Cilia, Flagella, and Microtubules

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In 1676 Leeuwenhoek sent to the Royal Society of London a letter describing his discovery of protozoa and their cilia and flagella. He wrote, "I also discovered a second sort of animalcules, whose figure was an oval, ... provided with diverse incredibly thin little feet, or little legs [cilia], which were moved very nimbly ..., and wherewith they brought off incredibly quick motions" (cf. translation of letter 18 [1]).

Cilia and flagella were observed on a variety of cells during the next two hundred years, and by the end of the 19th century several theories had been proposed to explain flagellar beating. For example, it was postulated that flagella were lifeless and were moved by elements within the cell body or were hollow structures into which fluid was injected and withdrawn. Alternatively, it was suggested that flagella contained a central contracting fiber or possessed a fibrillar substructure. This last theory had been formulated in 1868 by Engelmann, who proposed that the flagellum contained aligned fibrillar elements which shortened into a globular form during beating. Although Jensen in 1887 and Ballowitz in 1888 observed numerous fibrils in the fraying tips of sperm tails, the prevailing belief at the turn of the century was that the flagellum contained a solid contracting core (see reference 2).

In later studies, the fibrillar substructure of cilia and flagella was described in a variety of cells (2, 3), and these observations were confirmed with the development of the electron microscope (4–7). The number of fibrils was variously reported as between nine and twelve, and the diversity of ciliary and flagellar wave forms suggested no reason that the number of fibrils would be constant among species.

Axonemes: the 9 + 2 Pattern

Based on electron-microscope studies of plant sperm in a number of species (8–11), a diagrammatic reconstruction of the flagellum was proposed (10). Considering the resolution of the shadow-cast, whole-mount preparations used in these studies, the model was remarkable in its accuracy. During this same period, techniques were developed for embedding and sectioning biological material for electron microscopy, and in 1954 Fawcett and Porter published the first ultrastructural study of cilia (12). Regardless of origin, all cilia were observed to possess the same configuration, nine hollow, doublet fibrils equidistant from and radially surrounding a central pair of single fibrils. This structure was identical with that proposed for plant sperm flagella (10) and resulted in the recognition of the "9 + 2 axoneme" as the common structural organization of cilia and flagella. Later studies demonstrated, however, that there were notable departures from this format, particularly among the spermatazoa of insects (13, 14).

With the development of improved fixation and staining techniques, accessory structures within the axoneme could be visualized. By use of a high percent osmium solution to fix sea urchin sperm flagella, Afzelius (15) was first to describe the arms, later termed dynein (16), present in two rows along the larger fiber of each outer doublet. Noting their orientation toward the smaller fiber of the adjacent outer doublet, Afzelius (15) postulated that the dynein arms might be active in a sliding filament model of flagellar beating. The next 15 years were to prove this hypothesis correct. Afzelius also observed that the dynein arms imparted an asymmetry to the axoneme. Subsequently, Gibbons and Grimstone (17) demonstrated in several species that the arms always pointed clockwise around the axoneme when viewed from base to tip. They introduced the nomenclature terming the fiber bearing the dynein arms the A-subfiber, the other the B-subfiber.

Studies of axonemes in longitudinal sections demonstrated that the dynein arms, as well as the other major accessory structures, were regularly spaced along the length of the axoneme. The dynein arms in both the inner and outer rows had a center-to-center spacing of approximately 24 nm along the length of the A-subfiber (18–22). In addition, the inner and outer rows of arms were axially staggered with respect to each other (23, 24), and superimposition of the two rows of arms may have accounted for earlier reports of an arm spacing of 12–16 nm (17, 25).

Radial spokes were visualized in thin sections as projections from each A-subfiber to the central sheath (15). These spokes terminated in an enlarged head that was incorrectly identified as a fiber running the length of the axoneme (17). More recent studies verified the "spokelike" ultrastructure and revealed that the radial spokes occurred in pairs in *Chlamydomonas* (19, 22, 25, 26) or triplets in *Elliptio* gill and *Tetrahymena* cilia (18, 27), which were grouped at intervals of about 96 nm along the lengths of the A-subfibers.

