Cilia and Flagella of Eukaryotes

I. R. GIBBONS

> The simple description that cilia are "contractile protoplasm in its simplest form" (Dellinger, 1909) has fallen away as a meaningless phrase . . . A cilium is manifestly a highly complex and compound organ, and . . . morphological description is clearly only a beginning.
>
> Irene Manton, 1952

As recognized by Irene Manton (1) at the time that the basic 9 + 2 structural uniformity of cilia and most eukaryotic flagella was first becoming recognized, these organelles are sufficiently complex that knowledge of their structure, no matter how detailed, cannot provide an understanding of their mechanisms of growth and function. In our understanding of these mechanisms, the substantial advances of the intervening 28 years have, for the most part, resulted from experiments in which it has been possible either to correlate changes in structure with the changes in waveform that occur during a normal beat cycle, or to make experimental changes in the structure, chemical properties, or mechanical loading of the organelle, and then relate these to the consequent changes in its motility. Research has thus tended to concentrate on organisms in which the cilia or flagella are suitable for study by as many techniques as possible.

The number of organisms that readily yield sufficient cilia or flagella for protein chemistry is relatively small. Among these, the flagella of sea urchin spermatozoa have proved the most favorable for many purposes because their length of 40–50 μm is sufficient for detailed measurement of wave parameters, and they are rugged enough for the demembranated flagella to be reactivated easily with adenosine triphosphate (ATP). The availability of mutants with altered flagella makes *Chlamydomonas* also a highly favorable material, although observation of the detailed pattern of motility is more difficult because the flagella are only 12–15 μm long. Cilia of *Tetrahymena* have been used as a source of ciliary proteins, and cilia of molluscan gills can also be obtained in good quantity, but, in both cases, observations of their motility are difficult to achieve, and have been useful mainly for investigating conditions that induce ciliary reversal or arrest. For experiments that do not involve protein chemistry, a broader choice of organisms is available, including protozoa with a wide range of motility patterns and metazoa whose sperm flagellar structure differs substantially from the usual 9 + 2 organization.

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Early Developments

Among the most notable steps in the history of early studies on cilia and flagella were the initial light microscope observations of beating cilia on ciliated protozoa by Anton van Leeuwenhoek in 1675; the hypothesis proposed by W. Sharpey in 1835 that cilia and flagella are active organelles moved by contractile material distributed along their length rather than passive structures moved by cytoplasmic flow or other contractile activity within the cell body; and the observation in 1888–1890 by E. Ballowitz (2) that sperm flagella contain a substructure of about 9–11 fine fibrils which are continuous along the length of the flagellum (Fig. 1). More detailed accounts with full references to this early work and to other studies before 1948 can be found in the monographs of Sir James Gray (3) and Michael Sleigh (4). Several of the observations and hypotheses that are often regarded as recent were anticipated in this early work. However, it is fair to note that it is only in retrospect that the significance of these findings becomes apparent, and that they were by no means generally accepted at the time because the techniques required to confirm and extend them were, of course, not then available.

The foundations for many aspects of the more recent work on cilia and flagella were established, largely independently of each other, in the period 1949–1955. The use of dark-field light microscopy to study sperm motility was initiated by Lord Rothschild and Michael Swann in 1949 (5), and was extended in 1955 to photographic recording of the waveforms of the single flagellum of sea urchin spermatozoa by Sir James Gray (6). Formulations for calculating the hydrodynamic forces resulting from different flagellar waveforms were developed in 1951 by Sir Geoffrey Taylor (7), and were further developed and shown to account for the translational velocity of sea urchin spermatozoa by Gray and G. Hancock (8). The basic cylindrical 9 + 2 structural organization of the axoneme was first deduced in 1949 by G. Grigg and Allan Hodge from studies of splayed flagella of cock sperm (9). In 1952 the widespread uniformity of this 9 + 2 organization in cilia and eukaryotic flagella was recognized for algae and lower plants by Irene Manton and G. Clarke (10), and for cilia of invertebrate and vertebrate animals by Don Fawcett and Keith Porter (11); within a year, however, the occurrence of variations in this basic pattern in the sperm flagella of some animals had been shown by C. Challice (12) and by John Bradfield (13). The work of Hartmut Hoffmann-Berling (14) in 1955 showed that it was possible to reactivate flagellar motility by addition of exogenous ATP to cells in which the selective permeability

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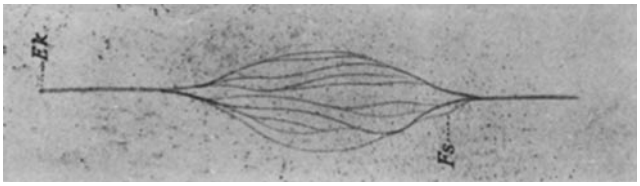

FIGURE 1 Drawing showing the substructure of the flagellar axoneme in a spermatozoon of the chaffinch, *Fringilla caelabs*. 11 "elementary fibrils" (Fs) are depicted in the frayed axoneme and presumably correspond to the 9 + 2 tubules known today. Additional density may have been contributed by the nine peripheral fibers associated with the nine doublet tubules in sperm of this species. The basal body (EK) is shown at the proximal end of the axoneme. The preparation was made by maceration of unfixed spermatozoa and stained with Gentian violet. Ballowitz, 1888 (2).

of the membrane has been destroyed by treatment with 50% glycerol. Studies of *Chlamydomonas* by Ralph Lewin in 1952–1954 (15–17), showed that mutants could be obtained with paralyzed flagella and laid the basis of complementation experiments in which the motility of the paralyzed flagella is rescued in the dikaryon formed by mating the mutant cells with those of wild type. In related studies at approximately the same time, Lewin demonstrated that uniflagellate cells of *Chlamydomonas* attached to a glass surface would glide steadily across the surface, flagellum leading, apparently because of interactions between the flagellar membrane and the axoneme within it. This work also provided a basis for the study of flagellar regeneration by showing that *Chlamydomonas* that had resorbed their flagella would grow new flagella within 90 min of being transferred to liquid medium in the light.

Knowledge of the principal proteins responsible for motility in cilia and flagella lagged behind the developments mentioned above by about 10 years. Several workers in the 1940s and 1950s, including V. Engelhardt (18), S. Burnasheva (19), Leonard Nelson (20), Hideo Mohri (21), Jack Tibbs (22), Frank Child (23), and Sir John Randall and co-workers (24), developed procedures for isolating flagella from spermatozoa of various animals and cilia from *Tetrahymena*, and demonstrated the presence of ATPase activity in the isolated organelles. However, attempts to characterize the axonemal proteins were hindered by the assumption that they were closely related to actomyosin from muscle, and by their apparent insolubility under mild conditions. In 1963, Ian Gibbons (25), using cilia isolated from *Tetrahymena*, found that this apparent insolubility was due to the ciliary membrane surrounding the axoneme. After the membrane had been removed with digitonin, the ciliary adenosine triphosphatase (ATPase) protein was extracted selectively and found to have properties very different from those of the muscle ATPase, myosin.

Knowledge of the physiology and biochemistry of cilia and flagella prior to the early 1950s is well summarized in the classic monographs of Sir James Gray (3) and of Thaddeus Mann (26). Among the many surveys of subsequent work are the 1962 monograph of Michael Sleigh (4) and its successor volume of review articles published in 1974 (27), the second edition of Mann's monograph (28), and the valuable reviews of sperm motility by David Bishop (29) and those of structure and function by Keith Porter (30), Don Fawcett (31), and Peter Satir (32). Detailed reviews of more recent work on ciliary and flagellar motility include those of John Blake and Sleigh (33), Michael Holwill (34), and Joseph Blum and Michael Hines (35).

Structure

To a large extent, the increasing knowledge of the structure of cilia and flagella during the past 28 years is the result of continued improvements in the techniques of specimen preparation for electron microscopy and for analysis of the resultant images. Although careful interpretation enabled a correct reconstruction of the basic axonemal structure of a cylinder of nine doublet tubules surrounding two central tubules from shadow-cast samples of splayed axonemes (Fig. 2) (1, 9, 10), this structural organization was more directly apparent to Fawcett and Porter in 1954 (11, 37) when they used the newly developed technique of thin sectioning to examine various ciliated epithelia. In addition to observing the 9 + 2 organization, they were able to say that only the nine outer tubules were doublets, whereas the two central tubules were singlets, and also to determine that the plane of beat was perpendicular to the plane of the central tubules (Fig. 3 *a,b*)

The enhanced contrast obtained by heavy-metal staining substantially increased the amount of structural detail visible in the axoneme. In 1959, Bjorn Afzelius (38) used a 40% solution of OsO_4 in CCl_4 to fix sea urchin spermatozoa, and was able to visualize an irregular double row of arms along one side of each outer doublet tubule in the axoneme, as well as sets of radial spokes that linked the arm-bearing component of each doublet to an undefined structure in the central region of the axoneme. Afzelius noted that the asymmetrical position of the arms made it possible to number the outer doublets in an unamibiguous manner (Fig. 3 *c,d*)

In the following year, Gibbons and A. V. Grimstone (42) obtained a further improvement in preservation and contrast by using epoxy resin, as developed by Audrey and Richard Glauert, to replace methacrylate as an embedding medium, and by staining the cut sections on a solution of uranyl acetate in 50% ethanol. Application of this procedure to flagellated protozoa confirmed the presence of most of the additional axonemal structures reported by Afzelius. In addition, it disclosed a more regular double row of arms along one side of each outer doublet tubule, a region of increased density near the middle of each of the nine radial spokes that was interpreted as a cross section through one of a set of longitudinally oriented "secondary fibers," and the presence of a central sheath, considered possibly helical, enveloping the two central tubules as reported earlier by Manton (1, 10, 36), but not previously observed in sectioned material. The large number of flagella in these protozoa and the regular arrangement of their attachments to the cell body made it possible to give a substantially more detailed account of both the structure of the basal body at the cytoplasmic end of each flagellum, and the transition zone between the basal body and the shaft of the flagellum. The basal body consists of a cylinder of nine triplet tubules, with the plane of each triplet skewed in toward the center of the basal body, and a cartwheel-like structure in the lumen of the proximal portion of the basal body. The three component tubules of each triplet in the basal body were designated as A, B, and C (Fig. 4 *a,b*). In the transition zone between basal body and flagellum, the C tubules terminate, whereas the A and B tubules continue into the flagellar shaft where the A tubule of each doublet acquires the double row of arms. The two central tubules of the flagellum terminate in the upper portion of the transitional region, and do not continue into the basal body.

A study of the gill cilia of the lamellibranch *Anodonta* (39) showed that the structural organization of the cilia, transition regions, and basal bodies was generally similar to that in the

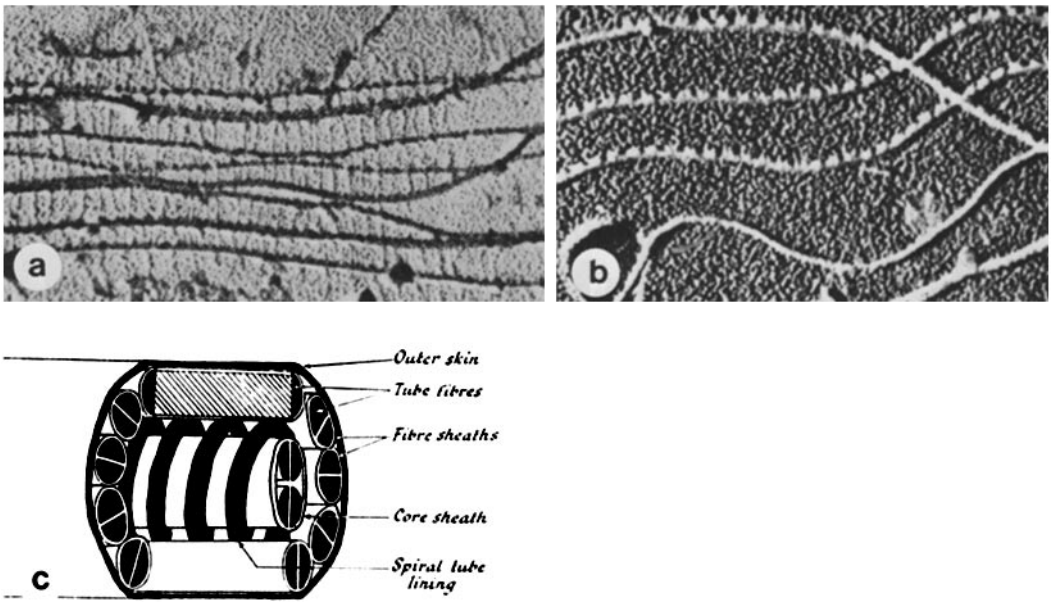

FIGURE 2 (*a*) Disintegrated flagellum of seaweed *Pylaiella*. Shadow-cast preparation showing "ladders" between doublet tubules, now interpreted as stretched nexin links. Manton, 1954 (36). (*b*) Same, with flagellum of moss *Sphagnum*, showing battlements on doublet tubules, now interpreted as paired radial spokes. Manton and Clarke, 1952 (10). (*c*) Diagrammatic reconstruction of flagellum of *Sphagnum* spermatozoid, prepared on the basis of *b*, and other contemporary micrographs. Manton and Clarke, 1952 (10).

flagellated protozoa; however, there were some differences in the pattern of linkages in the transition region; in the presence of a dense basal plate that ran across the lumen of the cylinder of doublets out to a constriction of the ciliary membrane, appearing to seal off the intraciliary matrix from the general cell cytoplasm; and in the presence of paired cross bridges spanning the gap between the central tubules in the cilia. The cilia also had a more definite structural polarity (Fig. 3 *e,f*), as indicated by a cross bridge between one particular pair of doublets (nos. 5 and 6) and a cross-striated, conical "foot" projecting from one side at the basal body. In all four types of ciliated cell on the gill epithelium, the direction of effective stroke in the ciliary beat cycle was toward the 5–6 bridge in the cilia and the foot on the basal body, with the plane of beat perpendicular to the plane of the two central tubules as reported previously by Fawcett and Porter (37).

