

# ACTIN FILAMENTS IN THE ACROSOMAL REACTION OF *LIMULUS* SPERM

## Motion Generated by Alterations in the Packing of the Filaments

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### ABSTRACT

When *Limulus* sperm are induced to undergo the acrosomal reaction, a process, 50  $\mu\text{m}$  in length, is generated in a few seconds. This process rotates as it elongates; thus the acrosomal process literally screws through the jelly of the egg. Within the process is a bundle of filaments which before induction are coiled up inside the sperm. The filament bundle exists in three stable states in the sperm. One of the states can be isolated in pure form. It is composed of only three proteins whose molecular weights (mol wt) are 43,000, 55,000, and 95,000. The 43,000 mol wt protein is actin, based on its molecular weight, net charge, morphology, G-F transformation, and heavy meromyosin (HMM) binding. The 55,000 mol wt protein is in equimolar ratio to actin and is not tubulin, binds tenaciously to actin, and inhibits HMM binding. Evidence is presented that both the 55,000 mol wt protein and the 95,000 mol wt protein (possibly  $\alpha$ -actinin) are also present in *Limulus* muscle. Presumably these proteins function in the sperm in holding the actin filaments together. Before the acrosomal reaction, the actin filaments are twisted over one another in a supercoil; when the reaction is completed, the filaments lie parallel to each other and form an actin paracrystal. This change in their packing appears to give rise to the motion of the acrosomal process and is under the control of the 55,000 mol wt protein and the 95,000 mol wt protein.

Actin appears to be a ubiquitous protein in both muscle and nonmuscle cells of eucaryotic organisms (see Pollard and Weihing, 1974, for references). Myosin as well has been described in a number of nonmuscle cell types which include the amoeba, fibroblasts, *Physarum*, and blood platelets. Once one has mentioned the fact that actin and myosin coexist in nonmuscle cells there is almost a "Pavlovian response"—everyone wants

to know where the sliding takes place, how long the myosin filaments are, etc. In fact, the sliding filament hypothesis of muscle is so firmly entrenched in our thinking that no other mechanism of motion even comes to mind, or, if it does, it is immediately dismissed as "irrelevant" or as occurring in an "oddball system." This is somewhat surprising as the myosin/actin ratio in most nonmuscle cells is very much less than that present in

muscle. In fact, in some cells such as sperm, myosin is not detectable at all and probably is absent although actin is one of the most abundant proteins in these cells (Tilney et al., 1973). I do not mean to imply that the sliding of filaments of myosin and actin does not provide motility in certain types of nonmuscle cells, but only to suggest that there may be other mechanisms of motion in nonmuscle cells which use actin as one of the "contractile" proteins. In essence, what I am saying is that since all eucaryotic cells contain actin, it is rather naive to take the most specialized cell, skeletal muscle, and expect all other cells to have an identical mechanism of motion. An example of a mechanism of motility which does not involve sliding of actin and myosin is in the formation of the acrosomal processes of certain sperm in which a 90- $\mu$ m long process is generated in about 10 s. The motion is produced by the transformation of the actin from the monomeric state (G) to the filamentous state (F) (Tilney et al., 1973).

In this paper I would like to describe a system, the acrosomal reaction of horseshoe crab (*Limulus*) sperm, in which motion is generated by the rearrangement of actin filaments. Myosin, tropomyosin, and the troponins do not appear to be present and sliding of the filaments past one another does not seem to occur. Two proteins besides actin are involved with the motion; both appear to be present in the skeletal muscle as well. This paper will be the first in a series of reports on *Limulus* sperm.

## MATERIALS AND METHODS

### *Obtaining Sperm*

*Limulus polyphemus* were collected by the supply department of the Marine Biological Laboratory, Woods Hole, Mass. They can be induced to discharge their sperm either by electrical stimulation (Shrank et al., 1967) or mechanical stimulation, i.e., by rubbing gently near the gonadopores. The semen, as it is released, is then collected by a Pasteur pipette. As there is no smooth muscle around the gonad proper as in sea urchin gonads (Shrank et al., 1967), organisms can be milked repeatedly since stimulation induces only the discharge of gametes from the ducts leading from the testes. In order to obtain sufficient quantities of sperm for the analysis of the proteins present a number of animals must be milked; I have found mechanical stimulation to be more effective. Sperm can be obtained throughout the year, but if *Limulus* are collected shortly after they have mated in nature, only small quantities of sperm can be obtained.

### *Isolation of the Bundle of Core Filaments in the False Discharge*

The terminology used in this paper to describe the different states of the core filament bundle is that of André (1965). All preparations were carried out on ice unless otherwise indicated.

Sperm were washed twice in sea water and pelleted at 750 g for 5 min. The pellet of washed sperm was suspended in at least 3 vol of 1% Triton X-100 (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), 0.1 mM EDTA, 3 mM MgCl<sub>2</sub>, and 30 mM Tris-HCl at pH 8.0 (Stephens, 1970). (This solution will henceforth be referred to as TTM.) The sperm were repeatedly sucked up and down in a Pasteur pipette in order to liberate the core filament bundle from the nucleus. The suspension was then centrifuged in a Sorvall RC 2B refrigerated centrifuge at 750 g for 5 min (2,500 rpm, using an SS 34 head, Ivan Sorvall, Newtown, Conn.). The supernate contained the core filament bundles (in the false discharge state), some flagellar axonemes, a few nuclei, and some caps (the region of the sperm anterior to the nucleus). The pellet, which was discarded, was composed mainly of sperm nuclei with attached flagellar axonemes and caps. The supernate was spun again to remove any remaining nuclei and caps. This was repeated as necessary. The false discharge and contaminating flagellar axonemes were pelleted by centrifugation at 12,000 g for 10 min. To free the preparation of flagellar axonemes (Stephens, 1968) the pellet was washed briefly in 10 mM Tris-HCl at pH 7.5 to eliminate the Mg<sup>++</sup> and pelleted. The pellet was resuspended in 10 mM Tris-HCl at pH 7.5 which contained 0.5% Sarkosyl (sodium lauryl sarcosine, Geigy Chemical Corp., Ardsley, N. Y.) for 30 min. The false discharges were then pelleted at 12,000 g for 10 min and washed twice in the buffered Sarkosyl solution. This pellet could then be used for negative staining, gel electrophoresis, and other procedures. At each stage in the procedure, purity was monitored by light microscopy.

### *Isolation of the Coil*

Sperm were suspended in a 1:1 solution of glycerol and sea water for 10 min at 0°C, pelleted (4,000 g for 5 min), and the pellet was resuspended in several volumes of TTM. By being passed repeatedly through a Pasteur pipette the coils would gradually "fall off" the flagella. Centrifugation at 600 g for 5 min would pellet the sperm heads and caps and most of the flagellar axonemes. The coils, some false discharge bundles, and some flagellar axonemes remained in the supernate.

Alternatively, coils could be obtained by treating freshly collected sperm with 0.1% Triton X-100 in 0.1 mM EDTA, 3 mM MgCl<sub>2</sub>, and 30 mM Tris-HCl at pH 8.0 for a few minutes and then treating the sperm with TTM. After the TTM treatment the coils were collected as outlined above.

### Preparation of Proteins

Acetone powders of whole sperm were prepared by extracting sperm with acetone at 4°C (three changes 5–10 min each). The powder was air dried and stored in a desiccator at low temperature. An acetone powder of rabbit muscle was also prepared (see Tilney et al., 1973, for details). Acetone powders of a pellet of isolated and purified false discharges were also prepared. Because of the small size of the sample the pellet was treated with acetone, the powder concentrated by centrifugation (12,000 g for 5 min), washed three times with acetone, and then air dried while still in the centrifuge tube. The centrifuge tubes containing the acetone powders of the false discharges were stored at 4°C in a desiccator.

The acetone powders were extracted for 30 min with water, with or without 10 mM Tris-HCl at pH 7.5, and the actin was polymerized by the addition of 0.1 M KCl. The F-actin could be pelleted by centrifugation at 80,000 g for 3 h.

Heavy meromyosin (HMM) was prepared from rabbit skeletal muscle and stored at –20°C in 50% glycerol or as a lyophilized powder (see Tilney et al., 1973, for details). One batch of HMM was kindly supplied by Dr. Tom Pollard.

For HMM binding, the HMM was dissolved in standard salt solution (Ishikawa et al., 1969). The purified core filaments of the false discharge were incubated in HMM (0.2–4.0 mg/ml) at room temperature for up to 4 h, put on a grid, and negatively stained. Also, actin which was polymerized from an acetone powder of the false discharges was incubated with HMM and then negatively stained.

### Gel Electrophoresis

5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was run using either 0.1 M phosphate buffer at pH 6.8 (Weber and Osborn, 1969) or 25 mM Tris-glycine at pH 8.3 (Bryan, 1974) as the running buffer. The gels were stained with Coomassie blue; the tracking dye was bromphenol blue. The samples were boiled in 1% SDS containing 1% mercaptoethanol and 0.01 M phosphate buffer at pH 6.8. Other samples were reduced and acetylated as described by Renaud et al. (1968). Standards used included muscle actin isolated from an acetone powder of chicken skeletal muscle and tubulin from flagellar axonemes (see Tilney et al., 1973).

### Light Microscopy

Sperm were studied under oil immersion ( $\times 100$ ) using a Zeiss phase-contrast microscope. Light micrographs were taken on Kodak plus X film and developed in Diafine (Acufine, Inc., Chicago, Ill.). Movies were taken with a 16-mm Bolex camera at 16 frames/s and processed commercially.

### Electron Microscope Procedures

Freshly collected sperm were washed once in sea water and then fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) in sea water for 1 h. The addition of the glutaraldehyde had no effect on the pH of the sea water. The sperm were then washed briefly in sea water and fixed in 1% OsO<sub>4</sub> in 0.1 M phosphate buffer at pH 7.0 for 1 h at 0°C, dehydrated in acetone, and embedded in Araldite. *Limulus* leg muscle was fixed and processed as outlined for sperm. Both transverse and longitudinal sections were cut.

A pellet of F-actin was fixed in 2% glutaraldehyde in 0.05 M phosphate buffer at pH 7.0 to which 0.0015 M CaCl<sub>2</sub> was added, washed briefly in 0.1 M phosphate buffer with CaCl<sub>2</sub>, and postfixed and processed as already mentioned.