The central sheath observed in whole-mount preparations by Manton and Clarke (10) and described in cross sections of cilia by Gibbons and Grimstone (17) consisted of two rows of projections spaced at 16-nm intervals along each of the two central fibrils (22, 25, 27, 28). The final axonemal structure to be described were the nexin links (29, 30), which connected the A-subfiber of one outer doublet to the adjacent outer doublet

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and were axially spaced at approximately 96-nm intervals (21, 22, 31).

Optical diffraction patterns of negatively stained axonemal fibers, microtubules, revealed the existence of a strong 4-nm as well as an 8-nm axial periodicity (23, 32–34) corresponding to the monomer and heterodimer subunit composition of the microtubules (33, 34). The relative intensity of the 8-nm repeat compared with the 4-nm repeat varied considerably with different types of microtubules, and suggested that accessory structures on microtubules might contribute to an amplification of the 8-nm diffraction pattern (23). The observations that the dynein arms, central pair projections, radial spokes, and nexin links, all bound to the flagellar microtubules at approximate multiples of 8 nm, supported this hypothesis, and it has been proposed that the axial spacing between adjacent binding sites on microtubules is 8 nm (20).

Dynein

Concurrent with a description of axonemal fine structure has been an elucidation of the molecular basis for ciliary and flagellar motility. Spermatozoa were shown to contain both measurable amounts of adenosine triphosphate (ATP) and ATPase activity (35), which later studies demonstrated was concentrated in the flagellum (36–38). Using techniques developed to study muscle contraction (39), Hoffman-Berling (40) discovered that addition of ATP to glycerinated sperm resulted in the reactivation of beating. The wave form resembled that of live sperm and indicated that the energy for motility was supplied by ATP hydrolysis. Reactivation specifically required ATP and Mg^{2+} and was inhibited by EDTA (38, 41, 42).

After the development of methods to isolate cilia in large quantities (43), Gibbons (29, 44) demonstrated by selective solubilization of *Tetrahymena* axonemes that the ATPase activity was localized in the two rows of arms on the A-subfiber. The enzyme was named "dynein," force protein, for its postulated role in the mechanochemical transduction of energy required for motility (16). Dynein specifically hydrolyzed ATP in preference to other nucleotides, required Mg^{2+} or Ca^{2+} for its activity, and was inhibited by EDTA, characteristics that correlated closely with those necessary for axonemal reactivation (45, 46). Although the inner and outer rows of dynein arms appeared to be functionally equivalent (47), they were morphologically distinct (48), had different solubilities (49), and were composed of different polypeptides (26, 50). The existence of more than one axonemal dynein has been demonstrated in a number of studies (29, 51–55).

A specific association between the dynein arms and the A-subfiber was indicated by the observations that the solubilized arms rebound to their original sites on the A-subfiber (29, 56). Recently, it was demonstrated that solubilized dynein arms also rebound to the B-subfiber, presumably to those sites with which the arms would normally interact during beating (57).

The role of dynein arms in motility, implicated both by their ATPase activity and orientation toward the adjacent outer doublet microtubule, was confirmed in studies that demonstrated that the beat frequency of reactivation was directly proportional to the number of dynein arms present within the axoneme (47, 56, 58). Furthermore, antibody prepared against dynein inhibited both its ATPase activity and axonemal reactivation (59–61). The existence of paralyzed flagellar mutants lacking dynein arms in both *Chlamydomonas* (26) and humans (62) also suggested a motile role for the dynein arms.

The Sliding Mechanism

Theoretical analysis of the mechanism generating the motive force within the flagellum indicated that shearing between adjacent outer doublets that resulted in microtubule sliding could account for the uniform propagation of waves along the axoneme (63, 64). Sliding between outer doublets was demonstrated first in sectioned axonemes (65, 66) and more directly by dark-field visualization of trypsin-digested axonemes supplied with ATP (67). These and later studies indicated that the sliding motions were mediated by dynein arms cyclically attaching to and causing a shearing force between adjacent outer doublet microtubules (68, 69) exerted toward the tip of the axoneme (70).

