STRUCTURE AND FUNCTION OF THE 
UNDULATING MEMBRANE IN SPERMATOZOAAN PROPULSION 
IN THE TOAD BUNO MARINUS 

M. A. SWAN, R. W. LINCK, S. ITO, and D. W. FAWCETT 

From the Department of Histology and Embryology, University of Sydney, New South Wales, 
Australia, 2006, and the Department of Anatomy, Harvard Medical School, Boston, Massachusetts 
02115 

ABSTRACT 

Accessory fibers in most sperm surround the axoneme so that their function in 
propulsion is difficult to assess. In the sperm of the toad BUNO MARINUS, an accessory 
fiber is displaced from the axoneme, being connected to it by the thin undulating 
membrane in such a way that the movement of axoneme and accessory fiber can 
be viewed independently. The axoneme is highly convoluted in whole mounts, 
and the axial fiber is straight. Cinemicrographic analysis shows that it is the 
longer, flexuous fiber, the presumed axoneme, that moves actively. The accessory 
fiber follows it passively with a lower amplitude of movement. The accessory fiber 
does not move independent of the axoneme, even after demembranation and 
reactivation of the sperm. 

On the basis of anatomical relations in the neck region, it appears that the 
accessory fibers of amphibians are analogous to the dense fibers of mammalian 
sperm. SDS polyacrylamide gel electrophoresis of demembranated toad sperm 
tails reveals two principal proteins in addition to the tubulins, the former probably 
arising from the accessory fibers and the matrix of the undulating membrane. 

The function of displacing an accessory fiber into an undulating membrane may 
be to provide stiffness for the tail without incurring an energy deficit large enough 
to require a long middle piece. A long middle piece is not present in toad sperm, 
in contrast to those sperm that have accessory fibers around the axoneme. However, the toad sperm suffers a reduction in speed of about one-third, compared 
with the speed expected for a sperm without an undulating membrane. 

There has been considerable speculation as to the role of accessory fibers or dense fibers in spermatozoan movement. These fibers occur in the sperm of most vertebrates and some molluscs and arthropods. Cleland and Rothschild (7) suggested that the fibers have a contractile function because in the bandicoot they are displaced some distance from the axoneme. This view was popular until it was found that accessory fibers are stabilized by S-S bonds in some mammals (6) and in various submammalian species, including the newt Noto- 
phthalamus, in which all of the dense material in the undulating membrane of the sperm tail appears to be stabilized by S-S bonds (1). This suggests that accessory fibers, including the dense fiber in the undulating membrane of amphibia,
have properties similar to those of keratin, which makes it doubtful that they contract and likely that they confer "elastic rigidity" to the tail (1).

It is difficult to determine whether accessory fiber motility is independent of the axoneme because in most sperm tails the fibers are located in a ring around the axoneme. However, in some amphibians, one of the accessory fibers is displaced far enough from the axoneme for its movement to be seen independently with the light microscope. This fiber has been called the axial fiber in the newt (9) and the membrane filament in Bufo arenarum (5). In the triton, Picheral (15) called it the supporting filament, but, in a review of urodele sperm structure, he (16) called it the axial fiber. We will call this structure in Bufo marinus the axial fiber to be consistent with references 9 and 16. The accessory fiber that is adjacent to doublet 3 in Bufo marinus we will term the juxta-axonemal fiber (see Fig. 9). This fiber was found in Bufo arenarum by Burgos and Fawcett (5) but not named. It is not present in the triton (15) or in other urodèles (16).

Burgos and Fawcett (5) studied the movement of the spermatozoa of the toad Bufo arenarum using normal film speeds and concluded that the undulating membrane moved rapidly, with a short wavelength, while the axoneme moved slowly, with a low amplitude and long wavelength. To follow the movement accurately and to eliminate stroboscopic effects, it is necessary to use a film speed of at least 150 frames per second (fps), a speed not available to the earlier study. In view of a revived interest in the function of accessory fibers resulting from the work of Bedford and Calvin (1), we decided to repeat the study of Burgos and Fawcett (5) using a higher film speed to record toad sperm movement. This work has been reported in abstract form by Swan et al. (19).

MATERIALS AND METHODS

Testes were dissected from adult male toads (Bufo marinus) and chopped with a razor blade in a hypotonic saline solution (containing 30 mM NaCl, 0.4 mM KCl, and 0.5 mM CaCl₂; pH 7.0). The resulting sperm suspension was pipetted off and either diluted further with distilled water for microscopic observation or demembranated with detergent, as will be described below.