The position of the arms on one side of the doublet tubules in cilia and flagella and the inward skew of triplet tubules in basal bodies give the structure an enantiomorphic asymmetry. In the studies of flagellate protozoa and gill cilia discussed above, as well as in a variety of other organisms surveyed (42, 43), the arms on the doublets have been found always to point clockwise, and, correspondingly, the triplets of a basal body are always skewed inward passing clockwise. (All orientations of structure and movement in this review are given as they would be seen by an observer looking outward along the organelle from its basal end.)

Application of the negative contrasting procedure, first described by Cecil Hall in 1955 (44) and developed for viruses by Sidney Brenner and Robert Horne, to the study of cilia and flagella enabled Jean André and Jean-Paul Thiéry (45) and Daniel Pease (46) to determine that the walls of flagellar tubules consist of longitudinally oriented protofilaments that were about 4 nm wide, and had a periodicity of about 8 nm along their length. More detailed information was obtained by Grimstone and Aaron Klug (47), who used optical diffraction of electron microscope images to analyze the arrangement of subunits in the walls; they reported that the surface lattice had a basic repeat of 4.0 × 5.0 nm, with a displacement in the relative radial positions of alternate subunits giving the actual repeat of 8.0 nm. Further development of the optical diffraction procedure by David DeRosier and Klug permitted three-dimensional image reconstruction by computer analysis of the digitized image of an object with helical symmetry. Application of this procedure to the singlet tubule portion of the doublets near their tip by Linda Amos and Klug (48) indicated that the wall of the singlet contained 13 protofilaments, and that dimers in neighboring protofilaments formed a staggered arrangement, equivalent to the lattice with 8-nm periodicity reported earlier. These dimers are believed to correspond to the α- and β-subunits of the tubulin molecule. Reconstruction of the B-tubule lattice from optically filtered images showed that the B tubule is also made up of 8.0-nm dimers but differs from the A tubule in that the dimers are lined up obliquely at a shallow angle, rather than in a staggered array. X-ray diffraction studies of tubules have been invaluable in providing a calibration indicating that the basic longitudinal periodicity is 4.0 nm in hydrated tubules (49). The number of protofilaments in the walls of the doublet tubules can be counted in thin sections of favorable material, and it was shown by Lewis Tilney and coworkers (41) that this substructure appears particularly clearly with negative contrast in sections of material fixed with a mixture of glutaraldehyde and tannic acid. These observations clearly illustrated that the A component of the doublet is a complete tubule with 13 protofilaments, whereas the B component is an incomplete tubule with 10 or 11 protofilaments (Fig. 3 *i*).

In addition to information about the flagellar tubules, negative contrasting has also provided much information about the other structures of the axoneme, particularly about the radial spokes and the appendages associated with the central tubules. The radial spokes appear to be rigid structures attached

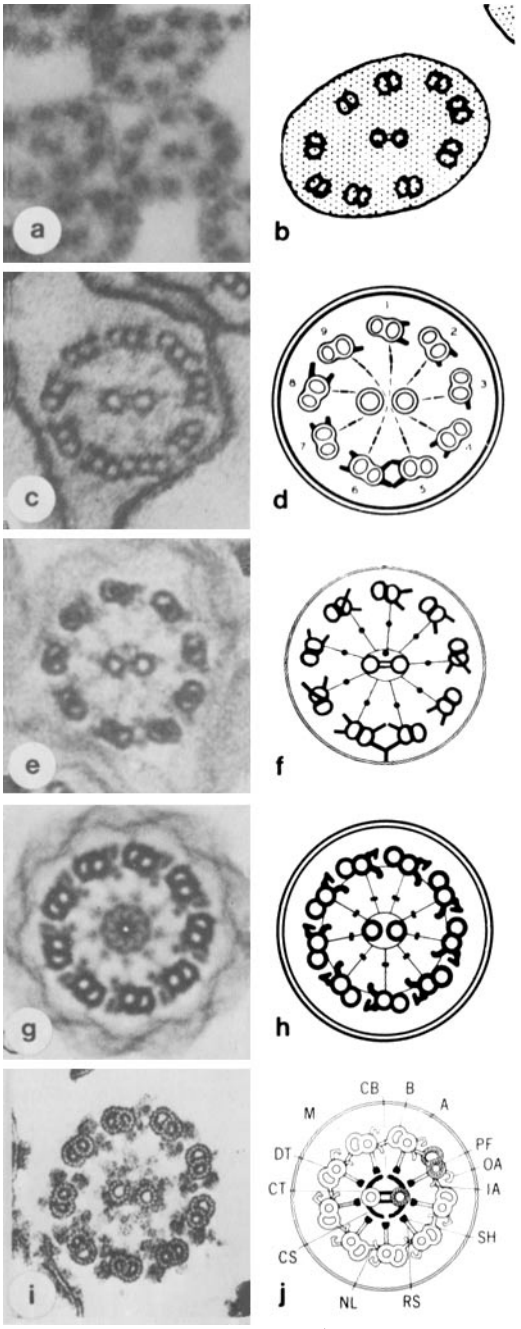

FIGURE 3 Increasing knowledge of flagellar and ciliary structure as depicted by electron micrographs of cross sections with contemporary diagrammatic interpretations. (*a*, *b*) Cilia on pharyngeal epithelium of frog. Plane of beat is vertical in figure. Fawcett and Porter, 1954 (37). (*c*, *d*) Flagellum of sea urchin spermatozoon. Afzelius, 1959 (38). (*e*, *f*) Lateral cilium of lamellibranch gill epithelium. Plane of beat is vertical in figure, with effective stroke toward bottom of page. Gibbons, 1961 (39). (*g*, *h*) Cilium of *Tetrahymena*, printed with ninefold Markham rotational translation. Structure of doublets and arms is reinforced. Structure of central tubules and central sheath which do not have ninefold symmetry is lost. Diagrammatic interpretation shows structure without rotation. Allen, 1968 (40). (*i*) Isolated axoneme from sea urchin sperm flagellum. Fixation with tannic acid and glutaraldehyde reveals protofilaments in tubule walls with negative contrast. Tilney and co-workers, 1973 (41). (*j*) Axonemal structures as currently known. M, membrane; DT, doublet tubule; A, A tubule of doublet; B, B tubule of doublet; OA, outer arm; IA, inner arm; PF, protofilament; CT, central tubule; CS, central sheath; CB, central cross bridge; NL, nexin link; RS, radial spoke; SH, spoke head. Modified from Holwill, 1977 (34). (With permission. Copyright by Academic Press [London] Ltd.)

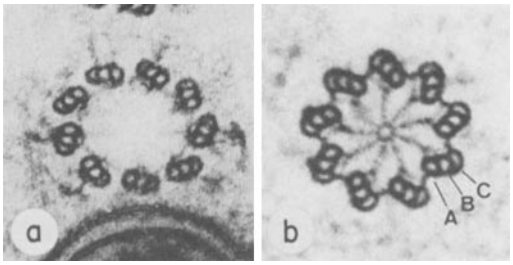

FIGURE 4 Cross sections of flagellar basal bodies in *Trichonympha*; (*a*) distal region; (*b*) proximal region. Gibbons and Grimstone, 1960 (42).

perpendicularly to the A tubule, and they are usually easily visible in axonemes where the tubules have splayed apart. In such specimens, the structures interpreted earlier as "secondary fibers" (39, 42) can be seen to consist of longitudinally oriented heads about 20 nm long, located at the centripetal end of each spoke. Although the heads on adjacent spokes sometimes appear to be in contact or to be joined by a thin fiber (50), the structures do not appear to have a general continuity along the length of the flagellum, and they are better described by the term "spoke heads." The studies of David Chasey, John Hopkins, Fred Warner, and others have shown that the spoke periodicity, originally given as about 27 nm (39), is in fact more complex and appears based on an overall repeat of 96 nm. In *Sphagnum* and *Chlamydomonas*, the spokes occur in pairs with alternate spacings of about 32 nm between members of a pair and 64 nm between adjacent pairs (10, 51, 52), whereas in *Tetrahymena* cilia, lamellibranch cilia, and rat sperm flagella, the spokes occur in groups of three, with spacings, passing from base to tip, of 32 and 20 nm between adjacent spokes, and 24 nm between adjacent triplets (Fig. 5*a*) (53–56). Regardless of whether the spokes on an individual doublet tubule occur as groups of two or of three, the spoke groups on the nine doublet tubules of the intact axoneme occur as a helix with a repeat of 96 nm. The structure in flagella of *Sphagnum* spermatozoids that Manton and Clarke (10) interpreted as a continuous helix, with a repeat of about 100 nm, situated between the outer doublets and the central tubules (Fig. 2*c*), is now interpretable as the discontinuous helix formed by paired radial spokes.

Recent studies of sectioned and negatively contrasted material have modified the description of the central tubule complex given by Gibbons in 1961 (39). The presence of paired, central bridges connecting the two central tubules at their nearest points, like double rungs on a ladder, has been confirmed in various cilia and flagella by Warner and others (56, 57). However, the helical structure described for the "central sheath" to which the radial spoke heads attach is incorrect, and from work originated by Chasey (55, 56), it is now clear that this structure is composed of two rows of projections arising from each central-pair tubule, with the projections on one tubule in close apposition to those on the adjacent tubule (52, 58).

The multiple photographic exposure procedure developed in 1963 by Roy Markham, S. Frey, and G. Hills (59) to enhance the visibility of periodic structures in electron micrographs has often proved useful in the study of cilia and flagella, particularly where the nature of the structure did not permit use of optical diffraction. This procedure was used with a ninefold rotation by Richard Allen in 1968 (40) to obtain substantially clearer images of the arms on the doublet tubules of *Tetrahymena* cilia. The resultant images showed that the outer and inner arms on each doublet had different profiles, with the

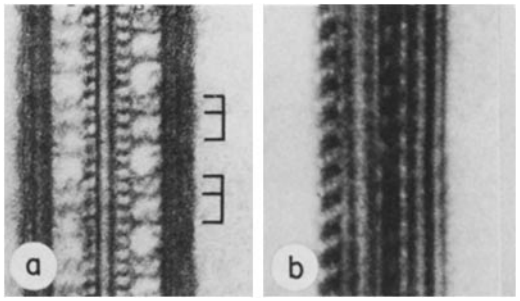

FIGURE 5 (*a*) Medial longitudinal section of lateral cilium of lamellibranch gill, showing triplet groups of radial spokes. Multiple-exposure Markham print with linear translation. Periodicity given as 86 nm at the time, but now believed to be ca. 96 nm. Brackets on right indicate two groups of triplets. Orientation is with ciliary basal end downward. Warner and Satir, 1974 (53). (*b*) Group of doublet tubules in ATP-disintegrated cilium of *Tetrahymena*, negatively contrasted with uranyl acetate. Note the basal tilt of the free arms on the doublet at left. Two-exposure Markham print with linear translation of 24 nm. Warner and Mitchell, 1978 (60).

outer arms extending out about 20 nm toward the membrane and then hooking sharply back toward the center of the axoneme, whereas the inner arm curved gently inward and had a small knob of increased density on its terminal end (Fig. 3 *g,h*); similar arm structures have been seen since then in cilia and flagella of many other species. The rotated images of *Tetrahymena* cilia also emphasized the presence of frequent connections between the doublet tubules and the ciliary membrane.

