SPLAYED *TETRAHYMENA* CILIA

A System for Analyzing Sliding and Axonemal Spoke Arrangements

WINFIELD S. SALE and PETER SATIR

From the Department of Physiology-Anatomy, University of California, Berkeley, California 94720

ABSTRACT

This study makes use of a procedure designed to illustrate, without serial section analysis, the three-dimensional changes in the ciliary axoneme produced by microtubule sliding, and to confirm essential features of the sliding microtubule hypothesis of ciliary movement. Cilia, isolated from *Tetrahymena pyriformis* by the dibucaine procedure, are attached to a polylysine substratum, and treated with Triton X-100. Critical point drying maintains three-dimensional structure without embedding. The detergent removes the membrane and many axonemes unroll, always in an organized fashion so that doublets follow one another in sequence, according to the enantiomorphic form of the cilium. The central pair of microtubules fall to the side as a unit. The parallel doublet microtubules retain relative longitudinal positions in part by interdoublet or nexin links. Spoke organization and tip patterns are preserved in the opened axonemes. We generalize the work of Warner and Satir (Warner, F. D., and P. Satir, 1976. *J. Cell Biol.* **63**:35-63) to show that spoke group arrangements are maintained for all doublets in straight regions, while systematic displacements occur in bent regions. The conclusion that local contraction of microtubles is absent in the axoneme is strengthened, and direct graphic demonstrations of sliding at the ciliary tip are shown. A morphogenetic numbering scheme is presented which results in a quantitative fit of the tip images to the images predicted by the equation for doublet sliding, and which makes possible new comparisons of structural parameters between axonemes and with cilia of other organisms.

The sliding microtubule model states that the basic motive force for ciliary motion is produced as the doublet microtubules of the ciliary axoneme, without changing length, move with respect to one another by means of their two rows of dynein arms, powered by hydrolysis of ATP. Sliding is resisted by additional components of the axoneme; in particular, the radially directed spokes, possibly with the aid of interdoublet nexin links, seem to be involved in converting sliding into local bending. Bending waves propagate along the axoneme to produce the typical forms of ciliary or flagellar motion. Although the sliding microtubule theory now rests on numerous pieces of evidence including studies of trypsin-treated axonemes (21, 22) and mutant analysis (1, 28), an understanding of changes in the organization of the ciliary axoneme throughout different stages of bending has been especially critical for the development of the model (17, 18, 27).

The quantitative aspects of these latter studies have been based on serial-section reconstruction of the tips of certain mussel gill cilia in various positions. This type of work is laborious and difficult to use generally, since it requires a reasonably complete understanding of the relation of fine structure to the direction of beat. However, with few exceptions, only such serial section analysis has provided detailed information on three-dimensional aspects of sliding. In the present study we introduce a new technique designed for the purpose of studying the three-dimensional organizational changes in the axoneme during sliding without the need for sectioning. We explore the usefulness of this technique, compare its methods of demonstrating sliding with the serial section analysis, and note certain limitations and difficulties.

In essence, the technique involves preparation and analysis of whole-mounted cilia that have been splayed apart to reveal the lattice organization of axonemal projections, and the form of the ciliary tip. We modify and extend earlier approaches of this sort. Particularly noteworthy is the original study of Manton and Clarke (13) on *Sphagnum* sperm, in which they delineated many features of structure by means of metal shadowing of whole-mounted, frayed axonemes. We now know that the "battlements" they described are in fact spoke groups, and the banding or helical wrapping between the central microtubules and the peripheral doublets which they based on the battlements is actually a consequence of spoke group arrangement, as discussed by Chasey (3) and as we will indicate further here. More recently, Chasey (4) used critical point dried material to describe a double helical arrangement of spoke groups around the *Chlamydomonas* axoneme.

One difficulty with previous interpretations of lattice arrangements, such as the form of the spoke group helix, has been that potential sliding of the axonemal microtubules has not always been taken into account. Warner (25) and Dentler (6) have especially been aware of this difficulty, and have produced elegant analyses of arrangements, based on interpretation of thin-sectioned or negatively stained material. This study confirms many of their findings regarding spoke arrangement, produces direct evidence to support some of their conclusions, and extends this approach to correlate ciliary tip pattern changes with changes in lattice arrangements. In this work, we use *Tetrahymena* cilia as a model. Using the dibucaine procedure of Thompson et al. (24), we can obtain a nearly pure fraction of cilia from this protozoan.

MATERIALS AND METHODS

Cell Growth and Cilia Isolation

Tetrahymena pyriformis (strain B-III) were grown axenically in the dark at 26°C in filtered 2% Proteose Peptone (Difco Laboratories, Detroit, Mich.) in either 500-ml Erlenmeyer or 2,500-ml low-form culture flasks. Cells are harvested in late log phase, then deciliated by the method of Thompson et al. (24): 200 ml of cells are pelleted at 200 *g* for 5 min, resuspended in 10 ml of fresh 2% Proteose Peptone; 0.5 ml of 25 mM dibucaine-HCl (CIBA Pharmaceutical Co., Summit, N. J.) is added, and the cells are gently mixed with a wide-mouth pipet. The cells are observed under the phase-contrast microscope until deciliation is nearly complete, usually within 1 or 2 min. The suspension is diluted to 30 ml with fresh 2% Proteose Peptone, the cells are pelleted at 1,500 *g* for 7 min; the supernate containing the cilia is removed, and the cilia are pelleted at 10,000 rpm for 8 min in a Sorvall RC-5, HB-4 rotor. The cilia are resuspended in 2–3 ml of 0.1 M Na cacodylate buffer, pH 7.3 (0–4°C) and sedimented once more at 10,000 rpm for 8 min. Cilia are resuspended finally in 1–2 ml of 0.1 M Na cacodylate pH 7.3 (0–4°C) and kept on ice for immediate use. The final protein concentration is approximately 0.5 mg/ml.

In some experiments isolated cilia were resuspended in a medium consisting of 0.15 M KCl, 0.5 mM mercaptoethanol, 0.5 mM EDTA, 4 mM $MgSO_4$, 2 mM Tris-Base pH 7.8 and 1% Triton X-100 (0–4°C) for 5 min. The extracted cilia were then sedimented at 10,000 rpm for 8 min, and resuspended in the same medium minus detergent at a protein concentration of approximately 0.5 mg/ml. This preparation was then stored until used within the hour.

Preparation for Electron Microscopy

One drop of 0.1% polylysine-1-HCl (80–100 kd) is added to a Formvar (0.25%)-coated, carbon-stabilized grid (14) for transmission electron microscopy (TEM) or clean glass slide surface for scanning electron microscopy (SEM). The treated surface is thoroughly washed with several drops of deionized water, followed by a few drops of the appropriate buffer. One drop of the suspension of cilia is then applied and allowed to settle for 15–20 s. It is important to note that the surface is never allowed to dry. After attachment of the cilia, the preparation is rinsed with two–three drops of buffer and fixed either immediately in 2% glutaraldehyde in 0.05 M Na cacodylate, pH 7.3 or after a drop of 1% Triton X-100 is added for about 30 s. The preparations are fixed in the cold for 45 min, rinsed once in 0.1 M Na cacodylate, postfixed (usually) in 1% OsO_4 in the same buffer,

rinsed in buffer followed by 50% ethanol, and stained in saturated uranyl acetate in 50% ethanol for 20 min. The preparations are then dehydrated in a graded series of ethanol and gradually infiltrated with Freon-TC by Freon-ethanol mixtures (1-3, 1-1, and 3-1, respectively) until the grids can be immersed in 100% Freon-TC. The grids are critical point dried in Freon 13 after the method of Cohen et al. (5). After drying, specimens are stored at room temperature in a dessicator. TEM images were taken with either the Siemens 1A or 101 at 80 kV. Preliminary observations were made on one preparation under a Jeolco HVEM at 300 kV; stereo images with a tilt of 16° were taken of these specimens. (We thank Dr. K. R. Porter and associates at the University of Colorado for the use of the HVEM facilities.) After platinum coating, the specimens for SEM were viewed with a Coates and Welter CWICSCAN 50.