It should be noted that all observations were made on testicular material because attempts to obtain sperm by injecting toads with human chorionic gonadotropin were unsuccessful.

All slides and coverslips were cleaned in detergent followed by sulfuric acid, rinsed in water and distilled water, and baked with silicone to prevent sperm adhesion and to reduce glass leaching. The coverslips were supported and sealed with a thick layer of petroleum jelly. All observations were made at room temperature (~21°C.) A Zeiss WL microscope with a 40 x nonfluor phase objective and a 4 x ocular with graticule was used in conjunction with a Locam camera (Redlake Corp., Photo Instrument Div., Campbell, Calif.) for recording sperm movement on Kodak Plus X 7276 16-mm movie film, which was reversal processed.

Films were analyzed using a Permafilm (Sydney) movie analyzer, which consisted of a Meopta projector modified for frame-by-frame projection from front-surfaced mirrors onto a drawing area. For analysis of movement, toad sperm were selected that were not close to other sperm and that had whole tails beating perpendicular to the line of sight. Sperm movement was analyzed from frame-by-frame cellophane drawings, aligned by means of the ocular graticule traced on each drawing. Tail wave amplitude was measured by drawing, as a reference, the ocular graticule and the axis along which the sperm was progressing.

The deviation from the progression axis of points at 10-μm intervals along the axoneme was measured with a grid marked off in millimeters after each drawing had been lined up with the ocular graticule. The point on the axial fiber closest to a corresponding point on the axoneme was chosen for amplitude measurement, a procedure that is permissible because the axial fiber is joined to the axoneme by the undulating membrane. Intervals along the tail were chosen such that toad sperm movement could be compared with ram and oyster sperm movement, for which points at intervals of 1/5A and 1A (wavelength) along the tail were chosen in a previous study (18). The wavelength of toad sperm proved to be ~20 μm, and, thus, intervals of 10 μm were selected. Tailwave frequency and phase difference between neighboring points on the axial fiber and axoneme were measured from the cyclic changes in amplitude with time, the latter being recorded by light emitting diode timing marks on the film.

By convention, the fiber with the lower amplitude (the axial fiber) was recorded as being either ahead of or behind the axoneme in phase. The forward velocity of the sperm was measured as the distance moved forward along the progression axis in a given time. Tail wave frequency was measured at 10-μm intervals on the axoneme and the axial fiber. However, inasmuch as no difference in frequency between different parts of the axoneme (or axial fiber), or between the axoneme and axial fiber, was detected by Student's t test, these measurements were pooled to give a mean tail wave frequency for each sperm.

Detergent Extraction and Reactivation

Toad sperm obtained as described above were demembranated by a tenfold dilution at room temperature with a solution consisting of 1% Triton X-100, 50 mM KCl, 4 mM MgSO₄, 1 mM CaCl₂, and 4 mM dithiothreitol in 10 mM phosphate buffer, pH 7.2. The demembranated sperm were diluted tenfold at room temperature in a reactivating solution containing all the above components (except Triton X-100), plus 4 mM ATP. This solution gave optimal reactivation and was arrived at largely by trial and error. It was subsequently found that equally good (but not improved) reactivation was obtained by omitting CaCl₂ from the reactivating solution. This trial was carried out because Brokaw et al. (3) found in sea urchin sperm that reactivation with solutions containing calcium ions led to asymmetrical tail movement. However, reactivated toad sperm swam along straight paths in solutions containing calcium ions.

To physically separate the axoneme and the axial fiber, some extracted toad sperm were sonicated for about 15 s in a cleaning bath sonicator, after which they were reactivated very well in the reactivating solution containing calcium ions.
FIGURE 1 Scanning electron micrograph of a Bufo marinus spermatozoon. (a) Axoneme. (b) Axial fiber. (c) Sperm head. Bar, 1 μm. × 6,000.

FIGURE 2 Diagram of the structure of the Bufo marinus spermatozoon (not strictly to scale). Enlarged transverse views are shown at eight levels of the spermatozoon.

**SDS Polyacrylamide Gel Electrophoresis**

SDS polyacrylamide gel electrophoresis was performed according to Bryan (4) and Stephens (18). A stock solution of 30% by weight acrylamide and 0.8% N,N'-bis-methylene acrylamide was prepared. The running gel was polymerized from a solution containing final concentrations of 7.5% acrylamide stock, 0.025 M Tris-glycine buffer, 0.05% N,N,N',N'-tetramethylenediamine, 0.1% SDS, and 0.1% ammonium persulfate. Tube gels (5 mm in diameter and 103 mm long) were layered with water-saturated n-butanol and polymerized. A preparation of rat sperm dense fibers (courtesy of G. E. Olson) and another of sea urchin sperm flagellar doublet tubule ribbons (prepared as described in reference 13) were run concurrently on separate gels.

