THE STRUCTURAL BASIS OF CILIARY BEND FORMATION

Radial Spoke Positional Changes Accompanying

Microtubule Sliding

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ABSTRACT

The sliding microtubule model of ciliary motility predicts that cumulative local displacement (ΔI) of doublet microtubules relative to one another occurs only in bent regions of the axoneme. We have now tested this prediction by using the radial spokes which join the A subfiber of each doublet to the central sheath as markers of microtubule alignment to measure sliding displacements directly. Gill cilia from the mussel *Elliptio complanatus* have radial spokes lying in groups of three which repeat at 860 Å along the A subfiber. The spokes are aligned with the two rows of projections along each of the central microtubules that form the central sheath. The projections repeat at 143 Å and form a vernier with the radial spokes in the precise ratio of 6 projection repeats to 1 spoke group repeat. In straight regions of the axoneme, either proximal or distal to a bend, the relative position of spoke groups between any two doublets remains constant for the length of that region. However, in bent regions, the position of spoke groups changes systematically so that Δl (doublet 1 vs. 5) can be seen to accumulate at a maximum of 122 Å per successive 860-Å spoke repeat. Local contraction of microtubules is absent. In straight regions of the axoneme, the radial spokes lie in either of two basic configurations: (a) the parallel configuration where spokes 1-3 of each group are normal (90°) to subfiber A, and (b) the tilted spoke 3 configuration where spoke 3 forms an angle (θ) of 9-20°. Since considerable sliding of doublets relative to the central sheath $(\sim 650 \text{ Å})$ has usually occurred in these regions, the spokes must be considered, functionally, as detached from the sheath projections. In bent regions of the axoneme, two additional spoke configurations occur where all three spokes of each group are tilted to a maximum of \pm 33° from normal. Since the spoke angles do not lie on radii through the center of bend curvature, and Δl accumulates in the bend, the spokes must be considered as attached to the sheath when bending occurs. The observed radial spoke configurations strongly imply that there is a precise cycle of spoke detachment-reattachment to the central sheath which we conclude forms the main part of the mechanism converting active interdoublet sliding into local bending.

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Microtubule sliding accompanies ciliary beat as evidenced by progressive displacement of doublet tubules at the ciliary tip (Satir, 1965; 1968). Displacement can be shown to be a strict consequence of the geometry of the bent region, provided that the doublet microtubules do not contract. Although sliding has been directly demonstrated in model axonemes (Summers and Gibbons, 1971), while gross contraction of microtubules has never been seen, the possibility of minimal local contraction occurring within bent regions has not been eliminated in prior tests of the sliding model.

Eq. 1 relates sliding displacement in relation to doublet no. 1 (Δl_n) to the bend angle $(\Sigma \alpha)$, where d_n is a constant indicating effective axonemal diameter (Satir, 1968).

$$\Delta l_n = (2\pi/360)d_n \Sigma \alpha \tag{1}$$

Eq. 1 can also be adapted to determine linear displacement between any two sliding surfaces (n) within the axoneme (Warner, 1972). A consequence of this equation, hitherto untested, is that all cumulative sliding displacements occur in bent regions of the cilium and conversely, Δl does not change or accumulate in straight regions of the axoneme.

The axonemal microtubules lie in a precise geometrical array, which is probably maintained by both the interdoublet or nexin links and the radial spokes (Linck, 1973 a; Summers and Gibbons, 1973; Warner, 1970, 1974). Since the radial spokes are the only well defined structures that lie normal (90°) to the direction of sliding, they may have a major role in the sliding-bending mechanism. Two major events in the sliding model are now well established. (a) The nine outer doublet microtubules of the axoneme slide relative to one another without measurable contraction during bend production (Satir, 1965, 1968; Summers and Gibbons, 1971). (b) Active sliding is generated by cyclic interaction of the dynein arms attached to the A subfiber of each doublet with sites on the B subfiber of the adjacent doublet. This process is accompanied by ATP dephosphorylation (Gibbons and Gibbons, 1972, 1973 a; Summers and Gibbons, 1971).

In the present study we direct attention to a different sliding couple, that which involves a given set of outer doublets and the central sheath (Warner, 1972), and extend the above statements to include a third major event of the sliding

mechanism. (c) The radial spokes joining the A subfiber of each doublet to the central sheath are a main part of the transduction mechanism that converts interdoublet sliding into local bending (Satir, 1972; Satir and Warner, 1973; Warner, 1970, 1972, 1974).

MATERIALS AND METHODS

Lateral (L) and laterofrontal (Lf) gill cilia (Fig. 1) from the freshwater mussel *Elliptio complanatus* were utilized in this study. For details concerning gill structure and isolation procedure, refer to Satir (1963).

The gill is stripped and activated in a solution of either 20 mM KCl, NaCl or 10 mM CaCl₂ in 5 mM Tris buffer at pH 7.5 and containing 1 mM 5-hydroxytryptamine creatine sulfate. Brief fixation (<2 min) with 2% unbuffered OsO4 preserves the metachronal wave (Satir, 1963). The gill is then transferred to 2% glutaraldehyde in 0.05 M sodium cacodylate buffer at pH 7.4 for 1 h at 4°C, and subsequently postfixed for 30 min in 1% cacodylate-buffered OsO, at 4°C. Tissue is embedded in Epon 812. Samples of tissue kept in deionized water as unactivated controls are fixed and embedded in parallel with the activated samples. Tissue is examined in the light microscope for preservation of the metachronal wave during the embedding procedure to ensure that multiple positions of the ciliary beat cycle are preserved. Bends on activated cilia may occur in either the direction of the effective stroke (E-pointing: positive, doublet nos. 5-6 at leading edge of the axoneme) or in the direction of the recovery stroke (R-pointing: negative, doublet no. 1 leading). Unactivated cilia all assume the R-pointing position with a basal bend (Satir, 1963).

For electron microscopy, thin sections were stained for 15 min in 5% aqueous uranyl acetate followed by 2 min in Reynolds' lead citrate. We find that the maximum usable section thickness for detecting accurately the radial spoke lattice is that of a gray interference-color section.

A Note on the Electron Micrographs

All electron micrographs were taken at direct magnifications of \times 30,000-60,000. Magnification was calibrated by using carbon-grating replicas. All figures of longitudinally sectioned cilia are oriented so that the base of the organelle is positioned at the bottom of the page: the marker for determining both orientation and component numbering may not always be visible in the figure.

Several structural markers within the axoneme permit accurate orientation and component identification (numbering). The most important markers are (a) radial spoke periodicity, (b) the doublet 5-6 bridge, (c) dynein arm position, and (d) the inclination angle of the central sheath projections.

Measurement of structural periodicity within the axoneme is done over a reasonable distance to reduce the

error inherent in individual measurements. For example, 86 mm of longitudinally sectioned central sheath on a micrograph at \times 240,000 contains 25 sheath projection periods. Simple division gives us a value of 143.3 Å per sheath period. Several such determinations are then processed for mean, standard error etc.

Periodicities in electron micrographs are often given to three significant figures, reflecting the precision of the above method of measurement; differences of less than 10 Å are real. Except where otherwise noted, values not measured as above are still given to three figures in order to avoid the bias inherent in rounding-off numbers and to permit a more critical comparison of certain numbers.

Major structural periodicities were measured at random orientations relative to the direction of knife travel. Section compression does not appear to seriously affect measurements, since all measurements for a given period are equivalent statistically to within the standard error indicated in Table I.

We consider the structural periodicities summarized in Table I to be very precise on a comparative basis; that is, measurements for all structures could sometimes be taken from a single electron micrograph. Combined with the method of measuring, the values reported in this study are therefore more accurate than any previously reported by us. We have not determined if the presently reported values are directly comparable to those obtained from other organisms, but certainly they fall into the range of 10% error.

OBSERVATIONS

General Axoneme Substructure

Extensive observations on microtubule organization and substructure in the gill cilia of E. complanatus have been published elsewhere (Satir,

1963, 1965, 1968; Warner and Satir, 1973). Fig. 1 is provided for orientation purposes and shows a transverse section of a single gill filament with its lateral (L), laterofrontal (Lf) and frontal (F) cilia. Different stroke positions are visible in the lateral cilia of the preserved metachronal wave. A transverse section of a single lateral cilium is shown in Fig. 2, and its various components are shown diagrammatically in Fig. 3. As is typical for all 9 + 2 axonemes, in *Elliptio* cilia, the nine outer doublet microtubules surround two single central microtubules in 40° or ninefold radial symmetry. Doublet nos. 5 and 6 are joined by structural bridges between the respective A and B subfibers (Figs. 2, 15). It appears that the bridge is part of the dynein arm complex between the doublets since both the bridge elements and the dynein arms repeat at 225 Å (Table I). In Elliptio the bridged doublets serve as an unerring marker for numbering all other doublet tubules (Satir, 1968), proceeding in the direction of dynein arm attachment to the A subfiber. Doublet 1 thus lies opposite doublets 5-6. In a cross section, an imaginary line, the ciliary axis, bisecting doublet 1 and passing between doublets 5-6, perpendicular to the aligned plane of the central tubules, represents the local direction of the ciliary beat (Gibbons, 1961; Satir, 1963; Tamm and Horridge, 1970): the direction of the effective stroke in Elliptio is toward doublets 5-6 (Satir, 1968).