For thin-section TEM, pellets of cilia are fixed in 2% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.3) for 1 h, washed two times in the same buffer, postfixed in 1% OsO_4 (Na cacodylate buffer, pH 7.3), dehydrated in a graded series of ethanol, and embedded in Epon 812. Sections were cut on a Reichert ultramicrotome, mounted on Formvar-coated, carbon-stabilized grids, and stained with uranyl acetate and lead citrate. Protein estimation was made by the method of Lowry et al. (12) with bovine serum albumin as a standard.

RESULTS

General Axonemal Structure After Isolation

The dibucaine method of cilia isolation from *Tetrahymena* into Tris or cacodylate buffers results in membrane-covered axonemes shaped like tadpoles, with tapered tails and rounded, blunt bodies. Fig. 1 is a negatively stained "tadpole." After dibucaine treatment, the point of scission in *Tetrahymena* has been shown to be a localized region just above the ciliary necklace (16). Above the point of scission, the membrane of the cilium closes around the basal end of the axoneme. The tadpole body is a result of the basal (proximal) end of the sheared cilium rolling back on itself within the ciliary membrane, as indicated by reversal of enantimorphic form (arm direction) in adjacent axonemal sections within single membrane profiles. This affects the proximal two-fifths of the axoneme. Often, the basal bends are very sharp and the axoneme is obviously broken. It is difficult to quantitate the total amount of bending of individual cilia produced by this coiling and breaking, but it is clear that bends of over 90° can be found. Coiling once begun occurs consistently in the same direction for one axoneme, because elaborate S-shaped curves are not seen. The remainder of the axoneme, distal to the coiled region, is usually straight, as in Fig. 1, but occasionally further bending with a radius of curvature much larger than that seen at the base is observed.

Ciliary tips are indicated by axonemal cross-sections with single peripheral microtubules after the criteria of Satir (17). The tip apparently simplifies in the manner usual for invertebrate cilia (i.e. some singlets persist longer than others, which gives domino-like appearances to tip cross-sections; see Fig. 2*a*). The usual linkers between axonemal doublets are present except at the tips: these include dynein arms, spokes, and nexin links. There is no bridge between doublets no. 5 and 6 in *Tetrahymena* cilia, but the axis of the cilium can be identified readily.

Spoke Organization in Whole-Mounted Axonemes

SEM is used to verify the maintenance of three-dimensional form in the critical point-dried specimens. The isolated cilia retain their membranes through subsequent handling (i.e. attachment to the grid, staining, and critical point procedures) unless detergent is employed. Both with and without membranes, axonemal staining is quite variable, but in favorable images internal structure including spoke group arrangements can be seen either with HVEM or usually at 80 kV (Figs. 6 and 7). After Triton treatment under our conditions, the majority of cilia fail to unroll except in local regions of the grid, and are seen as intact whole mounts. We have found no essential differences in axonemal arrangement between nondetergent- and detergent-treated intact whole mounts. It is generally more difficult to observe details at 80 kV with the membrane present.

The spokes are found in triplet groups (Fig. 8, inset) with an overall repeat of about 900 Å. The distances from S_1, the most basal spoke: S_1-S_2: 280 Å; S_2-S_3: 210 Å; S_3-S_1: 400 Å, agree closely with those of Chasey (3), Dentler (6) and Warner and Satir (27). In straight regions, the spokes, when clearly seen, lie perpendicular to their doublet of origin and to the central sheath in agreement with the parallel and tilted spoke 3 arrangements demonstrated by Warner and Satir (27). Often, the details of the arrangement of the subgroups are masked in these preparations by dense staining.

The overall impression in single images of straight regions of the whole mounted intact axo-

neme is that the spoke groups are arranged in three dimensions in a regular helix. This is confirmed by stereo-images such as Fig. 6, which shows the spoke groups arranged along a single helix with a pitch of 900 Å. Other images show what appear to be double helical arrangements. In a double helix, spoke groups on opposite sides of the axoneme will be positioned in direct opposition, and this is sometimes clearly observed (Fig. 7). Although we presumably see spoke groups from all nine doublets in such preparations, we are unable to ascertain that each doublet is in its proper helical lattice position for either a true single or double helix; indeed, we predict that this is not the case, and, to emphasize this point, we call these arrays quasi-helical. As we discuss below, in fixed axonemes the impression of a regular repeat of spoke groups in straight regions (i.e. quasi-helical arrangement) is a consequence of the geometry of sliding in that doublet relationships do not change along a straight region.

One single preparation contains straight regions of axonemes with either quasi-single or quasi-double helices, but only one type of helix is found along any one straight region. In images where adjacent bent and straight regions can be seen along the same axoneme, as the quasi-helix of the straight region enters the bent zone, systematic distortion occurs such that the side of the helix nearest the center of curvature of the bent region is elongated (i.e., displaced with respect to the opposite side). Some tilted spokes have been observed. In general, this picture agrees with the three-dimensional expectations of quantal bending developed by Warner and Satir (27) and Satir (19).

The Explanation of Organized vs. Unorganized Splaying

Splayed cilia are found after detergent treatment as either unorganized, randomly frayed axonemes (Fig. 8) or in organized, parallel doublets whose interior surface opens away from the grid (Figs. 3–5). Randomly frayed axonemes are broken before attachment to the polylysine surface. Where we apply Triton X-100 before attachment, essentially all opened axonemes on the grid appear to splay in an unorganized manner (Fig. 8) in that doublets do not generally run parallel to one another, so that their lattice positions and enantiomorphic arrangements are not maintained. The critical point method preserves depth in the doublets and their attached spokes (Fig. 3). This is also evident in Fig. 8 where some doublets overlie others and where, as a doublet twists, its spokes are seen en face (arrows). In some cases, twisting turns the doublets so that, in the same axoneme, spokes point to the right of one doublet and to the left of an adjacent doublet (illustrated in Fig. 2 of Mazia et al., reference 14). The triplet spokes of individual spoke groups are often seen to good advantage in such preparations. Spokes have a "T" or "Y" configuration with the spoke head somewhat enlarged.

In contrast to the above, the unique and somewhat surprising result of removing the membrane after attachment of the cilia to the polylysine surface is that the splayed axonemes, when seen, are

FIGURE 1 Tadpole-shaped isolated cilium, stained in 1% uranyl acetate. × 26,500.

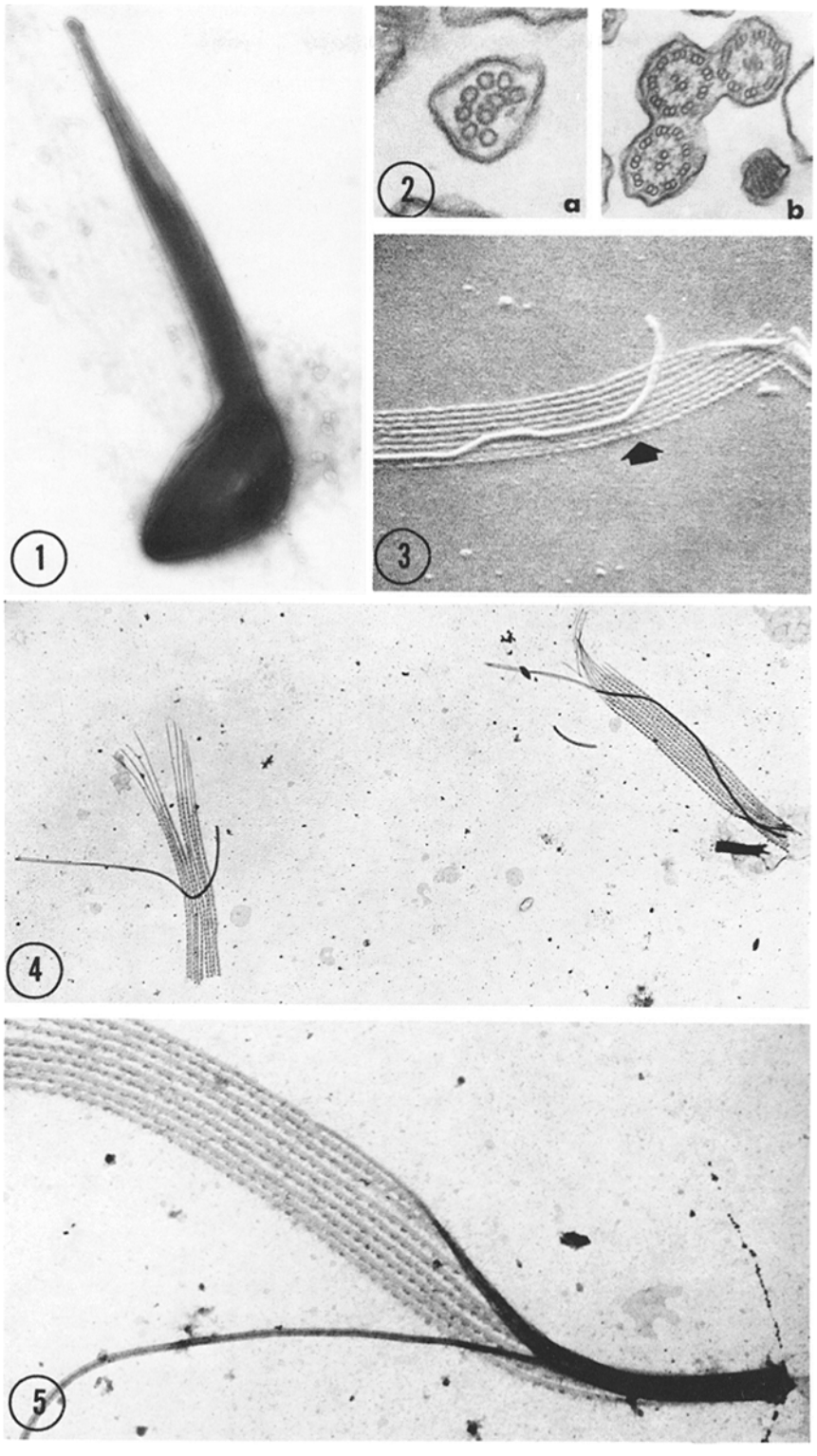
FIGURE 2 Cross-sections of isolated cilia. (*a*) Near the tip. × 93,000. (*b*) Near the base of a tadpole. Note the expected reversal of enantiomorphic form between adjacent sections of the axoneme within the membrane. × 45,000.

