Flagellar Gyration and Midpiece Rotation during Extension of the Acrosomal Process of *Thyone* Sperm: How and Why This Occurs

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Abstract. The midpiece of *Thyone* sperm contains a large mitochondrion and a centriolar pair. Associated with one of the pair, i.e., the basal body of the flagellum, are satellite structures which apparently anchor the flagellar axoneme to the mitochondrion and to the plasma membrane covering the midpiece. Immediately before and as the acrosomal process elongates, the flagellum and the midpiece begin to rotate at 1–2 rotations per second even though the head of the sperm, by being firmly attached on its lateral surfaces to the coverslip, does not rotate at all. This rotation is not observed in the absence of flagellar beating whose frequency is much greater than that of its gyration. To understand how the midpiece rotates relative to the sperm head, it is first necessary to realize that in *Thyone* the flagellar axoneme projects at an acute angle to the principal axis of the sperm and is bent towards one side of this axis. Thus movement of the flagellum induces the sperm to tumble or yaw in solution. If the head is stuck, the midpiece will rotate because all that connects the sperm head to the midpiece is the plasma membrane, a liquid-like layer. A finger-like projection extends from the proximal centriole into an indentation in the basal end of the nucleus. In contrast to the asymmetry of the flagellum, this indentation is situated exactly on the principal axis of the sperm and, along with the finger-like projection, acts as a biological bearing to maintain the orderly rotation of the midpiece. The biological purpose of flagellar gyration during fertilization is discussed.

ELUCIDATING the steps in sperm–egg fusion (fertilization) has occupied the careers of countless investigators. This seemingly simple event actually involves a large number of discrete steps, many of which are now at least partially understood. What is interesting is that the exact mechanism can differ significantly in different species. The most commonly studied group of organisms is the echinoderms and among the group, the sea urchins hold the limelight, largely for historic reasons.

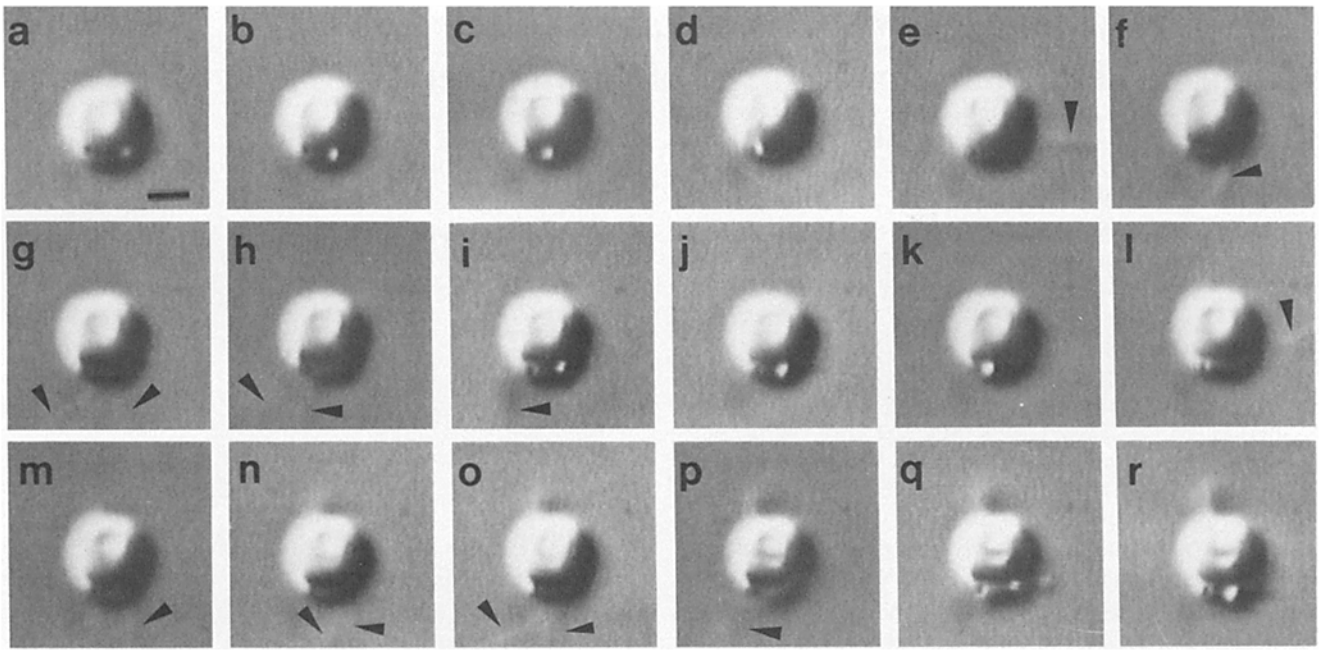
We have been studying the acrosomal reaction of living sperm of *Thyone briareus*, a sea cucumber, using high extinction video microscopy. During the course of these studies we noticed on our video sequences something which was not only remarkable but also inexplicable. Immediately before and as the acrosomal process elongates, the flagellum and the entire midpiece (which consists of its plasma membrane, a large mitochondrion, and a centriolar pair and associated satellite structures) begins to rotate even though the head of the sperm, by being firmly attached to the coverslip, does not rotate at all. The relative motion of the midpiece and the head proper is interesting on its own, but what is even more puzzling is that the rotary motion of the midpiece at 1–2 rotations per second is an order of magnitude slower than the rate of flagellar beat which, if *Thyone* sperm are similar to the sperm of sea urchins, is planar, not rotary (Gibbons and Gibbons, 1972).

In this paper we document the motion elicited in the midpiece, and from thin sections of fixed sperm, demonstrate how this type of motion could be generated with the complex equipment we find in the midpiece. In the discussion we will consider the biological function of this bizarre type of motion.

