A REINVESTIGATION OF CROSS-SECTIONS OF CILIA

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The cilium was one of the first cell organelles to be investigated with the electron microscope (see Manton and Clarke, 1952; Fawcett and Porter, 1954). Since then, this structure has been widely reinvestigated as improved techniques became available both to the electron microscopist and to the biochemist. In the study reported here, improvements in fixation and the use of the "Markham" rotation technique (Markham et al., 1963) have provided means for clearer observations on previously described appendages of the 9 + 2 complex of fibers and for a demonstration of additional structural details.

Among the most complete morphological de-

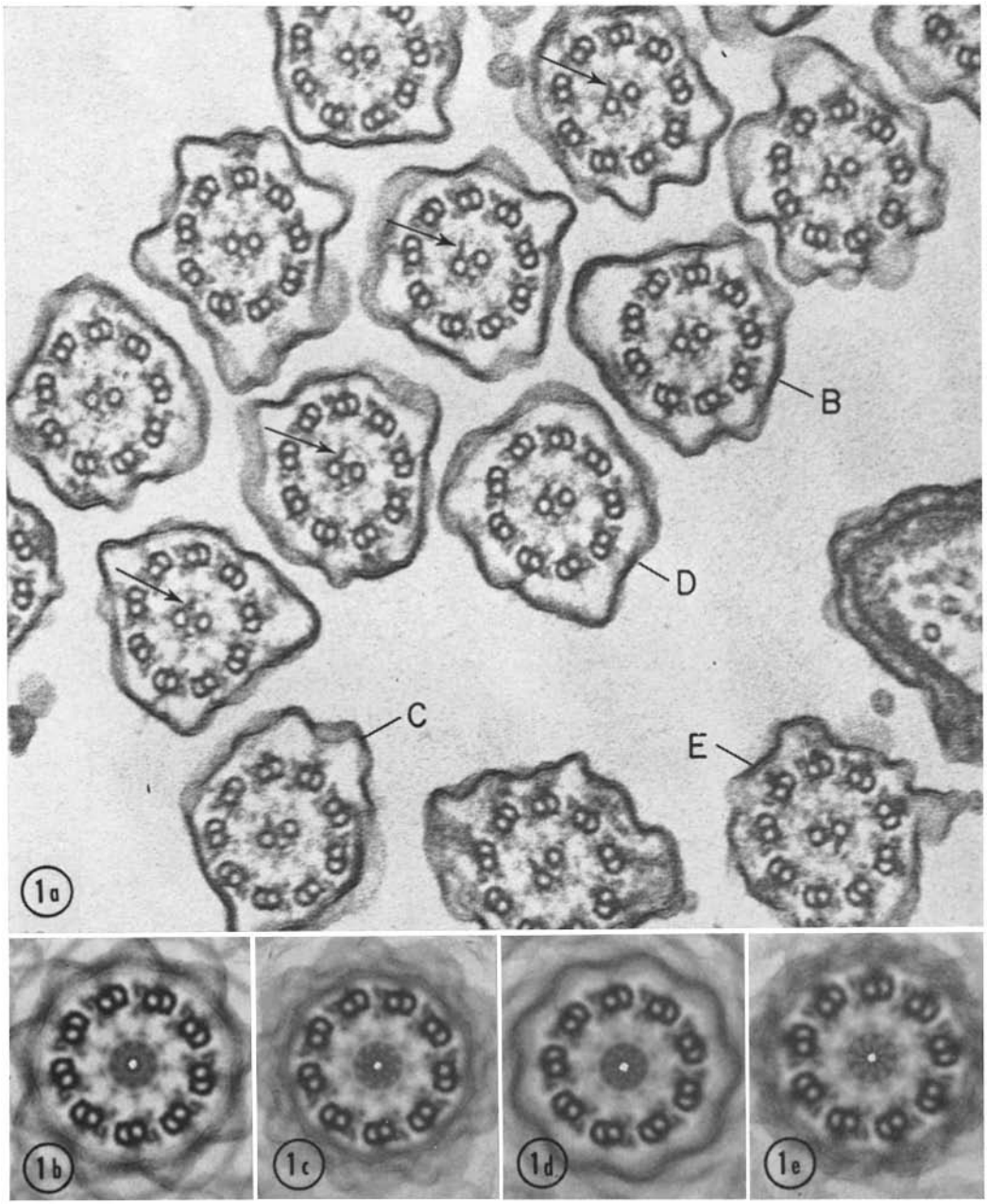

FIGURE 1 Cross-sections of cilia located within the oral groove of *Tetrahymena pyriformis*. Figs. 1 *b–d* and 1 *e* are Markham rotation images of cilia labeled *B*, *C*, *D*, and *E*, respectively, in Fig. 1 *a*.

FIGURE 1 *a* The typical 9 + 2 pattern of axonemal fibers is clearly evident. The two arms attached to subfiber A of each outer double fiber of the axoneme are easily seen and appear to have a more complex structure than simple, straight rods. Other structures previously identified within the cilium are not so clearly evident, but are suggested. These are the spokes between subfiber A and the central sheath, the thickenings along the spokes, the central sheath, and densities connecting the outer fibers and the ciliary membrane. This last structure is found only when the ciliary membrane lies close to the fibers.

scriptions of cilia and flagella are those published on flagella by Gibbons and Grimstone (1960) and Ringo (1967 *b*) and on the cilia of *Tetrahymena* by Gibbons (1963). The outer limiting membrane and the well known 9 + 2 array of fibers (microtubules)[1] of the axoneme predominate in cross-sections of these organelles. Other details of the cilium include a central sheath encircling the central two fibers, radially oriented links, or spokes, that extend from the central sheath to subfiber A (for axoneme terminology see Gibbons and Grimstone, 1960) of each of the outer fibers, dense thickenings near the middle of each radiating spoke which have been interpreted as cross-sections of a set of secondary fibers oriented longitudinally along the cilium, and two rows of short projections, or arms, attached to subfiber A of each of the outer fibers. These arms have been diagrammed as uncomplicated projections. They are reportedly 50 A thick and 150 A long and point in a clockwise direction on all the outer fibers of a cilium when the cilium is viewed from its base to its tip (for a recent summary see Gibbons, 1967).