FIGURES 3–5 Organized unrolling of ciliary axonemes.

FIGURE 3 SEM. Note that the critical point method preserves three-dimensional form of the individual doublets. The central pair lies above the opened peripheral doublet sheet. The beading (arrowhead) reflects spoke group periodicity. × 16,000.

FIGURE 4 Whole mounted, critical point-dried cilia. In the organized splays, the spokes are always on the left when the axoneme is viewed from base to tip. × 8,000.

FIGURE 5 Images of intermediate stages of axonemal unrolling allow reconstruction of the organized splaying of the axoneme on the polylysine-coated surface. The central pair usually falls off to the side as a unit. × 21,000.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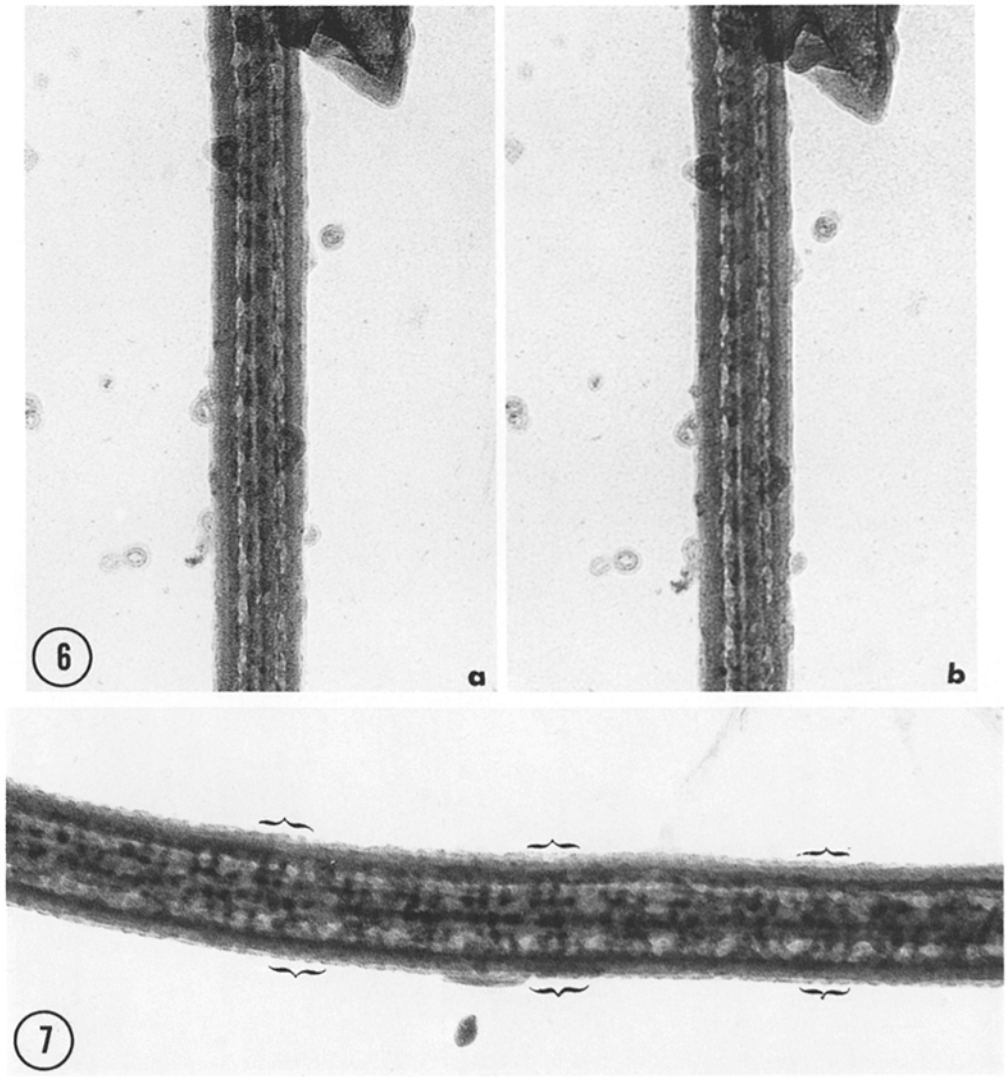


FIGURE 6*a* and *b* Stereo-pair of high voltage electron micrographs of an intact region of a critical point-dried axoneme. The micrographs were taken with a ±8° tilt between them. Note that the spoke groups generally follow a single helical arrangement. The relationship of the spoke groups on one side of the axoneme to the other side is constant throughout this straight region. × 86,000.

FIGURE 7 Region in which the axoneme is intact: the right hand side is the more basal direction as determined by spoke spacing. Corresponding spoke groups on opposite sides of the axoneme (below the bend) are marked by brackets. Note alignment suggestive of a double helix (see text). As the bend on the left begins, the spoke groups on the centripetal side of the bend move tip-ward relative to the corresponding groups on the outside of the bend. This is in contrast to the maintenance of the relative positions of the spoke groups along the straight region to the right. × 110,000.