Materials and Methods

Thyone briareus were collected by the Marine Resources Department (Marine Biological Laboratory, Woods Hole, MA). To obtain sperm, the testes were removed and minced in sea water. The suspension was filtered through cheese cloth and the supernatant centrifuged at 1,000 *g* for 5 min to pellet the sperm. To examine the midpiece rotation, we recorded the antics of *Thyone* sperm using a Leitz 100×/1.3 NA Smith T differential interference contrast objective lens with the condensor oil immersed and used at an NA of 0.91. The video tape, recorded at 60 fields per second in the "1.2 hour mode" on a time lapse recorder (Sony TVO-9000), was played back in the "72 hour mode." Each field was transformed during an interval free of noise onto a video laser disk recorder (Panasonic model TQ-2021 FBC high resolution monochrome optical disk recorder). Selected fields played back from the disk recorder were photographed off a monitor (Panasonic WV 5310) on 35-mm Plus-X film through a Ronchi grating. Details of the diffusion chamber, video apparatus etc., are found in Inoué and Tilney (1982) and Inoué (1986).

Since the sperm are thought to be immotile in the testes and to become



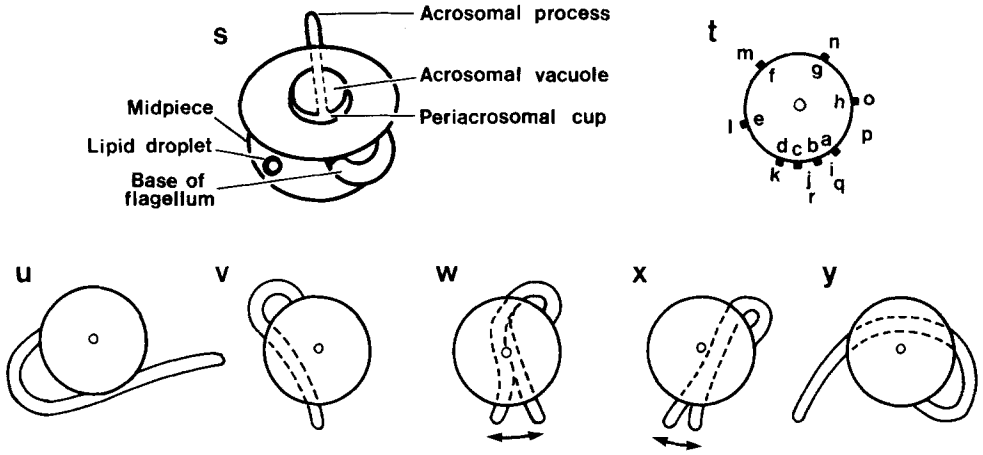


Figure 1. (*Top*) Video sequence illustrating the rotation of the midpiece in a living sperm in a slightly oblique side view. Each frame illustrated in this sequence is 1/15 s after the one preceding it. The bright spot on the midpiece in *a–d*, *i–k* and *q* and *r* is the basal end of the flagellum extending directly towards the viewer. Note how it shifts from right to left and then disappears, periodically. The arrows indicate other portions of the flagellum which have come into focus. The formation and extension of the acrosomal process occurs in *m–r*. Bar, 1 μm. (*Bottom*) *s* is an interpretation of *q*. Note the lipid droplet indicated which remains stationary in *a–r*. *t* is a composite top view of the sperm indicating the positions of the base of the flagellum. Their positions were determined from the lateral views where the bright spot was visible. Where the flagellar base was not visible (*e–h*, *l–p*), their positions were interpolated. The nonuniform speed of rotation is presumably due to the flagellum beating against the coverslip in *a–d* and *i–k*. *u–y* show top views of the basal portions of the flagellum reconstructed from *t* plus *e* and *l* (*u*), *f* and *m* (*v*), *g* and *n* (*w*), *h* and *o* (*x*), and *i*, *p*, and *q* (*y*).

motile by dilution in sea water and/or by substances liberated from the egg, we fixed small pieces of testis as well as mature sperm that had been liberated from the testes. Fixation was carried out by adding 1% glutaraldehyde (8% stock from Electron Microscopy Sciences, Fort Washington, PA) to the testis or sperm suspended in sea water. The sperm were fixed at room temperature for 30 min, concentrated by centrifugation, washed briefly in sea water, and postfixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.2 for 30 min at 0°C (Tilney and Inoué, 1982). The fixed sperm were then washed three times in cold water and en bloc stained with 0.5% uranyl acetate overnight. The sperm were then rapidly dehydrated in acetone and embedded in Epon 812. Thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate and examined in a Philips 200 electron microscope.

Results

Observations on Living Sperm

Some of the sperm introduced into the perfusion chamber attach to the clean surfaces of the slide or coverslip. While the attachment is secure enough so that most sperm, once attached, are not dislodged by additional perfusion, attachment seems to occur mainly by the side of the head, leaving the midpiece and the tail free. Thus, the tail of the attached sperm can beat at its normal frequency. Furthermore, the acrosomal process can elongate without hindrance of a glass surface because it extends from the apical, not the lateral surface of its head.

Close examination of the midpiece reveals that it is asymmetric, a feature which allows us to document the rotation of this region. Although it is difficult to understand the basis for this asymmetry exclusively by light microscopy, by a combination of light microscopy and thin sections we now know that the asymmetry is due to the asymmetric shape of the mitochondrion and the location of the flagellar axoneme

projecting basolaterally from the midpiece where the mitochondrion is either reduced in thickness or absent (see Figs. 1 and 3).