[1] Tubular appearing structures within cilia and flagella and in other parts of cells have been, at different times and in different cells, referred to as fibers, filaments, and microtubules. For a discussion of the terminology of these structures, refer to Ringo (1967 *a*) and to Porter (1966). In this paper, such structures in the cilia will be referred to as fibers, since this term was used in the earlier literature dealing with cilia.

MATERIALS AND METHODS

Tetrahymena pyriformis, strain W, was used in this investigation. The culturing procedure and details of preparation for electron microscopy are described elsewhere (Allen, 1967). Probably, the most significant difference in the fixation procedure as compared with the procedures usually employed by others in similar investigations is the use of glutaraldehyde followed by OsO_4 (Sabatini et al., 1963)—instead of OsO_4 alone—and relatively short fixation and dehydration times. Sections were cut on a Porter-Blum MT-2 ultramicrotome with a diamond knife and then mounted unsupported on copper grids. Staining was achieved with uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963). The sections were then examined in a Philips EM 200 or RCA EMU 3F electron microscope.

For the purpose of enhancing the density of any radially symmetrical structures in the photographs of cilia, use was made of the rotation technique first applied to electron micrographs by Markham et al. (1963). Particularly favorable images of cilia were selected for this purpose. However, since circularity is essential for the use of this technique, as has been pointed out by Ledbetter and Porter (1964) and Ringo (1967 *a*), and since many of the cilia showing fine detail were somewhat elliptical, as a result of compression during sectioning, a modification of the Markham rotation technique was employed. Instead of projecting the enlarged image on a horizontal surface, an easel that could be tilted was constructed and the image produced by the enlarger was then projected onto the inclined plane formed by the tilted easel. When this tilted easel is oriented

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An arm, not previously described, appears to arise from one of the two central fibers (arrows) in each cilium. This arm is oriented in the same general direction and inclination in most of the cilia in the picture. A second arm may arise from the opposite side of the same central fiber. × 100,000.