The sample of toad sperm for gel electrophoresis was prepared by obtaining a sperm sample as described above. The sample was centrifuged lightly to remove pieces of testis, sonicated with a probe to cause a break between the sperm head and tail, and centrifuged for 4 min on a bench centrifuge. This yielded a pellet of sperm heads, which was discarded, and a supernate containing sperm tails. Light microscope examination showed that the sperm tail preparation was of high purity. The supernate was treated with 1% Triton X-100 to solubilize the membranes. The tail components were centrifuged at 100,000 g and electrophoresed as described by Linck (13).
Figures 3 and 4  Longitudinal sections through the neck region of the *Bufo marinus* spermatozoon, showing that the accessory fibers are fused together in the neck region but are separated into two fibers, the juxta-axonemal and the axial, distally. (a) Axoneme. (b) Axial fiber separating from juxta-axonemal fiber. (c) Fibrous sheath. (d) Nucleus. Bar in Fig. 3, 0.2 μm. × 65,000. Bar in Fig. 4, 0.1 μm. × 11,000.
Figures 5 and 6 Transverse sections through the short middle piece of the *Bufo marinus* spermatozoon, where the accessory fibers are still fused. In Fig. 6, a section distal to Fig. 5, the axoneme and accessory fibers are completely separated from the mitochondria. The fibrous sheath is seen in cross section at a. Bars, 0.1 μm. × 125,000.
Electron Microscopy

Toad testis was cut into small pieces and fixed by immersion in fixative solution II of Furstmann et al. (11) or in the same fixative buffered with cacodylate buffer and diluted twofold with distilled water (final osmolarity, 1,100 mOsM). The latter gave good results. Some of this material was treated with 1% tannic acid before dehydration (17). Another fixative used was a formaldehyde-glutaraldehyde-trinitrocresol fixative (12) diluted threefold with distilled water. This fixative also gave good results. After fixation for 2 h at room temperature, tissues were washed in cacodylate buffer, osmicated with 1% OsO₄ in the same buffer, treated for 1 h with 1% uranyl acetate in maleate buffer (pH 5.2), and embedded in Epon-Araldite. Thin sections were stained with uranyl acetate and lead and viewed in the electron microscope.

For embedding, suspensions of sperm were vacuum filtered on Millipore filters (Millipore Corp., Bedford, Mass.) for ease of handling and then treated in the same manner as tissue blocks. For scanning electron microscopy, the sperm were vacuum filtered on SEP filters (Ernest F. Fullam, Inc., Schenectady, N. Y.) and dehydrated in alcohol followed by acetone. They were then critical-point dried and examined in a JOEL JSM-35 or JSM-U3 scanning electron microscope.

Demembranated sperm prepared as described above were negatively stained with 1% uranyl acetate in water and observed on carbon-coated grids by transmission electron microscopy.

RESULTS AND DISCUSSION

Fine Structure of the Spermatozoan Tail in Bufo marinus

In the scanning electron micrograph of the toad sperm in Fig. 1, it can be seen that one fiber (a) takes a tortuous course and is longer. Negatively stained demembranated whole mounts (Fig. 19), in which the microtubules of the axoneme can be...
easily identified and can be followed for some length, show that the convoluted fiber is the axoneme. The axial fiber (b in Fig. 1 and Fig. 19), runs straight and is shorter (Figs. 1 and 19) than the axoneme. Furthermore, the axial fiber also terminates before the axoneme (Figs. 14 and 15).

As is shown in Fig. 1 and in the diagram of toad spermatozoan structure in Fig. 2, the axoneme and axial fiber are close together near the head and at the end of the tail, being separated by a considerable distance (up to 1.9 μm) only in the midregion.

The undulating membrane stretches between the axoneme and axial fiber and contains dense material which, in transverse section, has a repeating period of 14 nm (SD, 3 nm) in the 14-nm space between the limiting plasma membrane (Figs. 9, 10, and 12). This dense material appears to run longitudinally along the axis of the sperm tail, as is seen in oblique sections (Fig. 12).