By playing the video tape back at various speeds, and by following the progressively changing location of the flagellum and the shape of the midpiece in successive frames of the video sequence, it is relatively easy to convince ourselves that the flagellum is gyrating and the midpiece is indeed rotating, rather than just swinging back and forth. A portion of such a sequence is illustrated in the top panel in Fig. 1 in which each frame is separated from its neighbor by 1/15 s. The discrete, bright spot on the midpiece is an optical section of the basal portion of the flagellum extending from the midpiece directly towards the viewer. The flagellum then curves around the sperm. These relationships are most easily understood by comparing the drawings in the lower panel of Fig. 1 with the appropriate video frames.

The flagellum beats so vigorously that, outside of the optical section at its base, only portions of it (see arrows) are visible in a single field of the video (1/60s), the rest appearing as a blur. On tape playback we see the whipping motion of the sperm tail, but definitive data on the gyration of the flagellum swinging completely around and around the sperm axis comes from sequential frame analysis of the movement of the base of the tail (the discrete, bright spot in *a–d*, *i–k*, and *q–r* in Fig. 1). The bright spot moves from right to left on successive frames in which the base of the tail is pointing towards the observer. Then it disappears for several frames until it reappears on the right side of the midpiece and the process repeats, again periodically (see composite top view, Fig. 1 *t*), at a frequency of approximately once every half second. The more distal part of the tail (arrows in *e–i* and *l–p* in Fig. 1) wraps partly around the midpiece as shown in the interpretive drawings (Fig. 1, *u–y*). Therefore the base of the tail that extends from the midpiece and the part that is presumably generating the thrust are pointing in different directions, thus producing a net torque which results in its gyration.

In the last six frames in the top panel of Fig. 1 (*m–r*), the acrosomal process is seen elongating out of the anterior end of the sperm.

From these reconstructions of the video sequence and others like it we have determined that the flagellum and midpiece invariably rotate in a clockwise direction as viewed from the sperm head towards the midpiece. The speed of midpiece rotation, which was measured in two sperm in detail (one is graphed in Fig. 2) and in several others where the asymmetry of the midpiece or swinging of the tail could be distinguished at least briefly, is 1–2 rotations per second.

In the video micrographs, the lipid droplet (labeled in Figs. 1 and 7) does not rotate with the midpiece but remains stationary. The droplet, which appears near the left margin of the midpiece (*a–s*) can be distinguished by its reversed shadowing (brighter to the right as in the acrosome owing to its lower refractive index; see Inoué and Tilney, 1982). We do not understand why the lipid droplet does not rotate with the rest of the midpiece; perhaps it is more firmly connected to the sperm head rather than to the midpiece.

Midpiece rotation has only been observed in sperm whose flagella are beating; however, not all sperm whose flagella are beating display midpiece rotation. In the latter case, both the midpiece and the head may be stuck to the glass surface.

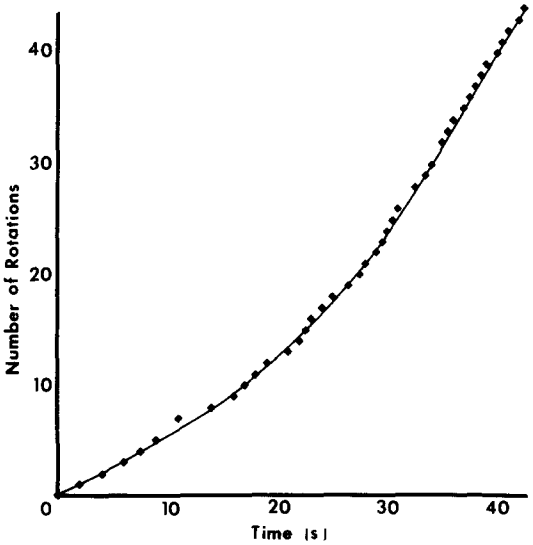


Figure 2. Graph depicting the rate of midpiece rotation. Each point on the graph is one complete rotation of the midpiece.

Electron Microscopic Observations

The Flagellum, Its Basal Body, and Associated Centriole Are Located not on the Principal Axis of the Sperm, but to One Side. In thin sections through sperm which were fixed while still in the testis, we find that the flagellum invariably extends from the basolateral end of the midpiece (Fig. 3). An identical morphology is encountered in unreacted sperm which have been liberated from the testis by mincing the gonad. Not only is the basal body of the flagellum (often referred to as the distal centriole) located off to one side of the major axis of the sperm, but also the proximal centriole is situated off axis. A curved finger-like projection extends anteriorly from the centriole into an indentation in the basal end of the nucleus. The free end of this projection lies on a line that extends posteriorly from the center of the acrosomal vacuole, the cup of profilactin, and the nucleus (Fig. 3). In short, the free end of this projection is situated precisely along the principal axis of the sperm in contrast to the proximal centriole and basal body of the flagellum, which are located to one side of this axis.

The Basal Body and Its Associated Satellite Structures. In thin sections cut through the basal body, we find nine spoke-like satellites that extend radially from the nine triplets. Each satellite bifurcates at a dense node to connect to two dense bodies (Fig. 4) which in turn are connected to a periodically striated substance (Figs. 5 and 6). This striated substance is in intimate contact with the plasma membrane along one of its surfaces and with the outer mitochondrial membrane along another (Fig. 6). The net result of this complex organization around the basal body is that this organelle and its associated axoneme appear to be firmly connected to not only the plasma membrane at this point, but also to the mitochondrion. Thus, gyration of the flagellum would result in rotation of the entire midpiece.

