisioned here, especially Fig. 10 in reference 6 which portrays most explicitly their prediction of analogous changes in cross-bridges between actin filaments.

Another aspect of the observed helical shape of axostyles is that differences in cross-bridge inclination ought to exist amongst adjacent laminae at all times (see Fig. 25). This notion comes directly from a theoretical consideration of helices: the different radii of curvature of the laminae on the inside and outside of a helix dictates that they must have different overall shapes (e.g., parallelograms with more or less inclination of their ends). One simple way to envision this is to grasp the upper right corner of this journal and bend it down toward the lower left corner. This creates a laminated helix analogous to an axostyle, in which each page can be likened to a lamina of microtubules. Doing this creates a substantial diagonal displacement of the corners of the uppermost pages vs. the last pages (Fig. 24); but in the axostyle we are assuming for the moment that all such "end displacements" are disallowed by terminal anchorages. This journal can only be bent into a helix, while keeping its ends in register, if the shapes of the pages are changed simultaneously. For example, by converting the uppermost pages into

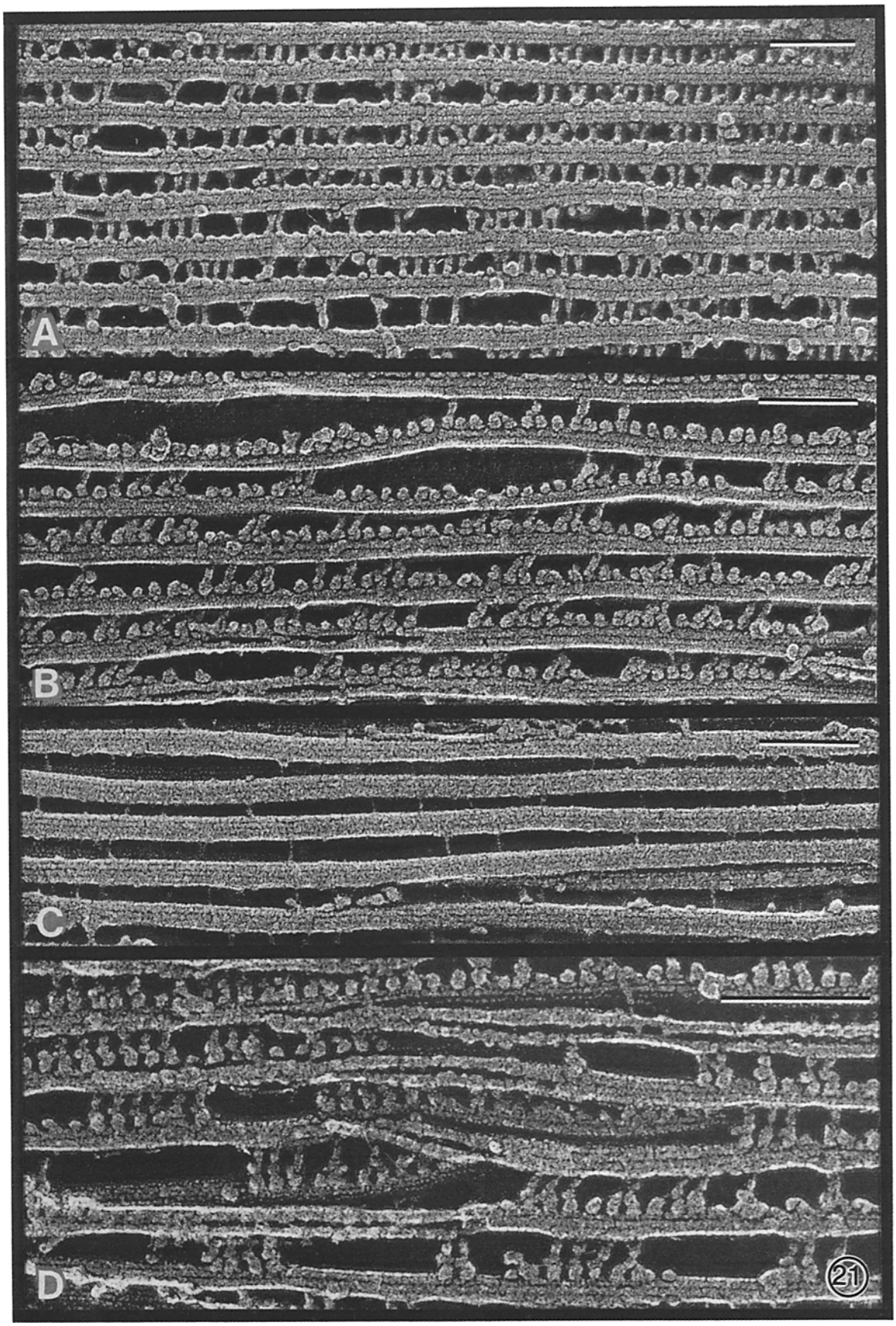


Figure 21. Partial extractions of intra-laminar cross-bridges that illustrate aspects of their subunit composition. *A–C* are from different *Saccinobaculus* axostyles and *D* is from a *Pyrsonympha* axostyle. Extraction was provoked in *A* and *B* by hypotonic shock (5 mM Pipes, pH 7.0, for 5 min at 4°C); in *C* by high salt extraction (0.3 M KCl for 5 min at 4°C); and in *D* by inadvertent exposure to high salt (the organism

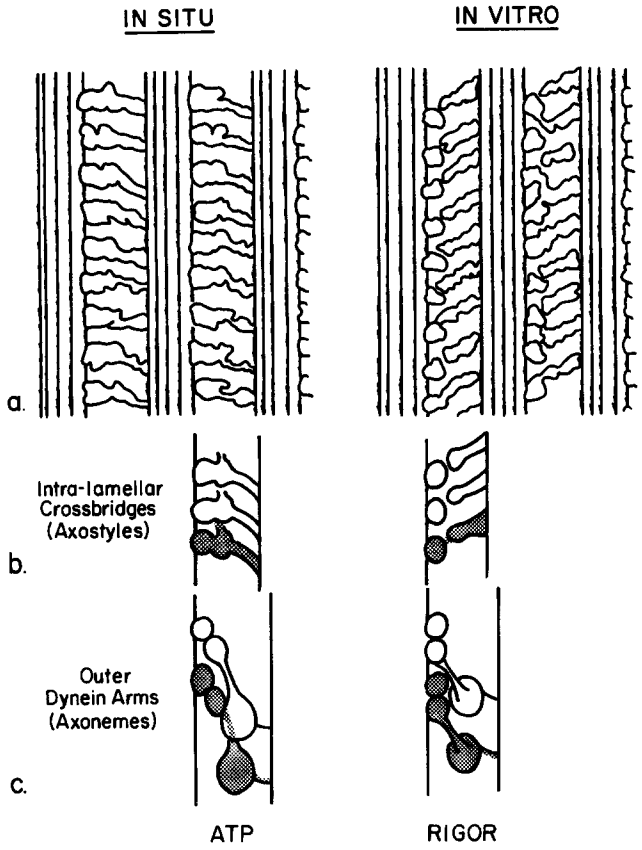


Figure 22. (*a*) Camera lucida tracings of intra-laminar cross-bridges in the two predominant states found in this report, from Fig. 9 (a *Pyrsonympha* axostyle in ATP) and from Fig. 13 (a *Pyrsonympha* axostyle in rigor). The vertical lines represent cracks between the protofilaments of the adjacent microtubules. (*b*) Three "idealized" cross-bridges drawn to illustrate our interpretation of this configurational change. The stippling represents one cross-bridge and its linker, for comparison with the overlapping configuration of dynein outer arms, diagrammed at the same size scale in *c*, as they look in ATP and in rigor (cf. references 11–14).