The arms on the doublets tend to become disrupted upon negative staining, and have been difficult to study with this procedure. Although in early studies the arms were reported to have a longitudinal periodicity of 13–16 nm (39, 42), most recent studies have found a periodicity of about 24 nm (51, 53, 60), and it has been suggested that the lower values obtained earlier may have been the result of superimposition of inner and outer arms staggered in their attachment by about half a period (55). However, the inner arms in *Chlamydomonas* have recently been reported to have a periodicity of 45 nm (61), whereas the structures thought to correspond to rows of detached outer arms from cilia of *Tetrahymena* had a periodicity of only 15 nm (62). The extent to which this confusion may be due to differences among different species is not yet clear. In the best-preserved preparations, the outer arms usually appear to be tilted relative to the longitudinal axis of the doublet (Fig. 5 *b*) (51, 60). The appearance of the arms changes with the angle from which they are looked at, and it may also vary depending on the presence or absence of ATP (60, 63).

In micrographs of thin sections of cilia and flagella fixed with most fixatives based on glutaraldehyde and/or OsO_4, the arms on the A tubule of each doublet extend only part way toward the B tubule of the adjacent doublet (Fig. 3). However, Nina Zanetti, David Mitchell, and Warner (64) have recently shown that, when fixation is performed in HEPES buffer with 5–10 mM Mg^{2+}, the arms appear to bridge completely the gap between the pairs of the doublet tubules. The relationship of these cross bridges to the transient cross bridges between doublets that are presumed to occur during normal movement (see below) is not yet clear.

In addition to the radial spokes and the arms, the axoneme is held together by a set of circumferential linkages that join the centripetal side of each doublet tubule to that of the next. These linkages are difficult to see in intact axonemes, and they were first noted in 1963 by Gibbons (25) in preparations of *Tetrahymena* cilia from which the arms, central tubules, and spokes had all been removed by chemical extraction (Fig. 6 *a*). Their presence was confirmed in intact cilia of *Tetrahymena* by Allen (40), and in sea urchin sperm flagella by Raymond Stephens (65), who tentatively identified them with a 160,000-dalton electrophoretic band and gave them the name "nexin." Studies on negative-contrasted material by Romano Dallai, F. Bernini, and Falco Giusti (66) and by Warner (57) showed that the nexin links are highly elastic, and that although their normal length is about 30 nm, they can be stretched to as much as 250 nm without breaking (57, 67). The longitudinal periodicity of the nexin links is about 96 nm, and, in retrospect, they can be seen clearly in the micrographs of shadowed flagella from *Pylaiella* spermatozoids published by Manton in 1954 (Fig. 2 *a*) (36).

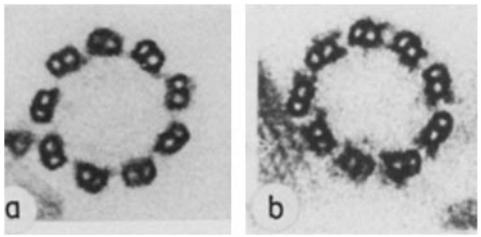

FIGURE 6 Cilia isolated from *Tetrahymena* and demembranated with digitonin. (*a*) Insoluble fraction after dialysis against 0.1 mM EDTA, 1 mM Tris/HCl, pH 8. Nexin links can be seen joining adjacent doublet tubules and are believed responsible for maintaining the integrity of the axonemal cylinder of nine doublet tubules after the central tubules, radial spokes, and dynein arms have been removed. (*b*) Same preparation after recombination with soluble ATPase fraction in presence of 2 mM Mg^{2+}; note reappearance of arms. Gibbons, 1963 (25).

The widespread uniformity of the 9 + 2 structural organization in cilia and flagella of different species is well known, and a current concept of the components visible in cross sections is presented in Fig. 3 *j*. However, variations do occur. The most common consist of additions exterior to the 9 + 2 structure, such as the mastigonemes found on many algal flagella (36), and the linkages that join the multiple axonemes of compound cilia (39, 68), rather than changes in the 9 + 2 structure itself. The sperm flagella of mammals, gastropods, and many insects contain an additional set of nine peripheral fibers situated centrifugal to the usual 9 + 2 axonemal core as well as an extension of modified mitochondria along much of their length. Whether the nine peripheral fibers are passive structures whose function is to strengthen the flagellum, or whether they contribute actively to flagellar motility is controversial. The studies of David Phillips (69) show that, among mammals, the sperm flagella of species in which the peripheral fibers are thickest have a relatively low amplitude of beating, suggesting that these fibers are major factors in flagellar stiffness; analysis of the isolated fibers by Baccio Baccetti, Vitaliano Pallini, and Anna Burrini (70) has shown that they are composed of a keratin-like protein with no detectable ATPase activity. The structure of the peripheral fibers, however, in electron micrographs appears similar to that of the single fiber in the undulating membrane of toad spermatozoa, and the work of Mario Burgos and Fawcett (71) has shown that this undulating membrane is motile with a beat frequency different from that of the flagellum proper. Similarly, the sperm flagella of several species of insect have been shown to propagate simultaneous bending waves of two different frequencies (72), suggesting the presence of two distinct motile mechanisms.

Variations in the basic 9 + 2 organization itself are less

widespread, although fairly numerous examples have been described (72, 73). Patterns reported in motile sperm flagella include 9 + 0, 9 + 1, 9 + 3, and 9 + 7; patterns of 12 + 0 and 14 + 0 have also been reported, but in these cases the doublets lack arms, and the spermatozoa are nonmotile. The 9 + 0 flagella in eel spermatozoa (74), and the 6 + 0 and 3 + 0 flagella in sporozoan gametes (75–77) are of particular functional interest (see below).

Composition

The first unequivocal characterization of the major protein components of cilia and flagella was performed by Gibbons and collaborators using cilia isolated from *Tetrahymena*. In a series of studies between 1963 and 1968, procedures were developed for isolation and characterization of the two major axonemal proteins, the ATPase protein responsible for mechanochemical energy transduction and the principal structural protein of the ciliary tubules, which account for about 15% and 70% of the total axonemal protein, respectively. A principal reason for the success of these experiments in opening up the field of ciliary and flagellar proteins for study was that the isolation of the cilia and the effects of successive extractions were monitored by the high-resolution electron microscopy permitted by heavy-metal staining of thin sections. This enabled the conditions to be adjusted to optimize the structural preservation of the cilia during their isolation and the selective removal of particular components during the successive extractions. After the ciliary membranes had been removed with digitonin, the proteins of the ciliary axonemes could be fractionated by dialysis against EDTA at low ionic strength, which solubilized almost all of the axonemal ATPase activity but only about 30% of the protein (25). Examination of the insoluble residue showed that it consisted of the outer doublet tubules alone, still largely arranged in cylinders of nine; the other structural components, including the arms, central tubules, and radial spokes, were almost completely removed (Fig. 6*a*). The axonemal structure could be partially reconstituted by restoring Mg^{2+} to the dialyzed preparation, which resulted in about half of the solubilized protein and ATPase activity becoming rebound to the doublet tubules. Electron microscopy showed that a high percentage of the arms had been restored to their original positions on the doublet tubules (Fig. 6*b*). This correlation of the presence or absence of ATPase activity with the presence or absence of the arms was taken to indicate that at least part of the axonemal ATPase was located in the arms.

Study of the physicochemical properties of the solubilized axonemal ATPase by Gibbons and Arthur Rowe (62) confirmed that its properties were quite distinct from those of the muscle ATPase, myosin; the name "dynein" (after dyne = a unit of force) was proposed for the axonemal ATPases and other related ATPases associated with microtubule systems. The dynein from *Tetrahymena* cilia occurred in two forms with sedimentation coefficients ($s^0_{20,w}$) of 14S and 30S, and average molecular weights of 600,000 and 5,400,000, respectively. Electron microscopy of shadow-cast particles showed that the 14S dynein consisted of globular particles measuring about 14 × 9 × 9 nm, whereas the 30S dynein consisted of rodlike particles of variable length, with a globular substructure repeating at a period of about 14 nm. Detailed examination of the recombination of the two forms of dynein to extracted axonemes indicated that only the 30S dynein was capable of rebinding and restoring the arms on the doublets, and that little 14S dynein became bound under the same conditions (78).

Extensive further studies have been performed on the enzymic properties of the two forms of dynein from *Tetrahymena*. The reports of Gibbons, of Blum and collaborators (35), and of Issei Mabuchi, Takashi Shimizu, and Ichiro Kimura (79) have shown that the ATPase activity of 30S dynein can be activated two- to sixfold by any of a number of treatments including high concentrations of salt, mild heating, acetone, SH reagents, and amino reagents, whereas the same reagents applied to 14S dynein usually cause only inhibition. Although 30S dynein can be broken down to 14S particles by sonication or by brief treatment with trypsin, the properties of these particles are not the same as those of the 14S dynein obtained directly by extraction at low ionic strength (35).

The development by A. Shapiro, E. Viñuela, and J. Maizel (80) of the technique of electrophoresis in polyacrylamide gels containing Na dodecyl SO_4, which enables easy analysis of the number and size of distinct polypeptides in a sample, had a major impact on studies of the composition of cilia and flagella. In 1970–1973, Richard Linck applied this technique to the study of axonemes and semipurified axonemal extracts containing 14S dynein ATPase from gill cilia and sperm flagella of the lamellibranch mollusc *Aequipecten*, and found that the presence of dynein ATPase activity was correlated with the presence of the upper of a closely spaced pair of slowly migrating electrophoretic bands with apparent molecular weights of 450,000–500,000 daltons (81). Subsequent studies have confirmed that at least a major part of the dynein ATPase copurifies with the slower migrating band, but have reported somewhat smaller values for the high molecular-weight polypeptides, in the range 300,000–350,000 daltons (82). The presence of one or more polypeptides in the 300,000–350,000 dalton range appears to be a characteristic property of dynein that distinguishes it from myosin and other ATPases.

Improvements in electrophoretic techniques, like improvements in electron microscopy, have revealed additional layers of complexity (Fig. 7). In 1976, Gibbons and colleagues (85) reexamined sea urchin sperm axonemes and were able to resolve four high molecular-weight bands in the same region as the two bands observed earlier. A further improvement in resolution was obtained by using the discontinuous-pH Na dodecyl SO_4 procedure of Ulrich Laemmli (86), and in 1979 Christopher Bell, Earl Fronk, and Gibbons (83) resolved as many as eight distinct high molecular-weight bands in this same region (Fig. 7*b*). A similar electrophoresis procedure applied to axonemes of *Chlamydomonas* flagella by Gianni Piperno and David Luck (84) resolved 10 bands with apparent weights between 300,000 and 330,000 daltons. Analysis of axonemes from spermatozoa of species in which the axonemal structure is simplified have shown a roughly parallel decrease in the number of high molecular-weight bands present (74). In these very high-resolution gel systems, the relative mobilities of different high molecular-weight bands vary even between closely related species, and it has not yet been possible to identify which bands correspond to functionally equivalent polypeptides in axonemes from different species.