To analyze the purity of the pellets of the false discharges and the coil, the pellet in question was fixed in 2% glutaraldehyde in 0.05 M phosphate buffer at pH 7.0 at 0°C for 1 h, then washed briefly in 0.1 M buffer, and postfixed, dehydrated, and embedded as mentioned above.

Thin sections were cut with a diamond knife on a Sorvall Porter-Blum ultramicrotome II, stained with uranyl acetate and lead citrate, and viewed in a Philips 200 electron microscope. The electron microscope was calibrated using calibration grids obtained from Ladd Research Industries, Inc., Burlington, Vt. We used two grids, one of which had 54,864 lines/inch, the other 28,800 lines/inch. Differences in magnification using these two grids were less than 1%.

For negative staining, a drop of a suspension of whole sperm, untreated or induced to undergo the acrosomal reaction by the addition of calcium to the sea water (see Results), was added to lightly carbonized colloidal-coated grids and negatively stained with 0.5% unbuffered uranyl acetate. Brief fixation with glutaraldehyde before negative staining was also carried out. Other sperm, both reacted and unreacted, were treated briefly with TTM and negatively stained. Isolated coils and the isolated false discharges were also added to grids and negatively stained.

### RESULTS

The basic morphology of *Limulus* sperm was described by André in 1965. Unfortunately, this excellent report is not readily available in this country—in fact, I had to procure it directly from the author. More recent reports have described the development of the sperm (Fahrenbach, 1973) or the attachment of the sperm to the egg (Shoger and Brown, 1970). Since André's paper is difficult to obtain and since this paper is the first in a series of papers on the actin filaments in *Limulus* sperm, I will describe the morphology of the sperm in some

detail, as such a description is necessary for a complete understanding of how the acrosomal reaction occurs. Therefore, the first half of the Results will describe the acrosomal reaction and its associated morphology, while the latter half will be concerned with actin and the proteins associated with the movement.

### Light Microscopy

The nearly spherical nucleus is covered anteriorly by the acrosomal vesicle (Figs. 1 *a* and 2); posteriorly are some mitochondria and a single flagellum (Figs. 1 *a* and 2). When sperm come in contact with sea water, a corkscrew-shaped process, which André (1965) has designated the false discharge, appears near the flagellum and usually at an acute angle to it (Fig. 1 *a*). The wavelength is about 2.8  $\mu\text{m}$  with a range of 2.6–3.0  $\mu\text{m}$ . The maximum length of this process is about 50  $\mu\text{m}$ . It

can be seen from direct observations and films that the false discharge rotates as it elongates, thus producing a screwing motion.

The formation of the acrosomal process with which the sperm penetrates the protective layers of the egg (Fig. 1 *b*), termed by André (1965) the "true reaction," can be seen in a few of the sperm as the sea water begins to evaporate near the edges of the cover slip. The true reaction also can be induced in about 10% of the sperm by the addition of  $\text{Ca}^{++}$  to the sea water (10–20% by volume of 0.5 M  $\text{CaCl}_2$  added to the sperm suspension) (Shoger and Bishop, 1967). The first response of sperm when perfused with excess  $\text{Ca}^{++}$  is to retract the false discharge. This process literally screws back into the sperm. A minute or so later the acrosomal vesicle ruptures, liberating its contents, and a long straight process emerges (Fig. 1 *b*) from the anterior end of the sperm. This process reaches its maximum length in a few seconds. Upon close

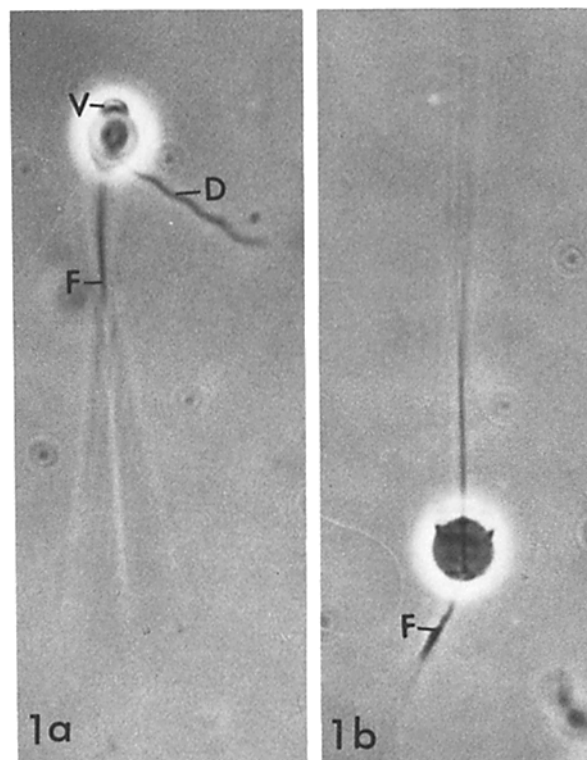


FIGURE 1 *a* Unreacted *Limulus* sperm in sea water. The acrosomal vesicle (*V*) sits on the nearly spherical nucleus. The false discharge (*D*) extends out at an acute angle to the flagellum (*F*).  $\times 1,800$ .

FIGURE 1 *b* *Limulus* sperm induced to undergo the acrosomal reaction with excess  $\text{Ca}^{++}$ . The core filaments in the acrosomal process can be seen extending through the nucleus. The flagellum (*F*) extends from the base.  $\times 1,800$ .

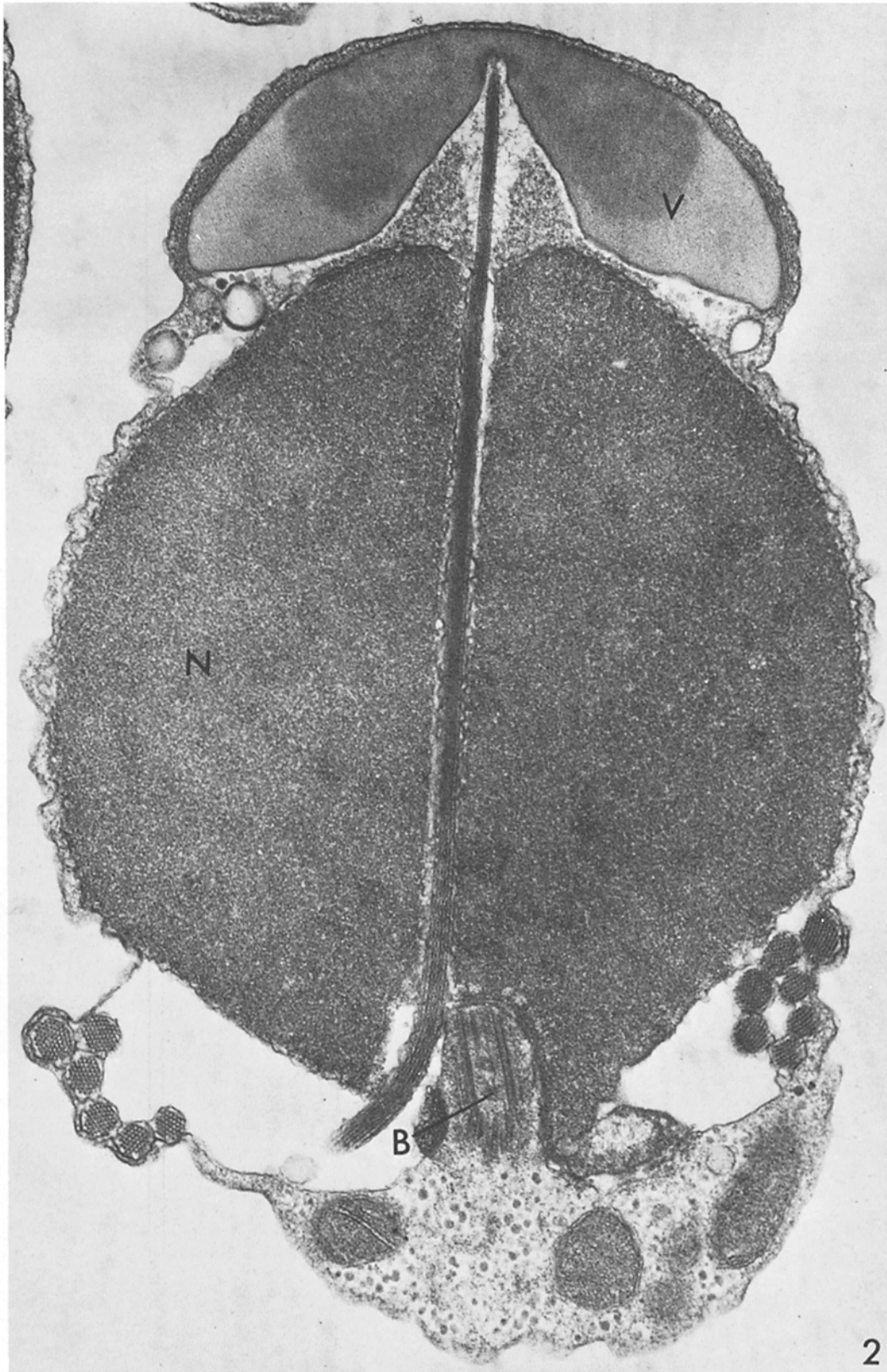


FIGURE 2 Unreacted sperm. The core filament bundle extends from the acrosomal vesicle (*V*) through a canal in the nucleus (*N*) and wraps around the base of the sperm. The basal body of the flagellum (*B*) is present at the base of the sperm.  $\times 58,000$ .

observation it is apparent that this process also rotates as it moves forward. I have been able to film this event and showed a movie at the 1973 American Society for Cell Biology meetings in Miami depicting the true reaction in several sperm (Tilney, 1973). I have been able to establish that the rotation is invariably right-handed so that as one looks toward the tip of the process it rotates clockwise. The reason that the screwing motion of the true and false discharges was not previously observed is perhaps due to the speed of discharge and to the difficulties in inducing the reaction.