Dynein arms present on intact axonemes or reattached to dynein-extracted axonemes projected from the wall of the microtubules at an angle of approximately 55°. In *Chlamydomonas*, the arms tilted toward the tip of the flagellum (19), whereas in mollusc gill and *Tetrahymena* the dynein arms tilted toward the base of the cilium (57, 70–72). To exert a directional force resulting in sliding (70), it has been postulated that the orientation of the dynein arms changes during the cross-bridge cycle (69), and a recent study supports this suggestion (73). Such a change in the orientation might account for the above differences in arm-tilt direction.

Transient ATP-dependent bridging between adjacent outer doublets was predicted from the orientation of the dynein arms and by their role in sliding, but these bridges were preserved and, therefore, visualized only when axonemes were fixed in rigor (74). The existence of a rigor state had been suggested by observations that bull sperm flagella became plasticized after ATP addition (75). Subsequent studies demonstrated that removal of ATP from reactivating axonemes caused them to enter rigor, characterized by a wave form frozen at the time of ATP depletion and maintained by dynein arm cross-bridges between adjacent outer doublets (74, 76). Release of the B-subfiber of the adjacent outer doublet by dynein as manifested by relaxation of the rigor wave required ATP binding, whereas subsequent reactivation required ATP hydrolysis (69, 76, 77). In other studies, however, dynein cross-bridges between adjacent outer doublet microtubules could be produced by fixing axonemes in appropriate buffers containing divalent cations (78). Nevertheless, addition of ATP to these cation-induced rigor axonemes resulted in relaxation of dynein cross-bridges between particular outer doublets (79). Although these observations conflict with studies demonstrating that reactivating axonemes entered rigor when either the Mg^{2+} (80) or ATP (76) concentration of the reactivation medium was rapidly lowered, they indicate that the dynein arms do, in fact, cross-bridge the adjacent outer doublet microtubule, and that the cross-bridges are ATP dependent.

Microtubules

Based on the observations of Van Beneden that protoplasmic fibrillae existed within the spindle and of Ballowitz that sperm tails contained minute fibrils, Wilson (81) postulated that the substance and outgrowth of the flagellar fibrils were comparable with those of the fibrils within the spindle. Moreover, he suggested that the contractile behavior of the spindle fibers noted by Boveri might also apply to the flagellum. It was not until the 1960s, however, that the relationship between cytoplasmic and flagellar microtubules was to be established, and it has not yet been determined if motility associated with

cytoplasmic microtubules is elicited by a mechanism similar to that of the flagellum.

Although early researchers using the light microscope had observed fibers within the spindle, the periphery of red blood cells, and in neurites, the existence of these structures had been a matter of controversy that was finally resolved with the advent of electron microscopy. By use of osmium as a fixative, fibrous structures were observed in the mitotic apparatus (82, 83) and in nerve axons (84), but their widespread distribution was only fully appreciated after the development of glutaraldehyde fixation (85). In one of the earliest uses of this technique, the fibers were described as microtubules whose similarity in morphology to the fibrils within the axoneme was apparent (86, 87).

Ultrastructural analysis revealed the presence of 13 longitudinal protofilaments comprising the walls of both cytoplasmic and axonemal central pair and A-subfiber microtubules (88–91). Moreover, optical diffraction studies of cytoplasmic microtubules demonstrated that they have both a 4- and an 8-nm axial periodicity (92), as had been observed previously for flagellar microtubules. In addition, the subunit dimers were axially staggered in adjacent protofilaments in both the A-subfiber and cytoplasmic microtubules, indicating that they were structurally similar. On the other hand, in the B-subfiber the dimers were lined up in adjacent protofilaments, thereby indicating a distinct surface lattice (23).