Fractionation of the axonemal proteins containing the various high molecular-weight polypeptides, either by differential extraction (81, 85) or by chromatography on hydroxyapatite as used by Kazuo Ogawa and Mohri (89, 91, 92), has indicated that several, although probably not all, of these proteins have ATPase activity. Gibbons and colleagues have distinguished dynein 1 and dynein 2 as two electrophoretically distinct isoenzymic forms from sea urchin sperm axonemes (85, 88). Dynein 1, which constitutes the outer arms, can be solubilized

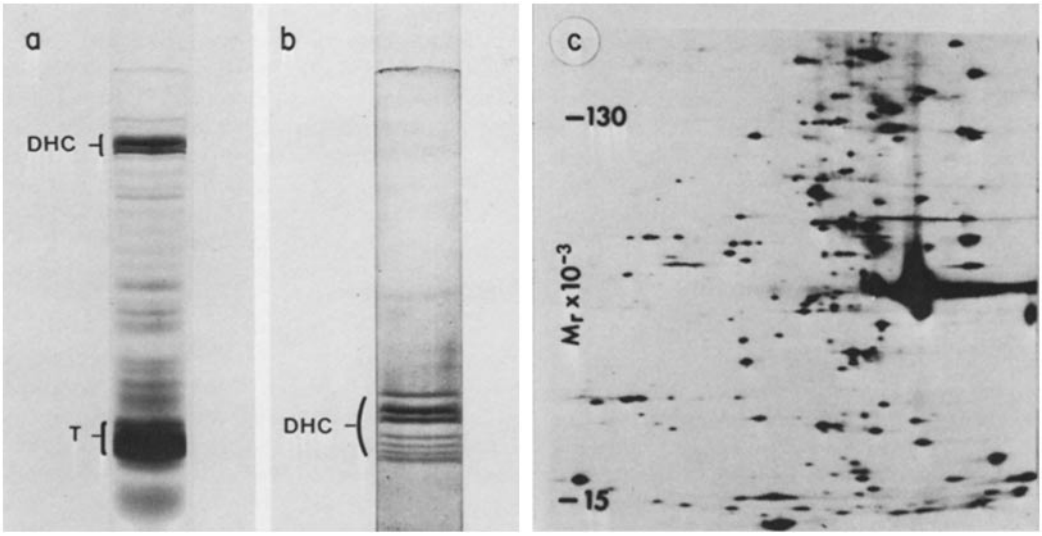

FIGURE 7 (a) Electrophoresis of axonemes from sea-urchin sperm flagella on 4% polyacrylamide gel in presence of 0.1% Na dodecyl SO_4, 50 mM phosphate buffer, pH 7.0. DHC indicates the group of dynein heavy chains migrating at a position corresponding to ca. 330,000 molecular weight; T indicates tubulin migrating at 55,000 molecular weight. Gibbons and Fronk, 1975 (unpublished). (*b*) Similar sample electrophoresed on Laemmli discontinuous pH Tris-Cl/Na dodecyl SO_4 system. Run was continued for twice time required for dye front to reach bottom of gel. Complex of high molecular weight bands containing dynein heavy chains now shows 8 bands. Bell, Fronk, and Gibbons, 1980 (83). (*c*) Autoradiogram of polyacrylamide slab gel used for two-dimensional separation of polypeptides in whole axonemes of *Chlamydomonas*. Horizontal separation between spots derives from isoelectric focusing (first dimension), with the more basic polypeptides lying on the right. Vertical separation derives from electrophoresis in the presence of Na dodecyl SO_4, and corresponds to differences in molecular weight. Only a portion of the original gel is shown, comprising the molecular weight range between 130,000 and 15,000 (see marker on left). The large streak is formed by the tubulin polypeptides, which are heavily overloaded in order to detect minor axonemal components. Piperno and Luck, 1979 (84).

as a 21S particle of 1,250,000 daltons with a complex polypeptide composition, and retains functional capability to rebind and restore the beat frequency of dynein-depleted sperm flagella (87, 90). This 21S form of dynein 1 can be dissociated into smaller particles that retain ATPase activity, but have lost the ability for functional recombination (87). Ogawa (91) has used trypsin digestion to isolate a 400,000 dalton fragment of dynein 1 that retains ATPase activity, but has lost the ability to rebind to dynein-depleted axonemes. An antibody prepared against this tryptic fragment inhibits the ATPase activity of dynein 1, but not that of dynein 2 (88, 92). The 14S and 30S dyneins from *Tetrahymena* cilia are also isoenzymic forms with electrophoretically distinct heavy chains (79). Dynein extracted from *Chlamydomonas* flagella by Takahiko Watanabe and Martin Flavin showed two forms sedimenting at 13S and 18S (93), and further chromatographic separation by Piperno and Luck (84) has indicated the presence of at least three isoenzymic forms of dynein with electrophoretically distinct heavy chains. In two *Chlamydomonas* mutants lacking outer arms, pf13 and pf22, the 13S and 18S ATPases are both missing, suggesting that the outer arms in these flagella contain two ATPase proteins. The set of polypeptides missing in a mutant lacking inner arms, pf23, shows no overlap with those missing in the outer arm mutants (61).

A characterization of the protein constituting the walls of ciliary tubules was begun in 1963 by Gibbons (25) and extended in 1966–1968 by Fernando Renaud, Rowe, and Gibbons (94, 95), who employed both acetone powders of whole cilia from *Tetrahymena*, and preparations of doublet tubules isolated by selective solubilization. The tubule protein was found to migrate as two closely spaced bands of equal intensity upon electrophoresis in polyacrylamide gels containing 8 M urea, to possess an amino acid composition resembling that of actin, and to exist as a 6.0S dimer of 108,000 daltons at low ionic strength and as a monomer of approximately 55,000 daltons in 8 M urea or in 5 M guanidine-HCl. Studies by Stephens, Renaud, and Gibbons (96) also showed that the dimer of tubule protein from cilia and sperm flagella contained 2 mol of mixed guanine derivatives, half of which were tightly bound. At this time, the tubule protein appeared to have many properties resembling actin, but shortly afterward the generally accepted weight of actin was revised sharply downward from 57,000 to 46,000 daltons (97), and it became clear that the two proteins were distinct.

At approximately the same time as the above work on cilia and flagella, Gary Borisy and Edwin Taylor (98) were studying the properties of a 6S colchicine-binding protein found in tissue culture cells and in several types of tissue containing high densities of microtubules, and they proposed that this protein was a subunit of microtubules. In a related study, Michael Shelanski and Taylor (99) used brief dialysis in the usual Gibbons fractionation procedure to isolate a 6S colchicine-binding protein from sea urchin sperm flagella, and identified it as the protein of the central tubules.

On the basis of its distinct amino acid composition, the protein of flagellar tubules was given the generic name "tubulin" by Mohri (100). Attempts to repolymerize tubulin from *Tetrahymena* cilia and from sperm flagella by Renaud et al. (94) and by Stephens (101) yielded only fibers and ribbons of protofilaments. Repolymerization of tubulin into intact tubules that had the same properties as naturally occurring tubules was first achieved with brain tubulin incubated in the presence of GTP at 37°C by Richard Weisenberg in 1972 (102). This discovery made it possible to purify tubulin by cyclic assembly/disassembly of tubules. An equivalent repolymerization of tubulin from cilia or sperm flagella was not achieved until

1976, when Ryoko Kuriyama (103) showed that tubulin solubilized from flagella by sonication would repolymerize under the standard conditions used to polymerize brain tubulin. The resulting tubules are singlets, and their stability is more like that of labile brain microtubules than that of flagellar doublets.

Although earlier workers had noted differences in the relative stability of different types of microtubules, these differences were first systematized by Olav Behnke and Arthur Forer (104), who distinguished four classes—cytoplasmic, ciliary or flagellar central pair, B tubule, and A tubule, in order of increasing stability. The subunit composition of the tubulin dimer was for a time confused with differences among these stability classes, but in 1971 several groups of workers independently showed that cytoplasmic tubules from a single source gave rise to two closely spaced bands of equal intensity when electrophoresed under appropriate conditions, and concluded that the 110,000 dalton 6.0S tubulin molecule was probably a heterodimer composed of α- and β-subunits (105). These α- and β-subunits were isolated electrophoretically from tubulin of chick embryo and of sea urchin spermatozoa by Richard Ludueña and Don Woodward (106), who then used cyanogen bromide peptide-mapping and a partial amino acid sequence to show that the α- and β-subunits were similar but distinct polypeptides, and that the sequence of each had been highly conserved during evolution. Microheterogeneity has been reported in the α- and β-chains of tubulins from different types of tubules and organelles (107), but it is not yet clear whether this results from multiple α- and β-tubulin genes or from posttranscriptional modifications.

In addition to the major components, dynein and tubulin, axonemes contain a large number of minor components present in relatively small quantity. The number of minor polypeptides is such that they can be resolved adequately only on a two-dimensional system (Fig. 7 *a*, *c*). The application of such techniques to flagella was pioneered by Piperno, Bessie Huang, and Luck (108), who analyzed ^{35}S-labeled axonemes from *Chlamydomonas* by an isoelectric-focusing/Na dodecyl SO_4-electrophoresis procedure modified from that developed by Patrick O'Farrell. In its present state of refinement, this procedure reveals as many as 180 polypeptides (Fig. 7 *c*) (84). Flagella from the paralyzed mutant pf14, which completely lacks radial spokes and spoke heads, are missing 12 polypeptides, whereas those from pf1, in which only the spoke heads are absent, lack 6 polypeptides that are a subset of the 12 missing in pf14. Subsequent study of flagella in which motility was rescued in the dikaryon formed by mating with wild type, together with analysis of UV-induced revertants, enabled identification of the two polypeptides that are the mutant gene products in pf1 and pf14 (109).

Piperno and Luck (110) have used chromatography on a DNase 1 affinity column to purify a component from *Chlamydomonas* flagella axonemes that appears identical with β-actin. It constitutes about 1.5% of the axonemal protein, corresponding to a molar ratio of 1:40 relative to tubulin and may be associated with one of the high molecular-weight polypeptides, but its significance in flagellar function is unknown. Other recent work by Gordon Jamieson, Thomas Vanaman, and Blum (111) has shown that chromatography on a chlorpromazine affinity column can be used to isolate calmodulin from *Tetrahymena* cilia. Calmodulin occurs partly associated with the 14S dynein fraction and is presumably involved in the mechanisms by which Ca^{2+} regulates the direction of beating.

Wave Parameters and the Hydrodynamics of Propulsion

As discovered by Gray in 1955 (6), the flagellar beating of marine invertebrate spermatozoa, such as those of sea urchin, is nearly planar and almost ideal for waveform analysis. When these spermatozoa encounter an obstructing surface, such as the bottom of an observation dish, they become trapped by it and, without actually being tethered, they swim in repeated circles, with their plane of flagellar beat parallel and close to the surface, and so remain constantly within the plane of focus. This circling movement makes possible extended observation of the wave parameters of an individual sperm flagellum; the degree of constraint on beating is much less than if the sperm head were tethered to the surface. Gray reported that the flagella propagated planar bending waves along their lengths at a beat frequency of 30–40 Hz, and that at certain stages of the beat cycle the flagellum had the form of a sine curve (Fig. 8 *a*), although there was an overall asymmetry in the degree of bending on the two sides of the flagellum. A subsequent analysis of sea urchin sperm flagellar movement by Charles Brokaw (113), who used a 100-μs flash to achieve improved spatial resolution, indicated that the waveform at any instant could be represented more accurately by a series of circular arcs joined by short, straight segments than by a sine curve; the departure of the flagella from a sinusoidal waveform is particularly evident in spermatozoa with tethered heads (Fig. 8 *b*, *d*) (112, 113). This "arc-line" waveform has been accepted by most subsequent workers as being a reasonably close approximation to actual flagellar waveforms, although it has often been noted that the curvature of bends at certain stages of the beat cycle is noticeably nonuniform (114, 115). An important basic parameter of the arc-line waveform is the total angle of each bend, for in a sliding-tubule mechanism (see below), the amount of sliding displacement in a bend is proportional to its angle. In situations where the arc-line curve does not represent a flagellar waveform with sufficient accuracy, the likelihood of significant end effects suggests that it may be more helpful to analyze the waveform numerically rather than by attempting to fit more refined analytical curves relatable to the underlying mechanisms involved.