Structures in the neck of Bufo marinus sperm are very similar to those described by Burgos and Fawcett (5) for Bufo arenarum. Surrounding the transverse centriole is a filamentous structure, which they termed the sheath (Figs. 3 and 4). This sheath extends distally and abuts on the accessory fiber material (Fig. 4). On the basis of these anatomical relations, the sheath would appear homologous to the connecting piece of mammals, and

TABLE 1

| Sperm | 1  | 2  | 3  | 4  | 5  |
|-------|----|----|----|----|----|
| Observed velocity, μm/s | 20.5 | 48.2 | 6.9 | 22.5 | 12.5 |
| Predicted velocity, μm/s | 33.9 | 65.9 | 21.5 | 22.5 | 17.5 |
| Predicted velocity, μm/s | 23.7 | 46.1 | 15.1 | 15.8 | 12.3 |
| Frequency, Hz  | 11.9 | 15.3 | 13.3 | 11.5 | 6.7 |

Amplitude at:
- 10 μm (axoneme), μm
- 10 μm (axial fiber), μm

Amplitude at:
- 20 μm (axoneme), μm
- 20 μm (axial fiber), μm

Amplitude at:
- 30 μm (axoneme), μm
- 30 μm (axial fiber), μm

Phase difference at 10 μm, radians
- 0.1
- 0.2
- 0.3
- 0.2
- 0.1

Phase difference at 20 μm, radians
- 0
- 0.2
- 0.3
- 0.2
- 0

Phase difference at 30 μm, radians
- 0.1
- 0
- 0
- 0
- 0.1

Number of frames analyzed
- 120
- 70
- 300
- 50
- 200

Film speed, fps
- 380
- 380
- 200
- 190
- 390

* From the empirical equation (8):

predicted velocity = \(k \cdot \frac{\text{frequency} \times (\text{amplitude of } \text{axoneme})}{\text{amplitude of fiber}}\),

where \(k = 1.0\).

† Where \(k = 0.7\) in the above equation.

Note: additional amplitude measurement for sperm 5 at 40 μm: 1.5 μm (axoneme) and 1.1 μm (axial fiber), with a phase difference of +0.1 radians.

FIGURES 9, 10 and 11 Transverse sections through the Bufo marinus sperm tail at progressively more distal points. The accessory fibers are widely separated in the midregion of the sperm tail (Fig. 9) but are close together toward the end of the tail (Fig. 11). (a) Juxta-axonomal fiber. (b) Axial fiber. (c) Periodic dense material with repeats of 14 nm within the undulating membrane. Bars, 0.1 μm.

FIGURE 12 Oblique section through the axial fiber (b) and part of the undulating membrane, showing the periodic dense material lying within the undulating membrane. At c the periodic dense material is cut in cross section, and at d it is cut obliquely; the dense material thus appears to run longitudinally with respect to the orientation of the spermatozoon. Bar, 0.1 μm.
the accessory fibers would seem homologous to the dense fibers of mammals. The accessory fibers in toad sperm are not divided into a cortex and medulla (Figs. 9–12), as is the case with mammalian dense fibers.

The juxta-axonemal and axial fibers are fused together in the neck region (Figs. 3 and 4) and begin to separate in the middle piece (Figs. 5 and 6). At this level, the undulating membrane is not present. As the fibers diverge farther, the undulating membrane appears. It is widest in the midregion of the tail (Figs. 9 and 10), becomes reduced distally (Fig. 11), and disappears with the axial fiber toward the end of the tail (Fig. 14). Finally, the juxta-axonemal fiber disappears also, and the central fibrils terminate (Fig. 15).

Occasionally, sperm with supernumerary accessory fibers are encountered. These supernumerary fibers are variable in position, being located adjacent to doublets 5–8. They are found at levels ranging from the middle piece to distal regions (Fig. 13), and have the same appearance as the other accessory fibers. Occasionally, spermatids with two spermtails implanted in the nucleus are seen.

The Normal Movement of the Spermatozoon

In tracings of sperm movement from cinemicrographs (Fig. 7), it can be seen that one fiber (marked with a bar in Fig. 7) is flexed more than the other and therefore is longer at all regions of the sperm tail. As discussed earlier, the axoneme assumes a convoluted course in negatively stained demembranated whole mounts. Thus, one fiber is longer than the other at neighboring regions of the sperm tail in both live and dead material, and in the latter this fiber is known to be the axoneme.