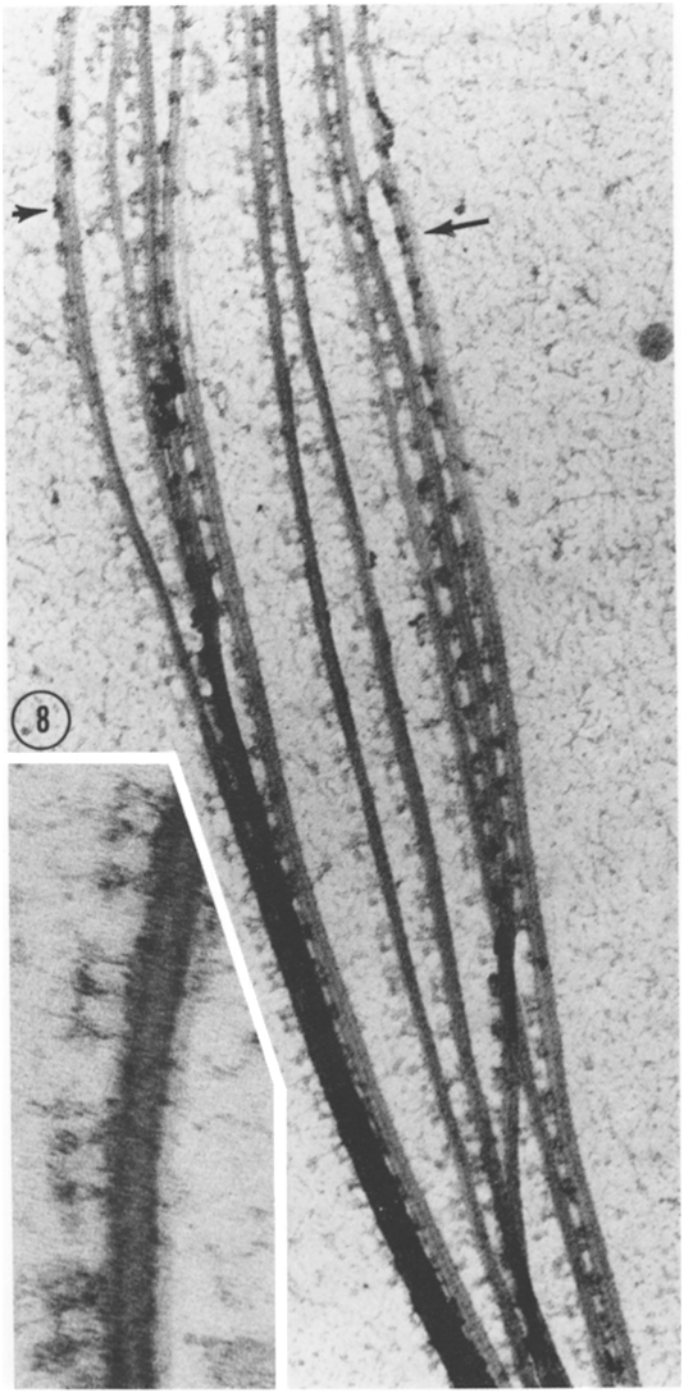


FIGURE 8 An axoneme disrupted before attachment to the polylysine-coated grid surface where enantiomorphic arrangement is not completely maintained. Individual spokes are seen standing normal to the doublet in regions where the A and B subfibers are superimposed (inset). Note the expanded spoke heads. As the doublet twists to an *en face* configuration (arrows), an "end on" view is seen. This is the usual image of the spoke groups in the organized splays. × 84,000; *inset* × 240,000.

always well organized with parallel-running doublets (Figs. 3–5). Many features of the axoneme are evident: subfibers A and B are readily distinguishable since the spoke groups lie along the A subfiber, and the midwall of the doublet can be resolved. The spoke groups appear as darkly stained nodes (Fig. 5) which have a period of ca. 900 Å. These groups resemble the more en face views of the spoke groups of Fig. 8, which is the image of the spoke group standing on a doublet that is lying flat against the grid. In some places, individual spokes or spoke heads can be resolved. When the axoneme is viewed from base to tip, the A subfiber is *always* to the left of the B subfiber (a total of 100 cases scored in which all nine doublets were distinguishable). This is the expected result if one were to attach a cilium firmly to a surface, remove its membrane, break open the axoneme at some point and allow it to unroll (as a carpet) so that the nine doublet microtubules lie in sequence while the central pair, as a unit, falls off to one side (Fig. 4). An intermediate stage of unrolling is shown in Fig. 5.

Careful examination reveals, in addition to the spoke groups and with the same periodicity, the connections between adjacent doublets characterized as interdoublet or nexin links (Figs. 9 and 10) (20, 26). (In using the term nexin, we do not imply that we have located the protein, but use the term for convenience in analogy with more completely characterized axonemes.) The nexin links are not readily seen in unorganized axonemes, and they apparently help to maintain the regular organization of doublets during unrolling. The nexin links are flexible and extensible, to a degree, in that even though the distance between successive doublets varies somewhat as one progresses along the axoneme, the links continue to span this distance. In general, they lie normal to the direction in which the doublet lies and they are not usually greatly tilted. Tilts predicted on the assumption that the links remain attached to adjacent doublets when the doublets slide relative to one another are occasionally observed (see Discussion).

The Organization of Splayed Cilia in Relation to Sliding Microtubules

Numbering the Doublets

If the axoneme is splayed in organized fashion, the doublets follow one another in strict sequence from one edge to the other in accord with the enantiomorphic form of the cilium. It is obvious that if we could identify one doublet by number in preparations such as Figs. 4 or 5, we could identify all doublets, and this would allow us to compare tip patterns or lattice position of projections such as spokes between splayed axonemes. Unfortunately, simple markers such as the bridge between doublets no. 5 and 6 are not seen in *Tetrahymena* cilia, and the orientation of the central pair is not readily ascertainable in the splayed image.

Satir (18) noted that, regardless of stroke position, there was a characteristic length for subfiber A of each doublet in mussel gill cilia. Since subfiber B's of these cilia behave as if they were all of equal length, the differences in subfiber A lengths show up in the length of the extension of that subfiber past the end of subfiber B (cf. Fig. 9 of Satir, reference 18). In gill cilia, this extension is shortest for doublet no. 5 and nearly 1 μm longer for doublets no. 3 and 8.

In our present preparations, tips are readily identifiable (Figs. 4, 9, and 10). Moreover, the point at which subfiber B of each doublet terminates is apparent, because the doublet is reduced in diameter at that point, the midwall disappears, and because this is also the approximate point at which spoke groups terminate. The B subfiber terminates quite abruptly rather than tapering gradually. The tip is also characterized by the central pair, the longest single microtubules of the axoneme which are capped together in this region. In *Tetrahymena,* the A subfiber is generally nearly 2 μm longer than the B subfiber; its exact termination is more difficult to ascertain, first because there is often a gradual taper at the end of the subfiber and second, because of possible artifact (i.e. breakage of tips due to lack of attachment to the surface). We assume that a gradual taper or a smooth rounding of the tip is a sign that we are approaching the true end of the A subfiber. Tips which are obviously broken are not used for numbering.

Measurement of subfiber A extension of doublets of individual tips used for numbering shows that one such extension is always shortest. In Fig. 13, we have plotted the results for three such individual tips. Clearly, there is considerable variability in our measurements, as might be expected, since in log phase cells new cilia are continuously being assembled; also, oral and somatic cilia are indistinguishable in our preparations. If we assume that in every case the short extension corresponds to doublet no. 5, then our results indicate that, on average, the other subfiber A

extensions in *Tetrahymena* are all approximately of equal length (1.88 μm), while the extension of doublet no. 5 is, on average, about 0.3 μm shorter. Doublet no. 5 is found at all positions of the unrolled axoneme (i.e., at the extreme left in Fig. 9, near the right in Fig. 10, etc.), indicating, as we would expect if our numbering is correct, that the axoneme attaches to the grid at random before it unrolls.

Spoke Arrangement

Warner and Satir (27) used the spokes as invariant markers of microtubule length. By photographic translation techniques, they showed, for pairs of doublets, that the relationship of one doublet to the next was constant along straight regions of the axoneme both proximal and distal to bends. Our preparations reveal the relationships of spoke groups on all nine doublets simultaneously. One preparation is shown in Fig. 12 where photographic translation by two spoke group repeats does not confuse the image of the spokes on at least six doublets for more than 10 periods. This preparation involves fixation and critical point drying but no embedding; moreover, the axoneme is fixed only after attachment to the polylysine substratum. Therefore, we conclude that the possibility of relaxation at fixation is minimized, and that, in support of Warner and Satir, none of the doublets of the splayed axoneme contracts locally.

Note that in Fig. 12 the spoke groups of one doublet are not always in exact horizontal alignment with those of the adjacent doublet; the longitudinal displacements seen remain constant from one spoke group period to the next. Between three or four adjacent doublets longitudinal displacements seem systematic, although this is clearly not true for all doublets. Systematic displacement would be expected for all doublets if the spokes lie along a regular helix, regardless of whether this is a single helix or a double helix. In that case, these preparations would reveal the unrolled helix in which the spokes would occupy regular lattice positions. Some longitudinal displacements fit these lattice positions. Since these are repeated from period to period, we might expect the intact axoneme to have regions of helix-like structure, as demonstrated in Figs. 6 and 7. Actually, the splayed images display the quasi-helical nature of the lattice most effectively. In Fig. 11 the axoneme to the left shows displacements between doublets that lie at a pitch resembling a quasi-single helix (broken lines), while the axoneme to the right resembles a quasi-double helix (refer to legend, Fig. 11).