The Relationship between the Proximal and Distal Centrioles. Unlike what one might predict from the literature on starfish (Sousa and Azevedo, 1983; Kuriyama and Kanatani, 1981) and sea urchin sperm (Longo and Anderson, 1969), in *Thyone* sperm the proximal and distal centrioles are not

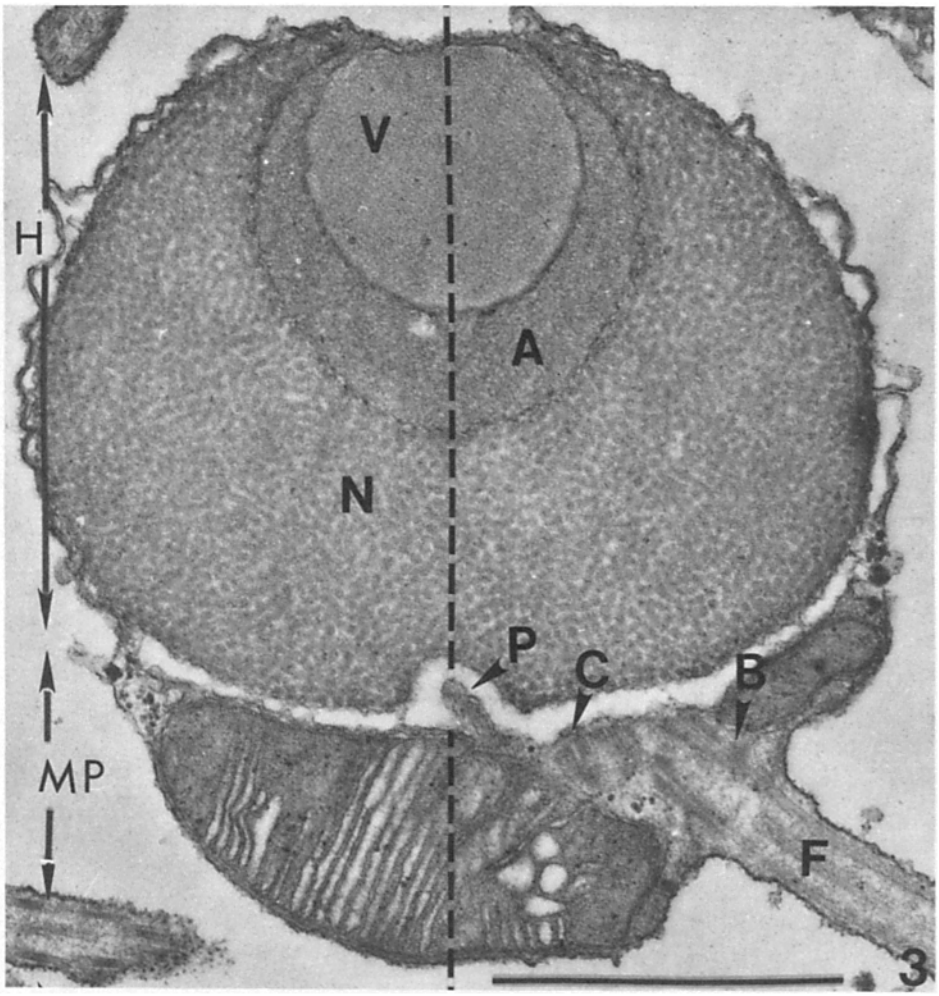


Figure 3. Thin section of a *Thyone* sperm. The head (*H*) and midpiece (*MP*) region are indicated. Located within an invagination at the anterior of the nucleus (*N*) is the acrosomal vacuole (*V*) and the profilactin region (periacrosomal cup: *A*). The flagellum (*F*) extends from the basolateral surface of the sperm. The flagellar axoneme is connected to the distal centriole or basal body (*B*); associated with this is the proximal centriole (*C*). A curved, finger-like projection (*P*) extends from the proximal centriole towards the major axis of the sperm (shown by the dotted line), its unassociated end fitting into an indentation in the nuclear envelope. Bar, 1 μm.