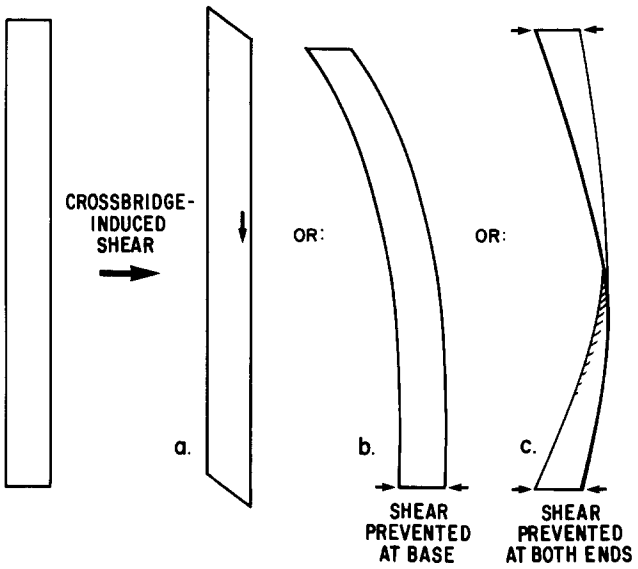


Figure 23. Illustration of the form and extent of overall lamellar shape changes expected to result from a two-step (i.e., 32-nm) "quantal" dislocation of microtubules. When summed across the ~100 microtubules in a typical 5-μm-wide axostyle, this would result in a ~3-μm downward displacement of its right edge. If unconstrained, this would create the change to a parallelogram shown in *a*. If constrained at the base (as in cilia and flagella), this would create the curvature shown in *b*. We propose in the text that if the axostyle were constrained at both ends, it would undergo neither of the above shape changes, but instead would assume a helical twist something like that shown in *c*.

parallelograms whose upper right corners are cut away, while leaving the last pages as rectangles, registration would be maintained. Of course, this could only be done to the journal with scissors; we wish to stress that it could be done to axostyle laminae by changing the inclination of their cross-bridges (Fig. 23).

To grasp how large these lamellar shape changes might be, and how much axostyle curvature they might provoke, imagine for example that in one area, inter-laminar cross-bridges changed from two-step upward registration (~33°) to two-step downward registration, a net displacement of 4 × 16 nm = 64 nm between each adjacent microtubule or 64 nm × 100 microtubules across the breadth of a whole lamina, a ~6-μm displacement of one edge versus the other. This could occur in an area <10 μm long (since local bends in the axostyle are seen to occupy areas no longer than that), so the above displacement would amount to a dramatic change in shape of the affected area, even without cross-bridge cycling. (For comparison, the page displacement generated by bending a corner of this journal all the way down is ~10% of its height, at its usual monthly thickness.)

One critical ingredient in this model is that axostyle laminae should not be able to slide passively relative to each other. Indeed, there is morphological evidence that laminae are anchored to each other at either end of the axostyle. The anterior end carries a special row of extensively cross-linked microtubules, orthogonal to the laminae and linked to each of their lateral edges (3, 15, 16, 28, 34). Geometrically, this "primary row" is in perfect position to bind laminae together and keep their ends aligned. At the posterior end, the laminae all wrap around each other to form concentric rings and end in a dense cytoplasmic "plug" (3, 14, 15, 28, 34). This would also appear to disallow any sliding displacement. In addition, laminae are cross-linked locally throughout the length of the axostyle by many inter-laminar links. These obviously hold the whole organelle together, but may also transmit shear forces generated by individual lamellar changes.

While discussing how lamellar shear could change axo-

ruptured and its axostyle was exposed to "artificial gut solution" described in Materials and Methods for 15 min at 4°C). *A* illustrates a random removal of whole bridges, with minimal change to the remaining ones. *B* illustrates nearly complete removal of the "stalks" but retention of one or both parts of their "heads". *C* illustrates complete removal of all bridges except a few that look like inter-laminar ones. *D* illustrates an interesting potpourri of remains, including half-heads, whole bipartite heads with no stalks, and some complete, quite normal looking bridges. Bars, 0.1 μm.