The obvious importance of the motility of mammalian spermatozoa in both human and veterinary medicine has led to fairly numerous studies of their flagellar waveforms. The initial

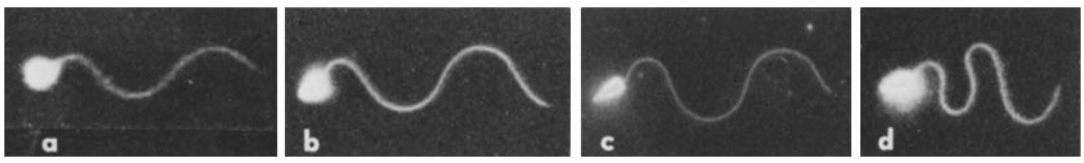

FIGURE 8 Flagellar waveforms of sea urchin spermatozoa. (*a*) Live spermatozoon in seawater. Species: *Psammechinus miliaris*. Gray, 1955 (6). (*b*) Live spermatozoon in seawater. Species: *Tripneustes gratilla*. Gibbons, 1974 (112). (*c*) Demembranated spermatozoon reactivated with 1 mM ATP. Species: *Tripneustes gratilla*. B. Gibbons, unpublished, 1980. (*d*) Live spermatozoon in seawater with head stuck to bottom of dish. Species: *Tripneustes gratilla*. Gibbons, 1974 (112).

studies of bull spermatozoa by Gray (116) and by Robert Rikmenspoel, G. van Herpen, and P. Eijkhout (117) showed that the amplitude of bending increases progressively along the tail toward the distal end, and that whereas the movement in the proximal region is planar, that of the distal region contains a significant three-dimensional component. More recent observations by David Phillips (69), and David Katz, R. Mills, and T. Pritchett (118) have shown that the pattern of flagellar movement in mammalian spermatozoa can undergo drastic change when they swim in close proximity to a surface or enter cervical mucus.

The motion of individual cilia on ciliated epithelia or on the surface of a ciliated protozoan is more difficult to visualize than that of flagella, partly because of their large number and close packing, and partly because the form of their beat is more complex. As a result of this difficulty, the early workers usually assumed that the motion of individual cilia was similar to that of the relatively large and easily observed compound cilia, which beat with a planar asymmetric movement composed of a rapid, effective stroke followed by a relatively slow recovery stroke. The first clear indication that this was not the case was obtained in an extended series of studies by Bela Párducz (119, 120). He modified the procedure for preserving ciliary waveforms by rapid fixation with a mixture of OsO_4 and $HgCl_2$ that had been developed in 1926–1927 by J. von Gelei, and used it to show that the beat of the body cilia in *Paramecium* is not planar, for, in the recovery stroke, the cilium sweeps out to the side and makes the overall beat cycle markedly three-dimensional, with the tip moving clockwise in an approximately semicircular path. This observation was not generally accepted at first because of the possibility that the rapid fixation might not be preserving accurately the waveform of the live cell. However, comparative studies of live and rapidly fixed waveforms have been made for *Opalina* by Sidney Tamm and Adrian Horridge (121), for the lateral cilia of *Elliptio* and of *Mytilus* by Peter Satir (122) and Edward Aiello and Michael Sleigh (123), and for *Paramecium* by Hans Machemer (124), and, in all cases, the three-dimensional waveforms seen in rapidly fixed preparations for light microscopy and scanning electron microscopy have appeared to be reasonably true representations of the waveforms in the live state. Somewhat surprisingly, the tips of the lateral cilia of *Mytilus* move in a counterclockwise direction (123), the mirror-image of that in *Paramecium*, in spite of the fact that structural enantiomorphism of the arms on the doublet tubules is clockwise in both cases (43). As emphasized by Párducz, the basic rotary motion of protozoan cilia is particularly evident in narcotized or moribund ciliates (e.g., *Paramecium*, *Colpidium*, and *Opalina*), in which, as the beat frequency falls to around 2 Hz, the cilia change from their normal beat pattern to a swiveling about their basal region in such a way that the ciliary shaft sweeps continuously around a wide-angled, conical envelope, moving clockwise (120).

Because of the close spacing of cilia on most ciliated cells, they need to move in a coordinated manner to work effectively. The coordination of cilia into propagated, metachronal waves used to be considered the result of a "neuroid" transmission process within the cell, but it is now generally thought to be the result of hydrodynamic forces acting on the autonomous beating of the individual cilia (124).

Sir Geoffrey Taylor appears to have been the first to realize that the propulsive forces of flagella and cilia result almost wholly from their viscous interaction with the medium (7), and that they cannot be modeled, even qualitatively, by the effect of the human arm in swimming in water. (Reynolds number, which is the ratio of inertial to viscous forces, has values of 10^{-4} to 10^{-6} for individual cilia and flagella, 10^{-2} to 10^{-1} for the body of ciliated protozoa, and of the order of 10^{4} for human swimming [33].) A rigorous treatment of the movement of flagellated microorganisms requires solution of Stokes equations with the appropriate boundary conditions. Equations appropriate for propulsion by flagellar waves of normal amplitude were developed by Hancock in 1953 (125), but the form of the equations is such that computation is not simple. This factor led Gray and Hancock (8) to develop a simplified computation based upon expressing the viscous force acting on a short element of flagellum in terms of normal and tangential coefficients of resistance, which is equivalent to assuming that the velocity field around the element is independent of the cell body and of the bending of other parts of the flagellum. They were then able to show that the forward velocity of the sperm computed from its observed flagellar waveform was in good agreement with that actually observed, and also to show that the viscous drag of the sperm head was small compared to that of the flagellum itself. This approach was extended by Brokaw (113, 126) to propulsion by flagella with nonsinusoidal waveforms of moderate asymmetry and showed that the computed time-averaged velocity and the angular velocity of yaw were both close to the actual measured values.

While the resistance coefficient model was being applied in this way, other workers were attempting to develop a more rigorously based hydrodynamic approach, and especially to consider the effect of the cell body on the fluid flow around the flagellum. A recent study of J. Higdon (127) used an iterative numerical procedure to consider the case of a spherical head propelled by planar sinusoidal waves and showed that minimal power consumption for locomotion of a given size head is obtained when the flageller length is 20–40 times the radius of the head, which agrees with the values found for actual spermatozoa. Comparison with results obtained using the resistive coefficient procedure indicated that the predicted swimming speed agreed within 10%, as was to be expected inasmuch as the predicted speed agrees with that of real sperm. However, the Gray-Hancock procedure appears to underestimate the power consumption by 30–50% for small cell bodies such as spermatozoa.

The hydrodynamic analysis of propulsion by large fields of cilia beating in metachronal rhythm requires a different approach from that of propulsion by a single flagellum. In the first approach to the problem by John Blake in 1971 (128), the ciliary motion was represented by a surface envelope containing the tips of the cilia, with the metachronal waves being modeled as nonsinusoidal undulations in this surface envelope. A second approach initiated by Blake (129) considers discrete cilia and involves calculating the velocity of fluid flow as a function of distance from the body surface, including both the ciliary sublayer and the exterior flow field. The calculated velocity profile for a spherical model of *Paramecium* is in reasonable agreement with the experimental observations of Theodore Jahn and J. Votta (130).

The substantial advances in hydrodynamic theory during the past few years have made it feasible to make detailed comparisons of the calculated and experimental propulsive velocities and flow fields associated with swimming and fluid propulsion by cilia and flagella in different organisms. Such comparisons may reveal something of the wide variety of ways that different organisms have exploited the basic uniformity of movement in cilia and flagella.

Theoretical Models

Various attempts have been made to create theoretical models that will reproduce the observed oscillatory beating of flagella and cilia as the result of balancing an active bending moment, M_a, which is dependent upon the parameters of bending, against the passive viscous and elastic resistances, M_v and M_e, according to the equation $M_a + M_v + M_e = 0$ at all locations along the length.

Initial work by Kenneth Machin (131) showed that waves generated by active bending moments located solely at the base of the flagellum would be highly damped by the viscous and elastic resistances distributed along the flagellar length, with the wave amplitude decreasing by 50% or more within half a wavelength of the proximal end. On the other hand, waveforms resembling those of real sperm flagella could be obtained by assuming generation of active bending moments by contractile elements distributed along the length of the flagellum, with these elements being activated by local bending after an appropriate time delay. Machin subsequently extended this work (132) to show that propagated bending waves could arise spontaneously on a flagellum, if changes in length of its contractile elements cause delayed changes in tension. The nonlinearities that must exist for the wave amplitude to remain finite were found to enable control of frequency and direction of propagation to be exercised from the proximal end, and indicated that two nearby flagella would tend to synchronize in frequency and phase.

The approach used by Rikmenspoel (133, 134) has been to balance the calculated external viscous resistance and the internal elastic bending resistance by an active moment specified as an arbitrary forcing function dependent upon time and position along the flagellum. A forcing function was found that reproduced the motion of a variety of cilia, but it required specifying two time constants as arbitrary parameters, as well as the observed velocity of bend propagation and the length of the bent region (133). In similar studies on the motion of flagella, Rikmenspoel reported that waves resembling those of sea urchin sperm flagella could be generated by a nonpropagated active moment varying sinusoidally with time, together with a propagated active moment of appropriate phase (133, 135).

Brokaw has developed several models of wave formation and propagation in flagella, most of which involve numerical solution of the equations of motion for a time-delayed active shear force proportional to curvature (136, 137). It was originally thought that four passive internal forces—viscous and elastic shear resistances and viscous and elastic bending resistances—were required to stabilize the motion, but more recent work has shown that the apparent need for viscous shear and bending resistances derived from problems with the numerical solution of the equations (35).

Investigations with these formal models have been useful in clarifying the constraints necessary for stable oscillations in a sliding-tubule system. However, more realistic models must consider the kinetic parameters of the cross bridges involved in producing sliding, and a thermodynamic framework for these parameters has been developed by Terrell Hill (138). The cooperative self-oscillating behavior of opposed cross-bridge systems is potentially interesting, because of the possibility of initiation and propagation of bending waves without need for control by a macroscopic variable such as curvature, but such models have so far been able to propagate bending waves only under conditions of high internal viscosity (139). More satisfactory results have been obtained with two-state cross-bridge models involving curvature-dependent rate functions, and Michael Hines and Joseph Blum (35, 140) have shown that such models will generate stable propagated waves with frequencies and amplitudes typical of sperm flagella. However, even the best of current models does not provide a completely satisfactory explanation of the mechanisms that control bending in flagella and cilia. They have particular difficulty in explaining the high curvature of developing bends at the basal end of flagella, and the observed independence between the waveform and the beat frequency.

Functional Mechanisms

The possible mechanisms by which the then-recently discovered, fine stuctural components of flagella and cilia might give rise to their motility were discussed in 1955 by Bradfield (141) and by Gray (6). On the strength of Gray's (1928) argument (3) that "a moving wave cannot provide the energy for propelling an organism and at the same time pass on with unreduced amplitude, unless the energy being lost is continually being replaced as the waves pass along," Bradfield concluded that the 9 + 2 tubule bundle plus its matrix and membrane produce much, if not all, of the force necessary for movement. On this basis, he advanced a hypothesis founded largely on the assumptions that the nine doublet tubules are capable of propagating active, localized contractions along their lengths, that the impulses producing contraction arise rhythmically at the basal end of one doublet, and that propagation of the contractile activity to the other doublets around the axonemal cylinder is unidirectional in cilia and bidirectional in flagella having a planar beat. In his 1955 study of beating in sea urchin sperm flagella, Gray supported his argument given above with experiments using celluloid models, and independently proposed a hypothesis explaining flagellar beating in terms of active localized contractions propagated along the doublet tubules. One of the most sriking features of these two reports is that they were almost wholly based upon localized contractions of tubules, an extension of William Astbury's well-established contractile fiber hypothesis (142), with little indication as to how the energy for repeated contractions might be supplied. The only mention of sliding (by Bradfield) was as a possible basis for tubule contraction with one component of each doublet tubule "sliding up on the other, without either shortening, in the manner suggested for muscle by Hanson and Huxley," thus reflecting the very tentative acceptance of what was then the radically new sliding-filament model for muscle.

By 1959, when Afzelius (38) described the arms and the radial spokes on the doublet tubules in sea urchin sperm flagella, the sliding-filament mechanism of muscle contraction was no longer a novelty and had gained widespread acceptance. The structural analogy between flagellar arms and the cross bridges on the thick filaments in muscle led Afzelius to suggest a sliding-tubule model in which flagellar bending was based upon relative sliding movement between adjacent doublets as a result of activity of the arms on the doublets. He calculated that a relative sliding movement of 0.23 μm would be sufficient to account for the observed bending, and noted that some pairs of doublets were better situated to produce bending than others.