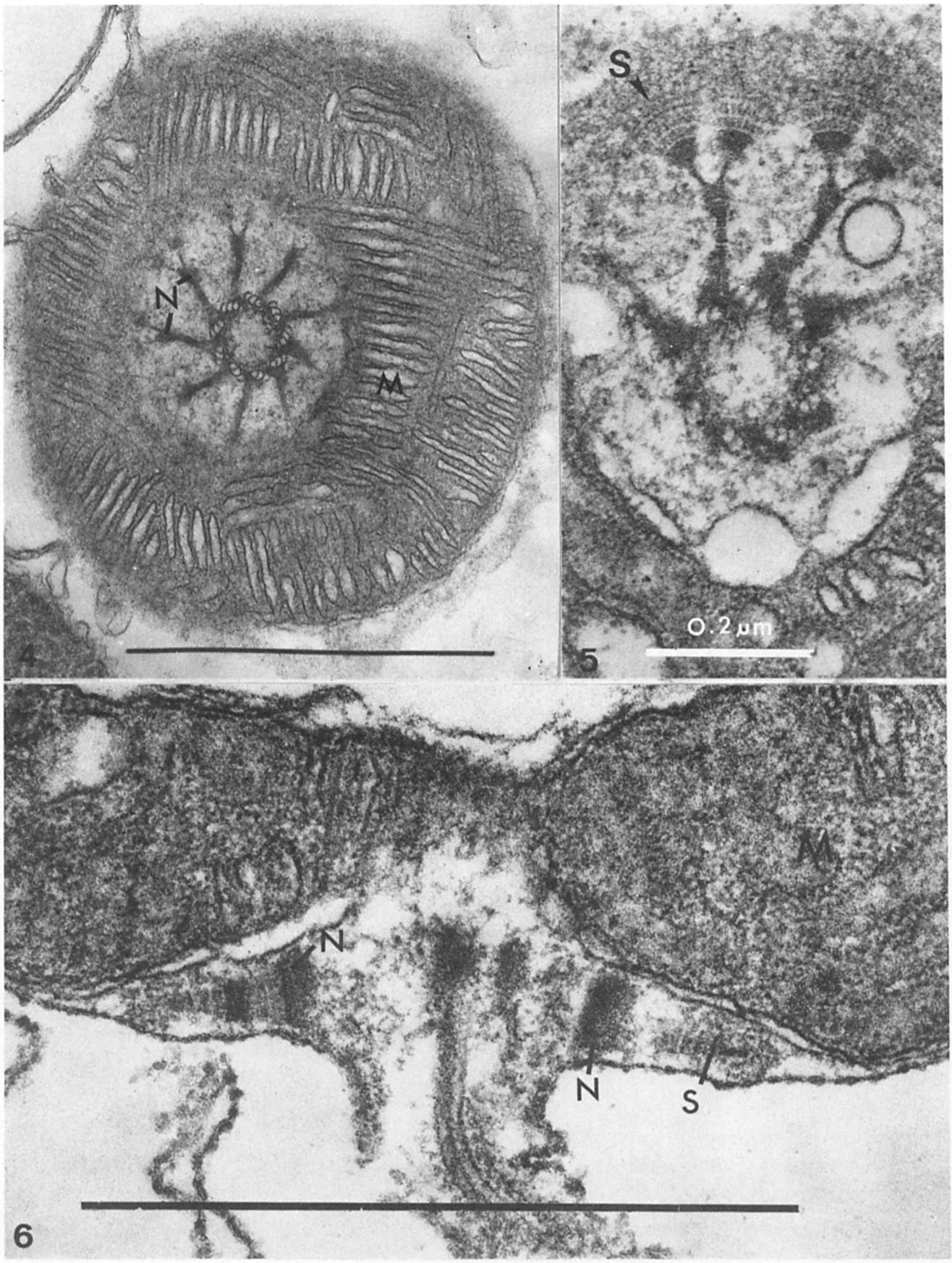
oriented at right angles to each other, but at approximately 30° to each other (Figs. 3 and 9). No obvious structures connect adjacent centrioles.

The Finger-like Projection Attached to the Proximal Centriole. Extending from the lateral surface of the proximal centriole is a curved, finger-like projection (Figs. 3, 9, and 10). Initially it parallels the basal surface of the nucleus (Fig. 8); it then curves anteriorly and enters an indentation in the mid-basal portion of the nucleus (Figs. 3, 8, and 9). It does not penetrate either the outer or inner nuclear envelope, but instead nests in this indentation (Figs. 8 and 9). Interestingly, the two nuclear envelopes at this position are connected together by fine filaments that span the space between these two membranes. Elsewhere the two membranes can be separated by varying amounts (Fig. 8). At high resolution, this finger-like projection is seen to be composed of light and dark bands whose periodicity is 110 Å. What will be significant later is the fact that in favorable sections the free end of this projection lies along the principal axis of the sperm.

Discussion

Two observations have convinced us that midpiece rotation is brought about by movement of the flagellum. First, midpiece rotation is only observed when the flagellum is also in motion, and second, in sperm the cytoplasm is so reduced in volume that there is not only no space for additional motile elements, but also none are found (see Tilney, 1985). If we accept that the flagellum provides the motile force for midpiece rotation which is seen when the sperm head is stuck