The two central microtubules are also numbered according to their position in the axoneme (Fig. 3). That tubule nearest the position of doublet 3 is

Important Axonemal Periodicities Number of Subunit size Corrected values ‡ Distance between sites Measured values subunits Å Å Å Radial spokes S1-S2 290 ± 8.5 § 7 41 285 222 ± 7.3 205 S2-S3 5 44 S3-S1 360 ± 10.9 9 40 370 S1-S1 860 ± 4.1 21 41 860 Sheath (α or β) 142 ± 0.8 143 219 ± 5.1 225 d_{5-6} bridge 215 ± 6.7 Dynein arms 225 Interdoublet links 866 ± 7.7

TABLE I

* Tubulin subunits per period based on Table IV.

‡ Based on average subunit size of 41 Å, and an exact 6:1 ratio of spoke to sheath period. Spoke values are rounded to the nearest 5 Å. These values are used in the text.

§ Standard error.



FIGURE 1 A single gill filament from *E. complanatus*. The transverse section plane reveals the three types of cilia associated with the filament: *f*, frontal cilia; *Lf*, laterofrontal cilia (cirri). The lateral cilia are seen in both recovery (*R*) and effective (*E*) stroke positions. Phase-contrast photomicrograph of a thick Epon section. \times 830.

designated cm_3 and that tubule nearest doublet 8 is designated cm_8 (Satir, 1973; Warner, 1974).

The nine doublet microtubules are joined by radial spokes (links) from the A subfiber (Warner, 1970) to the so-called central sheath around the two central tubules. The plane of the radial spokes delineates the nine 40° sectors of the axoneme (Figs. 2, 3). We describe below the structure and three-dimensional organization of both the spokes and central sheath, for the direct purpose of ascertaining their function in the motile process.

Substructure of the Straight Axoneme

For purposes of establishing a standard against which we can compare structural changes observed during motility, this section concerns axoneme substructure as it occurs in straight or unbent regions of the organelle. Since a cilium in an unbent stroke position rarely if ever occurs, our observations pertain to straight regions of the axoneme that lie either proximal or distal to a bend. We have used cilia in either quiescent (HOH) or activated (Na^{+2}, Ca^{+2}) stroke positions and either lateral (L) or laterofrontal (Lf) cilia for these observations. Although the distribution of straight and bent regions in E and R directions changes with cilia type and stroke postion, we have not noted any structural distinctions between the four categories in straight regions, and in this section we distinguish between them only as may be pertinent to a particular illustration.

RADIAL SPOKE ORGANIZATION: The radial spokes in Elliptio gill cilia have a structure and organization similar to that described in Sarcophaga sperm flagella (Warner, 1970). The spokes in Elliptio, however, occur in groups of three along the A subfiber of each doublet (Fig. 4). We have designated the spokes of each group as 1, 2, and 3, proceeding from base-to-tip (Figs. 4, 5, 10). Each spoke is about 50 Å thick, 380 Å long, and terminates in a small electron-opaque knob, the spoke head (Warner, 1970), at the perimeter of the central sheath. Each group of spokes repeats at an average of 860 Å along the A subfiber (Table I): the subperiods between adjacent spokes average 285/205/370 Å measuring from base-to-tip orientation (between spokes 1 and 2, 2 and 3, 3 and 1). The wider spacing (285 Å) between spokes 1 and 2 of any group is always nearest the base of the cilium (Figs. 4, 15) and thus serves as a constant marker for determining the orientation of cilia sectioned some distance from the base.

Using the criteria established for Sarcophaga sperm flagella (Warner, 1970), we find that the radial spokes in Elliptio gill cilia are also organized in the form of a helix in the axoneme matrix. When the axoneme is viewed near its base (proximal to a bend) in median longitudinal section, the consequences of double helical organization would included (a) progressive 190-Å displacement of spokes of adjacent doublets and thus (b) near alignment of spoke groups on opposite sides of the axoneme. A single turn helix would require that the spoke groups be displaced by about 430 Å on opposite sides of the axoneme in basal regions where sliding is absent. Since opposing spoke groups are usually in register at the base of the cilium, their helical organization can be regarded as double with an 860-Å period and 1,720-Å repeat. The slight displacement sometimes observed near the base (100-200 Å) can be accounted for by the necessary helical displacement of the



FIGURE 2 Transverse section of a single lateral cilium as viewed from base-to-tip. Doublet no. 1 (d_1) is in the 12 o'clock position and the d_{s-6} bridge is directly opposite. The plane between d_1 and d_{s-6} defines the local direction of the ciliary beat. In *Elliptio*, the direction of the effective stroke is toward d_{s-6} . See Fig. 3 for the structural details of the axoneme. \times 280,000.

spoke groups, since a median section cannot be a true halving of the 9-position helix. A small amount of sliding also may affect the value measured. Because of difficulties of following spokes of sequentially numbered doublets in thin sections, we have not been able to determine the handedness of the helix unequivocally. Since we know base-totip orientation, the helix appears to be left-handed with a pitch angle of 40° at the spoke head position and 30° at the spoke base or origin on the A subfiber. As with *Sarcophaga*, we emphasize that the helix occurs in form only, not in helically joined structure.

RADIAL SPOKE CONFIGURATION: In straight regions of the axoneme either proximal (doublet sliding absent) or distal (doublet sliding present) to a bend, the radial spokes lie typically at right angles or normal to the wall of subfiber A. For example, Fig. 4 shows an unbent region distal to a recovery-pointing bend (HOH lateral cilium)

where the bend angle α equals about 80°. Two configurations of spoke groups are seen here. In the first, which we designate the parallel 1 or 2 configuration (Fig. 5), all three spokes of a single group are normal (90°) to the tubule wall. The second configuration is designated the tilted spoke 3 (TS3) configuration. In this configuration, spoke nos. 1 and 2 of each group project at 90° from the tubule wall, but spoke no. 3 is often bent toward spoke 2, and forms an angle (θ) varying from about 9° to a maximum of 20° from perpendicular. Because both configurations can occur at the central sheath, the spoke heads along a straight region do not always maintain strict periodic spacing (Fig. 6), and fuzziness sometimes appears in photographic translation images in respect to spoke 3 (Fig. 4).

SUBSTRUCTURE OF THE CENTRAL SHEATH: The central sheath in 9 + 2 cilia and flagella has been described as a helical fiber(s)

surrounding the two central microtubules (Gibbons and Grimstone, 1960; Pedersen, 1970; Warner, 1970). However, recent studies on negatively stained axonemes from *Tetrahymena* and *Chlam*ydomonas (Chasey, 1969; Hopkins. 1970) show instead one to two rows of ~150-Å repeating projections along the central tubules. It appears that these projections are either unique structures or remnants of the aforementioned sheath.

Using thin-section microscopy, we have determined that the central sheath in *Elliptio* gill cilia consists of two rows of projections along each of the central microtubules (four rows in all). The single rows on each tubule nearest the doublet no. 1 side of the axoneme are designed $cm_{s,\alpha}$ and $cm_{s,\alpha}$: the single rows nearest the doublet nos. 5-6 side of the axoneme are designated $cm_{s,\beta}$ and $cm_{s,\beta}$ (see interpretive diagrams, Figs. 3, 10).

When the central region of the axoneme is viewed from lateral positions, i.e. from doublet no.

3 and 8-positions, the α and β rows of projections are clearly visible (Figs. 4, 7-9). It is obvious that one α and one β row is present on each tubule since they can be seen in longitudinal, but slightly oblique sections passing from cm_3 to cm_8 in the same section (Fig. 7).

Figs. 4 and 8 show that the α and β projections are in periodic register on each of the two central tubules. Further, they appear to span the lateral surface of each tubule and thus appear contiguous (Fig. 8, inset). The projections also appear to be in close proximity near their terminal ends (α - α , β - β) at the axoneme bend plane. However, to simplify interpretation of the organization for our purposes here, Figs. 3 and 10 indicate that the ends of the projections are not joined. Hopkins' (1970) study of negatively stained flagella indicates that the projection ends are joined and may hold the two central tubules together.

The α and β projections are spaced at an average



FIGURE 3 Diagram of a portion of the 9 + 2 axoneme as viewed from base-to-tip. The central sheath consists of paired projections (α, β) along each of the central microtubules (cm_s, cm_s) . Drawn approximately to scale (modified from Warner and Satir, 1973; and Warner, 1974).