More direct evidence for a sliding-tubule mechanism was obtained by Satir (122, 143, 144) in a series of electron microscope studies between 1963 and 1968. Using a modification of the rapid fixation procedure of Párducz to preserve the metachronal pattern in actively beating gill cilia of the lamelli-

branch, *Elliptio*, Satir focused attention on the structure of the tips of cilia fixed either at the end of their effective stroke or at the end of their recovery stroke, and found that in both cases, the tubules located on the inside of the bend in the cilium protruded beyond those on the outside of the bend, as would be expected if the tubules slide relative to one another, with their lengths remaining constant. In later work (144, 145), the amount of sliding displacement of each doublet was found quantitatively equal to that predicted by the geometry of a bend, on the assumption that the lengths of all doublets remain constant during bending, and that no sliding occurs at the basal end. This work provided the first experimental evidence for a sliding-tubule, as opposed to a contractile, mechanism of ciliary beating. Additional indirect support came from an observation of Brokaw on the movement of sea urchin spermatozoa in solutions containing thiourea (113).

The period 1955–1970 also saw the development of a powerful new approach to the functional mechanisms of flagella and cilia that was based upon removal of the membrane barrier so that the motile mechanism would be directly accessible to experimental manipulation. In 1955, Hoffmann-Berling (14) discovered that grasshopper sperm flagella, in which the selective permeability of the membrane had been destroyed by treatment with 50% glycerol, could be reactivated by addition of exogenous ATP. In spite of the limitation that the flagella in these preparations beat only rhythmically from side to side and did not propagate bending waves along their length, Hoffmann-Berling was able to demonstrate that beat frequency increased with ATP concentration up to about 1 mM, and that the presence of Mg^{2+} was essential for motility and could not be substituted for by Ca^{2+}.

Propagation of bends in reactivated flagella appears to have been first achieved by Brokaw in 1961, using glycerol-extracted flagella isolated from *Polytoma* (146). In similar preparations of glycerinated sea urchin spermatozoa (147), it was found that the rate of ATP hydrolysis by motile flagella was greater than that of the same flagella in which motility had been prevented by gentle homogenizing, and this difference in rates was termed the "movement-coupled ATPase activity" by Brokaw. In these reactivated preparations in which 25–50% of the flagella were motile, it amounted to about 40% of the total ATPase activity. However, measurement of O_2 uptake by live spermatozoa indicated that the fraction of motility-dependent metabolism was as high as 80% of the total metabolism (148).

In 1969, Barbara Gibbons and Ian Gibbons discovered that improved reactivation could be obtained by replacing glycerol with the nonionic detergent, Triton X-100 (polyoxyethylene isooctylphenol ether)—first tried at the suggestion of Raymond Stephens—which completely removed the membranes from the flagella of sea urchin spermatozoa (149, 150). The resulting demembranated spermatozoa became essentially 100% motile when subsequently reactivated with ATP, and their flagellar beat (Fig. 8*c*) was very similar to that of live spermatozoa. Probably because of their high motility, the percentage of motility-coupled ATPase activity in these preparations was found to be as high as 70–80% (150).

Shortly thereafter, conclusive evidence for the occurrence of active sliding between flagellar tubules was provided by the work of Keith Summers and Gibbons (151, 152), who isolated Triton-demembranated flagellar axonemes from sea urchin sperm and digested them briefly with trypsin. The subsequent addition of ATP caused a disintegration of the axoneme into separated microtubular doublets, and direct visual observation by dark-field light microscopy showed that this disintegration occurred by extrusion of tubules from the axoneme by a gradual sliding process (Fig. 9) and that the length, after disintegration was complete, ranged up to eight times that of the original axonemal fragment. The ATP requirement and divalent cation specificity for this sliding closely matched the requirements for normal beating in undigested axonemes. These observations also indicated that the presence of the centriole completely blocked the sliding of tubules at the basal end of the flagellum. Examination of the trypsin digestion as a function of time showed that the rate at which the axonemes were sensitized to disintegration by ATP paralleled the rate of disruption of the nexin links and the radial spokes, whereas the dynein arms and the tubules themselves were relatively resistant to disruption by trypsin (152). As a result, it was concluded that the dynein arms generate active shearing stress between adjacent doublet tubules, and that in the intact axoneme these shear stresses are coordinated and resisted by the radial spokes and the nexin links, leading to the formation and propagation of bending waves; whereas in trypsin-treated axonemes, in which the nexin links and radial spokes are disrupted, these shear stresses lead to unlimited sliding and the disintegration of the axoneme. In an extension of this work, Winfield Sale and Satir (153) used electron microscopy to study axonemes of *Tetrahymena* cilia that had undergone

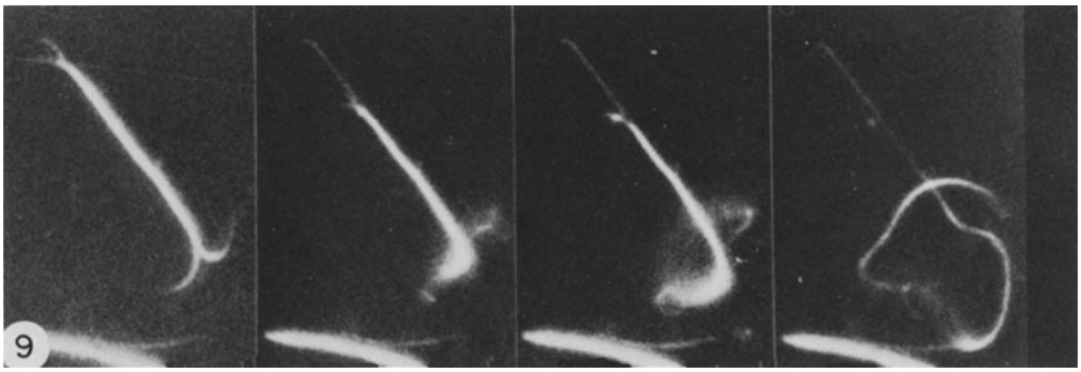

FIGURE 9 Dark-field light micrographs of trypsin-treated axonemes reacting to ATP. The successive micrographs from left to right were taken at intervals of 10–30 s. A large group of tubules is shown sliding toward the bottom right of the fields, leaving behind a smaller, stationary group of a few tubules attached to the coverglass. The free, forward end of the sliding group coils around out of the plane of focus, and in the final micrograph, it has coiled completely around and come back into the focal plane. Summers and Gibbons 1971 (151).

sliding disintegration, and found that the direction of sliding was always such that the arms on tubule A of one doublet pushed the B tubule of the adjacent doublet toward the tip of the cilium. The renaissance of interest in dark-field light microscopy has since extended its use to observe the movement of individual bacterial flagella (154) and to measure the growth rates of polymerizing microtubules (155) and the rigidity of individual actin filaments decorated with heavy meromyosin (156).

Further studies by Gibbons and Gibbons have shown that extraction of the demembranated spermatozoa of the sea urchin *Colobocentrotus* with 0.5 M KCl (157) results in a selective removal of the outer arms from the doublet tubules (Fig. 10), and that when the resultant KCl-extracted spermatozoa are reactivated with 1 mM ATP, their flagellar beat frequency is decreased in proportion to the number of arms removed while their waveform remains essentially unchanged; this suggests that the inner and outer arms on the doublet tubules are functionally equivalent and that the rate of sliding between doublets under these conditions is proportional to the total number of outer and inner arms present. A second type of experiment indicated that the sperm flagella could be set into stationary waveforms by reactivating them with 30 μM ATP and then rapidly diluting into a large volume of reactivating solution containing no ATP (158). By analogy to muscle in rigor mortis, these stationary flagellar waveforms have been termed "rigor waves." The rigor waves relax slowly (straighten) upon addition of 1–5 μM ATP, which is too low a concentration to support oscillatory bending, whereas higher concentrations of ATP cause resumption of normal beating. Qualitative study of the mechanical properties of flagella bent in rigor waves showed that they could easily be twisted by the viscous force of fluid flow, but that they are very resistant to straightening. These properties have been explained on the basis that the arms form fixed cross bridges between the doublet tubules in the absence of ATP. After appropriate fixation, these cross bridges can be visualized by electron microscopy (Fig. 11) (159). These two studies provided confirmation that the sliding between doublet tubules is produced by an ATP-driven cyclic interaction of the arms on the A tubule of the doublet with sites along the length of the B tubule of the adjacent doublet.

A local reactivation procedure has been used by Chikako Shingyoji, Akira Murakami, and Keiichi Takahashi (160) in an elegant confirmation of the sliding-tubule mechanism. These workers used iontophoresis from a micropipette to apply brief pulses of ATP to localized regions along the lengths of axonemes in demembranated sea urchin spermatozoa. Application of a pulse of ATP to the midregion of the axoneme caused formation of two bends of equal and opposite angle in the region where the ATP was applied, whereas the overall angle between the head and flagellar tip was unchanged. This result is exactly as would be predicted for a brief period of active sliding localized in the zone where the ATP was applied, with no sliding able to occur at the basal and tip ends where there was no significant ATP.

In 1974, Brokaw, R. Josslin, and Lynette Bobrow (161) showed that the asymmetry of the bending waves in reactivated sperm flagella is dependent upon Ca^{2+}. This effect of Ca^{2+} appears to involve two distinct processes, one being an apparently irreversible Ca^{2+}-dependent process that occurs during demembranation with Triton X-100, and the second, a reversible effect of Ca^{2+} concentration in the reactivating solution, with increased Ca^{2+} causing greater asymmetry. Recent extension of this work by Gibbons and Gibbons has shown that

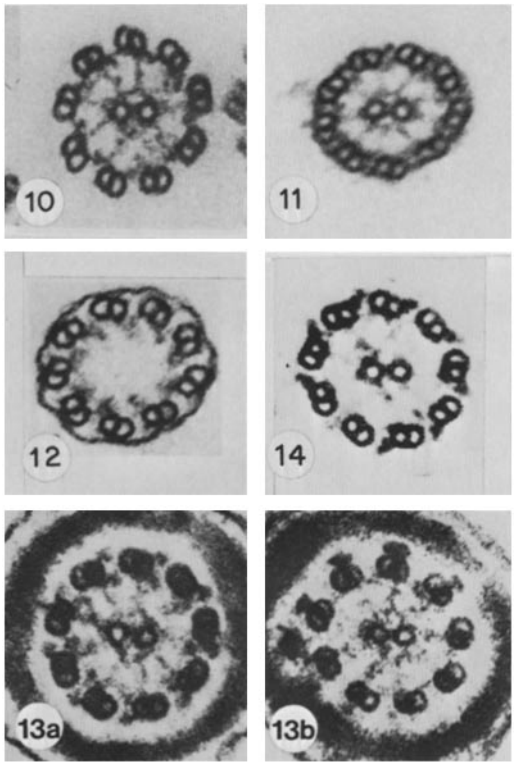

FIGURE 10 Axonemal cross section of sea urchin spermatozoon demembranated in 0.04% Triton X-100 containing 0.5 M KCl. Note absence of outer arms. Gibbons and Gibbons, 1973 (157).

FIGURE 11 Axonemal cross section of sea urchin spermatozoon fixed under conditions that preserve rigor waveforms. Gibbons, 1975 (159).

FIGURE 12 Cross section of 9 + 0 axoneme with only inner arms in spermatozoon of eel. Baccetti, Burrini, Dallai, and Pallini, 1979 (74).

FIGURE 13 Cross sections of principal piece of human sperm flagella. *(a)* Normal; *(b)* from patient with Kartagener's syndrome. Spermatozoa are nonmotile and appear to lack both inner and outer arms. Afzelius, 1976 (188). (Copyright 1976 by the American Association for the Advancement of Science.)

FIGURE 14 Cross section of isolated axoneme of *Chlamydomonas* mutant pf 23, which lacks inner arms. Huang, Piperno, and Luck, 1979 (61).

driving the sperm to an extreme degree of asymmetry causes them to become quiescent, with their flagella bent into a highly asymmetric cane-shaped form (67), and that the action of Ca^{2+} in causing asymmetrical bending and quiescence can be mimicked by low concentrations of methanol.[1]

A development of growing importance in the study of functional mechanisms has been the successive refinement of micromanipulation procedures for measuring the active bending moments produced by cilia and flagella and relating these to measured values of stiffness under different conditions. These procedures are based upon the early work of Haruo Kinoshita and Takeo Kamada with microneedles on the compound abfrontal cilium of *Mytilus* gill (162). In 1960, Mitsuki Yoneda (163) first succeeded in measuring the force exerted by this abfrontal cilium by the bending of a calibrated microneedle. Later work by Shoji Baba (164) measured the flexural rigidity and reported that, contrary to earlier ideas based largely upon

[1] Gibbons, B. H., and I. R. Gibbons. 1981. *Nature (Lond.)*. In press.

visual observation, the measured stiffness had an almost constant value irrespective of the stage of the beat cycle and of the direction of the force applied. A similar procedure was used by Charles Lindemann, W. Rudd, and R. Rikmenspoel (165) to determine the stiffness of individual bull sperm flagella. Extension of this work to the thinner flagella of echinoderm spermatozoa was achieved in 1979 by Makoto Okuno and Yukio Hiramoto (166), who showed that the stiffness of live flagella immobilized with CO_2 was only 5–10% of that of demembranated flagella in the absence of ATP. The stiffness of the immobilized, live flagella varied two- to three-fold when the spermatozoon was rotated about its long axis, whereas the stiffness of the demembranated rigor flagella was unaffected by such change in orientation. The change in stiffness of live flagella with orientation may reflect the relationship between the plane of the central tubules and the plane of bending. The high stiffness of rigor flagella presumably reflects the cross-bridges between doublets that are responsible for maintenance of rigor waves (158, 159).