Figures 4–6. (*Fig. 4*) Thin section cut through the basal body of the flagellum. Extending from each of the nine triplet microtubules are spokes, each of which bifurcates at a dense node (*N*) into two dense structures. These structures in turn contact the outer mitochondrial membrane and the plasma membrane. *M* indicates some of the cristae of the mitochondrion. Bar, 1 μm. (*Fig. 5*) Higher magnification of the basal body which shows the spokes in greater detail. Most interesting is that not only is each spoke striated, but also the nodes connect to a striated material (*S*) that is connected to both the plasma membrane and the mitochondrion (not visible in this figure, but in Fig. 6). Bar, 0.2 μm. (*Fig. 6*) Grazing, thin section cut parallel to the basal body but in front of it. In this section are two satellites, their nodes (*N*), and the striated material (*S*) that extends from the nodes. Of interest is that the striated material is in close contact with the plasma membrane and the surface of the mitochondrion (*M*). By comparing this micrograph with Figs. 4 and 5, and relating all this to the reconstructed drawing, Fig. 10, the reader can determine the orientation of the section. Bar, 1 μm.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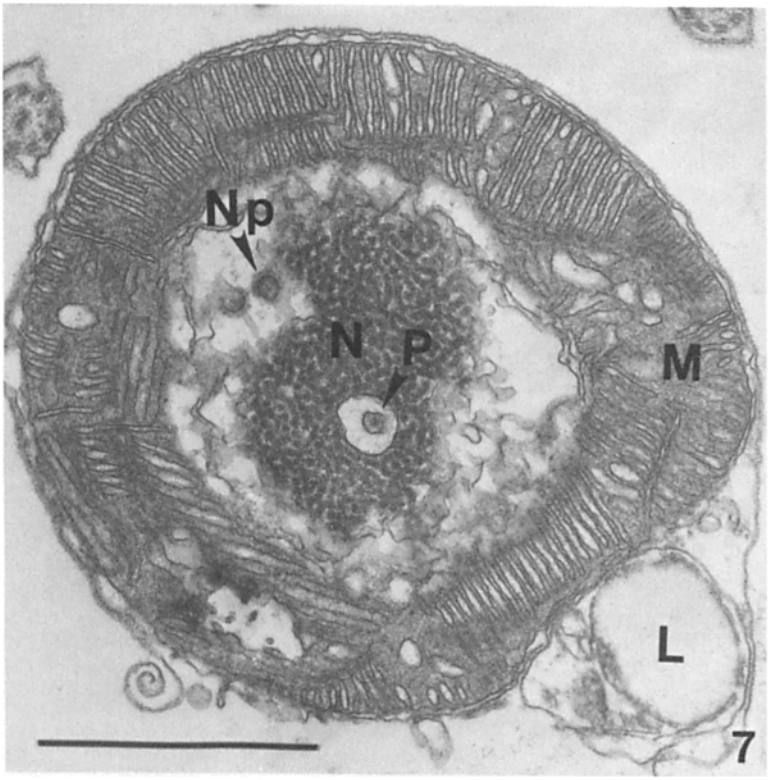
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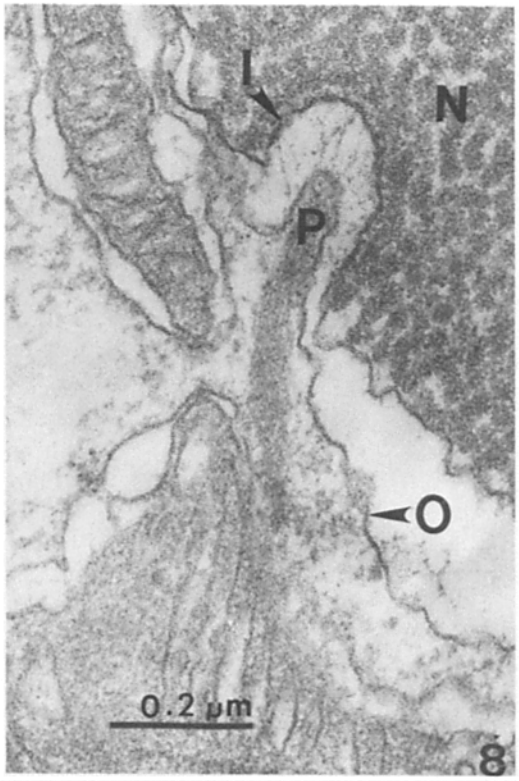
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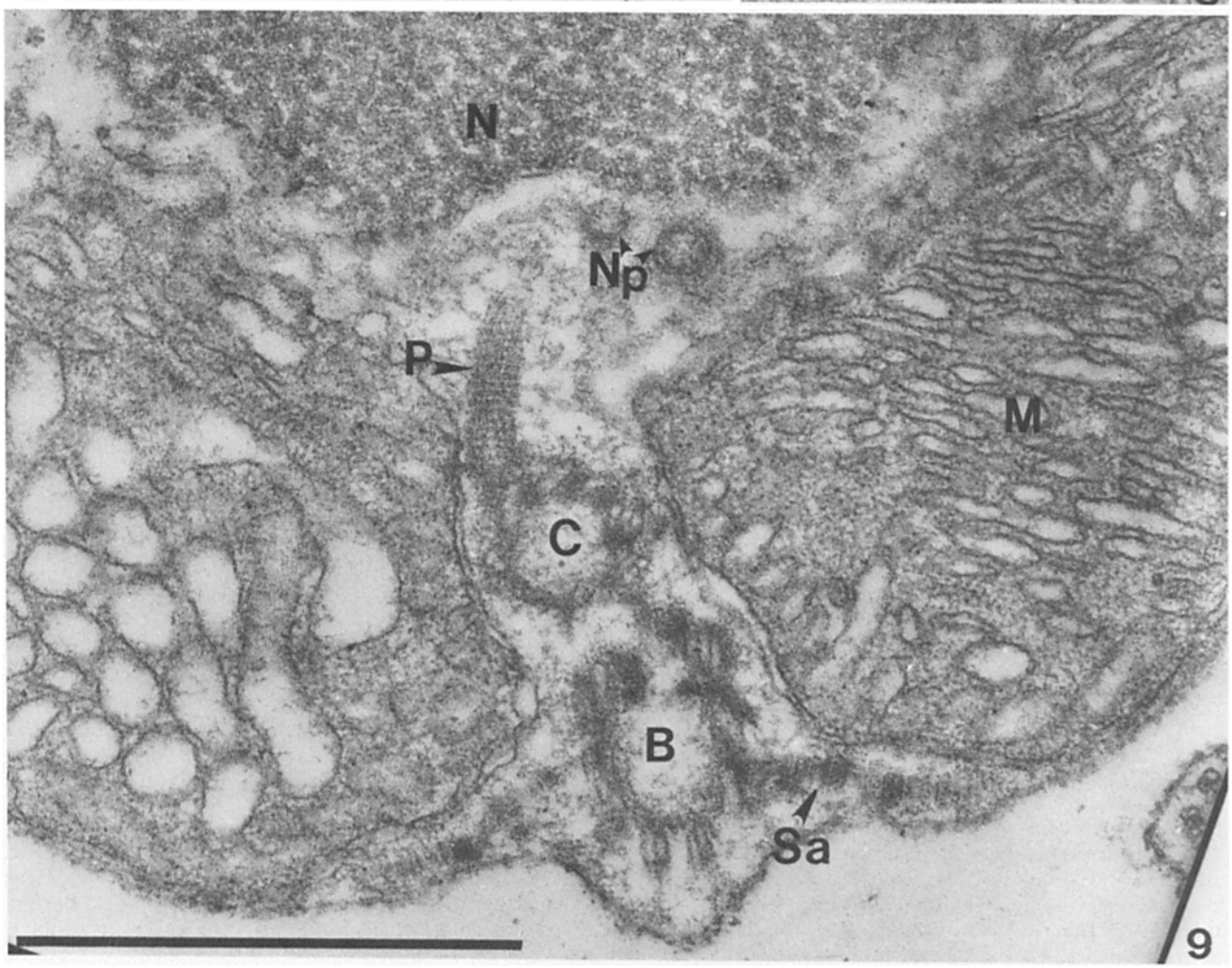
N
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to a slide or coverslip, one wonders how this occurs and why the frequency of rotation is a small fraction of the frequency of flagellar beat.