If it is assumed that cell death does not cause a sudden reversal in the relative lengths of the axoneme and the axial fiber at neighboring regions, it becomes possible to distinguish axoneme and axial fiber in cinemicrographs. Thus, in the tracing of toad sperm movement from cinemicrographs (Fig. 7), the longer axoneme (marked with a bar) draws the axial fiber behind it and, on reversing its direction of movement each half-cycle, crosses over the trailing axial fiber. This may also be seen in graphs of tail wave amplitude at neighboring points on the axoneme and axial fiber (Fig. 8). This suggests that the axoneme moves actively

| Table II |
| Tailwave Parameters of Toad Sperm after Demembranation with 1% Triton X-100 and Reactivation |

| Sperm | Frequency, Hz | Amplitude at: | Amplitude at: | Phase difference at 10 μm, radians | Observed velocity, μm/s | Number of frames analyzed | Film speed, fps |
|-------|--------------|---------------|---------------|---------------------------------|------------------------|--------------------------|---------------|
|       |              | 10 μm (axoneme), μm | 10 μm (axial fiber), μm | 20 μm (axoneme), μm | 20 μm (axial fiber), μm | 10 μm (axoneme), μm | 10 μm (axial fiber), μm | 20 μm (axoneme), μm | 20 μm (axial fiber), μm | 20 μm (axoneme), μm | 20 μm (axial fiber), μm |
| 6     | 15.2         | 1.1           | 0.8           | 1.9                           | 1.3                    | 0.1                      | 50            | 160          | 35.0         |
| 7     | 9.1          | 0.9           | 0.4           | 2.4                           | 0.5                    | 0.5                      | 50            | 180          | -            |
| 8     | 4.5          | 2.4           | 1.8           | -1.0                          | 1.8                    | 1.8                      | 80            | 160          | -            |
| 9     | 2.7          | 2.4           | 0.6           | -1.0                          | 0.6                    | 0.6                      | 100           | 170          | -            |

Sperm 9 had waves of two frequencies in the tail, and the 10-μm point on the tail was obscured by an immotile sperm head. Sperm 6 and sperm 9 were sonicated for 15 s (see text) and were stuck to the slide. Sperm 8 had no head.

**Figure 13** Occasionally, supernumerary accessory fibers are seen at various positions near the axoneme. In this micrograph one is seen alongside doublet number 7. (a) Juxta-axonemal fiber lying alongside doublet number 3. Bar, 0.1 μm.

**Figure 14** At a level equivalent to that of Fig. 13, no supernumerary accessory fiber is seen, and the axial fiber and undulating membrane have terminated. Only the juxta-axonemal fiber (a) and axoneme remain. Tannic acid postfixation. Bar, 0.1 μm.

**Figure 15** Several endpieces showing two, one, and, as the sperm tail terminates, no central fibers in the axoneme. Bar, 0.1 μm.
while the axial fiber follows it passively. The axoneme has been known for some time, from electron microscopy of sperm tails and from cilia containing axonemes only (2, 10), to be actively motile, whereas independent motility of the accessory fibers of sperm has not been established.

It can be seen from Table I that the tail wave amplitude of the axoneme in all five sperm studied is significantly greater than that of the axial fiber (by Student's t test, \( P = \leq 0.001 \)). This is also demonstrated by graphs of tail wave amplitude at neighboring points on the axoneme and axial fiber (Fig. 8). These measurements indicate that the axoneme moves actively and the axial fiber follows it passively.

The axoneme and axial fiber are separated by a maximum distance (as measured from cinemicrographs) of 1.9 \( \mu \)m and are joined by the undulating membrane. The phase difference between 10-\( \mu \)m (\( \frac{1}{2} \lambda \)) intervals on the axoneme is 90° or \( \pi \) radians. Thus, the phase difference between points 1.9 \( \mu \)m apart on the axoneme would be 0.6 radian (provided a wave were being propagated). The phase difference between the axoneme and the axial fiber (Table I) is not measurably different from zero (by Student's \( t \) test, \( P > 0.1 \) that the phase difference is zero), which means that they are effectively acting as one unit, i.e., as though they were joined together, with no propagation of a wave similar to the wave on the axoneme between them. This suggests that the undulating membrane lying between them does not actively generate movement, which is consistent with its electron microscopic appearance, already discussed. The undulating membrane does not contain any structures normally associated with movement, such as microtubules.

The effect of the undulating membrane on propulsion of the toad spermatozoon can be estimated by calculating what its velocity would be without this membrane and then comparing this velocity with the observed velocity.

The expected velocity of a toad sperm without an undulating membrane can be approximated by the empirical equation of Denehy (8), which applies to ram and oyster spermatozoa. The ram has a typical mammalian spermatozoon, and the oyster has a typical simple invertebrate spermatozoon with a tail that contains an axoneme only. The forward velocity of the toad sperm analyzed is lower by approximately one-third than expected from the empirical equation (Table I). The toad sperm follow the empirical equation fairly well if the constant in the equation is changed from 1.0 to 0.7 (Table I), suggesting that the undulating membrane (with its axial fiber) is dragging, reducing the sperm velocity.