FIGURE 4 Median longitudinal section of a straight region from a lateral cilium bent in an R-pointing position. The radial spokes are seen joining the A subfiber of the doublet (d_s) with the β row of projections along the central microtubule (cm_s) . The position of view is probably from the axis of the cilium looking out. The spokes lie in groups of three (1, 2, 3) with spacings of 285/205/370 Å and a major repeat of 860 Å. The wider spacings (285 Å) between spokes 1 and 2 is always nearest the base of the cilium. The linear translation (right) was translated in periods of the 860 Å spoke group repeat: note that both the radial spokes and the 143 Å repeating α, β sheath projections strongly reinforce. Many spoke groups are in the TS3 configuration (see Fig. 5) and the sheath projections (α, β) have the appearance of those associated with cm_s . Original micrograph and translation \times 240,000; inset \times 360,000.



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FIGURE 5 Interpretive diagram of the basic angular configurations formed by the radial spokes in both straight and bent regions of a cilium. Closed spoke heads are in alignment with the projections of the central sheath, while open spoke heads are misaligned and represent unattached spokes. Note that all heads in one period can be aligned with sheath projections only when one or more spokes are tilted. In straight regions this is seen in the TS3 configuration (Fig. 18 b, c). The TS3 configuration could be converted into the maximum tilt configuration seen on the concave side of bent regions by active sliding if all spoke heads remained attached during sliding (Fig. 18 e). Steps a-e represent one possible major sequence of the spoke tilting cycle. Note that sliding displacement (ΔI) is not associated with conversions postulated to be part of unbending (d-e). The last conversion (e-b) brings spokes back to a parallel configuration. Refer to the text for details. Drawn to scale.

of 143 Å center-to-center along the microtubule wall. Each projection is about 50 Å thick and extends out about 200 Å from the tubule surface. The projections are inclined at about 15° to the tubule wall: the inclination of both α and β projections on one tubule is in the same direction, giving either a Λ or V appearance in the plane of the tubule wall (Figs. 4, 7, 8). On cm_s both the α and β projections point proximally to the cilium base (A appearance) and on cm_8 they point distally (V appearance; see interpretive diagram, Fig. 10). This orientation provides a good marker for determining which of the central tubules is being seen in a longitudinal section, but it must be used with caution because the projection angle can be distorted. In some longitudinal sections of the sheath the α, β projections are clustered in groups of three to six (Fig. 9) by virtue of their angular orientation. This probably results from their attachment to the nearby spoke heads and suggests that the spokes are sometimes firmly attached to the sheath projections (see next section). Since our information on clustering is still preliminary, in this report, we do not attempt to relate such changes in angular orientation to bending. Sometimes the clusters of sheath projections appear to assume a large pitch helical orientation which parallels the helical organization of the spoke heads.

If the axoneme central region is viewed from either doublet no. 1 or 5-6 positions, the projections are inclined at about 15° so that a con-



FIGURE 6 Longitudinal section of a lateral cilium through two adjacent rows of radial spoke heads. The triplet grouping of spokes (brackets) is still apparent, but individual heads often vary in spacing because of the tilt of spoke 3 and attachment to the nearby sheath projections. This is clearly shown by comparison of spacing in the bracket marked TS3 with spacing in the adjacent

tinuous striation is superimposed over the tubules (Figs. 11, 12). When viewed from the doublet 1 position the $\alpha - \alpha$ inclination is from left to right: when viewed from doublets 5-6, the β - β inclination is from right to left (see interpretive diagram, Fig. 10). Because of both section plane and thickness, the α and β projections can appear as crossbridges between the two central tubules (Figs. 11, 12): as predicted by this interpretation in Elliptio cilia, as the section plane moves to the surface of the sheath, the crossbridges are in register with the projections (Fig. 12). However, this analysis must presently be regarded as equivocal, particularly since certain cilia and flagella are known to have periodic structures joining the two tubules in this region.

In summary, we find no evidence of a helically wound fiber(s) around the tubules. The lack of helicity to the tubule projections is definitely established by the observation that the angular inclination of the α and β rows on a single tubule is in the same direction when viewed from the doublet 3 or 8 positions. Helical organization would require that the α and β projections incline oppositely on the same tubule to form a single line from all positions of view, a previous claim to the contrary notwithstanding (Warner, 1970). Instead, we have demonstrated that the central sheath actually consists of two rows of 143-Å repeating projections along each of the central tubules.

SPOKE-CENTRAL RADIAL SHEATH STRUCTURAL RELATIONSHIPS: The enlarged radial spoke heads abut against the circular perimeter of the central sheath (diameter 660 Å) when the axoneme is viewed in transverse section (Fig. 2). When viewed in longitudinal section (Fig. 4) the spoke heads appear to join directly with the sheath projections. A precise mathematical or vernier relationship exists between the period of the sheath projections (143 Å) and that of the spokes (860 Å). If photographic linear translation is applied to the 860 Å spoke group repeat (Fig. 4), both the spokes and the α and β projections strongly reinforce. If the axoneme is translated in the 143-Å periods of the α - β repeat (multiples of 1-5), the projections

bracket. Note that the relative position of spoke groups between the two rows (p) is, however, constant throughout the figure, even though the region illustrated was distal to a nearby bend. \times 160,000.



FIGURE 7 Median longitudinal section of a single laterofrontal cilium which passes from cm_s to cm_s in the area illustrated. The position of view is probably from doublet no. 3 and d_1 is on the right side of the axoneme. The α,β projections of the central sheath point proximally (Λ appearance) on cm_s (lines) and distally (\vee appearance)

strongly reinforce, but the spokes are given a false spacing depending on the multiple used in the translation (not shown). Accordingly, the translation technique must be used with considerable discretion. If the axoneme is translated in multiples of six projection repeats, the spokes correctly reinforce (6×143 Å = 858 Å). Therefore, exactly six projection periods occur for each 860 Å group repeat.

Although the spoke head is rather diffuse, the thickness of a spoke is approximately the same as that of one of the sheath projections. A vernier alignment between spokes and sheath projections thus becomes possible so that spoke 1 of each spoke group is in direct line with the first sheath projection of that same period (see interpretive diagram, Fig. 5 a, projection 2). Accordingly, spoke 2 will be almost in direct line with the third sheath projection in each period (Fig. 5 a, projection 4), since 285 Å (spoke 1-2 distance) equals 2×143 Å (two sheath projection periods). Since spoke 2-3 distance is 205 Å, it is easily seen by similar reasoning that spoke 3 would ordinarily lie 62 Å distal to the fourth sheath projection (Fig. 5 a, projection 5); in fact, this spoke often is bent back to attach to the fourth projection of that period (Fig. 5 c, projection 6). By simple geometry, it can be seen that the spoke 3 angle (θ) when it is attached must be about 9°. This is the approximate angle found in the TS3 configuration (Fig. 5 c). The spoke configurations, TS3 and parallel, found in straight regions, are interconvertible if spoke 3 is considered to be flexible and extensible.

As noted previously, the spoke heads themselves always appear to be attached to the α and β projections, regardless of the doublet from which the spokes originate. Occasional sections obtained where the spoke heads appear detached can probably be accounted for on the basis of section plane. A single spoke head can span as many as two projection multiples and appear to be attached to one or two of the α,β projections (Fig. 4), depending on its position. Although it appears that there is no position where the spokes can be structurally dissociated from the sheath, as we

on cm_{\bullet} (lines). The overall orientation of the cilium is provided by the triplet grouping of the radial spokes (brackets). Where the section plane passes between the central microtubules, the α projections are cut transversely (arrows) \times 165,000.



FIGURES 8 and 9 Longitudinal sections of the central sheath in laterofrontal cilia. In Fig. 8 (viewed from the doublet no. 3 position), the repeating orientation (\land appearance) of the sheath projections (α , β) is clear along cm_3 . The linear translation (inset) enhances their orientation and shows that the projections are not only in register on opposite sides of cm_3 , but appear to be continuous as well, since they completely span the lateral edge (toward d_3) of the tubule. In Fig. 9 (probably viewed from the cilium axis looking out), the angle of the sheath projections of cm_3 is less uniform and the projections are clearly angled into groups of 3-6 (brackets) which reflects their attachment to nearby radial spoke heads (arrows). All figures, \times 204,000.