When one observes sperm in sea water under oil immersion, occasionally one can find both true and false discharge processes extending from the same sperm. Similar cases were reported by André (1965). Within each process is a phase-dense structure (core filaments) which is continuous from the tip of the false discharge through the nucleus (see Fig. 1*b*) and out to the tip of the true discharge, an observation confirmed by the electron microscopy of André (1965) and others. In a few of those sperm which had both the true and false discharges, I was able to watch the rupture of the nuclei as evaporation proceeded. A finding of particular interest is that as the substance of the nucleus disappeared, it became apparent that the transition between the corkscrew appearance of the false discharge and the linear profile of the true discharge occurred within what was initially the nucleus. Thus, the core filament bundle can exist in two states at the same time, part of it presenting a linear profile, the other an undulatory profile.

From the above description, fertilization must proceed in the following way. When the sperm comes in contact with the surface coats of the egg the acrosomal reaction is triggered. First, the acrosomal vesicle ruptures and then, as the acrosomal process elongates, it bores through the outer coverings of the egg by a screwing motion which appears to be generated by a change in the core filament bundle. This change seems to take place in the nucleus proper.

### *Electron Microscopy*

**UNREACTED SPERM:** As described by André (1965) and Fahrenbach (1973), the acrosomal vesicle takes the form of an inverted bowl which sits on the nearly spherical nucleus (Fig. 2). The filament bundle originates on the acrosomal vesicle and from there runs posteriorly through a canal in the nucleus (Figs. 2, 3, and 5) to form a coil just

anterior to the flagellum (Figs. 2 and 3). The number of turns in the coil can be variable, although usually there are six (Figs. 2 and 4). The posterior end of the filament bundle extends out into the false discharge (Fig. 6). The core filament bundle anterior to the nucleus lies free in the cytoplasm. As it extends posteriorly it is separated from the chromatin by the outer nuclear envelope and beneath it by the inner nuclear envelope (Figs. 2, 5, and 7). When the filament bundle reaches the base of the nucleus each turn of the coil is directly enclosed by the outer nuclear envelope; the inner nuclear envelope is reflected back over the posterior end of the nucleus. A second outer nuclear membrane encloses the entire coil. Thus, the false discharge should be covered by two nuclear envelopes and the plasma membrane. (A drawing of this complex membrane pattern is presented in Fig. 7.) This turns out to be the case. Yet, when the outer nuclear envelopes come into contact, they often adhere to each other, making a double layer (Figs. 2 and 6). Therefore the false discharge is covered by the plasma membrane and the double-thick nuclear envelope (Figs. 6 and 7). This description of the association of the core filament bundle with membranes is identical with that of Fahrenbach (1973) and is confirmed by the image obtained by freeze-fracture (unpublished observations). The only specialization of the nuclear envelope is at the anterior end of the nucleus; here the outer nuclear envelope is more electron dense (Fig. 2).

Where the filament bundle contacts the acrosomal vesicle, approximately 15 filaments form a single row around a central homogeneous core (see Fahrenbach, 1973). At the posterior end of the filament bundle, I have counted as many as 79 filaments. Thus, the filament bundle tapers; from sections at varying distances along the bundle it is clear that the taper is gradual. Most of the filaments are hexagonally packed (Figs. 4, 5, and 6*b*), but at the periphery of the filament bundle there are filaments which are not hexagonally packed (Fig. 5). Whereas the majority of the filaments in the bundle measure 80–85 Å in diameter, the peripheral filaments often are only 50 Å in diameter. These 50-Å filaments are often found in the nuclear canal (Fig. 5), but they can also be found in the core filament bundle as it coils around the base of the sperm. Since the 50-Å filaments are seen only at the periphery of the bundle and since the number of filaments in the bundle increases from 15 anteriorly up to 79

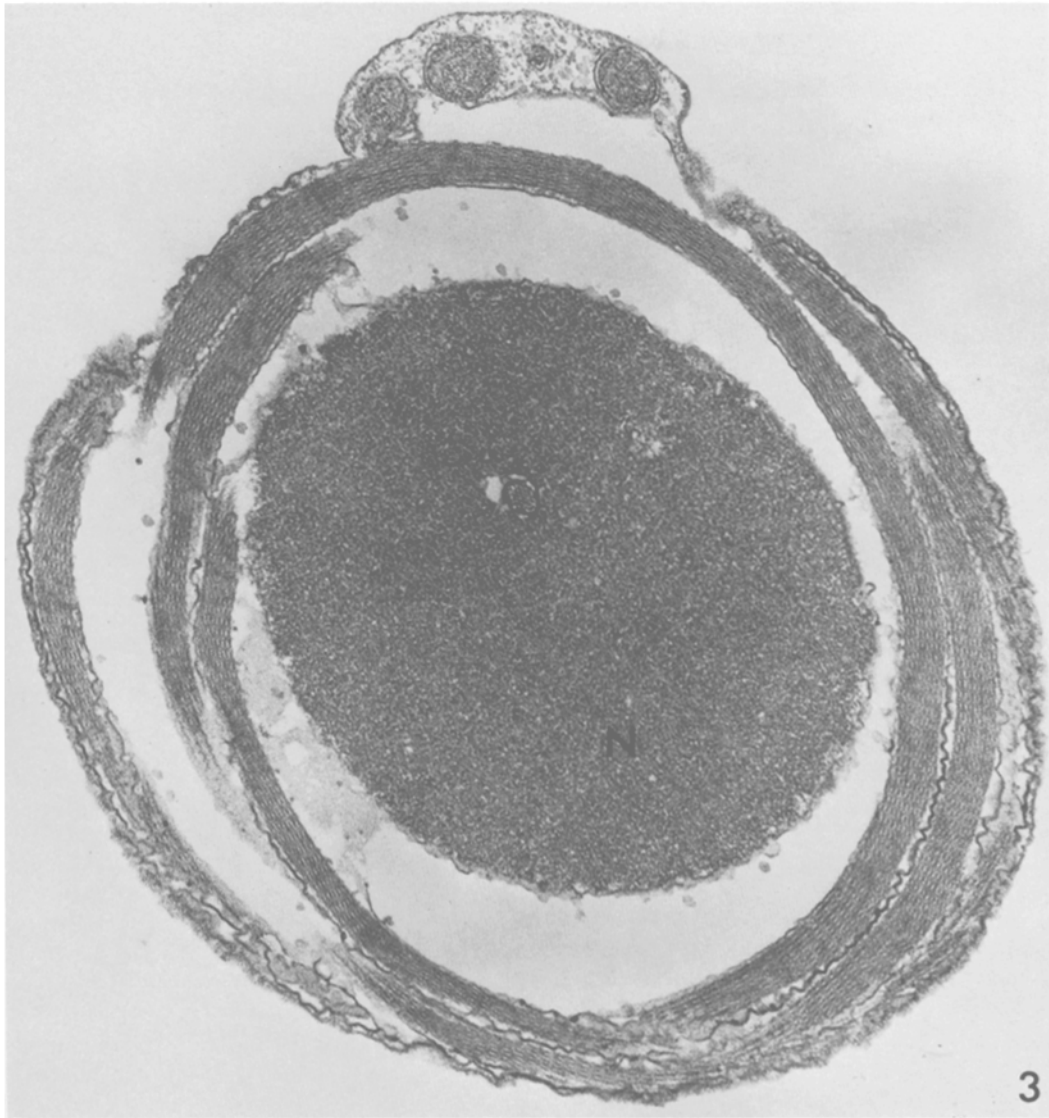


FIGURE 3 Unreacted sperm. This section is cut normal to Fig. 2. It passed through the basal end of the nucleus (*N*), cutting the core filament bundle in the nuclear canal in nearly transverse section. The core filament bundle in the coil can be seen in longitudinal section.  $\times 66,000$ .

posteriorly, it seems likely that the thin filaments represent the ends of filaments which, as one progresses posteriorly, will become part of the core filament bundle.

Basic to subsequent discussion is an accurate measurement of the diameter of the filaments in the coil, the false discharge, and the true discharge. With the exception of the thin filaments just described which are located peripheral to the bundle proper, the core filaments, irrespective of

their location, i.e. in the false discharge, in the coil within the sperm proper or in the nuclear canal, measured 80–85 Å in thin section. The outer doublet microtubules in the flagella measured 250 Å in diameter; thin filaments in *Limulus* skeletal muscle fixed and processed in a comparable manner measured about 70 Å in diameter, and filaments in thin sections of purified F-actin measured approximately 55 Å. At least 50 filaments were measured in each case.

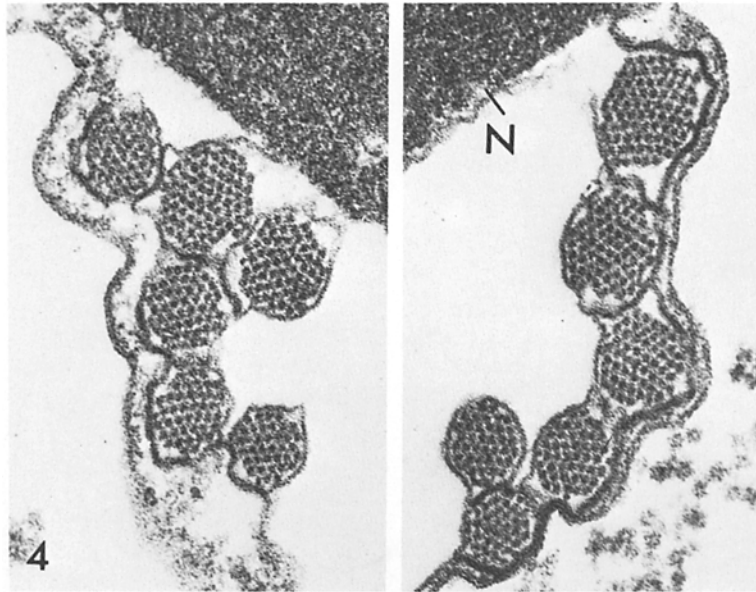


FIGURE 4 Transverse section through the core filament bundle in the coil at the base of the sperm. The plane of this section is similar to that of Fig. 2. The lower end of the nucleus (*N*) is included in this micrograph. Notice that the filaments in the bundle are hexagonally packed.  $\times 110,000$ .



FIGURE 5 Transverse section through the filament bundle while in the basal portion of the nuclear canal. A portion of the nucleus and the basal body of the flagellum (*B*) is included. Note that the most peripheral filaments of the filament bundle are smaller in diameter than those in the center of the bundle.  $\times 140,000$ .