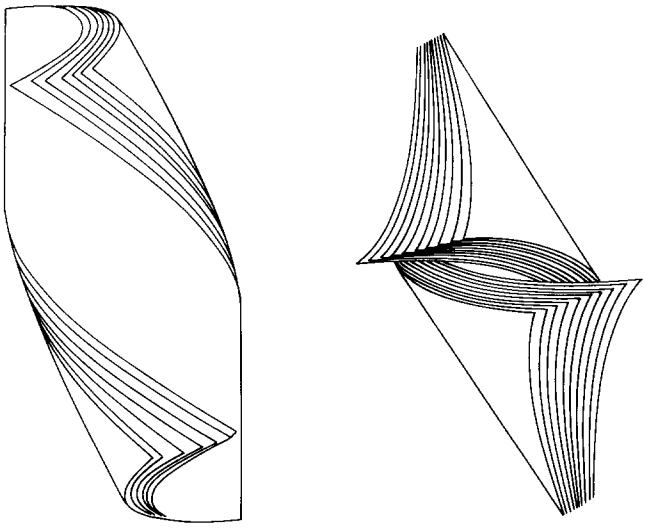


Figure 24. Illustration of the mis-registration that results from twisting a stack of rectangular laminae (e.g., journal pages) into a left-handed helix, first gently (*left*) and then more extremely (*right*). In the text, we propose that axostyle laminae become shaped like parallelograms in sharply curved regions, thereby preventing such mis-registration.

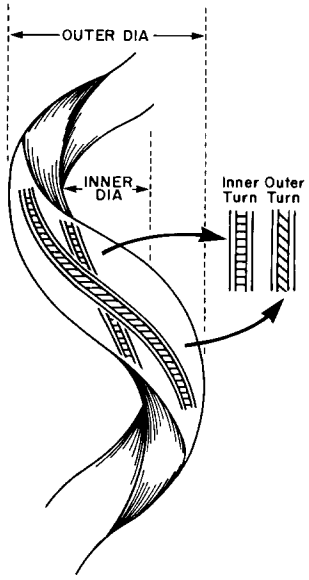


Figure 25. Illustration of the gradient of cross-bridge angles that ought to exist in an axostyle, to accommodate the differing degrees of helical twist of inner and outer laminae. In the text, we propose that active increases (or decreases) in the relative obliquity of cross-bridges in subjacent laminae could be the molecular driving force behind active changes in helical pitch. See references 5–7 for elaboration of this concept in another system.

style helices, we should also consider a concomitant structural change noted previously: increased longitudinal bending is accompanied by loss of the normal transverse curvature or "cupping" of the axostyle. McIntosh et al. (26) were the first to stress the significance of this change, likening it to the behavior of a carpenter's steel tape-measure, which is built with transverse curvature to make it stiff, but "pops" into a flat profile when flexed longitudinally. These authors considered the loss of transverse curvature in the axostyle to be a passive phenomenon that simply "permitted" the longitudinal bend to occur. We would offer, instead, that transverse curvature may also be controlled by active cross-bridge changes of the sort envisioned here. Until now, we have considered only the longitudinal vector of intra-laminar cross-bridge flip-flops, seen as a change in their inclination; but this change could also have a radial component, so that when cross-bridges tilted down to the right, they also elevated their rightward attachment points a bit toward the outer (convex) surface of the axostyle and thereby actively reduced its transverse curvature. Such a "down-and-out" cross-bridge movement would simultaneously create two (or more) vectors of force that could in principle be translated into complex shape changes in several different planes, depending on the different patterns of microtubule organization observed in different types of axostyles.

Clearly, experimental evidence for the view expressed here, that configurational changes in intra-laminar cross-bridges drive the overall shape changes of axostyles, awaits a method of dissociating axostyles into individual laminae and proving that a single one will undergo a shape change on its own when exposed to ATP. One indication that this experiment may be accomplished some day comes from the study of Langford and Inoué (25) who reported that axostyles in living cells occasionally split into several thinner ribbons that each continue to undulate on their own, albeit independently of each other.