FIGURE 10 Interpretive diagram of the central sheath-radial spoke complex as seen from several positions of view. For example, the doublet no. 8 view (d_8) is simply a 90° counterclockwise rotation of the $d_{6.6}$ view. When seen from $d_{8.8}$ positions the sheath projections (α, β) have a configuration similar to that seen in Figs. 4, 7, and 8. When viewed from $d_{1, 6.6}$ positions, the sheath is seen as it appears in Figs. 11 and 12. In the $d_{6.6}$ view, the aligned ends of the β - β projections are drawn as discontinuous in the plane between cm_8 and cm_8 , similar to the way they are illustrated in Fig. 3. This, however, may be an oversimplification of their true organization (refer to text for details). The radial spoke groups illustrated in the d_3 view are in the TS3 configuration. Drawn approximately to scale.

establish in the next section, they cannot be functionally bonded at all times. In transverse axoneme sections, the circular profile of the sheath perimeter and both lateral and terminal α - β projection continuity ensure that the spoke heads can maintain proximity to the sheath in 360°, that is, they are radially symmetrical. However, this means that certain spoke heads (from doublet nos. 3 and 8 in particular) are in quite close proximity to the central tubule wall, while others (doublet nos. 1, 5, and 6) are nearly the full α - β projection length away. Direct evidence about the curvature or scythe shape of the sheath projections as diagrammed in Fig. 3 is limited.

In summary, we have established the following important observations concerning radial spokecentral sheath organization in unbent regions of *Elliptio* gill cilia. (a) Radial spokes occur in groups of 3 with a basic repeat of 860 Å and subperiods of 285/205/370 Å along the A subfiber. (b) In straight regions spoke groups occur in two basic configurations: the parallel configuration where all spokes in a group project normal to subfiber A ($\theta_{S1-3} = 90^\circ$), and the tilted spoke 3 configuration where spoke 3 in the group bends toward spoke 2 ($\theta_{S1-2} = 90^\circ$; $\theta_{S3} = 9-20^\circ$). (c) The central sheath consists of two rows of 143-Å repeating projections along each of the central microtubules. (d) The α - β sheath projections occur in an exact ratio of six projection multiples to one spoke group repeat.

Structural Changes in Cilia during Bend Propagation

In this section our observations pertain only to activated lateral cilia sectioned in either R- or

FIGURES 11 and 12 Longitudinal sections of two lateral cilia as seen from the position of doublet no. 1. The α projections of the sheath (Figure 11 and inset *a*) are particularly clear superimposed over cm_3 (left tubule). In Fig. 12 and inset *b*, crossbridges (143-Å period) appear to join the two central microtubules although the image might be confused by superimposition of the β projections (or α projections in Fig. 11) (refer to text for details). The α projections in Fig. 12 (arrows) are visible for only a short distance since the section plane rapidly moves toward the other (d_{5-6}) side of the sheath. The 860 Å repeat (*p*) of the radial spoke groups is clear in Fig. 11. Note that the relative position of spoke groups is constant between the two doublets (d_{5-6}) . The insets (a, b) are linear translations moved at the 143-Å sheath projection repeat (marked lines). Figures, \times 160,000; insets \times 200,000.



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E-pointing stroke positions, although similar results were obtained for the laterofrontal cilia as well. Although it is clearly established that gill cilia bend in three dimensions (Aiello and Sleigh, 1972; Satir, 1963), single electron micrographs essentially present two-dimensional images of portions of the axonemes. Accordingly, in what follows, we often simplify our discussion to two dimensions, with the full realization of the geometrical complexity that is introduced by a nonplanar beat. Furthermore, when we discuss sliding displacements below, we are examining the results of active sliding between adjacent doublet microtubules, and we do not imply that any other interaction produces sliding. Nevertheless, as a response to active sliding, many other structures in the axoneme move, and it is convenient to refer to these movements in terms of sliding displacements as defined in Eq. 1.

RADIAL SPOKES AND MICROTUBULE SLIDING: The 860-Å radial spoke repeat provides an accurate measure of distance along a doublet tubule and enables us to test some of the predictions of the sliding microtubule hypothesis. When measured along subfiber A, spoke periodicity (285/205/370 Å; 860-Å repeat) remains the same in both bent and straight regions of the axoneme. This is shown by both direct measurement and by photographic translation techniques. Reinforcement of the triplet spoke group (Figs. 4, 13) implies invariance to within the precision of the translation, i.e., the spread of the image. In straight regions, the image of spoke 3 blurs slightly because of configurational changes, but reinforcement of spokes 1 and 2 retains the untranslated spoke thickness. In bent regions, reinforcement of the major period (860 Å) can be demonstrated throughout the bend, but the spoke subperiods blur because of angular configurational changes (Fig. 13). As expected, photographic rotation in bent regions which reinforces one side of the axoneme does not reinforce the other side. We conclude that in both bent and straight regions, neither local nor cumulative contraction of subfiber A is demonstrable: the radial spokes are attached to sites on subfiber A whose separation is unaltered in both bent and straight regions of fixed cilia.

Furthermore, in Figs. 4, 6, and 11, it is apparent that in straight regions of a bent axoneme, the relative displacement of successive spoke periods between the doublets on opposite sides of the axoneme does not vary systematically. The initial

displacement at the most proximal part of a straight region varies between different axonemes $(0\pm430$ Å) (Fig. 14). According to the sliding model, in a fixed cilium this value will vary depending on the original stroke position of the axoneme at the moment of fixation. Invariant or noncumulative spoke displacement graphs as a straight line with zero slope (Fig. 14), regardless of this initial displacement of the doublets along which the period is being measured (e.g. 1 vs. 5 or 3 vs. 8). This is also true regardless of whether the straight region is proximal or distal to a bend. Therefore, as predicted by the sliding model, Δl does not change from the initial value determined by proximal bending or accumulate as one moves more and more distally along straight regions of the axoneme.

If relative spoke displacement is measured in bent regions of the axoneme, quite a different situation becomes apparent. For example, Fig. 15 shows two R-pointing lateral cilia, longitudinally sectioned through doublets 1 and 5 in a bent region. If the relative spoke repeat of 860 Å is marked for the first group of spokes at the base of the axoneme and then successively (eight repeats) marked through the bend, it becomes obvious that there is a cumulative increase in the displacement of the spoke groups (Δl variant) proceeding through the bend. This sliding displacement (ΔI_{1-5}) accumulates in quantum steps of about 122 Å for each successive repeat measured in Fig. 15. The total Δl_{1-5} for the visible bend region in cilium 1 of Fig. 15 is 854 Å for seven successive spoke groups (950-Å spoke group no. 8 displacement minus 96 Å spoke group no. 1 displacement). The measured bend angle (α) for the axoneme is 30° and from Eq. 1, the calculated bend angle is 34° (d_{1-5} = 1,450 Å). Similar measurements can be made on axonemes sectioned in lateral positions (e.g., doublet 7 vs. 9) and a cumulative Δl is still apparent (Fig. 16). Several bent cilia similar to Figs. 15 and 16 were measured and are also graphed in Fig. 14. Occasionally we can successfully detect the transition between bent and straight regions on a single axoneme, but only in terms of cessation of accumulating Δl (Fig. 14). Successive sliding displacements through the bend plot as a straight line, indicating quantum increases in Δl per spoke group, although the slope of the line varies not only with α , but also with respect to doublet distance from the bend plane. That is, in a given stroke position, doublets in lateral positions (e.g. 7 vs. 9)



FIGURE 13 Rotational image reinforcement of a bent lateral cilium rotated in steps of the radial spoke group repeat of 860 Å (p). Since the bend is a circular arc and spoke periodicity is invariant, the 860-Å repeat (spoke no. 1, marked lines) strongly reinforces, demonstrating that no contraction of microtubules has occurred in the bend. Individual spokes of each group do not always strongly reinforce because of angular variations in their positions. Since the amount of rotation needed to move 860 Å is different for the convex (a) and concave (b) sides of the bend, each surface (at the inner surface of subfiber A) must be rotated separately at its individual radius (r) to reinforce the spoke periodicity. \times 110,000.

will show (geometrically) less sliding than those in the bend plane (Warner, 1972).

In Fig. 16, it is apparent that the first two groups of radial spoke heads near the base are displaced by about 145 Å and successive displacements still occur in quantum units. Thus the measurable Δl_{7-9} (spoke head position) for the visible bend region is 495 Å for successive spoke groups. Since spoke group 1 on the outside of the bend is positioned distally (145 Å) to spoke group 1 on the inside of the bend, the initial 145-Å displacement must be added to the 350-Å displacement measured at spoke group 9 for the total Δl of 495 Å. The reverse situation was apparent in Fig. 15 (preceding paragraph). The axoneme in Fig. 16 is graphed in Fig. 14 as doublets 7-9. It would appear that for doublets 7-9, 8-9, and 1-5 (Fig. 14), minimal sliding has extended into the basal body region

below the first group of radial spokes (see Discussion). Visible distortion of the basal plate also supports the notion of basally directed sliding (Fig. 15). Until proven otherwise, however, we regard this displacement as passive distortion resulting from the nearby shear forces of sliding. Excessive distortion in the basal region is probably prevented by the various anchoring or stabilizing structures associated with and occurring within the basal body.