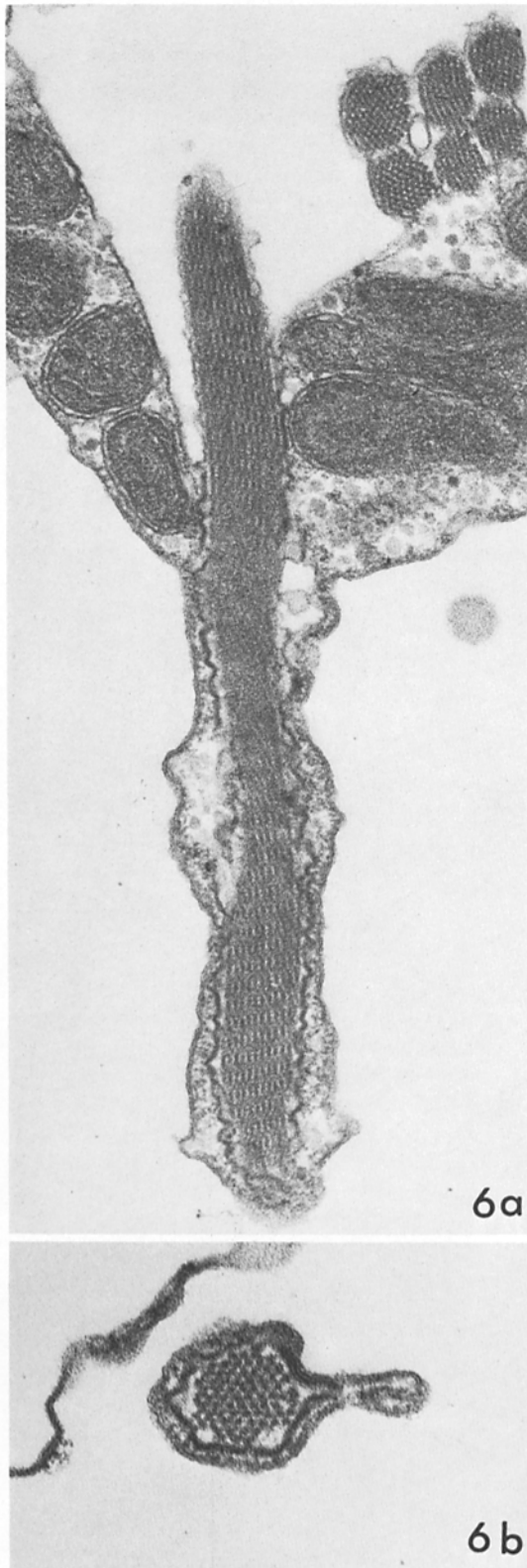
Close examination of the core filaments in longitudinal section in either the coil at the base of the sperm (Fig. 3) or through the false discharge (Fig. 6) reveals that there is a substructure present

in the filaments which gives rise to complex moiré patterns when one looks at the bundle as a whole. This will be discussed in more detail later, for the substructure of the filaments and their alignment is better seen in the negatively stained image.

**REACTED SPERM:** Washed sperm were induced to undergo the acrosomal reaction with excess  $\text{Ca}^{+}$  (15% by volume of 0.5 M  $\text{CaCl}_2$  added to sperm in sea water). They were then fixed. At the anteriolateral margin of the sperm is a collar (Figs. 1 *b* and 8) derived from the cytoplasm which was anterior to the acrosomal vesicle before its rupture. The amorphous extracellular material located anterior to the collar was derived from the contents of the acrosomal vesicle (Fig. 8). Longitudinal sections through the acrosomal process (true discharge) (Fig. 9) reveal a bundle of filaments which lie parallel to each other. The filament bundle now extends only to the posterior end of the nuclear canal (Figs. 1 *b* and 8). Thus, the core filament bundle making up the coil and the false discharge is now located in the acrosomal process.

Since the filaments in the acrosomal process are covered by the plasma membrane (Figs. 8 and 9) and since a membrane (the outer nuclear envelope) covers the core filaments in the coil before the acrosomal reaction, and yet no membrane remains after the reaction, Fahrenbach (1973) has sug-





gested that the outer nuclear membrane is directly converted into the plasma membrane which covers the acrosomal process. I have been unable to find any evidence of a connection between the plasma membrane and the outer nuclear envelope. Instead, the plasma membrane extends from the acrosomal process to the surface of the sperm and then over the collar, down the lateral margins of the cell, and out over the flagellar axoneme (Fig. 8). The specialization on the anterior surface of the nucleus does not change. Thus, in contrast to the conclusion of Fahrenbach (1973), the membrane which covers the acrosomal process does not seem to be *directly* derived from the outer nuclear envelope membrane.

As mentioned originally by Shoger and Brown (1970) the flagellum is retracted into the sperm after discharge. Thus, portions of the flagellar axoneme can be found in the cytoplasm (Fig. 8). From light microscopy, we know that the retraction of the flagellum is not linked temporally with the elongation of the acrosomal process; rather, the retraction of the flagellum takes several minutes, whereas acrosomal elongation just a few seconds. Thus, the membrane enclosing the flagellar axoneme cannot account for the formation of the plasma membrane of the acrosomal process.

#### *The Isolation of the Filament Bundle*

When sperm are treated with TTM the entire filament bundle rapidly transforms into the false discharge state (Fig. 10). Thus, filament bundles 50  $\mu\text{m}$  long can be collected. Examination of the sperm during treatment with TTM reveals that the false discharge literally screws out of the sperm. This motion generates considerable force, for if there is insufficient space for the elongating false discharge to come directly out of the sperm, the screwing motion of the false discharge is transferred to the sperm proper, causing it to move backwards and rotate at the same time. The undulatory profile of the demembranated false discharge and its wavelength is indistinguishable

FIGURE 6 *a* Longitudinal section through the proximal portion of the false discharge. Note the packing of the filaments.  $\times 73,000$ .

FIGURE 6 *b* Transverse section through the false discharge. The filament bundle is enclosed by two layers of the outer nuclear envelope that are fused and the plasma membrane.  $\times 110,000$ .

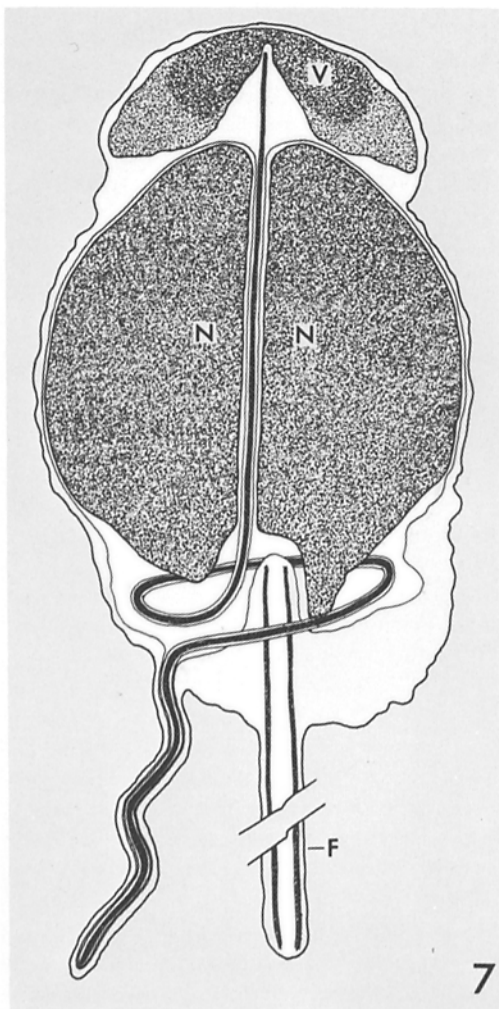


FIGURE 7 Drawing depicting the arrangement of the membranes. For simplicity, only one turn of the coil is included. The flagellum (*F*), the nucleus (*N*), and the acrosomal vesicle (*V*) are indicated.

from that seen in untreated sperm. The false discharge filament bundle can then be separated from other components such as the nucleus, the cap, and the flagella by differential centrifugation and a second detergent treatment (Fig. 12).

Alternatively, we have been able to isolate the coil from whole sperm by first treating sperm with 50% glycerol or low concentrations of Triton X-100 (0.1%) for a few minutes, then transferring the sperm to TTM (Fig. 11). Since the coil is wound around the base of the flagellum (Figs. 2 and 7), agitation is necessary to allow the coil to slide down the length of the flagellum (Fig. 11). I have fixed a preparation which contained large

numbers of coils and examined it by electron microscopy. The Triton removes the membranes completely. Thus, the demembrated filament bundle is stable in either the coiled state or as the false discharge state. Unfortunately, I have not been able to obtain a pure preparation of coils completely free from false discharges since, even with glycerol pretreatment, the TTM invariably stimulates some of the sperm to transform some of the coils or part of the coil into the false discharge state.

And, finally, if sperm are induced to undergo the acrosomal reaction by the addition of excess  $\text{Ca}^{++}$  to sea water, and then are placed into TTM, the filament bundle which would have been in the true discharge is freed from its investing membrane and from the sperm proper. The amount of "true discharge" filament bundles isolated in this way is dependent upon the percentage of sperm induced to undergo the acrosomal reaction. Obviously, if the sperm had not undergone the acrosomal reaction, the TTM would just induce the filament bundle to transform from the coil to the false discharge.

In summary, then, the filament bundle can exist and be isolated in three different states. The only state I have been able to isolate in pure form is the false discharge. Yet, since each state appears stable by itself, it is of interest to know what holds the filaments together and what differences there are in the packing of the filaments in each state.

#### *Negative Staining of the Core Filament Bundle*

The core filament bundle in each of the three states was examined by negative staining either by taking a sample of each state isolated as mentioned in the preceding section or by taking whole sperm and drying them down on a grid and negatively staining them. The three states can be readily distinguished by the characteristic profile of each state, even if the filament bundle comes loose from the body of the sperm. Neither the structure of the filament bundle nor the arrangement of the filaments is affected by fixation or detergent treatment.