FIGURE 1 *b* A rotation image of cilium *B* in Fig. 1 *a*. The outer arm extends out from subfiber A and then hooks sharply toward the center of the cilium. The inner arm curves in toward the center of the cilium, but does not have a hook. Spokes with a thickening along each spoke are present. Connections between subfiber A and subfiber B of immediately adjacent outer fibers, and connections between these same fibers and the ciliary membrane can also be seen. × 100,000.

FIGURE 1 *c* A rotation image of cilium *C* in Fig. 1 *a*. The inner arm bears a terminal knob of greater density than the remainder of the arm. × 100,000.

FIGURE 1 *d* A rotation image of cilium *D* in Fig. 1 *a*. A hook on the outer arm is apparent. × 100,000.

FIGURE 1 *e* A rotation image of cilium *E* in Fig. 1 *a*. A less dense area is present between the part of the outer arm adjacent to subfiber A and the terminal portion of this arm. A dense knob appears at the end of the inner arm. × 100,000.

so that the long axis of the elliptical image projected by the enlarger parallels a horizontal line drawn across the face of the inclined plane and the angle of the easel is adjusted appropriately, the image changes from an ellipse to a circle. A piece of photographic paper can then be placed on the tilted surface and rotated, as described by Markham. There is, of course, a limit to how much tilting is possible, and this limit is determined by the depth of focus of the optical system being used and the size of the image.

RESULTS

Preliminary study of electron micrographs of cilia cut in cross-section indicated that not all of the structural detail within these organelles had been described by earlier investigators. This was particularly evident in the arms attached to subfiber A of the outer fibers of the axoneme (Fig. 1 *a*). These arms seemed to have a more complex structure than the straight rodlike projections by which they are usually diagrammed. In order that as much information as possible could be obtained about these arms, recourse was taken to the Markham technique to average out the "noise" in the image and bring into clear view the substructure. Figs. 1 *b–d* and 1 *e* have been made by this technique from the cilia indicated in Fig. 1 *a*. Figs. 2 *b*, 3 *b*, 4 *b*, and 5 *b* have been made in a similar way.

This technique made it quite clear that the arms are more complicated than was thought and, in fact, that the outer and inner arms on subfiber A may be morphologically dissimilar. The outer arm extends out from subfiber A for a distance of 20–22 mμ toward the ciliary membrane. At its distal end, this arm appears to hook sharply toward the center of the axoneme (Figs. 1 *b*, 1 *d*, 2 *b*, 3 *b*, 4 *b*, 5 *b*, and 6). A 10-mμ portion of this arm adjacent to subfiber A and the 20–25-mμ portion hooked back toward the center of the axoneme appear more electron opaque than the region between these two parts. This density difference may represent a difference in diameter or it may indicate a change in the molecular composition at this point. The width of these denser regions is 8–10 mμ. The inner arm also extends out from subfiber A for a distance of 20 mμ, but does not appear to have so definite a hook as the outer arm. It curves in toward the center of the cilium and has a small knob of greater density on its terminal end (Figs. 1 *c*, 1 *e*, 2 *b*, 4 *b*