Because of tadpole formation, we are unable to see the arrangement of spoke groups at the base of the cilium in our preparations. Our data are consistent with three different morphogenetic arrangements of spoke groups in an unbent cilium: an intrinsic single or double helix or complete alignment of spoke groups around the unbent axoneme. The quasi-helices seen both in whole amounts and in splayed axonemes near the ciliary tip arise primarily because of constant Δl relationships between doublets in straight regions, that is, as a consequence of sliding rather than of any specific morphogenetic arrangement.

Tip Patterns and Sliding Microtubules

Many different patterns of termination of subfiber B can be seen after organized unrolling of the axoneme. Particularly noteworthy are the comparisons shown in Figs. 9 and 10. In Fig. 9, the axonemes have been selected so that they have unrolled in the same manner according to our numbering scheme (i.e., no. 5 to the left). Furthermore, although there are some differences in subfiber A lengths for identical doublets in the two cilia, for doublets no. 5 and 9 these lengths in left vs. right axonemes are virtually identical. In the left axoneme, Δl 5 vs. 9 is approximately zero based on subfiber B terminations, while no. 9 is about 0.2 μm longer than no. 5 on the basis of subfiber A terminations. In the right axoneme, 9 has moved tipward by some four spoke group periods, as indicated both by the increased Δl at the subfiber B endings and by displacement at the tip. This is a direct graphic demonstration for *Tetrahymena* cilia of sliding at the ciliary tip, comparable to the reconstructions for mussel gill cilia.

When we plot Δl_n as defined by Satir (18), where Δl_n is the displacement of subfiber B of doublet no. n compared to doublet no. 1, for representative axonemes such as those of Figs. 9 and 10, we get the curves shown in Fig. 14. Note that, in Fig. 10*a*, Δl_n reverses sign when compared to Fig. 10*b*, which is a requirement of the sliding model where bends occur on opposite sides of the axoneme. The curves are virtual reproductions of the reconstructed values given for mussel gill cilia in Fig. 10 of Satir (18). Moreover, the amount of bend ($\Sigma\alpha$; Satir, reference 18) can be calculated for each doublet n by the equation:

$$\Delta l_n = d_n \Sigma\alpha$$

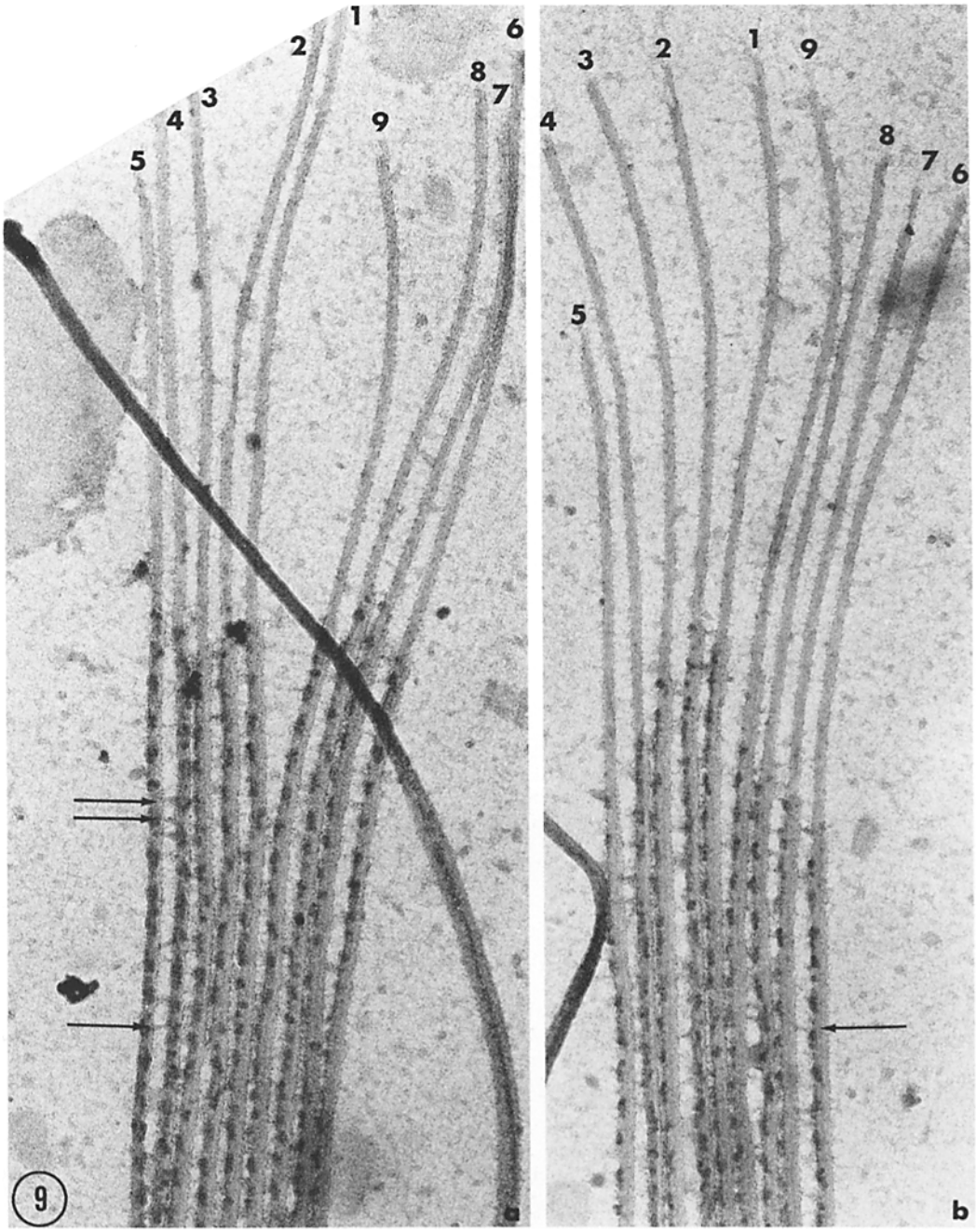


FIGURE 9-10 Representative ciliary tip patterns from organized, splayed axonemes. The axoneme attaches and opens at random in the process of splaying out. The nexin links (arrows) appear to maintain doublet alignment. × 48,000.

FIGURE 9 (*a*) The B-subfibers of all nine doublets end at approximately the same level (Δl = zero). Also, the spoke groups end approximately at the level of the B-subfiber termination. (*b*) The axoneme has opened in the same way as in Fig. 9*a*; however, doublet no. 9 now appears to be longer than doublet no. 5 by about four spoke groups or 0.36 μm. This amount of Δl_n would correspond to a bend of just over 100° (see text). Note that the A-subfiber of doublet no. 5 ends in a taper.