Not only do the filaments in the true discharge lie parallel to each other, but also the helical substructure of adjacent filaments is in register. Thus the crossover points of one helical filament are perfectly aligned with the crossover points of all the adjacent filaments (Fig. 13 *c*). The precision in the packing of these helical filaments is even more amazing when one recognizes that the bundle

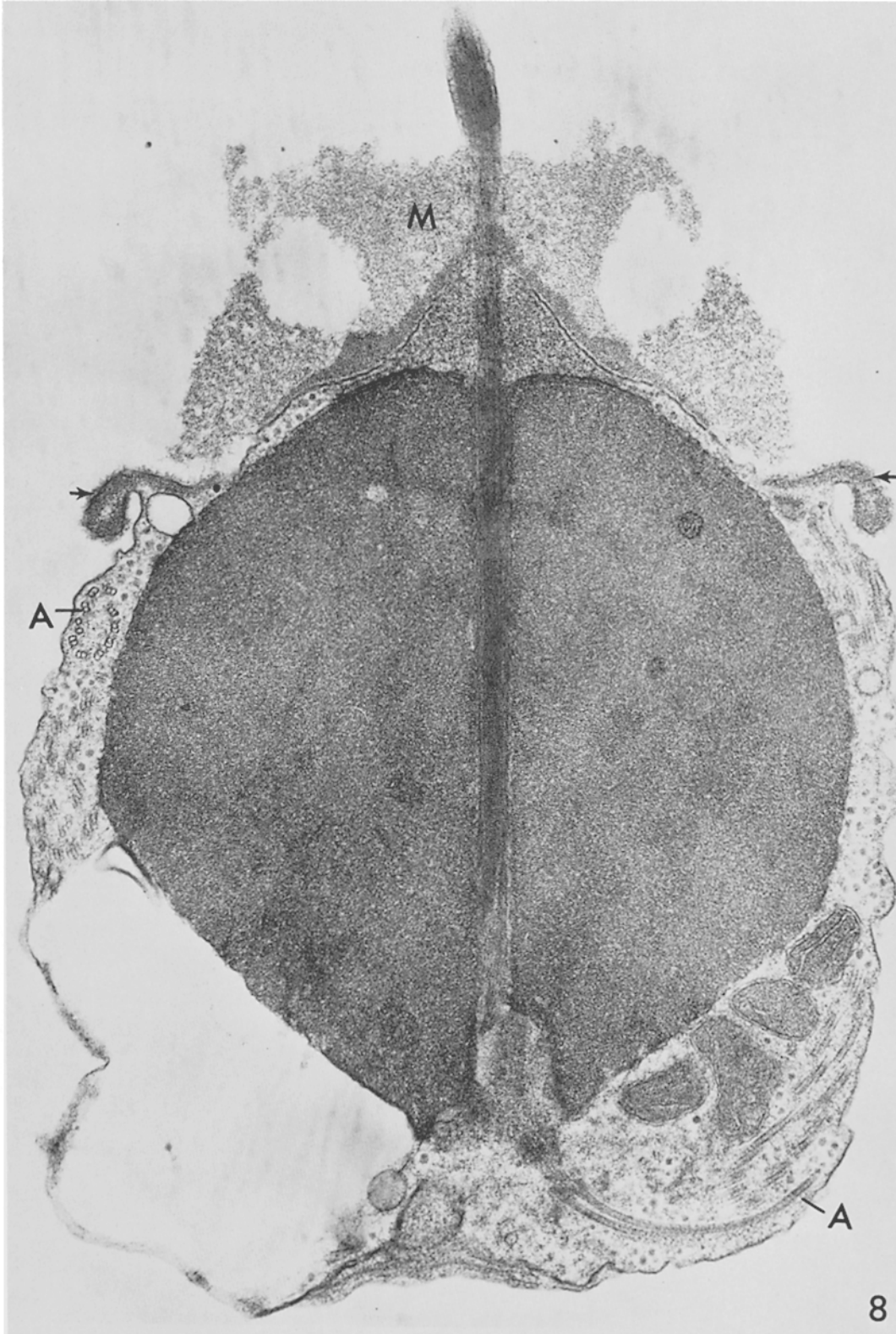


FIGURE 8 *Limulus* sperm induced to undergo the acrosomal reaction with excess  $Ga^{++}$ . The spongy material (*M*) outside the anterior end of the sperm are the remains of the acrosomal vesicle. The "collar," indicated by the arrows, formerly extended around the acrosomal vesicle. The flagellum has retracted and the axoneme (*A*) is present in the sperm cytoplasm.  $\times 50,000$ .

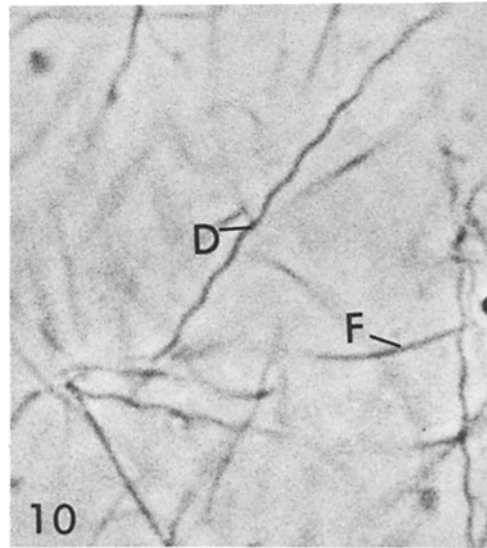
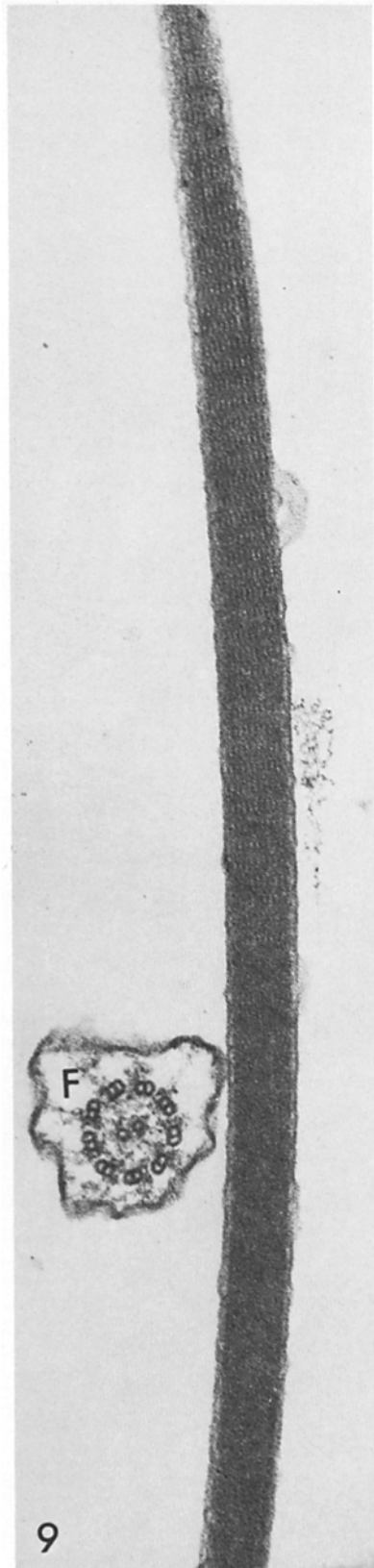


FIGURE 10 A pellet of the false discharge (*D*) and contaminating flagellar axonemes (*F*) prepared by differential centrifugation of sperm which had been treated with Triton X-100.  $\times 2,000$ .

has thickness so that when one sees one filament by negative staining, one is actually seeing the superposition of six or seven filaments. Thus the filaments in the true discharge appear as a paracrystalline array. The image, in fact, is remarkably similar to the optically filtered, reconstructed image obtained by Hanson et al. (1973), Hanson (1973), and O'Brien et al. (1971) of paracrystals of purified actin induced by high  $Mg^{++}$ . The helical periodicity of the filament bundle in the true discharge (385 Å) is comparable to the periodicity of an actin paracrystal (355-375 Å) (O'Brien et al., 1971). The center-to-center spacing of adjacent filaments measured from 10 different micrographs was 85 Å. Measurements of the center-to-center spacing of actin filaments in an  $Mg^{++}$  paracrystal determined from micrographs published by O'Brien et al. (1971) revealed a value of 50 Å, whether or not the measurement was made from the micrograph or from the reconstructed image. On the other hand, an actin paracrystal which contained troponin and tropomyosin measured 85 Å, again from the work of O'Brien et al. (1971). I

FIGURE 9 Longitudinal section through the acrosomal process. The core filaments are enclosed in a membrane. The flagellum (*F*) from another sperm is cut in transverse section.  $\times 83,000$ .

should emphasize that, without any special procedures, the negatively stained true discharge resembles the *reconstructed* or *filtered* and noise-free image of an  $Mg^{++}$  paracrystal of Hanson and her co-workers (1973), not her original micrographs. This fact plus the spacing of the filaments in the true discharge filament bundle indicates that the actin filaments in the true discharge are held in register by protein molecules similar in size to troponin and tropomyosin. As will be demon-

strated later, the filament bundle is *not* a  $Mg^{++}$ -induced paracrystal of actin, nor is it composed of troponin or tropomyosin, but rather it is an actin paracrystal held together by two other proteins. And, finally, if one carefully sights down a micrograph of the true discharge filament bundle, it is apparent that there is a very slight twist to the whole structure.

In contrast to the substructure of the true discharge, the arrangement of the filaments in the false discharge is strikingly different. In the false discharge the filaments do not lie parallel to one another but twist over each other at regular intervals (Fig. 13 *b*). Because the filament bundle has thickness, the image that one sees is very complex due to the superposition of filaments. This gives rise to complex moiré patterns. In favorable regions the double helical nature of individual filaments can be resolved, but in most places intrinsic filament substructure is eliminated due to superposition.