The key to understanding how the flagellum induces the rotation of the midpiece is the observation that the basal body of the flagellum is not located along the principal axis of the sperm, but instead is situated off to one side and is bent at its base so that it projects at an angle to the head axis. The result of this is that when the flagellum beats, irrespective of the type of motion, albeit by planar waves, by three-dimensional waves, or by some combination, the sperm head would not only be induced to move forward but at the same time there would be a component to the motion that would tend to make the sperm tumble or yaw. The frequency of the tumbling or yawing would bear no obvious relationship to the frequency of flagellar motion because it would be dependent on a variety of factors such as the angle of the flagellum relative to the principal axis and the resistance of the sperm head to midpiece rotation.

When the sperm head is stuck to the slide, a portion of the plasma membrane covering the midpiece would be induced to move because it is attached to the gyrating flagellum by the satellite structures. Yet another portion of the same membrane, that covering the nucleus, would be stationary being stuck to the slide. One might wonder where the shear zone is located on the membrane and if the membrane is specialized in this region. Fortunately for us there is a biological precedence for rotary motors. The most relevant case is the devescovinid protozoa in which one part of the plasma membrane, the head, rotates relative to a neighboring part, the body (Tamm and Tamm, 1974). By electron microscopy (Tamm and Tamm, 1974), conventional freeze fracture (Tamm and Tamm, 1980), and analysis of the sterol composition of the membrane at the point of rotation (Tamm and Tamm, 1983), it has been shown that this is not a unique membrane. Rather shearing is a natural consequence of the fluid nature of plasma membranes. Thus, different parts of the plasma membrane of this same organism can rotate with respect to other parts. A similar situation, albeit documented in much less detail occurs during the acrosomal reaction of *Limulus* sperm (Tilney, 1985). Thus, by analogy to these systems, midpiece rotation with respect to the nucleated portion of *Thyone* sperm could occur. Because the satellite structures associated with the basal body morphologically are connected to both the plasma membrane and the outer mitochondrial membrane, it seems likely that the entire midpiece (with the possible exception of the lipid droplet) would rotate as a unit relative to the nuclear half of the sperm, which is stuck. Logically then the shear region would be situated on the plasma membrane at or near the juncture between the midpiece and nucleated portion of the sperm. We cannot be more accurate than this as we do not have enough markers on the lateral surface of either the plasma membrane or the nuclear envelopes to pinpoint the position of shear more accurately.

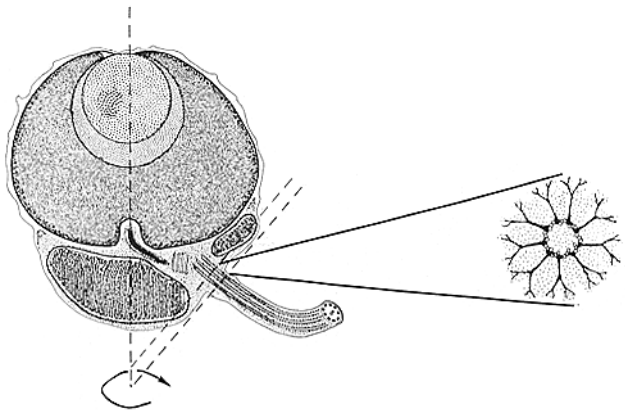

Figure 10. Drawing illustrating a *Thyone* sperm. The single broken line indicates the principle axis. The pair of broken lines indicates the section one would need to visualize the basal body of the flagellum and its satellite structure. Extending from the proximal centriole is the finger-like projection which nestles into an indentation in the nucleus. Notice that the tip of this projection is aligned on the principle axis. It acts as a pivot around which the midpiece can rotate by gyration of the flagellum. Notice that the flagellum extends from the basolateral surface of the sperm and then wraps around the midpiece. If the sperm head were stuck, motion of the flagellum would induce a rotation of the midpiece.

If the plasma membrane is fluid, as we are lead to believe, and if it is the major connector of the midpiece and the nucleated half of the sperm, then one wonders why the midpiece always remains basal to the head. Why doesn't it creep around as it moves relative to the head? The answer to this question seems related to the finger-like projection that extends from the proximal centriole into an invagination in the nucleus. Recall that the projection fits into the indentation at a point that is located along the principal axis of the sperm (Fig. 10). It is this point on which the midpiece rotates relative to the nucleated portion of the sperm. Thus the finger-like projection and indentation in the nucleus form the biological equivalent of a pivot and sleeve whose major function is to maintain the location of the midpiece relative to the nucleated portion of the sperm. As a further point of interest, it is specifically at this indentation and only there that the outer and inner nuclear envelopes are connected together by fine filaments. This provides a biological bushing. Elsewhere these two membranes can separate to varying amounts.