In 1974, Warner and Satir (53) made a detailed study of the radial spokes in straight and bent regions of gill cilia fixed while beating. Their results indicated that the relative positions of the triplet spoke groups on any two doublet tubules remain constant in straight regions of the axoneme, either proximal or distal to a bend. However, in bent regions the positions of the spoke groups change systematically up to a maximum of 12 nm per group for tubules on opposite sides of the axoneme, whereas individual spokes tilted at angles up to 33° from their usual position roughly normal to the doublets. These observations confirmed Satir's earlier evidence that the lengths of the tubules remain constant during bending (143, 144), and provided direct evidence to support the earlier suggestions that the radial spokes play a major role in resisting sliding and converting it to bending (151). The limited range of tilt angles of the spokes in bent regions of the cilia suggested that the radial spokes are able to detach and reattach their connections to the projections of the central sheath. The factors influencing this cyclic detachment and reattachment of the radial spokes, and whether it is a passive process or an active process driven by ATP, remain unknown. The nexin links, on the other hand, appear to be elastic and to have permanent attachments to the tubules (53, 57). Their function may be to prevent the occurrence of excessive sliding displacement between tubules. When the sliding displacement between adjacent tubules attains a level of about 140 nm, the elastic limit of the nexin appears to be reached, and no further displacement occurs unless the flagellum is forced to such an extent that its structure is permanently damaged (67).

The hypothesis that the radial spokes and the central sheath complex to which they attach play a major role in coordinating sliding among the outer doublets and converting it into bending is supported by the work of George Witman, J. Plummer, and G. Sander (50), showing that the flagella of three mutants of *Chlamydomonas*, which lack either the radial spokes or the central tubules and sheath, are all paralyzed, although they are all capable of sliding disintegration after trypsin digestion, showing that the dynein arms remain capable of generating shear stress between doublets. Although the radial spokes and central sheath complex appear to be required for motility in *Chlamydomonas* flagella, it is nevertheless possible for flagella lacking these structures to show a simple form of oscillatory bending, as demonstrated by the slow helicoidal bending waves reported in the 9 + 0 flagella of eel spermatozoa by Baccetti and colleagues (Fig. 12) (74), and in the 6 + 0 and 3 + 0 flagella in male gametes of certain gregarine sporozoa by Joseph Schrével, Stuart Goldstein, and colleagues (75–77). There must be, therefore, a mechanism capable of coupling dynein ATPase activity to bending that exists even in these structurally reduced flagella. Perhaps the most likely hypothesis is that suggested by G. Douglas (167), which postulates that bending of a doublet microtubule causes a change in the subunit lattice that modulates the capability of the subunits to interact with the dynein arms. This conformational change could constitute the essence of a curvature control of dynein ATPase activity, of the type that appears required in theoretical models of flagellar bending. Support for this hypothesis is provided by the observation of Marie-Paule Cosson and Gibbons (168) that nonmotile sea urchin spermatozoa with their flagella fixed into normal waveforms by brief treatment with *N*-ethyl maleimide show an augmented ATPase activity that is lost when the flagella are homogenized.

Although a mechanism of this type appears capable of explaining slow helicoidal bending waves, it is evident that a more complex regulatory mechanism involving the central tubules and sheath, and the radial spokes is required to explain the more usual beat patterns of cilia and flagella. The work of Charlotte Omoto and Ching Kung (169), and of Robert Jarosch and Bernhard Fuchs (170) suggests that in some cases the pair of central tubules may rotate within the cylinder of nine doublets. Such an arrangement appears generally consistent with the semi-three-dimensional beat pattern of many cilia, and it might also explain the slow rotation of the plane of beat reported in flagella of some porifera by E. Kilian (171). On the other hand, the work of Tamm (172) has shown that no reorientation of either the cylinder of nine doublets or the pair of central tubules occurs during ciliary reversal in ctenophores. Moreover, the lack of bilateral symmetry in the axonemal structure, resulting from the arrangement of the dynein arms and their apparently unidirectional power stroke (43, 153, 158), make it difficult to envisage how such a pattern of activation would have sufficient torsional stability to generate the almost planar waveforms typical of echinoderm sperm, in which the nonplanar component is too small to be visualized and appears to have a propulsive effect of around 1% of the main force in the plane of bending (6, 173). It seems necessary to explore further the possibility that the arms in intact flagella may be capable of a bidirectional power stroke, for the unidirectional power stroke observed in disintegrating cilia and flagella might be due to a loss of normal regulation under these conditions.

Studies of reactivated cilia and flagella of other organisms have had particular value in illuminating the varied roles that Ca^{2+} plays in regulating movement. Reactivation of cilia of *Paramecium* was first achieved by Yutaka Naitoh (174) using a glycerol procedure. Improved results were achieved by Naitoh and H. Kaneko (175) who used a modification of the Triton X-100 procedure of Gibbons and Gibbons (149), in which the concentration of Triton was reduced to 0.001%, at which it destroys the selective permeability of the cell membrane system while leaving the structure of the cell cortex intact and the cilia still attached. With this system, Naitoh and Kaneko were the first to demonstrate a regulatory role for Ca^{2+} in reactivated cilia: at Ca^{2+} levels of 0.1 μM and below, the direction of swimming is forward, whereas at Ca^{2+} levels above 1 μM, the cells swim backward because of reversed beating of the reactivated cilia. This lent strong support to the hypothesis proposed by Roger Eckert (176) to explain the backward swimming induced by mechanical or electrical stimulation in *Paramecium*. In solutions containing ATP but no Mg^{2+}, the

cilia of Triton-treated cells do not beat, but they change their direction from pointing posterior at low-Ca^{2+} levels to pointing anterior at Ca^{2+} levels above 1 μM. This result was taken to suggest the presence of two motile components: one activated by $MgATP^{2-}$ responsible for cyclic beating, and a second, activated by $CaATP^{2-}$, that governs the orientation of the effective stroke (175).

An analogous regulation by Ca^{2+} in *Chlamydomonas* has been described for isolated pairs of flagella by Jeremy Hyams and Borisy (177), and for individual flagella by Matthew Bessen, Rose Fay, and Witman (178). The regulation of pairs of *Chlamydomonas* flagella is of particular interest because the beat cycle changes from a typical ciliary pattern at low Ca^{2+} to a typical flagellar pattern at higher Ca^{2+}, indicating that, at least in this case, a single organelle has the potential to produce both ciliary and flagellar beat patterns.

Studies by Holwill and collaborators (179, 180) on the motion of the trypanosomid *Crithidia* have shown that the flagellum has the unusual property that, during normal forward swimming of the organism, bends are formed near the flagellar tip and then propagate toward the base. During backward motion of the organism the direction of flagellar bend propagation reverses, so that bends propagate from base to tip. In demembranated preparations reactivated with ATP, tip-to-base propagation is observed at Ca^{2+} concentrations below 0.1 μM, while at higher concentrations base-to-tip propagation only is seen.

In most ciliated epithelia of invertebrates and vertebrates, nervous control appears to be limited to activation or arrest of beating. The studies of Tatsuo Motokawa, Murakami, and Takahashi (181) have shown that the arrest response of lateral cilia of live muscle gill is dependent on the presence of extracellular Ca^{2+}, suggesting that arrest is due to an increased level of intracellular Ca^{2+} as a result of opening of voltage-sensitive Ca^{2+} gates upon depolarization of the ciliary membrane. This hypothesis has been confirmed by Teizo Tsuchiya (182) and by Marika Walter and Satir (58) using reactivated cells as well as live cells treated with the divalent-cation ionophore A23187.

The above results make clear that Ca^{2+} exerts a regulatory role on the beating of cilia and flagella in many organisms. The detailed effect of Ca^{2+} on beating varies considerably from one organism to another, with a particularly striking instance of this variation being that increased Ca^{2+} causes increased asymmetry in sea urchin sperm and decreased asymmetry in *Chlamydomonas* flagella. The rule appears to be that, rather than having a single effect, it is always the low-Ca^{2+} form of beating that is the "normal" one for the organism, whereas the high-Ca^{2+} form occurs during taxis or an avoidance response, etc. The mechanism by which Ca^{2+} exerts its influence remains to be determined, but the recent discovery of calmodulin in *Tetrahymena* cilia (111) suggests strongly that this ubiquitous regulator will be somehow involved.

The basal bodies of each of the two pairs of flagella in *Platymonas* are anchored to the plasmalemma by a thick cross-striated fiber, and the recent work of J. Salisbury and G. Floyd (183) has shown that this fiber, which is about 2.2 μm long when the organism is fixed in the absence of Ca^{2+}, contracts to as little as 0.9 μm when fixed in the presence of 1 mM $CaCl_2$. The contraction of this fiber may be responsible for changes in angular orientation of the basal region of the flagella during swimming. A similar cross-striated fiber joins the two basal bodies in *Chlamydomonas* (184), which shows a decrease in the angle between the basal regions of the flagella upon addition of Ca^{2+} to either beating or nonbeating flagella pairs (177). Although the relationship of these apparently contractile fibers to other forms of cell motility remains to be clarified, their structural resemblance to the basal foot and to the cross-striated rootlet-type structures associated with basal bodies in other organisms has effectively reopened the whole question of a possible active role for these structures whose activity was much debated earlier, but that have recently been relegated to an inactive supporting role.

Since the discovery that, in many cases, demembranated flagella can be reactivated to apparently normal motility by supplying them with exogenous ATP, there has been a tendency to neglect the possible importance of flagellar and ciliary membranes. Recent work by William Dentler, Melanie Pratt, and Stephens (185) suggests that this may be an oversimplification. In 1977, Stephens (186) compared the compositions of the membrane fractions from gill cilia and sperm flagella of the scallop, *Aequipecten*, and found that a large fraction of the ciliary membrane protein appeared to be a glycosylated tubulin, whereas the flagellar membranes contained a major glycosylated protein of about 350,000 daltons with little or no tubulin. Similar differences in membrane composition appear to occur among protozoa and algae. These differences in composition may underlie some difference in membrane function, for photochemical cross-linking by the cleavable lipophilic agent 4,4′-dithiobisphenylazide in vivo causes inhibition of motility in cilia of *Aequipecten* and of *Tetrahymena* (185), whereas it has no apparent effect on the motility of *Aequipecten* sperm flagella.[2] Electron micrographs of partially disintegrated cilia suggest that the structural effect of the cross-linking is to stabilize the attachment of bridges between the doublet tubules and the membrane. The functional action of these bridges in untreated cilia is not clear, but the fact that their stabilization appears correlated with inhibition of ciliary motility suggests that ciliary membranes may in some cases play a more active role in overall function than the reactivation of motility in demembranated organelles might suggest.

A characteristic form of motility in the flagellar membrane of *Chlamydomonas* becomes apparent when a cell is attached to a solid substratum by just one of its two flagella. Under such conditions the cell glides continuously across the substratum, flagellum leading, at a speed of about 2 μm/s, and as noted by Lewin (15), gliding is particularly apparent in mutant strains with paralyzed flagella. The relationship of this gliding to the saltatory movements, at about the same speed, of particles attached to the flagellar membrane in *Chlamydomonas* described recently by Robert Bloodgood and co-workers (187) is not yet clear. It has long been known that the flagellar membranes in *Chlamydomonas* play an important active role in the pairing of cells during mating (15), and interest in these forms of flagellar membrane motility has been accentuated recently by their possible relationship to membrane-microtubule interactions in the cytoplasm (Haimo and Rosenbaum, this volume).