The filaments in the coiled state show a packing arrangement similar to that of the false discharge, but with subtle differences (Fig. 13 *a*). The negatively stained coil does not present a smooth, gently curving profile, but rather the bundle has a straight region, then a bend, then a straight section, then another bend, etc. I have measured approximately 14 bends per turn of the coil. This polygonal nature is related to changes in the packing of the filaments which twist over each

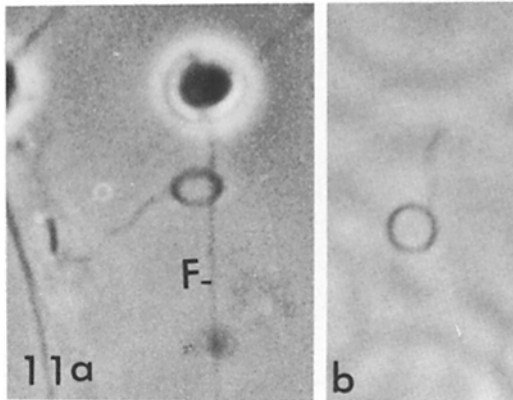


FIGURE 11 *a* Light micrograph of a sperm which had been treated with glycerol and then Triton X-100. Note that the coil, formerly located at the base of the sperm, is "falling off" the flagellum (*F*).  $\times 1,800$ .

FIGURE 11 *b* Isolated coil.  $\times 1,800$ .

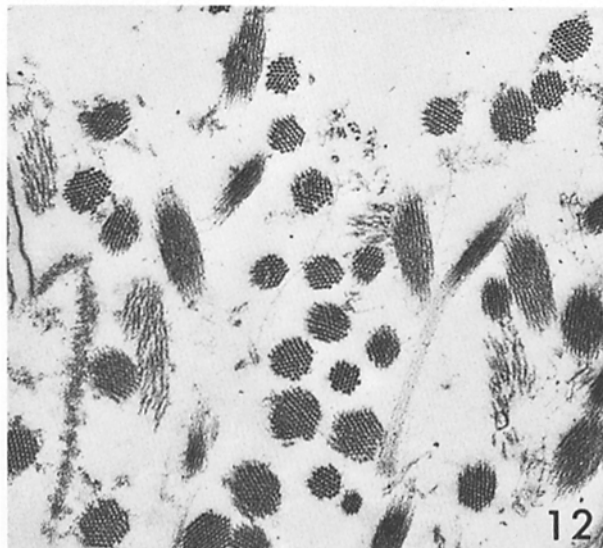


FIGURE 12 Section through a purified pellet of false discharges. No flagellar axonemes remain. The only contamination is some membrane fragments.  $\times 48,000$ .





**FIGURE 13** Negative staining of the portions of the demembrated: (a) coil, (b) false discharge, (c) true discharge.  $\times 125,000$ .

other more markedly at the bends. In the false discharge the packing arrangement changes for each bend in the undulatory profile but, unlike that in the coil, it seems to reverse. A more rigorous treatment of these three states will be a subject for further investigation in which we are examining the arrangement of the filaments using replicas so that the problem of superposition can be eliminated.

#### *Purification and Composition of the False Discharge Filament Bundle*

After treatment with TTM the false discharge filament bundles were isolated by differential centrifugation (Fig. 10). Contaminating flagellar axonemes were solubilized by a second detergent, Sarkosyl, and after washing, a pellet of the filament bundles in the false discharge was obtained. Purity was substantiated by thin sections cut through the pellet (Fig. 12). The only contaminant present was an occasional membrane fragment. Whereas TTM has no effect on the morphology of the false discharge filament bundles even if these structures remain in TTM for several hours at room temperature, prolonged storage in Sarkosyl gradually breaks them apart. However, if the purified bundles are stored as a pellet after Sarkosyl treatment, they appear stable for a number of days at 0°C. The wavelength of the isolated and purified false discharge is indistinguishable from that found for the wavelength of the false discharge *in vivo*. The average wavelength is 2.7  $\mu\text{m}$  with a range of 2.4–2.8  $\mu\text{m}$ .

When a purified preparation of the false discharge is run by SDS gel electrophoresis, only three bands appear on the gels; their molecular weights are 43,000, 55,000, and 95,000 (Figs. 14 and 15). Two of the bands (43,000 and 55,000) appear to be in approximately an equal molar ratio if the increase in Coomassie blue staining due to differences in molecular weight is taken into account. The third band (95,000) is present in much smaller amounts. Because of the similarity between the molecular weight of the 55,000 band and that of tubulin, which could be a contaminant from the microtubules in the flagellar axoneme, we ran the preparation of purified false discharges by SDS gel electrophoresis using Tris-glycine at pH 8.3 as the running buffer. In a separate group of experiments, we ran this preparation on urea gels. In both these gel systems tubulin is resolved into its  $\alpha$  and  $\beta$  component molecules (Renaud et al., 1968; Bryan, 1974). It is apparent from Fig. 15 and

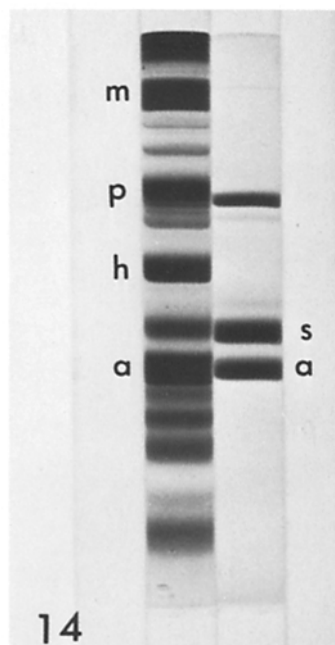


FIGURE 14 Conventional SDS gel electrophoresis of a *Limulus* myofibril (left gel) and a pellet of purified false discharge (right gel). On the left gel are actin (*a*), hemocyanin (*h*), paramyosin (*p*), and myosin (*m*). On the right gel are three bands: actin (*a*), the 55,000 mol wt protein (*s*), and the 95,000 mol wt protein.

from the urea gels that the 55,000 mol wt protein cannot be tubulin because: (*a*) it does not split into the  $\alpha$  and  $\beta$  components, and (*b*) its *R*<sub>z</sub> in these gel systems is different from that of either component of tubulin.

In SDS gels of myofibrils prepared from *Limulus* leg muscle there are bands whose molecular weights are indistinguishable from those of the proteins which make up the false discharge (Fig. 14). One of these, of course, is actin, which is no surprise on the basis of the morphology of the filament bundle in negative staining. The 95,000 mol wt component corresponds to the  $\alpha$ -actinin in the muscle gel and the 55,000 mol wt component corresponds to a prominent 55,000 mol wt band in muscle. The latter is not tubulin because if the myofibril preparation is run using the Tris-glycine buffer system, the 55,000 mol wt band from muscle does not split as tubulin does.

#### *HMM Binding*

Because of the remarkable similarity in the substructure of the filaments in the true discharge

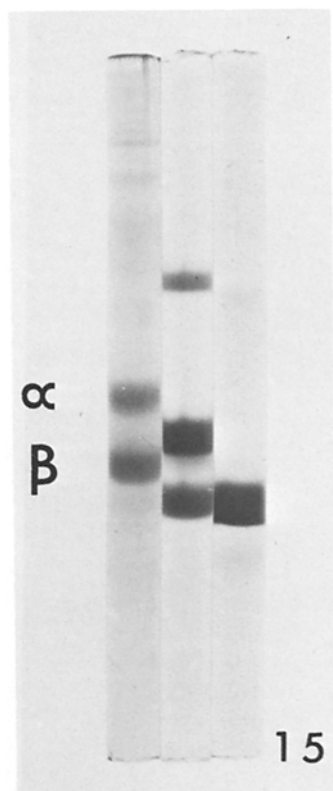


FIGURE 15 SDS gel electrophoresis using Tris-glycine as the running buffer. Left gel is a flagellar axoneme, middle gel is a pellet of the false discharge, and the right gel is purified rabbit muscle actin. The  $\alpha$  and  $\beta$  components of tubulin in the left gel are indicated.

and in actin paracrystals (see O'Brien et al., 1971, and Hanson et al., 1973), and the similarity in the molecular weight of a major band in the false discharge and of actin, I tried to decorate with HMM the filaments making up the false discharge. Much to my surprise, the filaments in the false discharge would *not* decorate with HMM even though muscle actin isolated and polymerized from an acetone powder of skeletal muscle would decorate beautifully. However, if the isolated and purified false discharge filament bundles were acetone extracted, air dried, and the acetone powder reextracted with water, filaments indistinguishable from actin could be polymerized (Fig. 16). These filaments would now decorate with HMM (Fig. 17). When I ran the water extract of the acetone powder of the false discharge, before HMM addition, on SDS gels, two bands were present, actin (43,000) and the 55,000 mol

wt band; but, unlike the situation before acetone extraction, the 55,000 mol wt band was present only in minor amounts. Thus the 55,000 mol wt protein seems to inhibit decoration with HMM.

Measurements were made of the diameter of the repolymerized actin filaments in the negatively stained image. If our measurements were made over holes, the filaments were about 55Å in diameter; if they were made just on the colloidin, they varied from 50 to 80 Å depending on the degree of flattening.

#### *Attempts to Transform the False Discharge Filament Bundle or the Coil into Another State*

Since the false discharge can transform *in vivo* into the coil and the coil into the true discharge, attempts were made to transform the isolated false discharge filament bundle into the coil, and the coil into either the false or true discharge *in vitro*. The conditions necessary for this transformation then might tell us something about the trigger and thus the mechanism of changes in filament packing *in vivo*.