RADIAL SPOKE DETACHMENT IN STRAIGHT REGIONS OF THE AXONEME: Consider the straight region of an axoneme distal to a bend: it has been pointed out in previous studies (Satir, 1972, 1973; Warner, 1972) that if the radial spokes were connected permanently to both subfiber A and to the central sheath, as a bend formed the cumulative ΔI would be translated through the

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straight region so that all the spokes would become stretched and angularly inclined to accommodate this sliding. Some predicted doublet (spoke head) displacements relative to the surface of the central sheath (Warner, 1972) calculated from Eq. 1 are shown in Table II. Eq. 1 predicts that spoke heads in the bend plane (from doublets 1 and 5-6) will have maximal displacement relative to the sheath, while spoke heads in lateral positions will show correspondingly less displacement as their distance from the bend plane (e.g. from doublets 3 and 8) becomes greater. Table II is calculated for a bend angle (α) of 100° for which, at the sheath, a maximum Δl of 660 Å occurs: about the maximum measured for gill cilia.

Fig. 4 shows a straight region of the axoneme distal to a bend of about 80°. From Eq. 1 we calculate that the ΔI for spoke heads from doublets 1 and 5 relative to the surface of the central sheath would be about +528 Å and -503 Å, respectively (R-pointing cilium, see Table II). If the spokes were permenently attached, from Eq. 2 the necessary spoke stretch or extension (s) can be calculated where s is the direct distance from

subfiber A to the sheath surface (380 Å) and y is the displacement of doublet 5 relative to the sheath surface (s).

$$\Delta s = (s^2 + y^2)^{\frac{1}{2}} - s \tag{2}$$

For example, Δl_{5-8} of 503 Å would require a spoke stretch of 250 Å or 66%. Similarly, from Eq. 3 the hypothetical resulting spoke angle θ from normal can be calculated:

$$\theta = \tan^{-1} y/s \tag{3}$$

 θ thus would equal 53° for Δl_{5-s} of 503 Å.

Some predicted numerical relationships between the ciliary bend angle (α), radial spoke-sheath displacement (ΔI), spoke angle (θ), and spoke stretch (Δs) are summarized in Table III. For radial spokes 1 and 2, angular tilt (θ) of greater than 2° is never observed in straight regions of a bent axoneme (Satir, 1973; Warner, 1972, 1974). In either the parallel or TS3 configuration, spokes 1 and 2 of each group are always seen to be nearly perpendicular to the A subfiber, while spoke 3 is



FIGURE 14 Plot of the radial spoke repeats (860 Å) (p) measured between various doublet microtubules in both straight (open symbols) and bent (closed symbols) regions of several different cilia. In straight regions the relative position of spoke groups between any two doublets remains constant as indicated by the straight lines with 0 slope. The relative position of spoke groups in bent regions changes systematically and plots as a straight line with variable slope which can change to 0 slope at the leading edge of the bend (\oplus , \blacksquare). For bent regions, the numbers for p represent successive spoke groups and pl is the first group at the base of the cilium. For straight regions, p numbers are arbitrary and reflect only successive groups. Measurement of spoke periodicity (860 Å) was standardized by measuring only the position of spoke no. 1 where its origin lies on a projected radius through the center of bend curvature.



FIGURE 15 Median longitudinal section in the bend plane through 2 R-pointing cilia. The d_{s-s} bridge can be seen on the convex side of the bend in the first (right) cilium (arrows). The radial spoke groups (brackets) are clear on both sides of the bend. By numbering successive spoke groups along each doublet, it can be seen that systematic displacement of opposing groups occurs. This represents visible evidence that doublet sliding accumulates in the bend region. For cilium 1 (right) sliding displacement (ΔI) accumulates to approximately 854 Å within the eight successive spoke groups visible in the bend. See text for exact calculation and further explanation. In cilium 2 (left), ΔI increases to about 500 Å within five successive spoke groups. Note the distortion of the basal plate (arrows) in cilium 2 which indicates that some proximally-directed sliding may have occurred. \times 140,000.



FIGURE 16 Longitudinal section of an R-pointing lateral cilium as seen from the position of doublet no. 8. The radial spoke heads (brackets) are marked and a Δl of 350 Å is visible between doublets 7 and 9 at the position of the ninth spoke groups. The initial position of spoke groups 1 is displaced by about 145 Å. The relative position becomes aligned at spoke groups 4 and then begins to displace once again, resulting in the variable slope plot for d_{7-9} in Fig. 14 and a total Δl of 495 Å. \times 130,000.

TABLE II Predicted Δl between Doublets and Central Sheath for an E-pointing Cilium

doublet	d_n^*	$\Delta l_n/100^{\circ}$
n	Å	Å
I	378	-660
2	254	-443
3	43	-75
4	212	+ 370
5	360	+628
6	360	+628
7	212	+370
8	43	-75
9	254	-443

* The distance d is measured from the base of the radial spoke on the A subfiber to the intersect of the spoke on the central sheath perimeter, after both sites are projected to the effective bend plane.

‡ Positive values for Δl reflect distally directed sliding of the doublets. Negative values reflect distally directed movement of the central sheath relative to the doublets. The signs would be reversed in an R-pointing cilium.

moderately tilted $(-9 \text{ to } -20^\circ)$ in the TS3 configuration. As we have described above, in a straight region above a bend, Δl has a constant value at any one time but the value changes as the proximal bends change in magnitude ($\Sigma \alpha$ changes). We conclude that since stretching and spoke tilting are extremely limited when Δl changes from 0 in a straight region distal to a bend, the radial spokes must be considered to be functionally detached at one of their ends for at least part of the sliding cycle.

Since the subfiber A distances between spokes are invariant, while the distance between spoke heads is variable, it is likely that the subfiber A side (origin) of the spoke is permanently attached to the microtubule wall under physiological conditions, while the sheath side (head) is detachable. This notion is also supported by both the solubility and morphogenetic sequences (Warner, 1971, 1972) of the radial spoke-central sheath complex.

RADIAL SPOKE CONFIGURATION IN BENT REGIONS: If the radial spokes are examined in bent regions of the axoneme, they are seen to undergo characteristic angular changes that are undoubtedly related to the accumulating sliding displacement described in the previous section. Significantly, the spoke angular tilt does not follow radii (r) through the center of bend curvature as would be expected if they were passive supporting structures. For example, Fig. 17 shows cilium 1 from Fig. 15 and views doublets 1 and 5 in longitudinal section. Through most of the length of the bend, two new configurations of the spoke groups are seen. The first is the parallel tilt configuration (Figs. 5, 18) which occurs throughout most of the bend (Fig. 17) and is characterized by the inclination of spokes 1–3 to a maximum of $20-26^{\circ}$ from perpendicular. The second is the maximum tilt configuration which occurs at the leading (distal) edge of the bend (Fig. 17, spoke group 6, concave) and is characterized by the further inclination of spoke 3 to a maximum of 33° from perpendicular.

These spoke angles are maintained for successive spoke groups near the leading (distal) edge of the bend. Since Δl in this axoneme is increasing in quantum steps of about 120 Å throughout the visible bend, and the radius of curvature is near minimal, it appears that the 26° spoke tilt for spokes 1 and 2, and 33° tilt for spoke 3 may also be both quantal and the maximum that can occur. From Eq. 3 where s is 380 Å, θ equals 26° when y = 185 Å (1.29 sheath periods), and θ = 33° when y = 247 Å (1.73 sheath periods). Similarly from Eq. 2, the spoke extension is 43 Å or 11% when θ = 26°

TABLE III

Predicted Relationships Between Bend Angle (α) , Radial Spoke-Sheath Displacement (Δl) , Spoke Angle (θ) , and Spoke Stretch (Δs)

α	$\Delta l_{5-6}^{\clubsuit}$	θ‡	Δs§	Δs
degrees	Å	degrees	Å	%
10	63	9	.5	1.3
15	94	14	11	2.9
20	126	18	20	5.3
25	157	22	31	8.2
30	188	26	44	11.6
35	220	30	59	15.5
40	251	33	75	19.7
60	377	45	155	40.5
80	503	53	250	65.8
100	628	59	354	93.2
180	1131	71	813	213.9

Values above the horizontal lines lie in the range of actual values observed in electron micrographs.

* Calculated from Eq. 1 for doublet 5 (d = 360 Å).

‡ Calculated from Eq. 3 (s = 380 Å) and rounded to the nearest degree.

§ Calculated from Eq. 2 (s = 380 Å).