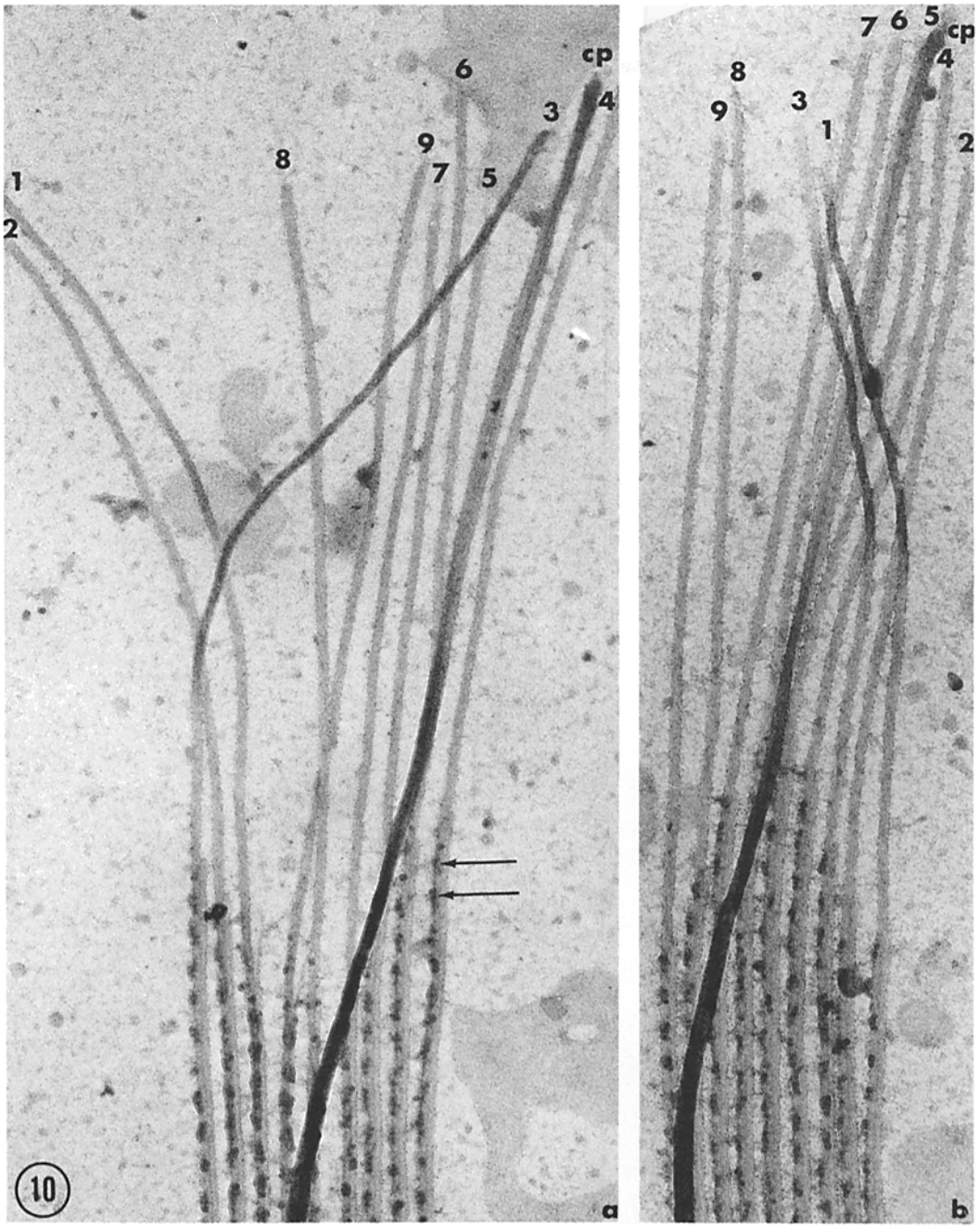
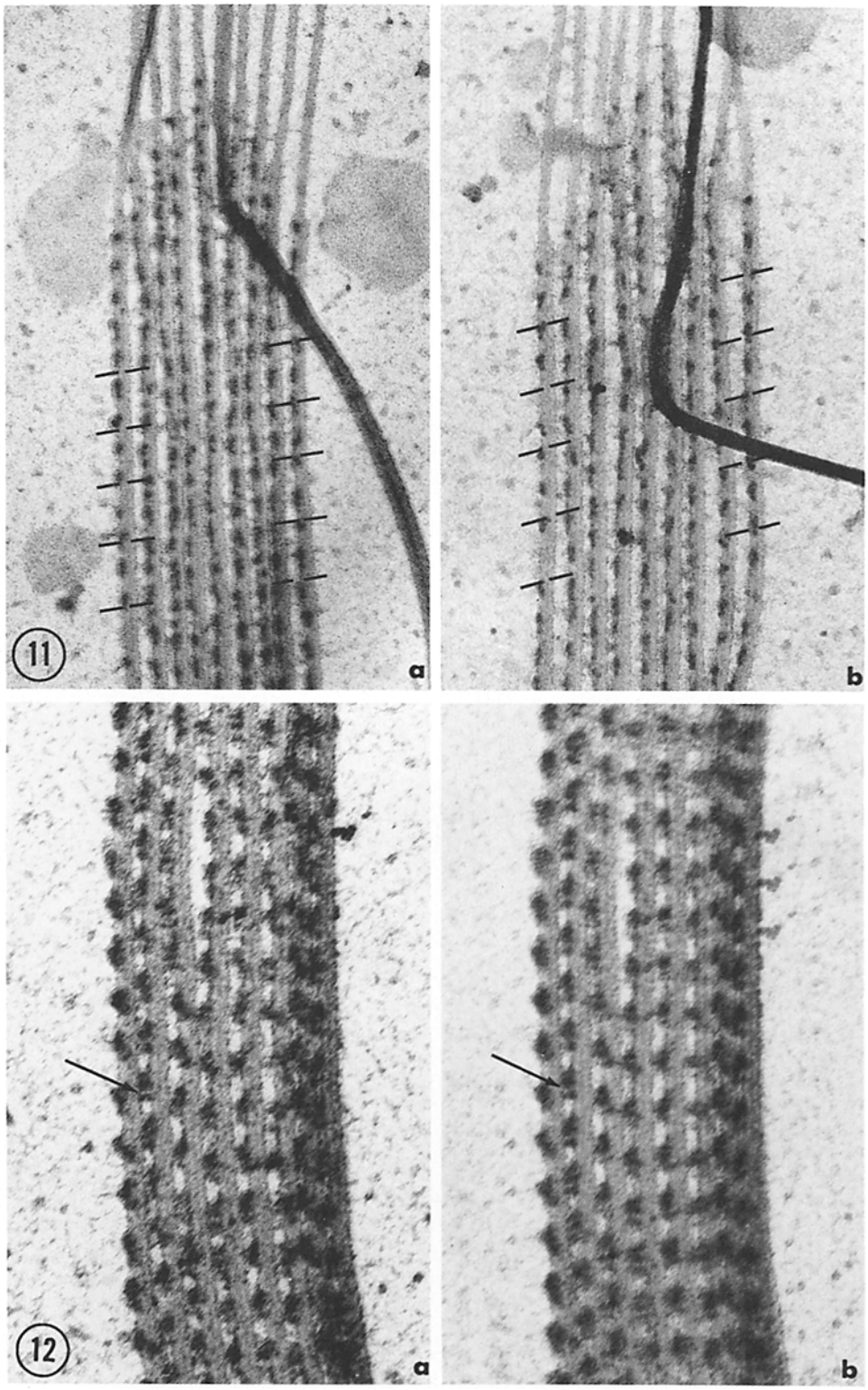


FIGURE 10 *a* AND *b* Doublet no. 5 is in the middle of these axonemes in contrast to those of Fig. 9. The B subfibers of *a* terminate in a manner which is the inverse of *b*, i.e. the sign of Δl is reversed. × 48,000.

where d_n is a constant characteristic of doublet n. For the tips we have examined, $\Sigma\alpha$ calculated for various doublets of a single axoneme within limits of error of measurement of Δl_n (± 0.1 μm) gives constant values that are within expectations of the amount of bend being predicted by the whole mounted images showing tadpole formation (refer to legend, Fig. 14).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DISCUSSION

Because serial sectional analysis of cilia is laborious and limited, we have developed a new technique involving organized unrolling of the ciliary axoneme with which to study the three-dimensional arrangement of the axoneme and changes in such arrangement due to sliding. The present study employs this technique to confirm in a direct graphic way for *Tetrahymena* many of the conclusions derived from serial section analysis of mussel gill cilia. Especially, the work of Warner and Satir (27), where the consequences of the sliding microtubule hypothesis were explored by examination of spoke arrangements between selected pairs of doublets, is generalized in this study simultaneously to all nine doublets of single ciliary axonemes. The conclusion that local contraction of microtubules is absent in the axoneme is strengthened, and both qualitative and quantitative aspects of the sliding model developed by Satir (18) are confirmed. Moreover, we are able to demonstrate that spoke arrangement in the axoneme near the ciliary tip is quasi-helical as a consequence of microtubule sliding rather than any intrinsic morphogenetic arrangement.