Thus far, most of our attempts at transformation *in vitro* have been unsuccessful. Although our attempts were unsuccessful in transforming the false discharge or the coil, the stability of the filament bundle and the tightness of the association of the 55,000 mol wt and the 95,000 mol wt components to the actin filaments is demonstrated by these experiments. The effect of each agent on the isolated false discharge filament bundle or the coil was monitored by light microscopy under oil immersion. As can be seen, the false discharge and coil are stable to changes in pH, low or high salt, an excess or absence of divalent cations and presence of ATP (Table I). Even 0.6 M KI, a reagent which breaks down thin filaments in skeletal muscle, had no effect as was true of heat (45°C) (see the disassociation of tropomyosin from actin, Tanaka and Oosawa, 1971), sonication, and phosphotungstic acid (see Costello, 1973, for an effect on the ciliary axoneme). In fact, breakdown of these filament bundles could only be achieved in SDS, 8 M urea, or 0.5 M divalent salts. The only way to effect transformation of these filament bundles into another state (in this case the false discharge can be converted to the true discharge) is to age a preparation of the false discharge *in Sarkosyl* for several days and, then, add about 50



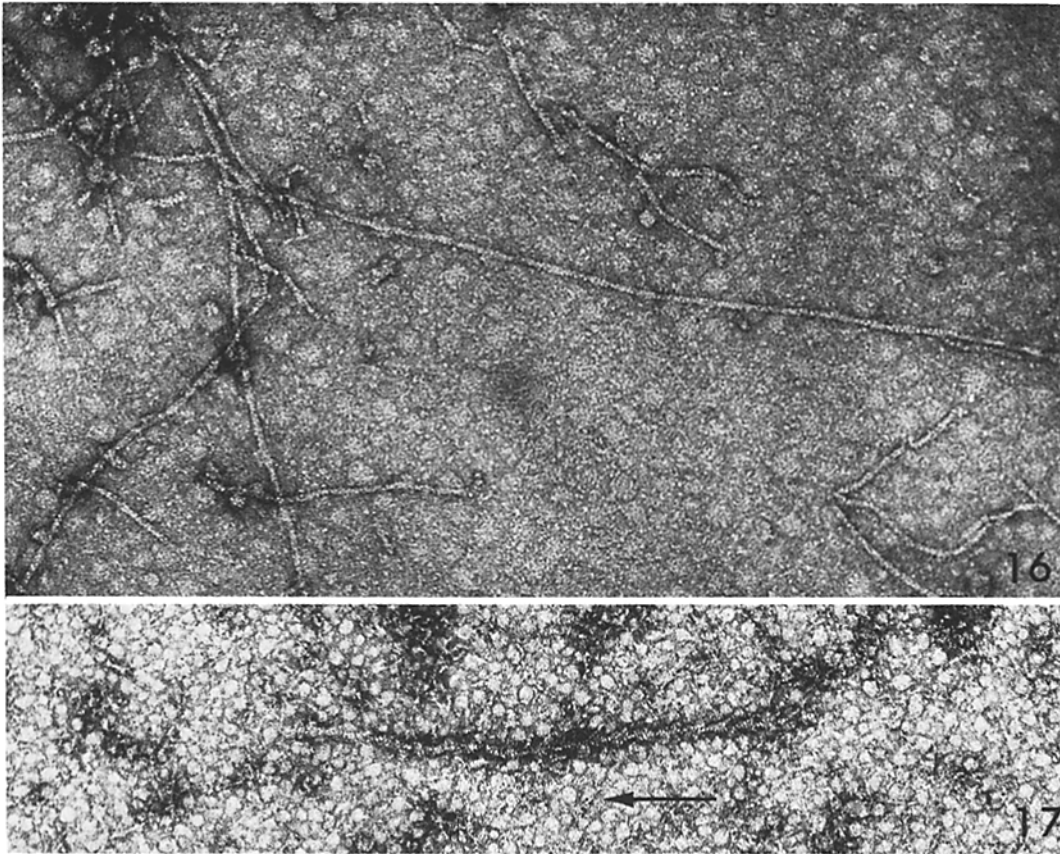


FIGURE 16 An acetone powder of the purified false discharge was made, extracted with water, and the actin polymerized with salt. Fig. 16 presents typical examples of the filaments.  $\times 110,000$ .

FIGURE 17 Some of the filaments seen in Fig. 16 were decorated with HMM. The arrow indicates the direction of the arrowheads  $\times 84,000$ .

TABLE I

*Stability of the Filament Bundles*

No change in the false discharge was found with following treatments	No change in the coil was obtained with the following treatments
1. changes in pH from 5.5 to 9.5	1. 50 mM EGTA at pH 7.0
2. 1 mM Tris pH 8.0 or distilled H <sub>2</sub> O	2. 50 mM EDTA at pH 7.0
3. 1 M KCl	3. 1 mM Tris-HCl at pH 8.0
4. 0.1 M EDTA pH 7.0	4. 1 M KCl
5. 50 mM EGTA at pH 7.5	5. 50 mM CaCl <sub>2</sub> or 40 mM MgCl <sub>2</sub>
6. 50 mM CaCl <sub>2</sub> or 50 mM MgCl <sub>2</sub>	6. 5 mM ATP at pH 7.0
7. ATP (0.25–10 mM)	
8. 0.6 M KI	
9. 2 M urea	
10. sonication	
11. 45°C for 10 min	
12. 1% phosphotungstic acid	

mM divalent salts. By light microscopy the undulatory profile of the false discharge disappears and in its place we see the linear profile of the true discharge. And by negative staining, the packing of the filament bundles resembles that of the true discharge. If aging in Sarkosyl is allowed to progress too far, then the filaments fall apart completely.

## DISCUSSION

Evidence is presented that the rapid elongation and rotation of the acrosomal process in *Limulus* sperm is brought about by changes in the packing of the actin filaments. No myosin seems to be present; instead, two other proteins, whose molecular weights are 55,000 and 95,000, appear to be involved in the packing or lateral association of the filaments. Let me first discuss the composition of the filament bundle, relating the proteins present to what is known about homologous proteins in skeletal muscle, and then compile the evidence indicating where these proteins are located in the paracrystalline array of actin filaments which form the true discharge.

### *Composition of the Filament Bundle and the Homology of these Proteins with Muscle Proteins*

After gel electrophoresis, the isolated and purified false discharge consists of three proteins: actin, a 55,000 mol wt protein and a 95,000 mol wt protein. Thus far, only four proteins have been described which bind to actin: myosin, tropomyosin, troponin, and  $\alpha$ -actinin.  $\alpha$ -Actinin has a molecular weight of about 95,000 (Schollmeyer et al., 1973) and thus might correspond to the 95,000 mol wt protein in the false discharge. It is interesting that  $\alpha$ -actinin is a major component of the "Z" line in skeletal muscle and is found in the dense bodies of smooth muscle (Schollmeyer et al., 1973). Its function in muscle seems to be to hold the actin filaments together. A similar function for the 95,000 mol wt protein in *Limulus* sperm seems probable. Schollmeyer et al. (1974) using a fluorescein-labeled antibody against  $\alpha$ -actinin indicate that  $\alpha$ -actinin is present along the length of the isolated false discharge filament bundle. Thus, the 95,000 mol wt band seems to correspond to  $\alpha$ -actinin.  $\alpha$ -Actinin also seems to be present in fibroblasts and in the brush border.

A band whose molecular weight is indistinguishable from the 55,000 mol wt band is also present in *Limulus* myofibrils. Recent studies by Lehman et

al. (1973) and Lehman and Szent-Györgyi (1972) using SDS gel electrophoresis of *Limulus* muscle show that this band is attached to the thin filaments. Their studies also demonstrate that this protein is present on the thin filaments of other organisms, namely, the water bug *Lethocercus* and the brachiopod *Glottidia*. Similarities in the binding affinity of the 55,000 mol wt protein to actin in the false discharge filament bundle and of a 55,000 mol wt band to actin in *Lethocercus* muscle is suggested by recent work of Bullard et al. (1973). These investigators could not purify *Lethocercus* muscle actin free from a 55,000 mol wt protein by either cyclic polymerization and depolymerization or by column chromatography. They also demonstrated that this protein was not tropomyosin because *Lethocercus* has tropomyosin as well. (Similar observations were made by Lehman and Szent-Györgyi, 1972, for *Limulus* muscle.) Furthermore, this protein cannot be a troponin since muscle extracts containing troponin and tropomyosin are not contaminated with this 55,000 mol wt protein. Bullard et al. (1973) speculated that the 55,000 mol wt protein might be tubulin which polymerizes along with the actin. Evidence was presented here which shows that this interpretation is incorrect. If *Limulus* myofibrils are run on SDS gels using Tris-glycine as the running buffer, tubulin will split into its  $\alpha$  and  $\beta$  components, but the 55,000 mol wt band from *Limulus* muscle neither splits nor does it have the same  $R_f$  as either component of tubulin. We are left, therefore, with the following information: a 55,000 mol wt protein is present in the thin filaments of skeletal muscle of arthropods and brachiopods which is not tubulin, tropomyosin, or troponin, and which purifies with actin, indicating that it binds to actin; it is similar to the 55,000 mol wt protein in *Limulus* sperm in its molecular weight and its binding behavior with actin. We are currently peptide mapping the 55,000 mol wt protein from *Limulus* sperm and the 55,000 mol wt protein from *Limulus* muscle. Thus far, the peptide maps indicate that the proteins are indeed similar. One wonders if the 55,000 mol wt protein also behaves as a glue like  $\alpha$ -actinin and, thus, if it is not a component of the Z lines in certain types of muscle.

### *Location of the 55,000 mol wt and the 95,000 mol wt Proteins in the Filament Bundle*

The following facts are known concerning the 55,000 mol wt protein and the filament bundle: (a)

the core filaments by negative staining closely resemble in their ultrastructure purified actin, (b) the 55,000 mol wt protein is approximately equal in molar ratio to actin, (c) the 55,000 mol wt protein inhibits the decoration of the core filaments by HMM, (d) the core filaments are tightly bound to one another and disruption can occur only under fairly drastic conditions, (e) in thin sections through the core filament bundle at the periphery there are often thinner filaments each measuring 50 Å in diameter—the diameter of F-actin in thin sections—whereas the bulk of the filaments in the bundle measure 80–85 Å in diameter; and (f) the center-to-center spacing of the core filaments in negative staining of the true discharge measures 85 Å, while the center-to-center spacing of the filaments from a Mg<sup>++</sup> paracrystal of actin measures 50 Å, and a troponin-tropomyosin-actin paracrystal measures 85 Å. (Data collected from the micrographs of O'Brien et al, 1971.) On the basis of these facts, the most probable arrangement of actin and the 55,000 mol wt protein can be selected from the several possible arrangements that could exist. Let me begin by listing the possible arrangements. First, actin filaments might have the 55,000 mol wt protein attached to their surfaces such that one molecule of the 55,000 mol wt protein attaches to each actin subunit in such a way as to inhibit HMM binding. A bundle of filaments would then be held together by adjacent molecules of the 55,000 mol wt protein interacting with each other. Secondly, the actin and the molecules of the 55,000 mol wt protein might alternate along the length of a filament making a heteropolymer. Finally, one might visualize actin filaments interspersed between the filaments of the 55,000 mol wt protein. This last possibility can be eliminated immediately as the core filaments in thin section are all the same size, with the exception of a small number of thin filaments peripheral to the main core. Since in thin section the bulk of filaments measure 80–85 Å in diameter and F-actin measured only 55 Å, the filaments in the sperm are too thick to be pure actin. A similar argument can be made for the spacing of the filaments in the negatively stained image. The spacing of actin in an actin paracrystal is 50 Å; that of an actin-troponin-tropomyosin paracrystal is 85 Å; while that in the true discharge of sperm is also 85 Å. This means that the actin filaments in the sperm must have substances attached to their surfaces which increase the center-to-center spacing.