FIGURE 17 An enlargement of cilium 1 in Fig. 15 which shows the angular configurations assumed by the radial spokes in bent regions of the organelle. The spokes do not lie on radii (r) through the center of bend curvature, but form an angle θ which varies here between $\pm 20-33^{\circ}$ from normal. Radii are normal (90°) to the A subfiber of the doublet: the numbers of spoke groups (1–6; spoke no. 1 is marked) are positioned at the radii lines. On the concave side of the bend, spoke groups 1–5 are in the parallel tilt configuration while group 6 is in the maximum tilt configuration. The insert defines spoke angle θ and the parameters of Eq. 3. \times 180,000.

and y for spoke 1 = 185 Å, and 73 Å or 19% when $\theta = 33^{\circ}$ and y for spoke 3 = 247 Å. Since these figures are near the maximum we have observed in the bend plane where Δl is maximal, they may represent the limits of spoke distortion due to accumulating Δl and thus be part of the feedback mechanism (Brokaw, 1971; Satir, 1973) that regulates bending (see Discussion). Because of the diffuse nature of the spoke head, we do not measure spoke stretch directly, but rely on measurements of θ . Actual spoke stretch may be restricted by changes in axonemal diameter such as those found by Satir (1963), by changes in the sheath projection (α , β) angle (Fig. 9), or by possible changes in spoke head tilt.

At the base of the bent region in Fig. 15, cilium 2, the spokes are clearly in the parallel 1 or 2 configuration where $\theta_{s_{1}.s} = 90^{\circ}$. A second parallel or parallel tilt configuration also occurs near the base (proximal end) of bent regions where $\theta_{s_{1}.s} \simeq 20-26^{\circ}$ (Figs. 5, 17, 18). This configuration is obviously an intermediate between the maximum tilt and other configurations and we think this may be a transition position (Fig. 18).

ACTION OF RADIAL SPOKES AT OPPO-SITE SIDES OF THE AXONEME: On the concave side of a bent axonemal region, as well as in all straight regions, spoke tilt direction is uniform. Tilted spokes always point proximally: that is, the spoke head lies nearer the cilium base than the spoke origin at subfiber A does. By convention, θ is given as a negative value when spokes point in this direction (Fig. 18). Positive values for θ have not been measured in either straight regions or on doublets to the concave sides of bent regions: on doublets to the convex side of bent regions (Fig. 17), θ is usually positive. Since doublets move relative to each other during sliding, we account for this appearance if, as doublets on one side of the bend move actively in one direction, doublets on the opposite side of the axoneme move passively in the opposite direction. Fig. 19 shows a chevron configuration where θ is obviously negative to both sides of the central sheath in the bent region. We can account for this occasionally observed image if sliding in the same direction has occurred on opposite sides of the axoneme, possibly not synchronously. It seems likely that this sliding is in the active direction, and therefore that this direction is the same for doublets at opposite sides of the axoneme; but we cannot rule out the possibility that the chevron configuration may be the passive

response of both doublets in the figure to active sliding of unseen doublets. Asynchronous active sliding of the doublets is a possibility that would explain how, if spokes are attached in a propagating, completed distal bend, sliding changes could be seen at the tip of the cilium during development of a more proximal bend on the opposite side of the axoneme.

DISCUSSION

The sliding microtubule model of ciliary motility, developed for mussel gill cilia (Satir, 1965, 1967, 1968, 1972), is confirmed and enlarged in this present work. Specific features of the model to which our work pertains are: (a) The nine peripheral doublets of the axoneme slide relative to one another without measurable contraction during bend production. (b) Sliding is generated by the cyclic interaction of the dynein (ATPase) arms attached to the A subfiber of one doublet and sites probably on the B subfiber of the adjacent doublet, accompanied by ATP dephosphorylation.

Since sliding may be uncoupled from bending in membraneless model axonemes by trypsin digestion of radial spokes and other linkages within the axoneme (Summers and Gibbons, 1971), and since the radial spokes are the one significant periodic structure in the axoneme that lie normal to the direction of sliding (Warner, 1970), it is reasonable to enlarge these statements by the assumption that: (c) The radial spokes are a central part of the mechanism by which sliding is converted into bending. There follows a discussion of this assumption as it is supported by our present evidence.

Structural Periodicities

The reported radial spoke (group) periodicity in a variety of organisms is 840-960 Å (eg., Burton, 1973; Chasey, 1972; Hopkins, 1970; Warner, 1970), and all values fall into the range of 10% difference; it is not improbable that all reported values simply represent a common figure in the hands of different investigators. Although early studies of the radial spokes in *Chlamydomonas* and *Sarcophaga* (Hopkins, 1970; Warner, 1970) showed the spokes to be grouped in pairs, subsequent investigations have revealed a three-spoke or triplet grouping in other organisms and Chasey (1972) suggests that the triplet pattern may also be characteristic of *Chlamydomonas*. It may be that



FIGURE 18 Selected regions of longitudinally sectioned lateral cilia, each of which includes two adjacent groups of radial spokes in equivalent configurations (compare with Fig. 5). The top figures (a-c) were taken from straight regions. Fig. 18 *a* shows spoke groups in the parallel 1 configuration, while (*b*) and (*c*) show spoke groups in the TS3 configuration but with variable angles for θ . The bottom parts of the figure (18 e-f)were taken from the concave sides of bent regions. Fig. 18 *d*, taken from the convex side of a bend, shows the parallel 1 configuration; Fig. 18 *e*, the maximum tilt configuration; and Fig. 18 *f*, the parallel tilt. All figures $\sim \times 300,000$.



FIGURE 19 Longitudinal section of a bent lateral cilium, probably seen from the doublet no. 8 position (stroke position unknown). Although the individual radial spokes are not clear, their 860-Å group repeat (p) and angle θ are still apparent (lines). The spoke angle is directed to the cilium base on both sides of the bend, thereby imparting a chevron configuration to the region. Note that sliding displacement between opposing spoke groups still accumulates through the bend (compare positions of lines marked p). \times 140,000.

the third spoke is sometimes solubilized during negative staining or otherwise obscured in sectioned organelles, and that a triplet grouping is a common feature of cilia, flagella, and sperm tails.

Confirmation of the Geometry of Sliding

The general equation of sliding, Eq. 1 (see Introduction), formulated by Satir (1968), has been tested previously only by correlation of ciliary

tip position vs. accumulated Δl . In this study, we demonstrate for the first time (Figs. 14-16) that the Δl displacements of the axonemal doublets predicted by Eq. 1 occur locally in an axonemal bend as expected and that, also as predicted, Δl is always constant when the axoneme is unbent (Figs. 6, 11, 14). This demonstration is possible because with improved techniques and very thin sections (Warner, 1970) we are able to visualize the complex periodicity of the radial spokes that accurately marks microtubule alignment in these axonemes. The spokes occur as triplets with a major repeat (p) of 860 Å and subperiods of 285/205/370 Å along subfiber A of each doublet. This periodicity is maintained along subfiber A at all times in every cilium studied, regardless of stroke position. It is reinforced by photographic translation techniques along both straight (Fig. 4) and bent (Fig. 13) portions of the axoneme. Also, as we would expect from the geometry of a bend, photographic translation of spokes on one side of the bend fails to reinforce the period on the opposite side. We conclude that the invariability of spoke periodicity, even in bent regions, rules out even minimal contraction of the axonemal microtubules.

The predicted sliding displacements (ΔI) between two opposing doublets varies according to the doublets examined (Warner, 1972). Maximal displacement in any given stroke position should occur between doublets at opposite sides of the cilium axis (1 vs. 5-6). The maximum amount of displacement between doublets that we have observed per spoke period (p) is approximately 122 Å or 0.14 p (Figs. 14, 15). This occurs, as expected, between doublets 1 vs. 5 when the cilium is bent so that the radius of curvature is close to minimal. According to the analysis of Satir (1967), the empirically derived Δl per spoke period for a minimal radius of curvature ($\alpha = 51^{\circ}$) is 0.1 p. Taken together, these findings add new strong support to the sliding microtubule model of ciliary motion.

Three possible formulations of the sliding event can be conceptualized: (a) Δl begins to accumulate at the base of a bent region and increases moving distally to it (i.e., sliding occurs towards the tip, rather than towards the base of a cilium) (Satir, 1968); (b) Δl can accumulate both proximal and distal to a bent region (sliding is symmetrical around the center of the bend) (Sleigh, 1968); or (c) Δl can accumulate only proximal to the bent region, but not distal to it (sliding occurs

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primarily towards the base of the cilium). For mussel gill cilia, data on tip displacement vs. Δl (Satir, 1968) are consistent with formulation (a), and this is supported by images such as Fig. 15 where opposite sides of the radial spoke double helix are clearly in close alignment at the base of the cilium. In Fig. 14 some cilia graphed are not consistent with formulation (a) in that an apparent initial decrease in Δl with distance is measured. This apparent decrease is, however, only a positional effect of the spoke groups being measured: quantal Δl is always maintained throughout the bend. For example, in Fig. 16, the first group (1) of radial spokes on the outside of the bend is more distally positioned than the first group on the inside of the bend. As sliding progresses through the bend, the displacement between the relatively positioned spoke groups approaches 0 (spoke group 4) and then begins to accumulate once again. The cilium in Fig. 16 is graphed as doublets 7-9 in Fig. 14. We interpret this positional effect to mean that a small amount of sliding of certain doublets may occur at the base of the cilium. This is also supported by the previously noted distortion of the basal plate (Fig. 15). Such sliding is, however, restricted to only a few percent of measurable Δl in any given bend. A second possibility for explaining this positional effect is that the helical positional relationships between spoke groups may not be precisely as we envision them. For example, a single strand helix could account for an initial 430-Å spoke displacement (spoke group 1) between doublets 1 and 5.