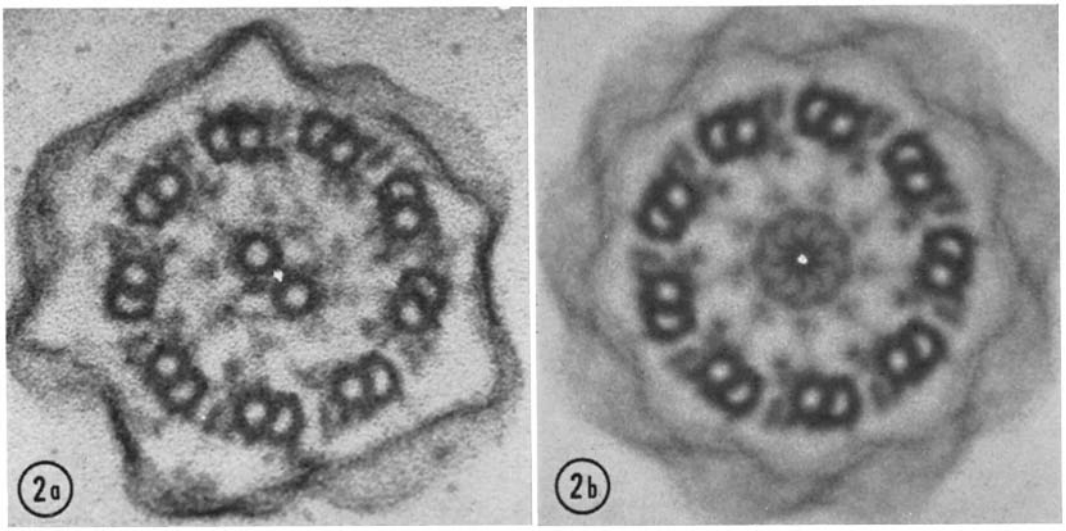
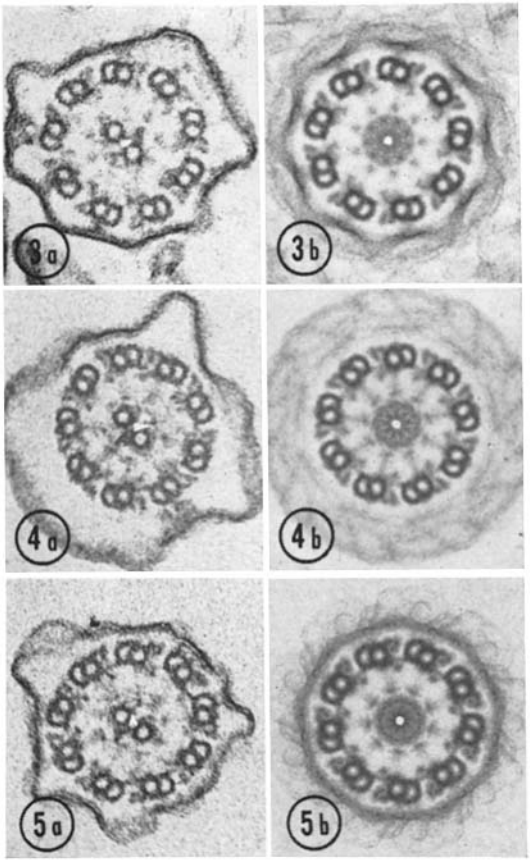


FIGURE 2 *a* An enlarged image of a cilium projected onto a tilted easel to make the image round rather than ellipsoid. Arms are evident, a suggestion of the central sheath appears, and spokes and thickenings along the spokes may be seen. × 200,000.

FIGURE 2 *b* A rotation image of the cilium in Fig. 2 *a*. The structure of the arms, the presence of spokes and a thickening on each spoke, and the connections between adjacent outer double fibers are much clearer and more evident than in Fig. 2 *a*. The inner arm appears to terminate in a dense knob. × 200,000.