The use of the drug colchicine has been basic to the understanding of the chemical composition of microtubules. Noting that colchicine caused a reversible loss in birefringence of spindle fibers, Inoué (93) postulated that the drug bound to the subunit of these fibers. Later, Taylor (94) demonstrated that colchicine was reversibly bound by a substance within the cell, and in subsequent studies a colchicine-binding protein was isolated and shown to be the subunit of both cytoplasmic (95, 96) and flagellar (97, 98) microtubules. Characterization of the purified colchicine-binding protein from brain tissue revealed it to bind 2 mol of guanosine triphosphate (GTP) per mol of protein and to have a sedimentation coefficient of 6S and a native molecular weight of about 110,000 daltons (99), properties identical with those of the colchicine-binding protein in the mitotic apparatus and axoneme. The protein was given the name "tubulin" (100). Electrophoretic analysis revealed tubulin to be composed of two closely migrating 55,000-dalton polypeptides present in equal amounts (101). Recent experimentation has shown that 6S tubulin is a heterodimer composed of these two components (102). Although similar in subunit composition and structure, flagellar microtubules differed from cytoplasmic microtubules in their ability to form doublets and in their relative stability; the biochemical basis for these differences has not been determined.

Assembly In Vitro: the Role of Accessory Proteins and Microtubule Polarity

With the discovery of conditions that permitted the in vitro assembly of tubulin into microtubules (103), it was possible to study their biochemistry. Microtubule assembly occurred at 37°C from homogenates of brain in buffers containing Mg^{2+}, GTP, and a calcium chelator. Tubulin has subsequently been assembled from a number of other sources, including flagellar outer doublet microtubules (104–106).

Electrophoretic analysis of the protein composition of brain microtubules assembled in vitro revealed the presence of several protein species in addition to tubulin (107–109). A prominent class of polypeptides having a high molecular weight (ca. 300,000 daltons) copurified stoichiometrically with tubulin through several cycles of assembly (110, 111). Separation of the 6S tubulin from these proteins inhibited its ability to polymerize into microtubules except at high protein concentration (112) or unphysiological solvent conditions (113–115). Readdition of the high molecular-weight MAPs (microtubule-associated proteins) to 6S tubulin stimulated both the rate and extent of polymerization by lowering the critical concentration of tubulin necessary for assembly (110, 116). MAPs stabilized the microtubules (117) by lowering the reverse rate constant for assembly (118). Another class of proteins that copurified with tubulin, termed tau, was also shown to stimulate microtubule assembly (109, 119).

By examining the incorporation of radioactive precursors into regenerating flagella, it was determined that flagella assembled principally at their distal tips (120, 121). Tubulin obtained from cytoplasmic microtubules also exhibited directional assembly. For example, addition of brain tubulin onto isolated basal bodies (122), centrioles (123–125), or axonemes (19, 126) resulted in microtubule polymerization predominantly onto the distal ends of these organelles. Similarly, addition of brain tubulin onto microtubule pieces resulted in preferential assembly onto one end of these pieces (108, 127). Recent studies of microtubule assembly in vitro have indicated that polymerization occurred at one end of the microtubule and depolymerization occurred at the opposite end (128). These studies suggest that the two ends of the microtubule have different critical concentrations for assembly, and at polymerization equilibrium the rate of tubulin addition onto one end of the microtubule would equal the rate off the other end. Other experimentation, however, has demonstrated that microtubules assembled from kinetochores or centrosomes polymerized and depolymerized at the same end (129).

The polarity of microtubules, as manifested in their directional polymerization, may permit them to function in directional intracellular movements. Of these movements, those exhibited during mitosis have generated the most interest and have been the subject of several different models (130–135). Both chromosomes (123, 136, 137) and centrosomes (124, 125, 136) served as nucleation sites for microtubule assembly in vitro. Studies of the direction of this assembly have indicated that both kinetechore (129, 138) and centriolar (129, 139) microtubules added tubulin subunits at the microtubule end distal to the organizing center. These observations suggest that each half-spindle of the mitotic apparatus is composed of microtubules present in an antiparallel array.[1]

While axonemes could be reactivated to beat in vitro and, accordingly, their movements analyzed biochemically, no equivalent assay has been developed to study the movements associated with cytoplasmic microtubules, although work on the reactivation of mitotic movements is progressing (140). Nevertheless, recent studies have provided some insight into the mechanism by which motility is elicited within the cytoplasm.