For *Elliptio* cilia, we can rule out formulation (c) but not formulation (b) at this time. Even rather minimal sliding at the ciliary base might produce important changes in the microtubule-

membrane complex and the ciliary necklace, as suggested previously by Gilula and Satir (1972).

Vernier Alignment and Attachment of Radial Spokes

A significant finding of this study is the precise vernier between the central sheath period and radial spoke period so that the ratio of periods is exactly 6:1. In straight regions, where spoke 1 is normal to the A subfiber, it may also be in direct alignment with one sheath projection (Fig. 5, parallel 1 configuration). A feature of the vernier is that spoke number 2 is precisely two sheath periods distant from spoke 1, and therefore in this configuration it is also in direct alignment with a sheath projection. Finally, spoke 3 is distinctly set so that it is not an integral number of sheath periods distant from spoke 2, and therefore is not in alignment with a sheath projection. Alternatively, if spoke 3 is in alignment with a sheath projection (Fig. 5, parallel 2 configuration), spokes 1 and 2 will be equally out of alignment.

Spoke periodicity must reflect distance between spoke origin sites on the A subfiber, directly. It is reasonable to suppose that these distances are integral numbers of tubulin subunits apart along those protofilaments (Fig. 3) forming the sites (i.e., the relationship between attachment site and underlying protofilament structure is the same at all site loci). Table IV compares measured ratio vs. those predicted by this supposition. The size of the tubulin subunit calculable from this data is 41 Å (Table I). The configuration of subunits responsible for spoke binding at their origin is not known, but might now be looked for in negative stain preparations, spaced 5, 7, and 9 subunits

 TABLE IV

 Integral Ratios between Radial Spoke Distances along Subfiber A

	Measured ratios‡			
Distance involved*	(a)	(b)	(c)	Predicted integral ratios§
\$2-\$3: \$1-\$2	0.712	0.766	0.719	5:7 = 0.714
S2-S3: S3-S1	0.587	0.617	0.554	5:9 = 0.556
S1-S2: S3-S1	0.824	0.806	0.770	7:9 = 0.778

* S1-S2 is the distance from spoke 1 to the adjacent spoke 2, S2-S3 from spoke 2 to 3 etc.

 \ddagger (a) Direct measurement of 11 complete spoke periods. Ratio measurements are free of microscope and enlargement calibration error. (b) Ratio of uncorrected values for distances from Table I. (c) Ratio of corrected values for distances from Table I.

§ Based on an average tubulin subunit size of 41 Å.

apart. There are a total of 21 subunits in one major spoke period.

The central sheath period is not an integral multiple of tubulin subunit distance. The fixed ratio between the two quantities is 1:3.5. We assume that the sheath projections contain sites to which the spoke heads intermittently attach. If in morphogenesis the first head attachment site is aligned to spoke 1 of a doublet at the base of the cilium, all spoke 1 and spoke 2 sites will be in identical alignment with attachment sites, but all spoke 3 sites will be equally misaligned. It seems likely that such a finely regulated vernier would not only be useful in morphopoiesis, but might also be functional, in that sliding and bending could be regulated by relative displacements of spokes in relation to attachment sites at the sheath. The difference in alignment of spokes 1 and 2 vs. spoke 3 would be critical in this regard. Spoke 3 would be in alignment with the sheath projection period if the doublet moved relative to the sheath by about \pm 62-81 Å. Within one spoke period, ΔI 's of such magnitude might be measured by changes in alignment of spoke 3, such as those we now discuss.

The Significance of Radial Spoke Tilt θ

In this study we have described several different appearances of the basic spoke period with regard to θ . These are diagrammatically summarized in Fig. 5. All angles θ are measured with respect to changes from the perpendicular direction (Fig. 17). The parallel 1 and parallel 2 spoke configurations occur when spoke 3 is found roughly parallel to spokes 1 and 2 and all spokes are normal to subfiber A ($\theta_{S_{1-3}} = 90^\circ$). Parallel spoke configurations (Figs. 4, 11) occur in straight regions and near the proximal (trailing) edges of bends (Fig. 15, cilium 2, spoke period no. 1). Distinctive tilted spoke configurations are also found: the TS3 configuration, where $\theta_{S1-2} = 90^\circ$, $\theta_{S3} = -9$ to -20° , characterizing straight regions only (Figs. 4, 18), and the maximum tilt configuration where θ_{S1-2} = -20 to -26° , $\theta_{ss} = -30^\circ$ to -33° , characterizing bent regions only (Fig. 17, spoke period no. 6, concave). The clear correlation of maximum tilt and TS3 configurations with bent vs. straight regions suggests that these configurations are important intrinsic features of these regions with physiological significance. It is very unlikely that such specific patterning could be artifactual in the sense that it had no relation to the ciliary beat.

The TS3 configuration can be accounted for by simple geometry if spoke 3 is angled so that the spoke head is aligned with the proximal head attachment site at the central sheath. The TS3 configuration is therefore one in which all spoke heads appear in direct alignment with head attachment sites (Fig. 5 c) so that presumably some attachment must be possible. There is no simple way to account for any systematic spoke tilt at all in either straight or bent regions if spokes are never attached to the central sheath sites. Permanent attachment to these sites is clearly ruled out by our findings since distal to a bend this would require increasing permanent tilt and stretch of spokes as Δl increased with progressive sliding (Table III) (Satir, 1972; Warner, 1972). The maximum spoke angle (spoke 3, maximum tilt configuration) observed is about 33° ($\Delta l = 247$ Å) which would necessitate (as in Table III) a spoke stretch of 73 Å or 19%. Values greater than this have never been observed. Therefore the TS3 position must be an intermittent one in straight regions. One can readily construct a cycle of detachment-reattachment of spoke heads to attachment sites at the central sheath whereby in a straight region spokes move from the TS3 to successive parallel configurations and back to TS3 as sliding proceeds. We visualize the TS3 configuration to occur because as the moving microtubule slides past successive sheath attachment sites, spoke 3 heads have a tendency to adhere to these sites so that the parallel 2 configuration converts into TS3.

The maximum tilt configuration in bent regions is accounted for by continued distally directed movement of the microtubule while all spokes remain firmly attached to the sheath, which produces progressive distortion from the TS3 position. The amount of movement from the TS3 position necessary to give the angles observed in the maximum tilt configuration is 1.29 sheath periods (185 Å) (Fig. 5). The origin of spoke 3 is thus out of alignment with its head attachment site by 1.73 sheath periods (247 Å) and, therefore, is stretched maximally by about 73 Å. The origins of spokes 1 and 2 are out of alignment with their head attachment sites by only about 1.3 sheath periods and are less extended.

Consider the edge of developing bend, where proximal spokes are in the maximum tilt configuration and distal spokes are in a TS3 configuration. The distance between the head of spoke 3 of the last maximum tilt period and the head of spoke

1 of the first TS3 period must be four sheath periods instead of the usual three between adjacent TS3 periods. The strain on spoke 3 of a maximum tilt unit could be lessened if the spoke head detached so as to move distally by about one-half period; this would produce the parallel tilt configuration shown in Figs. 5, 17, and 18, where all spokes are moderately tilted in the bent region (Fig. 17, spoke period no. 3, concave side). Finally, new sliding could convert the TS3 unit adjacent to the bent region into a maximum tilt unit. The attachment of spoke heads to sheath during this process may provide the sole or major source of resistance to sliding shear that must act along the axis of the cilium to produce bending. According to this postulation, when all spokes in a bent unit detached from the central sheath, the region of the unit would straighten.

Our results therefore suggest that there are cycles of radial spoke attachment associated with the development, maintenance, and possibly the propagation of axonemal bends. One probable major cycle is illustrated in Fig. 5, but other variations may also occur. Our micrographs provide the first direct evidence concerning the mechanism of bend production within the axoneme. Particularly, we conclude that in straight regions spokes are functionally detached, in that extensive proximal sliding produces no angular changes in spokes 1 and 2 and little change in spoke 3, while in bent regions spokes are functionally attached to sheath sites, in that sliding produces pronounced angular changes. The details of the conversion between bent and straight regions outlined above of course will await further verification. It is nonetheless clear that the vernier arrangement and intermittent attachment of spokes embody enough flexibility to account for significant feedback features of ciliary motion, including the control of radius of curvature of bends (Satir, 1967).