The mean forward velocity was 136 \( \mu \)m/s for 49 ram sperm and 169 \( \mu \)m/s for 14 oyster sperm (8), whereas it was only 22 \( \mu \)m/s for five toad sperm (range, 7-48 \( \mu \)m/s). The slower speed of toad sperm was due mainly to a lower tail wave frequency (only 12 Hz, compared with 29 Hz for ram and 43 Hz for oyster). To a much lesser extent it was due to a lower tail wave amplitude. The radius of the sperm heads for a sphere of equivalent

---

**Figure 16** Transverse section of the *Bufo marinus* sperm tail treated with 0.1% Triton X-100 to remove the plasma membrane. This treatment removed the membrane around the axoneme only. (m) Plasma membrane still remaining around the undulating membrane and axial fiber after treatment with 0.1% Triton X-100. Bar, 0.1 \( \mu \)m.

**Figure 17** The membrane is removed from the entire sperm tail by treatment with 1% Triton X-100. The dense material in the undulating membrane (a) usually keeps the axoneme and axial fiber together. The axial fiber of a neighboring sperm tail is seen in longitudinal section at (b). Bar, 0.1 \( \mu \)m.

**Figure 18** 7.5% SDS polyacrylamide gels from (a) isolated, demembranated toad sperm tails, (b) rat sperm dense fibers (courtesy of G. E. Olson), and (c) sea urchin sperm flagellar doublet microtubule ribbons. The position of the four rat sperm dense fiber proteins (\( C_1, C_2, M_1 \), and \( M_2 \)) and of \( \alpha \) and \( \beta \)-tubulin (\( T, T \)) of doublet microtubule ribbons are indicated beside gel a. Rat sperm dense fibers are composed of a medulla (with the medullary proteins \( M_1 \) and \( M_2 \)) and a cortex of two proteins (\( C_1 \) and \( C_2 \)), as described by Olson et al. (14). Gels of demembranated toad sperm tails thus reveal four major protein components: two tubulins (55,000 daltons) arising from the microtubule axoneme and two proteins comigrating near the rat \( M_1 \) (11,000 daltons) and \( M_2 \) (25,000 daltons) proteins whose structural origin is less certain. It seems most likely that these are derived from the toad accessory fibers, but there may be some contribution from the dense material of the undulating membrane, which still connects the accessory fibers in demembranated toad sperm (Fig. 17). In the toad sperm tail there are no major proteins with mobilities corresponding to those of the proteins located in the cortex of rat sperm dense fibers.
volume was approximately the same for toad, oyster, and ram sperm (1.7, 1.2, and 1.6 μm, respectively), and, thus, the slower speed of toad sperm was not due to drag from the head. Unrelated to the absolute value of toad sperm velocity is the fact that the velocity is lower than predicted by the empirical equation, which suggests that the undulating membrane is dragging.

Accessory fibers in sperm tails are normally associated with an increased number of mitochondria, usually located around the axoneme in a long middle piece. This is not the case with Bufo marinus, which has only a few mitochondria arranged in two or three turns of a helix forming an extremely short (about 1.3-μm-long) middle piece (Figs. 3 and 4). This suggests that the undulating membrane may have the function of increasing the stiffness of the tail without incurring an energy penalty heavy enough to require a long middle piece. Thus, the undulating membrane may reduce the sperm's energy requirements, as compared with an arrangement of accessory fibers around the axoneme, but it does not result in speedier movement.

The Movement of Detergent-extracted and Reactivated Toad Sperm

Treatment with 0.1% Triton X-100 did not remove the plasma membrane from the undulating membrane and its axial fiber, although it did remove it from the axoneme (Fig. 16).

When sperm were extracted with 1% Triton X-100, all of the membrane was removed (Fig. 17); under these conditions the sperm were completely immotile. On dilution with the reactivating solu-

---

**Figure 19** A complete toad sperm tail with part of the head at the upper margin. The specimen was demembranated with 1% Triton X-100 and negatively stained with uranyl acetate. The axial fiber (f) runs relatively straight and terminates before the axoneme (a). The axoneme is flexuous, crosses over the axial fiber four times, and is longer than the axial fiber. Bar, 1 μm. × 3,700. The three enlarged views are higher magnifications of the indicated areas of the same sperm. The upper figure clearly reveals that the axoneme is to the right of and is separated from the axial fiber by the undulating membrane. The center enlargement shows the axoneme crossing over the axial fiber from the upper right to the lower left corner. The bottom view shows the curved axoneme (to the right) and the axial fiber with the narrowed undulating membrane terminating in this region. Bar, 0.2 μm. × 43,000.
tion containing 50 mM KCl, 4 mM MgSO₄, 1 mM CaCl₂, 4 mM ATP, and no detergent, the sperm resumed vigorous movement.