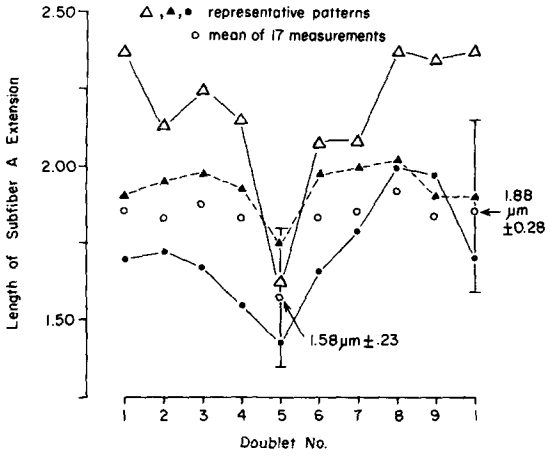

FIGURE 13 Assignment of doublet number. Lengths of the A subfiber extension beyond the B subfiber termination vary considerably for doublets of different ciliary tips. Three representative patterns are shown. However, on average, the subfiber A extensions of doublets no. 1–4 and 6–9 are of equal length (open circles). One subfiber A (assigned to doublet no. 5) is, on average, 0.3 μm shorter than the rest.

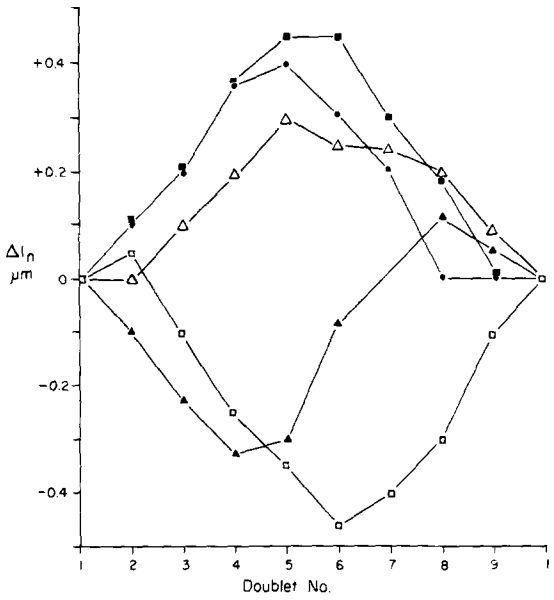

FIGURE 14 Typical curves plotting Δl_n vs. doublet number reconstructed from selected splayed tips, numbered as indicated in Fig. 13. These curves correspond quite closely to the curves predicted by the geometry of the sliding microtubule model, except that not all curves are symmetrical about doublets no. 5 and 6 (see Discussion). (The curve with the open triangles [△] represents a cilium with a bend of approximately 83°.) We have selected tips whose Δl_n values correspond to the correct magnitude for ciliary bends. Larger magnitudes are seen and are probably due to tadpole formation.

FIGURE 11 Examples of quasi-helical arrangements of spoke groups in unfolded axonemes. × 48,000 (*a*) Spoke group positioning in which the lattice approximates a single helical ("quasi-single helix") organization with a pitch of approximately 900 Å. (*b*) The spoke group arrangement in which the lattice approximates a double helical ("quasi-double helix") organization with a pitch of approximately 1,800 Å. Note that in both cases (*a* and *b*) certain doublets do not fall on the helical path: thus quasi-helical. Also the lines marked on each image appear to have a somewhat similar slant because of lateral spread of the doublets in *b* relative to *a*.

FIGURE 12*a* and *b* Spoke periodicity along each doublet is constant (900 Å). Photographic translation of the splayed cilium (Fig. 9*b*) by two spoke group periods does not blur but instead reinforces the image on all doublets. Individual spokes are reinforced (arrow). × 80,000.

The critical point method suitably maintains three-dimensional structure of the axoneme without embedding. The axoneme is stained positively before drying. Although the staining is somewhat variable so that not all images are equally useful, many axonemes show adequate contrast within the unrolled whole axoneme. For viewing the specimens at either 80 kV or 300 kV, the lack of embedding probably contributes to the usefulness of the images, especially at the lower voltage, and distortions which can be caused during resin infiltration and polymerization are avoided, although a different set of potential artifacts is introduced. For the study of microtubule sliding, the advantages of tacking down some doublets and viewing the remainder in spatial positions relative to the attached doublets are obvious.

When the axonemes are tacked down on polylysine according to the method of Mazia et al. (14) before treatment with Triton to remove their membranes, a significant proportion of them unroll to reveal the exact tip pattern and spoke lattice arrangement. Other workers (cf. reference 11) have noted strings or sheets of microtubules in their preparations after detergent treatment, whose appearances were used to indicate that nexin linked adjacent doublets around the axoneme even after dynein extraction. Triton apparently increases the "fragility" of the axoneme so that the probability of unrolling by interaction with the cationic polylysine surface is increased. It is well known (cf. reference 7) that, at lower concentrations, Triton does not affect the reactivation of the axoneme by ATP, and therefore Triton probably does not extract crucial axonemal components.

It is clear from this study that an attached axoneme always opens in an organized manner. The central complex falls away and the spoke heads stand free. Therefore, interactions between spoke heads and central sheath must be transitory in the unrolled axonemes, and central attachments must be absent or readily broken in the regions observed. It may be that we see mainly unrolled, straight portions of axonemes for this reason, since Warner and Satir (27) have postulated that spoke heads are functionally unattached to the central sheath in straight regions of the axoneme but are attached in bent regions.

The parallel microtubule sheets formed by the outer doublets upon unrolling retain their positions and integrity in part via nexin links. These preparations offer some of the best images of such links (Figs. 9 and 10) and may be useful in studying their detailed contribution to motility. The nexin links appear to be attached to the doublets and to be extensible as discussed by Warner (26). However, in general, if the links are attached permanently to doublets at either end, they are not tilted to the degree expected by the amount of sliding seen in the unrolled axonemes. This may be artifactual, since the tilts are sometimes observed, but it is also possible that nexin, like the spokes, is capable of detaching and reattaching to sequential sites as sliding progresses. If this is the case, the links, unlike the spokes, must retain an attached position in straight regions of the axoneme after several detachment-reattachment cycles.

Under conditions of organized unrolling, the enantiomorphic form of the cilium is preserved. Using the simple assumption, derived from the studies of Satir (18) on mussel gill cilia tips, that the distance from the termination of subfiber B to the termination of subfiber A of doublet no. 5 is always the shortest of such distances in the axoneme, we are able to number each doublet in sequence. We predict that (*a*) axonemes should be tacked to the polylysine at random and that (*b*) they will probably open at random. If the assumption is correct, doublet no. 5 should be found in different positions in different unrolled axonemal lattices. This has proven to be the case (Fig. 9 vs. Fig. 10).

Unlike mussel gill cilia tips, in *Tetrahymena*, as far as we are able to measure, except for doublet no. 5, all other subfiber A's within the axoneme are, on average, of equal length. Individual tips, however, have highly variable patterns (Fig. 13). One disadvantage of our method is that the subfiber A extensions are extremely fragile and can be lost or broken during unrolling or drying. We have attempted to use in our analysis only tips where subfiber A is tapered or rounded, since breaks should give abrupt ends to the singlets. Satir (18) has shown that, in *Elliptio*, subfiber A tapers, while subfiber B is capped, and we confirm this result for *Tetrahymena*. We explain the variability of length between corresponding doublets of different axonemes by differences in cilia type (somatic vs. oral) and morphogenetic stage in asynchronous growth of *Tetrahymena* cilia in our cultures. In addition, doublet length controls in *Tetrahymena* cilia may simply not be as stringent as we imagine.

Pitelka (15) has devised a numbering scheme for doublets of ciliate cilia based on the relationship of the doublets to structures of the cell cortex such as microtubule ribbons and kinetodesmal fibers associated with basal bodies. This system places doublets nos. 5–6 at the leading edge of the cilium during its effective stroke in normal forward swimming. In other cilia and sperm tails where careful correlation of stroke form and axonemal arrangement has been attempted, the direction of beat always lies along the axis, a plane passing between the central pair and also between doublets no. 5 and 6 (1, 8, 9, 23). Whether this is the case during ciliary reversal in protozoa is still uncertain; in ciliates, the effective stroke direction is not fixed and thin-section tip analysis has not yet been accomplished. We are unable at this time to correlate our method of numbering the axoneme directly with either the direction of beat or the axis of the ciliary cross-section. However, we can show that our numbering scheme leads to curves of tip displacement (Δl_n) vs. doublet no. as in Fig. 14, which are virtual reproductions of the curves found for mussel gill cilia, where doublets no. 5–6 are at the leading edge of the cilium during the effective stroke, and which are consistent with a simple sliding model embodying that assumption. As predicted, different axonemes are found with different tip patterns, and Δl_5 is both positive (effective pointing) and negative (recovery pointing). It would be interesting to compare curves constructed from splayed axonemes obtained after different amounts of ciliary reversal to see whether doublets no. 5–6 always remain at the leading edge during the effective stroke regardless of its spatial direction.