FIGURES 3 *a–5 b* Figs. 3 *a*, 4 *a*, and 5 *a* were projected onto a tilted easel to make the images as round as possible. Figs. 3 *b*, 4 *b*, and 5 *b* are rotation images of 3 *a*, 4 *a*, and 5 *a*, respectively. In Figs. 3 *a*, 3 *b*, 5 *a*, and 5 *b*, the arms extend from the outer fibers in a clockwise direction since these cilia are being viewed from their bases outward toward their tips. Figs. 4 *a* and *b* have the arms extending in a counterclockwise direction since this cilium is being viewed from its tip toward its base. The outer arms in the rotation images are clearly hook-shaped. The two denser regions of this arm, the portion adjacent to subfiber A and the portion hooked back toward the center of the axoneme, are particularly evident in Fig. 3 *b*. In this figure, there is a definite light area between these two densities. The inner arms curve in toward the center, but differ from the outer arms in the absence of a hook. They seem to have a denser knob on their terminal end in Figs. 4 *b* and 5 *b*. The spokes are particularly evident in Fig. 4 *b*, while the thickenings along the spokes are present in all three rotation images. Connections between outer double fibers are suggested in Figs. 3 *b* and 4 *b*, but are particularly evident in Fig. 5 *b*. Connections between the outer fibers and the ciliary membrane can be seen in Fig. 3 *b*. × 100,000.

5 *b*, and 6). The proximal portion of this arm has a uniform density and measures 8–10 mμ in width.

Besides demonstrating the arms, the Markham technique confirms the presence of spokes extending from the inner margin of subfiber A toward the center of the axoneme (Figs. 1 *b*, 2 *b*, and 4 *b*). However, since the center of the axoneme does not have 9-fold radial symmetry, the detail in this portion of the axoneme is lost. The presence of a sheath encircling the central two fibers, although frequently suggested as in Figs. 1 *a* and 2 *a*, was never convincingly evident.

The thickening along each spoke is, however, confirmed. It lies about 33 mμ from the margin of subfiber A. It usually appears elongated with its long axis set perpendicular, or at least almost so, to the long axis of the spoke. It varies from 10 to 18 mμ in length (Figs. 1 *b*, 2 *b*, 3 *b*, 4 *b*, 5 *b*, and 6).

Filaments appear to connect the A subfibers of each outer double fiber with the B subfibers of immediately adjacent outer double fibers. Although suggested in all the Markham images, this connection is particularly evident in Figs. 2 *b*, 4 *b*, and 5 *b*. Gibbons (1965) reported a similar density connecting adjacent A subfibers in isolated axonemes of *Tetrahymena* and Ringo (1967 *b*) describes similar connections in the proximal portions of the flagella in *Chlamydomonas*.

Electron-opaque material extends from the

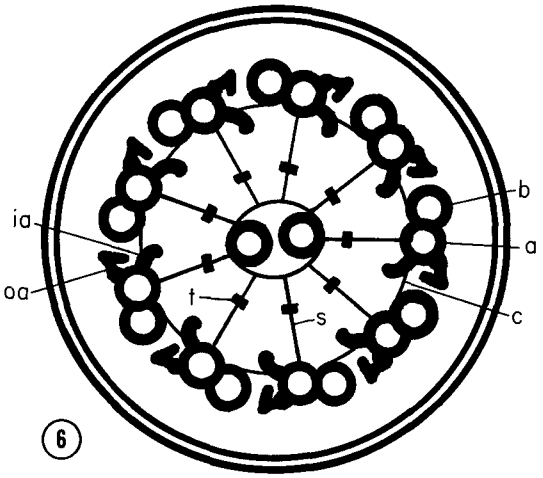


FIGURE 6 A diagram of a cross-section of a cilium. Except for the central fibers and central sheath, the diagram is based on Markham rotation images and is drawn approximately to scale. *a*, subfiber A; *b*, subfiber B; *c*, connection between outer double fibers; *oa*, outer arm; *ia*, inner arm; *s*, spoke; *t*, thickened region along spoke. × 210,000.

middle of the outer double fibers to the ciliary membrane (Figs. 1 *b*, 1 *c*, 1 *e*, and 3 *b*) in some of the Markham images. These connections can also be seen in Fig. 1 *a*, but here they only appear when the membrane lies close to the fibers. Ringo (1967 *b*) has reported similar connections in the transitional region of flagella and has reviewed their occurrence in other organisms as reported in the literature.