Toad sperm were demembranated, and some were also sonicated and reactivated, in an attempt to separate the movement of the axial fiber from that of the axoneme. In no case was the axial fiber observed to move independent of the axoneme, and its amplitude was always less than that of the axoneme.

As is seen from Table II, the tail wave frequency of the reactivated sperm was high. There was no significant difference in frequency, as determined by Student’s t test (P > 0.1) between the untreated and reactivated sperm. However, in one sonicated and reactivated sperm there was a small amplitude fast tail wave of 35 Hz in the axoneme superimposed on a larger amplitude slow wave of 2.7 Hz. Why this sperm should have a 35 Hz wave, unusually fast for toad sperm, is not known. A double wave was not observed in any other reactivated sperm.

The tail wave amplitudes of extracted and reactivated sperm were slightly depressed, compared with untreated sperm. Although there was no significant difference between the axonemal amplitudes of treated and untreated sperm at 10 µm (0.1 > P > 0.05), the amplitudes were significantly different at 20 µm (0.05 > P > 0.02), where an additional sperm was included. These probabilities also applied in the case of the axial fiber amplitudes at 10 µm and 20 µm.

As was found in untreated sperm, the amplitudes of the axoneme and the axial fiber were significantly different as determined by the t test (0.02 > P > 0.002) in reactivated demembranated sperm.

The average maximum separation between axial fiber and axoneme in untreated sperm (as measured from cinemicrographs) was 1.9 µm. The two demembranated and reactivated sperm that had the largest phase difference between the axoneme and the axial fiber (−1.9 and 1.0 radian) also had the largest separation between these structures (2.7 and 2.6 µm, respectively). Probably, the undulating membrane had disintegrated in these regions, leaving the axial fiber to follow the point to which it was joined to the axoneme. Thus, it was shifted in phase with respect to the point on the axoneme from which it had separated. Because of this shift, the phase differences between axoneme and axial fiber for reactivated sperm (Table II) were significantly different from zero as determined by the t test (0.05 > P > 0.02), whereas there was no significant difference for untreated sperm.

**SDS Polyacrylamide Gel Electrophoresis**

Fig. 18 shows the stained gels prepared from isolated, demembranated toad sperm tails (a), rat sperm tail dense fibers (b), and sea urchin sperm flagellar doublet microtubule ribbons (c). Toad sperm tails contain tubulin from the microtubule axoneme, as well as two additional proteins with molecular weights of 11,000 and 25,000. The latter two proteins correspond in molecular weight to the two proteins of the rat sperm dense fiber medulla (14). It seems likely that at least one of these two *Bufo marinus* proteins arises from the accessory fibers, the homologue of the rat dense fiber medulla; the other may arise from the dense material associated with the undulating membrane. In demembranated sperm this intermembranous material still connects the two accessory fibers (a in Fig. 17). The two proteins, with molecular weights of 17,000 and 87,000 (14), that make up the rat dense fiber cortex, which is preferentially solubilized by brief urea extraction, are absent in the toad sperm tail preparations. As seen in sectioned material (Figs. 9-12 and 17), there is no division of the accessory fibers into cortex and medulla, in contrast to mammalian sperm.

**Conclusion**

Studies of the normal movement of *Bufo marinus* spermatozoa, combined with studies of fine structure, show that the axoneme moves actively while the axial fiber follows it passively with a lower amplitude of movement. In an attempt to separate the movement of the axoneme from that of the axial fiber, sperm were demembranated and reactivated, and some were also sonicated before reactivation. Although the sperm reactivated well, on no occasion did the axial fiber move independent of the axoneme, an observation that is at variance with the report by Burgos and Fawcett (5) on *Bufo arenarum*, which suggested active, independent movement by the membrane filament (here designated the axial fiber).

As suggested by Fawcett (9) for a urodele spermatozoon and as supported by the present study, the accessory fibers of amphibians appear to be homologous with the dense fibers of mammalian sperm, on the basis of anatomical relations in the neck region. In *Bufo marinus*, the juxta-axonemal fiber, which is associated with doublet 3, fuses
with the axial fiber in the neck region. Thus, these two accessory fibers are presumably of the same composition; they are also indistinguishable in their fine structure.

SDS polyacrylamide gel electrophoresis of demembranated *Bufo marinus* sperm tails reveals two major proteins in addition to the tubulins; these probably arise from the accessory fibers and the dense material associated with the undulating membrane.