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References

1. Amos, L. A., and A. Klug. 1974. Arrangement of subunits in flagellar microtubules. *J. Cell Sci.* 14:523–549.

2. Bloodgood, R. A. 1975. Biochemical analysis of axostyle motility. *Cytobios.* 14:101–120.

3. Bloodgood, R. A., K. R. Miller, T. P. Fitzharris, and J. R. McIntosh. 1974. The ultrastructure of *Pyrsonympha* and its associated microorganisms. *J. Morphol.* 143:77–106.

4. Bloodgood, R. A., and K. R. Miller. 1974. Freeze-fracture of microtubules and bridges in motile axostyles. *J. Cell Biol.* 62:660–671.

5. De Rosier, D. J., and L. G. Tilney. 1984. How to build a bend into an actin bundle. *J. Mol. Biol.* 175:57–73.

6. De Rosier, D. J., L. G. Tilney, E. M. Bonder, and P. Frankl. 1982. A change in twist of actin provides the force for the extension of the acrosomal process in limulus sperm: the false discharge reaction. *J. Cell Biol.* 93:324–337.

7. De Rosier, D. J., L. G. Tilney, and P. Flicker. 1980. A change in the twist of the actin filaments occurs during the extension of the acrosomal process in limulus sperm. *J. Mol. Biol.* 137:375–389.

8. Fitzharris, T. P., R. A. Bloodgood, and J. R. McIntosh. 1972. The effect of fixation on the wave propagation of the protozoan axostyle. *Tissue & Cell.* 4:219–225.

9. Gibbons, I. R. 1966. Studies on the ATPase activity of ^{14}S and ^{30}S dynein from cilia of Tetrahymena. *J. Biol. Chem.* 214:5590–5596.

10. Gibbons, I. R., and E. Fronk. 1979. A latent ATPase form of dynein 1 from sea urchin sperm flagella. *J. Biol. Chem.* 254:187–196.

11. Goodenough, U. W., and J. E. Heuser. 1972. Substructure of the outer dynein arm. *J. Cell Biol.* 95:798–815.

12. Goodenough, U. W., and J. E. Heuser. 1984. Structural comparison of purified proteins with *in situ* dynein arms. *J. Mol. Biol.* 180:1083–1118.

13. Goodenough, U. W., and J. E. Heuser. 1985. Substructure of inner dynein arms, radial spokes, and the central pair/projection complex. *J. Cell Biol.* 100:2008–2018.

14. Goodenough, U. W., and J. E. Heuser. 1985. Outer and inner dynein arms of cilia and flagella. *Cell.* 41:341–342.

15. Grasse, P. P. 1956. L'ultrastructure de Pyrsonympha vertens: les flagelles et leur coaptation avec le corps, l'axostyle contractile, le paraxostyle, le cytoplasme. *Arch. Biol.* 67:595–611.

16. Guy-Brugerolle, M. 1970. Sur l'ultrastructure et la position systematique de Pyrsonympha vertens (Zooflagellata Pyrsonymphina). *C.R. Acad. Sci. Paris D.* 270:966–969.

17. Grimstone, A. V., and L. R. Cleveland. 1965. The fine structure and function of the contractile axostyles of certain flagellates. *J. Cell Biol.* 24:387–400.

18. Heuser, J. E. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* 84:560–583.

19. Heuser, J. E. 1983. Procedure for freeze-drying molecules adsorbed to mica flakes. *J. Mol. Biol.* 169:155–195.

20. Heuser, J. E., and R. Cooke. 1983. Actin–myosin interactions visualized by the quick-freeze, deep-etch replica technique. *J. Mol. Biol.* 169:97–122.