MAPs, Arms, and Movement

The high molecular-weight MAPs, which copurified with brain tubulin, had an electrophoretic mobility similar to that of flagellar dynein, leading to speculation that they might be

[1] Direct visualization of microtubule polarity has recently revealed the half-spindle to be composed of parallel microtubules (139*a*, 139*b*).

functionally equivalent (108). Moreover, a flagellar fraction containing dynein was shown to stimulate the assembly of brain tubulin (141) while, in other studies, in vitro assembly of flagellar outer-doublet tubulin was stimulated by the addition of brain MAPs (105). Early work indicated the presence of a low level of ATPase activity associated with brain microtubules assembled in vitro, although contamination by mitochondrial ATPases could not be precluded (142, 143). More recent studies have demonstrated the presence of a dyneinlike ATPase in the cytoplasm of unfertilized sea urchin eggs (144) and associated with the mitotic apparatus (145). In other studies, a protein that had properties similar to dynein was purified from brain microtubules (146), and the activity of an ATPase associated with brain microtubules was stimulated by addition of tubulin (147).

MAPs have been visualized as filamentous projections (110, 112) that exhibit an axial periodicity of 32 nm (148, 149) along microtubules assembled in vitro. Similar projections have been observed on brain microtubules *in situ* (150). The presence of arms on cytoplasmic microtubules from a number of sources has been well documented (see reference 20), and arms periodically cross-bridging microtubules have been observed within the mitotic apparatus of *Barbylonympha* (151). At present, however, it has not been determined if these arms have a motile function.

Other studies have suggested that a dyneinlike ATPase might be implicated in movements associated with cytoplasmic microtubules. Lysed mitotic mammalian cells were capable of continuing anaphase motions if ATP was present (152). These movements were blocked by vanadate, an inhibitor of ATPases, at concentrations that inhibited dynein, but not myosin, ATPase activity (140). In addition, antibodies prepared against sea urchin flagellar dynein prevented chromosome movements in isolated mitotic apparatuses, whereas those prepared against myosin had no effect on these movements (153). Moreover, fluorescently labeled antibody against dynein stained the mitotic apparatus (154). Recently, it has been demonstrated that cytoplasmic microtubules have the capacity to bind dynein (155). A specificity of flagellar dynein binding to brain microtubules assembled in vitro was indicated by the 24-nm axial periodicity of the bound arms along the microtubules. This spacing is identical with that of dynein arms present on axonemal microtubules. Furthermore, flagellar dynein caused brain microtubules to become cross-bridged together. Subsequent ATP addition dissociated these bridges. Together, these data suggested the possibility that ATP-dependent dynein cross-bridges between microtubules, which results in outer doublet sliding within the axoneme, may also be involved with movements occurring within the cytoplasm.

Flagellar dynein bound to in vitro assembled microtubules provided a direct means by which microtubule polarity could be determined (155). Application of this technique to microtubules within the cell would be analogous to the use of heavy meromyosin bound to actin filaments to determine their polarity (156, 157), and has the advantage that microtubule polarity could be determined in transverse, as well as in longitudinal, sections. By decorating the mitotic apparatus with dynein, for example, it could be determined if microtubules of opposite polarity are adjacent to each other during anaphase and, therefore, likely to interact during mitotic movements. Dynein, both in its role in motility and in its use as a probe for determining the polarity of microtubules, may therefore provide information about the mechanism by which movements occur in association with microtubules.

This brief presentation is not meant as a thorough review of the microtubule literature. The reader is directed to the recent review by Bloodgood and Kelleher (158), which lists most of the major reviews and books on microtubules up until 1976, beginning with Porter's initial review in 1966. In addition, several excellent and exhaustive reviews have appeared within the past three years (159–166). Rather, the foregoing account attempts to trace the overall direction that research on microtubules has taken over the past quarter-century. With the development of electron microscopy as a routine laboratory procedure came the ability to observe microtubules first in cilia and flagella and later in other organelles and in a great variety of cell types. Studies on the biochemistry of ciliary and flagellar microtubules and the colchicine-binding protein of brain and other tissues were soon followed by successes in the reactivation of microtubule-based motile systems, particularly the cilia and flagella and, more recently, the mitotic apparatus. Finally, the development of techniques to assemble microtubules and some of their associated proteins in vitro, and to visualize systems of microtubules and related cytoskeletal elements by labeled antibody procedures and by high-voltage electron microscopy, has provided the impetus for continued progress in this rapidly moving field.

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