Cohen and Satir (in preparation) have shown that the skew of actual data in Fig. 10 of Satir (18) is probably the result of twisting of the axoneme during the beat. Gibbons (10) has observed twists in sea urchin sperm tail axonemes studied with HVEM. Although our data on this point are preliminary, a skew similar to that observed by Satir (18) is suggested by several of the curves of Fig. 14 and by similar results not plotted here. The *Tetrahymena* axoneme, like other axonemes, is undoubtedly capable of considerable twisting, at least during normal swimming since the beat is three-dimensional.

Skew in the data of Fig. 14 could also be produced if subfiber B's of the doublets in the axoneme were not of strictly morphogenetically equal lengths but ended systematically with displacements along a left-handed doublet helix. This is the most probable of the morphogenetic spoke arrangements according to Warner (25), Chasey (4), and Dentler (6). In that case, the number of spoke groups on one doublet should also be a constant. Our data do not support this conclusion, but it is possible that staining of the most distal spoke groups, which is highly variable, or their loss during preparation has influenced our interpretation.

In the unrolled axonemes, the tip displacements (Δl_n) seen are usually of a magnitude consistent with tadpole formation on deciliation, and probably do not directly reflect the magnitude of bending in the living cilium before dibucaine treatment. The sliding displacements observed are not solely the result of mechanochemical activity, but rather are also a function of the possibly passive (and in a sense artifactual) coiling which occurs at the base of the organelle. However, it is clear that sometimes the measured tip displacement fits well with prior geometrical predictions (see Fig. 14).

Since the tadpoles are generally broken at the base, this method so far lends itself best to studying arrangements along the length of the axoneme or at the tip. As noted above, it has previously been concluded that the spoke groups near the base of the axoneme in *Tetrahymena* and elsewhere fall along a left-handed double helix. We have been able to demonstrate that, whatever the morphogenetic arrangement of the spokes, this will be distorted as the doublets slide; because of the geometry of sliding, true helical lattice positions will not be preserved for all nine doublets, and at the distal side of the bend, the spoke group arrangement will be quasi-helical. In theory, for any axoneme, we should be able (as W. L. Dentler has attempted) to move the tip pattern back so that Δl_n is zero for every doublet and to reconstruct the morphogenetic helix. When we attempt to do so, we find that errors in our measurements of about half a spoke group period obscure the answer and we are unable to confirm the true morphogenetic arrangement of the spokes on the basis of our data. What we are able to demonstrate is that the quasi-helix seen in different axonemes changes in a manner consistent with sliding. Whatever the morphogenetic arrangement of the spokes, the helix observed is ephemeral. It would be interesting to know

whether the three-dimensional arrangement of the spokes (i.e., helically arranged) acts as a feedback system in control of bend formation. Taken all in all, although some limitations to interpretation must be considered, the present work provides strong confirmation of the static picture of doublet sliding during ciliary bending. The method we have developed should also be suited to a dynamic study of sliding using ciliary models, and to comparative studies of tip structure and internal arrangement of cilia from a variety of cells.

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REFERENCES

1. AFZELIUS, B. A. 1959. Electron microscopy of the sperm tail. Results obtained with a new fixative. *J. Biophys. Biochem. Cytol.* **5**:269–278.
2. ALLEN, C., and G. G. BORISY. 1974. Flagellar motility in *Chlamydomonas:* reactivation and sliding *in vitro*. *J. Cell Biol.* **63**(2, Part 2):5*a* (Abstr.)
3. CHASEY, D. 1972. Further observations on the ultrastructure of cilia from *Tetrahymena pyriformis*. *Exp. Cell Res.* **74**:471–479.
4. CHASEY, D. 1974. The three-dimensional arrangement of radial spokes in the flagella of *Chlamydomonas reinhardii*. *Exp. Cell Res.* **84**:374–380.
5. COHEN, A. L., D. P. MARLOW, and G. E. GARNER. 1968. A rapid critical point method using fluorocarbons ("Freons") as intermediate and transition fluids. *J. Microscopy.* **7**:331–342.
6. DENTLER, W. L. 1973. Molecular cytology of *Tetrahymena* cilia. Ph.D. Thesis. University of Minnesota, St. Paul, Minnesota.
7. GIBBONS, B. H., and I. R. GIBBONS. 1972. Flagellar movement and adenosine triphosphate activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.* **54**:75–97.
8. GIBBONS, I. R. 1961. The relationship between fine structure and the direction of beat in gill cilia of a lamellibranch mollusc. *J. Biophys. Biochem. Cytol.* **11**:179–205.
9. GIBBONS, I. R. 1963. A method for obtaining serial sections of known orientation from single spermatozoa. *J. Cell Biol.* **16**:626–628.
10. GIBBONS, I. R. 1975. The molecular basis of flagellar motility in sea urchin spermatozoa. *In* Molecules and Cell Movement. S. Inoué, and R. E. Stephens, editors. Raven Press, New York. 207–232.
11. LINCK, R. W. 1973. Comparative isolation of cilia and flagella from the lamellibranch mollusc. *Aequipectin irradians*, *J. Cell Sci.* **12**:345–367.
12. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
13. MANTON, I., and B. CLARKE. 1952. An electron microscope study of the spermatozoid of *Sphagnum*. *J. Exp. Bot.* **3**:265–275.
14. MAZIA, D., G. SCHATTEN, and W. S. SALE. 1975. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. *J. Cell Biol.* **66**:198–200.
15. PITELKA, D. 1968. Fibrillar systems in protozoa. *In* Research in Protozoology. Vol. 3. T. T. Chen, editor. Pergamon Press, New York. 282–388.
16. SATIR, B., W. SALE, and P. SATIR. 1976. Membrane renewal after dibucaine deciliation of *Tetrahymena*. *Exp. Cell Res.* **97**:83–91.
17. SATIR, P. 1965. Studies on cilia. II. Examination of the distal region of the ciliary shaft and the role of the filaments in motility. *J. Cell Biol.* **26**:805–834.
18. SATIR, P. 1968. Studies on cilia III. Further studies on the cilium tip and a "sliding filament" model of ciliary motility. *J. Cell Biol.* **39**:77–94.
19. SATIR, P. 1975. Ciliary and flagellar movement: an introduction. *In* Molecules and Cell Movement. S. Inoué, and R. E. Stephens, editors. Raven Press, New York. 143–149.
20. STEPHENS, R. 1970. Isolation of nexin—the linkage protein responsible for maintenance of the ninefold configuration of flagellar axonemes. *Biol. Bull.* **139**:438.
21. SUMMERS, K. E., and I. R. GIBBONS. 1971. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea urchin sperm. *Proc. Natl. Acad. Sci. U.S.A.* **68**:3092–3096.
22. 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.
23. TAMM, S. L., and G. A. HORRIDGE. 1970. The relation between orientation of the central fibrils and the direction of beat in cilia of *Opalina*. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **175**:219–233.
24. THOMPSON, G. A., L. BAUGH, and L. F. WALKER. 1974. Nonlethal deciliation of *Tetrahymena* by a local anesthetic and its utility as a tool for studying cilia regeneration. *J. Cell Biol.* **61**:253–257.
25. WARNER, F. D. 1970. New observations on flagellar fine structure. The relationship between ma-

trix structure and microtubule component of the axoneme. *J. Cell Biol.* **47**:159–182.
26. WARNER, F. D. 1976. Ciliary inter-microtubule bridges. *J. Cell Sci.* **20**:101–114.
27. WARNER, F. D., and P. SATIR. 1974. The structural basis of ciliary bend formation. Radial spoke positional changes accompanying microtubule sliding. *J. Cell Biol.* **63**:35–63.
28. WITMAN, G. B., R. FAY, and J. PLUMMER. 1976. *Chlamydomonas* mutants: evidence for the roles of specific axonemal components in flagellar movement. *In* Cell Motility. R. Goldman, J. Rosenbaum, and T. Pollard. *Cold Spring Harbor Symp.* In press.