Largely as a result of the evidence summarized above, it is now widely accepted that the normal beating of flagella and cilia results from active sliding movements between adjacent doublets of the axoneme, with this sliding being powered by an ATP-driven mechanochemical cycle in which dynein arms on one doublet interact with successive binding sites along the B tubule of the adjacent doublet, and are coordinated and resisted by the radial spokes and nexin links that convert the sliding into bending.

The importance of flagellar and ciliary function in human

[2] Dentler, W. L., and R. E. Stephens. Personal communication.

medicine has become more apparent recently as the result of the discovery by Afzelius (188) and by Henning Pedersen and Heinrich Rebbe (189) that the respiratory difficulties and male infertility found in the hereditary defect known as Kartagener's syndrome are the result of immotile cilia and sperm flagella. This lack of motility is associated with lack of both inner and outer dynein arms on the doublet tubules of the flagellar and ciliary axonemes (Fig. 13*a*, *b*). Kartagener's syndrome appears to constitute one form of a broader immotile cilia syndrome, and a second form involving immotile cilia with defective radial spokes has been described by Jennifer Sturgess and colleagues (190).

Among other genetic variants are the mutants of *Chlamydomonas* having paralyzed flagella that lack either their inner or their outer arms (Fig. 14) (61). In view of the motility reported in two other instances in which the axonemal structure lacks outer arms (74, 157), the basis for the lack of motility in these *Chlamydomonas* flagella is not yet understood.

Evidence regarding the steps in the dynein cross bridge cycle is preliminary. In the absence of $MgATP^{2-}$, the arms appear to form fixed cross bridges between the doublet tubules, as indicated by the stability of flagellar rigor waves (158) and by the high stiffness of the flagellum under these conditions (166). The observations of Masami Takahashi and Yuji Tonomura (191) that 30S dynein from *Tetrahymena* cilia will bind to either the A or B tubules of isolated doublets, but that the addition of 1 μM ATP causes dissociation of the dynein from B tubules, while having no effect on the dynein bound to A tubules, suggests that $MgATP^{2-}$ causes detachment of the dynein cross bridges in intact axonemes. This is supported by the finding of Sale and Gibbons (192) that addition of $MgATP^{2-}$ to trypsin-treated axonemes in the presence of the inhibitor vanadate (193, 194) results in disintegration of the axonemes by a passive peeling apart of the doublets, rather than by the active sliding seen in the absence of vanadate, which suggests that vanadate does not interfere with the ATP-induced detachment of the arms but binds to the detached arm and inhibits reattachment. This conclusion is further supported by the finding of Okuno that the stiffness of axonemes in the presence of $MgATP^{2-}$ and vanadate is only about 5% of that in the rigor state (195). The presteady-state kinetics of the hydrolysis of ATP by dynein (196;[3]) indicate the occurrence of an early burst of ATP hydrolysis of around 1 mol per mol of active site, suggesting that the rate-limiting step in the overall reaction may be product release. These observations are consistent with an ATP-driven cross bridge cycle for dynein similar to that believed to occur in the myosin cross bridge cycle in muscle (197). However, the evidence supporting this mechanism for dynein ATPase is still quite limited, and the recent report suggesting the presence of two distinct ATPases in the outer arms of *Chlamydomonas* flagella (61) indicates the necessity for continued caution in drawing parallels between dynein and myosin.

The general stability of the oscillatory movements of flagella and cilia, as manifested by their capability to form and propagate uniform bending waves over a wide range of mechanical and chemical conditions and by their rapid recovery from transitory mechanical disturbances (e.g., collisions between sperm), indicates the presence of at least one feedback loop in the regulatory mechanisms. The report by Brokaw and Tom Simonick (198) of abrupt transitions between two oscillatory modes, one in which bends are propagated normally along the full length of the flagella and a second in which the amplitudes of the bending waves decrease rapidly as they propagate, suggests the presence of distinct feedback loops associated with bend initiation and bend propagation.

There are two general types of approach to the study of the regulatory mechanisms: one involves perturbing the beating flagellum with a wide variety of agents and then comparing their effects on the various wave parameters; the other involves study of flagella under nonoscillatory conditions in which the feedback loop has been opened to facilitate examination of its individual components. Survey of the effects of a wide variety of perturbing agents on the wave parameters of sea urchin sperm flagella by Gibbons (112) has suggested that two largely independent mechanisms are responsible for regulating the beat frequency and the waveform. The mechanism regulating beat frequency appears to be closely related to the mechanochemical cycle of dynein that causes active sliding between tubules and is relatively insensitive to the hydromechanical forces on the axoneme, whereas the mechanisms regulating waveform appear relatively more sensitive to the mechanical boundary conditions at the flagellar base and to the properties of the radial spokes, nexin links, and the tubules themselves, which are together presumed responsible for converting active sliding into a particular pattern of bending (199).

An example of the second approach of interrupting the feedback loop is the study of Summers and Gibbons in which digestion by trypsin was used to uncouple sliding from bending. More recent studies by Brokaw, Barbara Gibbons, Goldstein, and Flavin and their collaborators (67, 193, 194, 200, 201) have identified several agents—including Ca^{2+}, methanol, CO_2, decreased pH, and vanadate—that can be used to inhibit reversibly the normal oscillatory beating. The use of these agents makes it possible to study the bending of flagella that occurs upon addition of ATP to preparations in which oscillatory beating is inhibited. The preliminary reports by Goldstein (201) and by Gibbons and Gibbons (67) indicate that substantial amounts of active bending can occur in flagella inhibited by decreased pH, vanadate, or Ca^{2+}. This general approach of investigating the bending and straightening of demembranated flagella under nonoscillatory conditions may be a useful way to learn about the factors regulating the activity of dynein cross bridges at different positions on the flagellum as well as about the viscoelastic properties of the structural components that resist active sliding and convert it into bending.

Although, as indicated above, most evidence indicates that movement associated with microtubules occurs as a result of sliding, there are some indications that significant changes in microtubule length may occur in certain cases. Electron microscopic data suggesting that single microtubules in protozoan axostyles are capable of shortening by as much as 25% has been reported by Richard McIntosh (202), but more information is needed before the physiological significance of this finding can be interpreted.

Considerable evidence for small differences of the order of 1% in the lattice spacings of the A and B components of flagellar doublet tubules is provided by the work of Summers and Gibbons (151), Donald Costello (203), and Richard Zobel (204) showing that the doublets have a marked tendency to assume uniform helical forms as a result of bending approximately within the plane containing the centers of the A and B tubules, usually with the A tubule on the outside of the bend. Recent studies by Taiko Miki-Noumura and Ritsu Kamiya (205) have shown that small changes in pH or in Ca^{2+} concentration appear to cause discrete changes in pitch and diameter

[3] Evans, J., and I. R. Gibbons. Unpublished data.

of these tubule helices, and these factors, as well as organic solvents such as methanol are known to have substantial effects on the asymmetry of flagellar bending (205). It is possible that these agents may function by modifying the changes in lattice structure associated with a basic curvature-controlled regulation of dynein arm activity as discussed above. Since microtubules contain a variety of minor protein components in addition to tubulin, it is not clear whether the above factors act directly on the tubulin, in a manner analogous to the action of hydrodynamic stress, pH, and organic solvents in effecting transitions between the various polymorphic forms of flagellin in bacterial flagella (206–208), or indirectly through accessory proteins, as in the effect of Ca^{2+} on the structure of thin filaments in striated muscle (209), but the fact that mild trypsin digestion desensitizes the axonemes to Ca^{2+} (199) suggests that at least part of their action is indirect.

Growth Mechanisms

Investigation of flagellar and ciliary growth mechanisms is greatly facilitated by use of organisms in which the time of growth can be synchronous. For this reason, most studies have involved the regeneration of new flagella or cilia on cells from which the organelles have been either shed or resorbed, or the growth of flagella in cells that can be induced to undergo an amoeba-flagellate transition.

The early work of Lewin showed that *Chlamydomonas* that had resorbed most of their flagella as a result of being kept on agar in the dark, would regenerate full-length flagella within 1–2 h of being transferred to fluid medium. This work was extended by Malvine Hagen-Seyfferth (210), who showed that *Chlamydomonas*, after having been deflagellated completely by exposure to a pH shock or to ethanol, would regenerate new flagella within about 1 h.

Rosenbaum and Child (211) amputated flagella of *Euglena*, *Astasia*, and *Ochromonas* by mechanical agitation, and found that in all cases regeneration was characterized by an initial lag period, after which regeneration occurred at a rate that decelerated as the original length was approached. In these species, inhibition of protein synthesis by cycloheximide at the time of amputation resulted in almost complete inhibition of regeneration. However, *Chlamydomonas* flagella can regenerate up to one-third of their normal length (212), and cilia from the embryo of the sea urchin can regenerate to full normal lengths (213), both in the absence of protein synthesis, indicating the presence of significant pools of precursor proteins during normal growth in these cells. Using *Chlamydomonas* gametes that have a low basal level of protein synthesis, Paul Lefebvre and co-workers (214) have been able to detect deflagellation-induced synthesis of tubulin, dynein, and flagellar membrane protein, as well as of about 20 minor axonemal proteins. The factors responsible for triggering synthesis of flagellar proteins upon deflagellation are not clear, but it is notable that the same pattern of synthesis occurs upon induced resorption, even when assembly of the new protein into flagella is inhibited with colchicine.

As described originally by Schardinger in 1899 and more recently in greater detail by E. Willmer (215), the cells of *Naegleria gruberi* undergo transformation from an amoeboid form to a flagellated form upon being transferred from their growth environment to a nonnutrient buffer solution. This amoeba-flagellate transformation has been used in an extended series of studies of flagellar morphogenesis by Chandler Fulton and Alan Dingle and their collaborators (216, 217), who have shown that a burst of synthesis of new proteins precedes the appearance of visible flagella, and have suggested that a change in the compartmentalization of intracellular Ca^{2+} may be responsible for triggering the transformation.

The lengths of cilia and flagella are under close control by the cell, so that different cilia on a single cell may have greatly different lengths—as exemplified by the components of the compound laterofrontal cilium in lamellibranch gills, which range from 2 to 12 μm in length (39). This control by the cell permits even the resorption of certain flagella while others on the same cell are growing longer—examples are the studies by Tamm showing the resorption of the parental leading flagellum with simultaneous growth of the two new daughter leading flagella that occurs prior to cell division in *Peranema* (218) and the work of Rosenbaum (212) and of Randall (184) showing that in *Chlamydomonas* cells from which just one flagellum has been sheared off, the remaining old flagellum is partially resorbed at the same time as the new flagellum begins regenerating. The factors by which cells regulate the length of their flagella are not known, but several studies have shown that the presence of divalent-cation chelators in the medium causes partial or complete flagellar resorption in *Chlamydomonas*, and that this effect can be reversed by addition of Ca^{2+}, Sr^{2+}, or Mn^{2+} to the medium (219). Pulse labeling, followed by autoradiography, has shown that during flagellar growth in vivo most of the subunits are added to the distal region of the growing flagellum, although about 20% appear to be added within the proximal region (211, 212). Studies by Dentler and Rosenbaum (220) involving polymerization of brain tubulin onto partially disrupted flagella of *Chlamydomonas* have indicated that polymerization onto the outer doublet tubules occurs at their distal (+) ends, whereas polymerization onto the central tubules occurs at their proximal (−) ends, apparently because their distal (+) ends are blocked by a cap attached to the tip of the flagellar membrane. These results suggest that, during normal growth in vivo, the doublet tubules grow at their distal ends while the central tubules grow at their proximal ends. The full implications of this asymmetrical growth pattern are not yet clear, but it may be noted that in many organisms the proximal ends of the central tubules appear unattached—as perhaps they must be if they are to rotate in the way described for *Paramecium* and *Synura* (169, 170).

Knowledge of flagellar assembly mechanisms is still largely at a descriptive stage. Repolymerization of tubulin to form singlet tubules (103), and rebinding of dynein to extracted axonemes (78) are the only steps that have yet been accomplished in vitro. Study of the conditions under which mutants of *Chlamydomonas* with structurally defective flagella can be rescued as dikaryons may provide some more detailed information. However, if, as seems likely, the process of assembly for flagella is as complex as that of, for example, bacteriophage T2, then progress will be hard to come by until more of the assembly steps can be reproduced in vitro.

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