Coordination of the Radial Spoke Cycle and Other Questions

We have presented evidence in favor of a detachment-reattachment cycle of radial spoke heads relative to the central sheath projections in the ciliary axoneme, and have suggested that this cycle plays a crucial role in bend formation and propagation during ciliary beat. The cycle would involve repeated distortion of radial spokes and delicate controls of attachment strength at the spoke head. Very little is known about the chemistry of spoke and sheath material, and nothing is known about the molecules involved in attachment. However, recently some evidence has appeared regarding a dynein-like ATPase not located in the doublet arm. Particularly, dynein has been shown to be present in two forms within the axoneme, one form of which is stoichiometrically reduced by 50% when the outer arms are removed (Linck, 1973 b). It is reasonable to assume that the remainder of this form is associated with the inner arms and that the second form of dynein is elsewhere, perhaps in the spoke heads or the sheath projections. Similarly, histochemical studies have shown the localization of an ATPase reaction product not only near the dynein arms, but near the spoke head-sheath interface as well (Anderson and Personne, 1968; Burton, 1973). Such findings would provide an energetic basis for the spoke cycle.

Model cilia where the outer dynein arms are removed beat with only half the normal frequency but with normal bending (Gibbons and Gibbons, 1973 a). This result is consistent with our conclusions that the spoke cycle is responsible for bend formation in 9 + 2 cilia and that stable attachments between spoke heads and sheath attachment sites are formed in bent regions. It would be interesting to know whether complete detachment of interdoublet dynein disturbs the bend form of axonemes in rigor (Gibbons and Gibbons, 1973 b). The spokes are intact in such models.

In gill cilia, our results show that sliding displaces spokes far from the bent region. This suggests that active sliding may not be local (Brokaw, 1971; Rikmenspoel, 1973; Satir, 1973). A reasonable alternative proposal is that sliding of a particular doublet occurs by cyclic dynein arm interaction along the entire doublet length. This is consistent with present conceptions of myosin cross-bridge activity in muscle, as well as with the behavior of trypsin-treated flagellar models (Summers and Gibbons, 1971). Under this alternative, sliding would be all or none; to produce motion, a particular doublet would slide actively against its neighboring doublet only during a certain phase of the beat cycle.

In our model, asynchrony of active sliding is necessary to explain how changes at the ciliary tip could occur when a proximal bend develops on an axoneme possessing an already completed propagating distal bend. Although we cannot as yet strictly relate our present observations to phases of the beat cycle, independent sliding movements of opposite doublets may occur in sequential stages that could not only produce two bends in opposite directions on the same axoneme, but also could regulate radius of curvature of the bend.

Bent and straight regions of the axoneme are intrinsically different, at least with respect to spoke attachment. We interpret this to imply that although sliding may be all or none, bending is locally regulated. Bending originates near the base of the cilium when attached spokes are distorted maximally by sliding. It would appear that for these cilia, active sliding and bend propagation are separable phenomena (Satir, 1972), in that the latter phenomenon involves local adjustments of alignment that do not reflect activity of the dynein arms, while the former requires hydrolysis of ATP by the dynein arms.

Asynchrony of sliding activity is picked up in our experiments as a variable relationship between spoke angle (θ) on opposite sides of the axoneme. This is particularly illustrated by comparing Fig. 17, where the spokes are aligned on opposite sides of the central sheath, with Fig. 19, where the spokes appear as chevrons. The chevron position indicates that sliding in the same direction has occurred at opposite sides of the axoneme. With the reservations noted previously (see Observations), we believe that this probably defines the direction of active sliding between adjacent doublets: towards the ciliary tip.

Sliding-Bending and the Interdoublet Links

The presence of an interdoublet or nexin link connecting adjacent doublets near the region of the inner dynein arm (Fig. 3) has been pointed out numerous times although their precise structure and organization remain elusive (see Warner, 1974, for a discussion of these structures). Summers and Gibbons (1971, 1973) point out that the partial digestion of axonemes with trypsin disrupts both the interdoublet (nexin) links and the radial spokes at about the same time as the axoneme becomes susceptible to disintegration in the presence of ATP. We have not yet studied the interdoublet links in detail, but reasoning similar to that presented in our discussion of the radial spokes suggests that the links must be inherently detachable at one end or else be elastic to a seemingly improbable degree to permit doublet sliding (Warner, 1972). Importantly, it is not yet

clear if the interdoublet links are a connection associated functionally with the inner dynein arms.

If the links are unrelated to the inner arms, their presence might considerably increase the complexity of macromolecular interaction during the sliding-bending cycle: the links must be displaced in some manner to accommodate interdoublet sliding. Data from columns α , θ and Δ_s (%) from Table III are also applicable to the interdoublet links $(d_{3-4, 7-8})$ since the values d and s for the links is exactly one-half of the same values for the radial spokes. For example, maximum interdoublet displacement (doublets 3-4, 7-8; Warner, 1972) for a cilium with a bend angle of 100°, from Eq. 1, is approximately 314 Å (d = 180 Å). From Eq. 2, the interdoublet link would have to stretch by about 177 Å (93% increase in length) to accommodate this displacement. And while a 100° bend angle is about the maximum that occurs in cilia, many sperm flagella may have bends of up to 180°. Evidence exists, however, suggesting that considerable stretching of interdoublet connections might be possible. Dallai et al. (1973) describe interdoublet connections between the respective A and B subfibers in the non-9 + 2 axonemes of Sciara sperm flagella. When negatively stained these connections still join adjacent doublets even when the A and B subfibers are separated by as much as 2,000 Å (our measurements). The normal doublet separation in sectioned Sciara sperm is only about 180 Å. Similar (and perhaps artificial) elasticity has never been seen in negatively stained radial spokes, and the stretch capability (spoke angle θ) observed in our study, even if a real property of the spokes per se, does not exceed 20%. If linkage stretching such as that observed in Sciara were shown to be a naturally occurring property of the interdoublet or nexin links of 9 + 2 axonemes, it might obviate the necessity for a complex cycle of detachment-reattachment for these structures.

At this time the contribution of the interdoublet links to the sliding-bending interconversion is unknown. However, it should be mentioned that non-9 + 2 sperm tails and microtubule bundles such as the axostyle of termite flagellates are capable of producing at least some forms of propagated bending without participation of the elaborate radial spoke-central sheath interaction of 9 + 2 axonemes (see Philips, 1974, for discussion of non-9 + 2 axonemes). Unfortunately, the differences in bend form and propagation between other microtubule-based systems and true cilia and

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flagella, including 9 + 2 sperm tails, have not yet been analysed. The widespread occurrence of 9 + 2 axonemes throughout the phyla may be taken as evidence of the evoluntionary efficiency of the pattern (Satir, 1962), including the radial spoke cycle as described, in producing regulated bending motion in these organelles.

SUMMARY

With respect to the sliding microtubule model of ciliary motion, we have shown the following: (a) Local contraction of the doublet microtubules is absent in bent, as well as in straight regions of the axoneme. (b) Sliding displacements (ΔI) accumulate in bent regions as the bend angle (α) increases. The amount of sliding per unit of microtubule length is constant throughout the bend. (c) ΔI is constant throughout straight regions, both proximal and distal to a bend. These conclusions are consistent with the sliding model and serve to verify important predictions of the model. With respect to the structure of the ciliary axoneme in our material, we have shown that: (a) The central sheath consists of four rows of projections, one α and one β row per central microtubule. The inclination of the two rows is such as to produce a A configuration on cm_3 and a \vee configuration on cm_8 , which permits identification of these structures in thin sections. (Additional information from transverse sections and negative staining is needed to exclude the residual possibility that the central sheath might consist of more than four rows of projections.) (b) The α , β projection repeat is 143 Å. (c) Each cradial spoke group in Elliptio cilia consists of three spokes with an overall repeat of 860 Å and subperiods of 285/205/370 Å. (d) Accordingly, a precise vernier of 6:1 is formed between sheath and spoke group periods, such that when spoke I of one group is in alignment with a sheath projection, all spokes 1 and 2 along a particular doublet are also in alignment with corresponding projections, and all spokes 3 are misaligned.

With respect to the attachment and inclination of radial spokes, we conclude the following: (a) In straight regions, both proximal and distal to a bend, spokes occur in two basic configurations: parallel configurations, where all spokes lie normal to subfibers A, and the tilted spoke 3 configuration, where spoke 3 is inclined toward the nearest proximal sheath projection. (b) In bent regions, additional configurations are observed where all spokes are inclined. The maximum tilt configuration occurs on the concave side throughout part of the bend, particularly at the leading edge. The parallel tilt configuration occurs near the trailing edge of the bend. (c) Spokes in straight regions distal to a bend are functionally detached as sliding proceeds. Spokes behave as if they were continuously attached on the concave side of the bend at the transition between bent and straight regions.

On this basis, we postulate cycles of radial spoke configuration changes that occur during bending. We finally conclude that our evidence supports the hypothesis that: (d) The radial spokes are a main part of the transduction mechanism that converts interdoublet sliding into local bending in 9 + 2 cilia.

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