The next question that should be raised is this: is midpiece rotation of physiological interest or importance to the sperm or is it just a consequence of sperm being stuck to a glass surface? Obviously it would be difficult if not impossible to

Figures 7–9. (*Fig. 7*) Thin section cut perpendicular to the principle axis of the sperm through the basal end of the nucleus (*N*). Two nuclear pores (*Np*), the mitochondrion (*M*), and an eccentrically positioned lipid droplet (*L*) are seen in this section. Of interest to this report is the projection (*P*) that extends from the proximal centriole. This projection extends into an indentation in the nucleus. It is limited by the outer nuclear envelope. Bar, 1 μm. (*Fig. 8*) Thin section through a portion of the basal end of the nucleus (*N*). Extending into an indentation in the nucleus is the projection (*P*). Notice that it does not penetrate either the outer (*O*) or inner (*I*) nuclear envelopes. Fine strands connect these two nuclear envelopes at this point of indentation but are absent from the other parts of the nuclear envelopes. Bar, 0.2 μm. (*Fig. 9*) Thin section through a portion of the midpiece. Included in this section are the basal end of the nucleus (*N*) with its nuclear pores (*Np*), a portion of the mitochondrion (*M*), the proximal centriole (*C*) with its associated nuclear projection (*P*), the basal body of the flagellum (*B*), and a portion of one of its satellites (*Sa*). Of interest is that the projection is striated. Bar, 1 μm.

determine if midpiece rotation ever occurs in free swimming sperm. What is important is whether midpiece rotation occurs during fertilization. We do not yet know the answer to this question, but we hope our study will stimulate others to examine it.

Unlike the sea urchin (e.g., *Arbacia* or *Strongylocentrotus*), the jelly surrounding the sea cucumber (*Thyone*) and starfish (e.g., *Asterias* or *Marthasterias*) egg is so rigid that when a sperm makes contact, its forward motion is arrested. One of the first steps in the acrosomal reaction is to firmly attach the sperm to the surface of the jelly layer so that as the acrosomal process extends, the sperm proper is not translocated backwards away from the jelly layer. Adhesion is presumably accomplished by a "bindin-like" protein, formerly present in the acrosomal vacuole (see Vacquier and Moy, 1977). An additional way to keep the sperm attached to the egg is for the flagellum to maintain its motion so that it continuously pushes the sperm towards the egg. In fact, many investigators (e.g., Chambers, 1930; Dan, 1954) have stated that the flagella of fertilizing sperm continue to beat during the acrosomal reaction. Thus, during the steps which immediately precede fusion of sperm and eggs, the flagellum of the fertilizing sperm is beating maximally which is what one would have predicted. If the flagellum were to move with a rotary motion, then the sperm would be pressed continuously and consistently towards the surface of the egg, a situation that would be ideal. However, if the flagellum is beating with a planar wave as it does in sea urchin sperm (see Gibbons and Gibbons, 1972), then the sperm head might be dislodged on the jelly layer as it oscillates backward and forward in one axis. (To inhibit this dislodgement it is possible that the pattern of bend generation is different at the junction of the flagellum with the sperm head, but we lack evidence for this.)

Irrespective of the motion of the flagellum, albeit with a planar wave or a three-dimensional wave, bindin must fix the head of the sperm to the jelly sufficiently tightly so that the head will remain stationary. The midpiece, however, could rotate independently of the attached head and if so, convert the gyrating flagellar beat into a forward thrust, a thrust needed to combat the backward directed force induced by elongation of the acrosomal process. In short, it seems to us as if the midpiece of *Thyone* sperm is perfectly designed to function in fertilization.

Consistent with the above speculation are observations made on the eggs and sperm of different groups of echinoderms. Sea urchin and sand dollar sperm such as *Arbacia* (Longo and Anderson, 1969), *Paracentrotus*, *Strongylocentrotus*, and *Echinarachnius* (Christen et al., 1982; Lee et al., 1983; Summers and Hylander, 1974) all have short acrosomal processes (<1 μm in length) and all have flagella whose basal bodies are situated on the principal axis. Furthermore, in all cases the flagella extend directly posteriorly, not posterolaterally as in *Thyone*. The jelly of the eggs of these organisms is soft so that the sperm can swim through it. On the other hand, sperm which produce long acrosomal processes, such as sea cucumbers (Colwin and Colwin, 1956; Tilney, 1985), brittle stars (Hylander and Summers, 1975), and starfish (Tilney, 1975, Sousa and Azevedo, 1985) have eccentric flagella and the jelly of the eggs of the same species is sufficiently hard so that the sperm cannot swim through it.

Thus, if the acrosomal process is long, e.g., more than a few micrometers, then the posteriorly directed force produced by the anterior extension of the acrosomal process must be compensated for by a force in the opposite direction which pushes the sperm towards the egg. This force is presumably produced by the flagellum which in all cases of sperm that form long acrosomal processes is eccentric in its location. Thus, a propeller type movement might be indicated. On the other hand, sperm with short acrosomal processes do not have to be concerned with a force directed posteriorly and accordingly do not need a propeller type movement.

Many investigators have noticed that sperm which have undergone the acrosomal reaction can be readily identified by the midpiece which tends to round up; concomitantly the flagellum reorients, now projecting laterally from the point of connection of the midpiece and the sperm head proper (Chambers, 1930; Dan, 1952; Tilney, 1975; Schroeder and Christen, 1982). Since sperm "taken directly from the testis are sluggish and frequently motionless, but become active when diluted in sea water" (Chambers, 1930), it is possible that the trigger of this activity in turn induces the motility by changing the position of the flagellar complex. It turns out that this is not correct. Dan (1954) could see no difference in the morphology of nonmotile and motile sperm and in her study the fine structure of the midpiece region in the sperm examined in pieces of fixed testis was identical to that of those fixed while suspended (presumably motile) in sea water. Furthermore, careful analysis of our video tapes show that the rounding up of the mitochondrion and the reorientation of the flagellum is a late event, occurring after the completion of extension of the acrosomal process. Thus, the eccentric location of the flagellar basal body, which we presume leads to the rotary motion of the midpiece is present in nonmotile, motile, and fertilizing sperm.

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