The central two fibers of the axoneme, which are 29 mμ in diameter, appear to be asymmetrical with respect to each other. One of these fibers appears to bear a prominent arm which extends from the fiber to the region of the central sheath in a direction more or less perpendicular to an imaginary line connecting the centers of the two central fibers. In a group of ciliary cross-sections in the same picture, this arm is present in the same general position and inclination in each cilium (arrows, Fig. 1 *a*). A second arm may arise from the same central fiber on its opposite side; however, it is usually less distinct. These details are, of course, lost in the Markham images.

DISCUSSION

Fixing *Tetrahymena* with glutaraldehyde followed by OsO_4 has preserved detail within the cilia which OsO_4 alone does not preserve. A fast dehydration time may also help to prevent leaching out of easily solubilized materials. Thus, better preservation combined with the use of the Markham technique has made it possible to add further information to the morphology of cilia. With the Markham rotation technique, it has been possible to confirm the presence of previously described, but not always observable, structures such as the spokes and thickenings along the spokes. The occasionally reported connections between adjacent outer subfibers and between these double fibers and the ciliary membrane have been more firmly established, and the structure of the arms has been expanded and elucidated.

Another structure which appeared too consistently to be overlooked is the arm or possibly the pair of arms on one of the two central fibers. This structure causes the central two fibers to be asymmetrical with respect to each other. The significance of these arms is not clear.

The molecular composition of cilia, particularly of the outer fibers and their arms, has yielded to biochemical studies in recent years. Adenosine triphosphatase (ATPase) activity has been localized in the arms (Gibbons, 1963, 1965). The protein having the ATPase activity has been called "dynein." Dynein appears in two forms having sedimentation coefficients of 14S and 30S.

Electron micrographs of 30S dynein show it to be a rodlike particle of variable length. These rodlike particles are 70–90 A wide and 400–5000 A long. They have a periodicity of 140 A along their lengths. The 14S dynein approximates the shape of an ellipsoid 90 A in diameter and 140 A in length. From the above measurements, Gibbons and Rowe (1965) suggest that "the 14S dynein molecules form the individual units of the arms and that these units are linked in the longitudinal plane of the cilium." This polymer of 14S molecules is thought to make up the 30S fraction. Grimstone and Klug (1966) have also observed rods in preparations of outer fibers from flagellated protozoa. These rods measured 70 A in diameter and have a mean length of 400 A. Single particles 70 A in diameter and 100 A in length which were also observed are thought to correspond to the 14S monomers of Gibbons and Rowe.

When the above information is compared with the observations in the present study, two problems are evident: (*1*) the arms in the present study, although of about the same diameter, are longer and of more complex structure than those described above, and (*2*) the outer and inner arms attached to subfiber A at the same cross-sectional plane of the cilium seem to show morphological dissimilarity which may indicate a biochemical or functional dissimilarity or both. The outer arm as viewed in cross-section appears to be formed of more than one subunit. The dense portion next to subfiber A has dimensions similar to those described by Gibbons and Rowe (1965) and even more closely similar to those described by Grimstone and Klug (1966). Thus, it seems that the portion of the outer arm hooking back toward the center of the axoneme may be lost in the experimental procedures employed by these workers. It is more difficult to correlate the image of the inner arm as seen in this study with the biochemical subunits described above. These inner arms, as viewed in a cross-section of a cilium, may be composed of a combination of two 14S monomers, or it may be that their structure is similar to that of the outer arms but that because of their position this structure is more labile and has become distorted during fixation and dehydration.

This work has shown again the usefulness of the

Markham rotation technique to improve resolution where radial symmetry is present. It also raises questions about previous interpretations correlating electron microscope images of the arms on subfiber A with the images of isolated dynein protein.

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