Analysis of the movement of *Bufo marinus* spermatozoa strongly suggests that the axial fiber, which is probably homologous with the dense fibers of other sperm, is not an independently motile organ. We can only speculate on the function of displacing the axial fiber into an undulating membrane, but it is possible that it provides stiffness for the tail without incurring an energy deficit large enough to require a long middle piece. Unlike other sperm that have accessory fibers around the axoneme, the sperm of *Bufo marinus* can propel itself without a long middle piece associated with the axoneme. However, it suffers a reduction in speed of about one-third, compared with the speed expected for a sperm without an undulating membrane.

Received for publication 3 January 1980.

REFERENCES

1. **Bedford, J. M., and H. I. Calvin.** 1974. Changes in S-S-linked structures of the sperm tail during epididymal maturation, with comparative observations in sub-mammalian species. *J. exp. Zool.* 187:181-204.
2. **Bradfield, J. R. G.** 1953. New features of protoplasmic structure observed in recent electron microscope studies. *Q. J. Microsc. Sci.* 94:35-367.
3. **Broughton, C. J., R. Josselin, and L. E. Borg.** 1974. Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa. *Biochem. Biophys. Res. Commun.* 58:95-107.
4. **Bryan, J.** 1974. Biochemical properties of microtubules. *Fed. Proc.* 33:152-157.
5. **Burgos, M. H., and D. W. Fawcett.** 1956. An electron microscope study of spermatozoon differentiation in the toad, *Bufo arenarum* Hensel. *J. Biophys. Biochem. Cytol.* 2:223-240.
6. **Calvin, H. I., and J. M. Bedford.** 1971. Formation of disulfide bonds in the axoneme and accessory structures of mammalian spermatozoa during maturation in the epididymis. *J. Reprod. Fertil. (Suppl.)* 13:65-75.
7. **Cleland, K. W., and Rothschild.** 1959. The bandicoot spermatozoon: an electron microscope study of the tail. *Proc. R. Soc. Lond. B Biol. Sci.* 150:24-38.
8. **Densley, M. A.** 1975. The propulsion of nonmotile ram and oyster spermatozoa. *Biof. Reprod. 13*:17-29.
9. **Fawcett, D. W.** 1970. A comparative view of sperm ultrastructure. *Biol. Reprod. (Suppl.)* 2:90-127.
10. **Fawcett, D. W., and K. R. Porter.** 1954. A study of the fine structure of ciliated epithelia. *J. Morphol.* 94:221-281.
11. **Fordyssen, W. G., S. Ito, E. Weide, A. Aoki, M. Dyn, and D. W. Fawcett.** 1977. An improved perfusion fixation method for the testis. *Anal. Rec.* 188:307-314.
12. **Ito, S., and M. J. Karnovsky.** (1968). Formaldehyde-glutaraldehyde fixatives containing trinitro derivatives. *J. Cell Biol.* 39:2, Pt. 23:689 a (Abstr.).
13. **Link, R. W.** 1976. Flagellar doublet microtubules: fractionation of minor components and a-tubulin from specific regions of the A-tubule. *J. Cell Sci.* 20:465-469.
14. **Olsen, G. E., D. W. Hamilton, and D. W. Fawcett.** 1976. Isolation and characterization of the fibrous sheath of rat epididymal spermatozoa. *Biof. Reprod. 14*:157-230.
15. **Picheral, B.** 1972. Les éléments cytoplasmiques au cours de la spermiogenèse du triton *Platydeles walii* Michah. III. L'évolution des formations caudales. *Z. Zellforsch. Mikrosk. Anat.* 133:399-416.
16. **Picheral, B.** 1979. Structural, comparative and functional aspects of spermatozoa in urodèles. In *The Spermatozoon. Maturation, Motility and Surface Properties.* (D. W. Fawcett and J. M. Bedford, editors). Urban & Schwarzenberg, Inc., Baltimore. 267-287.
17. **Simionescu, N., and M. Simionescu.** 1976. Gallyérphofenion of low molecular weight as mordant in electron microscopy. 1. Procedure, and evidence for mordanting effect. *J. Cell Biol.* 70:608-621.
18. **Stevens, R. E.** 1975. High-resolution preparative SDS-polycrylamide gel electrophoresis. Fluorescent visualization and electrophoretic elution concentration of protein bands. *Anal. Biochem.* 65:369-379.
19. **Swan, M. A., R. W. Linck, D. W. Fawcett, and S. Ito.** 1970. The function of the accessory fiber in spermatozoan propulsion. *J. Anat.* 128:642-643 (Abstr.).