21. Heuser, J. E., and M. W. Kirschner. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* 86: 212–234.

22. Hirokawa, N. 1986. Microtubule-associated proteins in crayfish peripheral nerve axons. *J. Cell Biol.* 103:33–39.

23. Ip, W., and J. E. Heuser. 1984. Structural correlates of pigment granule translocation observed in quick-frozen erythrophores. *J. Ultrastruct. Res.* 86: 162–175.

24. Langford, G. M. 1975. The plane of bending in the axostyle of *Pyrsonympha vertens*. *Biosystems.* 7:370–371.

25. Langford, G. M., and S. Inoué. 1979. Motility of the microtubular axostyle in Pyrsonympha. *J. Cell Biol.* 80:521–538.

26. McIntosh, J. R. 1973. The axostyle of *Saccinobaculus*. II. Motion of the microtubule bundle and a structural comparison of straight and bent axostyles. *J. Cell Biol.* 56:324–339.

27. McIntosh, J. R. 1974. Bridges between microtubules. *J. Cell Biol.* 61: 166–187.

28. McIntosh, J. R., E. S. Ogata, and S. C. Landis. 1973. The axostyle of *Saccinobaculus*. I. Structure of the organism and its microtubule bundle. *J. Cell Biol.* 56:304–323.

29. Mooseker, M. S., and L. G. Tilney. 1973. Isolation and reactivation of the axostyle. Evidence for a dynein-like ATPase in the axostyle. *J. Cell Biol.* 56:13–26.

30. Nalepa, C. A. 1984. Colony composition, protozoan transfer, and some life history characteristics of the woodroach *Cryptocercus punctulatus* scudder (Dictyoptera: Cryptocercidae). *Behav. Ecol. Sociobiol.* 18:31–40.

31. Ritter, H., Jr., S. Inoué, and D. Kubai. 1978. Mitosis in *Barbulanympha*. I. Spindle structure, formation and kinetochore engagement. *J. Cell Biol.* 77: 638–654.

32. Ritter, H., Jr. 1974. A fluid system for the cultivation, light microscope examination and manipulation of obligate anaerobes. *J. Protozool.* 21:565–568.

33. Satir, P. 1968. Studies on Cilia III. A "sliding filament" model of ciliary motility. *J. Cell Biol.* 39:77–94.

34. Smith, H. E., and H. J. Arnott. 1974. Axostyle structure in the termite protozoan *Pyrsonympha vertens*. *Tissue & Cell.* 6:193–207.

35. Souto-Padron, T., W. DeSouza, and J. E. Heuser. 1984. Quick-freeze, deep-etch rotary-replication of Trypanosoma cruzi and Herpetomonas megaseliae. *J. Cell Sci.* 69:167–178.

36. Summers, K. E., and I. R. Gibbons. 1973. Effects of trypsin digestion on flagellar structures and their relationship to motility. *J. Cell Biol.* 58:618–629.

37. Tilney, L. G., J. Bryan, D. J. Bush, K. Fujiwara, M. S. Mooseker, D. B. Murphy, and D. H. Snyder. 1973. Microtubules: evidence for 13 protofilaments. *J. Cell Biol.* 59:267–275.

38. Trager, W. 1974. The cultivation of a cellulose-digesting flagellate, trichomonas termopsidis and of certain other termite protozoa. *Biol. Bull.* 66: 182–190.

39. Tsukita, S., S. Tsukita, J. Usukura, and H. Ishikawa. 1983. ATP-dependent structural changes of the outer dynein arm in Tetrohymena cilia: a freeze-etch replica study. *J. Cell Biol.* 96:1480–1485.

40. Woodrum, D. T., and R. W. Linck. 1980. Structural basis of motility in the microtubular axostyle: implications for cytoplasmic microtubule structure and function. *J. Cell Biol.* 87:404–414.

41. Yamin, M. A., and S. L. Tamm. 1982. ATP reactivation of the rotary axostyle in termite flagellates: effects of dynein ATPase inhibitors. *J. Cell Biol.* 95:589–597.