The second possible arrangement of actin and the 55,000 mol wt protein, that of a heteropolymer, puts a number of restrictions on the binding behavior of the 55,000 mol wt protein to actin. First, we know that the double helical substructure characteristics of an actin filament remain. Thus, the molecules of the 55,000 mol wt protein must bind to adjacent actin subunits in the same manner that actin binds to actin in order to maintain this structure. Secondly, in some way the molecules of the 55,000 mol wt protein must cover all the actin-binding sites for HMM. If they did this by changing the conformation of the actin, the double helical structure with its characteristic 385 Å repeat would most likely be lost, which it is not. Finally, this possibility would not account for the thin filaments peripheral to the filament bundle. These filaments, because of their diameter, must be actin. Although none of these arguments completely eliminates the possibility of a heteropolymer of the 55,000 mol wt protein and actin, they make it extremely unlikely.

Thus, the most probable location for the 55,000 mol wt protein is the first possibility, that is, attached to the surface of the actin subunits. This would account for the increase in filament diameter and the 85 Å spacing of filaments in a paracrystal, similar to the way in which tropomyosin and troponin increase the diameter of thin filaments in muscle. It would also account for the inhibition of HMM binding.

From the work of Schollmeyer et al. (1974), antibodies to  $\alpha$ -actinin (the 95,000 mol wt protein) bind along the length of the false discharge filament bundle. We do not know if, in fact, this protein is in the true discharge at all. It may be present only in the false discharge and the coil, and thus perturb the system sufficiently to form this supercoiled structure. If this is the case, then the 55,000 mol wt protein may operate in all three states simply as a "glue" to forcefully hold together the filaments.

The packing of the filaments in the true discharge is very precise, much more so, in fact, than the packing in an actin paracrystal induced by magnesium (Hanson et al., 1973; O'Brien et al., 1971). The alignment of the filaments in the true discharge is as good, if not better than, the image obtained after optical filtering the diffracted image of a Mg<sup>++</sup> paracrystal. In both these cases the filaments are arranged in register, with the cross-over points of the actin helices transversely aligned. This indicates that probably the 55,000

mol wt protein, and perhaps the 95,000 mol wt protein as well, amplify the substructure of actin, essentially producing an image that is "noise-free" in negative staining. Thus if the 55,000 mol wt protein is a glue, it must glue together actin filaments at specific sites in order to amplify the substructure of the filament. Likewise, because of the stability of the filament bundle resisting agents such as KI, which will break down thin filaments in muscle, the 55,000 mol wt protein must bind adjacent filaments together not only precisely but very strongly.

#### *The Need for an Additional Component or Components for the Transformation of the Filaments from One State to Another*

Thus far, I have been unable to change the state of the filament bundles in vitro, transforming, for example, the coil into the false or true discharge, or the false discharge into the coil. Either I have not yet managed to find the exact ionic conditions necessary for transformation from one state into the next, or some substance or substances necessary for a change in the packing of the filaments have been removed from the filament bundle during isolation. Thus, the false discharge filament bundle may be a stable state by itself, and unless something is added, which was removed by the detergent, reversal cannot occur. It is interesting in this regard that the coil can be isolated only if the cell is pretreated with glycerol before treatment with TTM; or, if TTM is used, a very low concentration of Triton X-100 is used initially (10 times lower than is normally used). This observation is consistent with the fact that detergent treatment, by removing the membranes, allows some material crucial for transformation to diffuse away. Yet, the true discharge is never produced by detergent treatment and, in fact, is *only* generated out of the anterior end of the sperm. The transformation of the packing of the filaments in this bundle occurs within the nuclear canal. This indicates that there may be a second factor present in the nuclear canal which enables the transformation to occur here locally. Excess calcium induces true discharge, so that perhaps calcium is a cofactor. This is consistent with the effect of divalent cations and "aged" Sarkosyl-treated false discharges. This treatment allows the false discharge to transform into the true discharge.

#### *Membrane Generation*

The Colwins, in a classic series of papers on fertilization of two invertebrate organisms, the annelid worm *Hydroides* and the hemichordate *Saccoglossus*, described how the membranes surrounding the acrosomal vesicle fused with the limiting plasma membrane of the sperm. They also described how fusion between the plasmalemma surrounding the acrosomal process of the sperm and the egg oolemma took place. (See their excellent review for references, Colwin and Colwin, 1967.) Whereas the fusion between the acrosomal vesicle membrane and the plasmalemma in *Limulus* appears similar to what has been previously described in *Hydroides* and *Saccoglossus* (André, 1965; Shoger and Brown, 1970), there is some circumstantial evidence that the nuclear envelope may be involved in the formation of the acrosomal process membrane in *Limulus*. If this is true, then such an association is unique in the biological literature. In unreacted sperm, surrounding the core filaments there is a membrane composed of the outer nuclear envelope (Fahrenbach, 1973). This tubular membrane is not left behind in the circumnuclear cisternae during the acrosomal reaction, but instead disappears. Fahrenbach (1973) argues that this indicates that the outer nuclear envelope is directly converted into the membrane surrounding the acrosomal process. If this were to occur, then there should be a connection between the outer nuclear envelope and the plasma membrane. Thus far I have been unable to find any connection between these membranes. Since I have never found a connection between the nuclear envelope and the plasma membrane, and since in the formation of the acrosomal process in other sperm, such as echinoderm sperm or mollusc sperm where the acrosomal process is of comparable length or up to twice as long as that in *Limulus* (Tilney et al., 1973), there is no reason to suspect that the nuclear envelope has a direct connection with the generation of the acrosomal process membrane. Another source for the acrosomal process membrane might be the membrane which encloses the flagellum, since sperm attached to *Limulus* eggs (Shoger and Brown, 1970) or sperm induced to discharge their acrosomes by excess calcium resorb their flagella. Unfortunately, this cannot be the case as flagellar retraction is a much slower event than the formation of the acrosomal process.

Other evidence clearly indicates that membranes must be formed and broken down rapidly in *Limulus* sperm. There are two examples of this. First, during the formation of the false discharge a plasma membrane must be formed to cover the corkscrew process, which under ideal conditions can be 50  $\mu\text{m}$  in length. It is not formed from the flagellar membrane as this structure remains intact, nor is there any change in the nuclear membrane which covers the core filaments. Alternatively, the cell can retract all 50  $\mu\text{m}$  of this false discharge which it does before the true discharge. Thus, the membrane covering the false discharge must be generated rather rapidly and broken down equally rapidly. Secondly, the plasma membrane which covers the axoneme must be broken down during flagellar retraction as after retraction is complete this membrane has "disappeared." Thus, *Limulus* sperm are capable of rapid mobilization and dissolution of membrane. This must mean that new membrane is produced on these sperm either by "stretching" the existing membrane, a rather unlikely situation due to the relative amount (about half again as much membrane is produced as covers the head of the sperm during true or false discharge), or that membrane is produced from soluble precursors. Since an increase in the amount of membrane occurs during the false discharge, and since there is no connection between the nuclear envelope and the plasma membrane during the true discharge, I conclude that the plasma membrane formed during the true discharge forms *de novo*. Perhaps some of the precursors of the nuclear membrane are used in the formation of the acrosomal process membrane. This interpretation is more consistent with the formation of membrane from soluble precursors during the acrosomal reaction in other sperm in which there are no excess membranes in the cell before the acrosomal reaction (Tilney et al., 1973).

It is my great pleasure to acknowledge the invaluable help of my colleagues, Drs. Ray Stephens, Roger Lucas, and John Fuseler. I am particularly grateful to Ray Stephens who saw immediately the similarities between a  $\text{Mg}^{++}$  paracrystal of actin and the true discharge and who, I hope, survived many stimulating discussions and use of his equipment. Roger Lucas pointed out to me the similarities between the proteins in the false discharge and muscle proteins, and also spent endless hours in discussion. John Fuseler helped initiate the project by showing me how to easily obtain sperm from the

*Limulus*. It is also my great pleasure to thank Doris Bush for excellent technical assistance.

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## BIBLIOGRAPHY

- ANDRÉ, J. 1965. A propos d'une leçon sur la Limule. *Ann. Fac. Sci. Clermont*. **26**:27-38.
- BRYAN, J. 1974. Biochemical properties of microtubules. *Fed. Proc.* **33**:152-157.
- BULLARD, B., R. DABROWSKA, and L. WINKELMAN. 1973. The contractile and regulatory proteins of insect flight muscle. *Biochem. J.* **135**:277-286.
- COLWIN, L., and A. L. COLWIN. 1967. Membrane fusion in relation to sperm egg association. In *Fertilization I*. C. B. Metz and A. Monroy, editors. Academic Press Inc., New York. 295-367.
- COSTELLO, D. P. 1973. A new theory on the mechanics of ciliary and flagellar motility. I. Supporting observations. *Biol. Bull. (Woods Hole)*. **145**:279-291.
- FAHRENBACH, W. H. 1973. Spermiogenesis in the horseshoe crab, *Limulus polyphemus*. *J. Morphol.* **140**:31-52.
- HANSON, J. 1973. Evidence from electron microscope studies on actin paracrystals concerning the origin of the cross-striation in the thin filaments of vertebrate skeletal muscle. *Proc. R. Soc. Lond. B Biol. Sci.* **183**:39-58.
- HANSON, J., V. LEDNEV, E. J. O'BRIEN, and P. M. BENNETT. 1973. Structure of the actin-containing filaments in vertebrate skeletal muscle. *Cold Spring Harbor Symp. Quant. Biol.* **37**:311-318.
- ISHIKAWA, H., R. BISCHOFF, and H. HOLTZER. 1969. The formation of arrowhead complexes with heavy meromyosin in a variety of cell types. *J. Cell Biol.* **43**:312.
- LEHMAN, W., J. KENDRICK-JONES, and A. G. SZENT-GYÖRGYI. 1973. Myosin-linked regulatory systems: comparative studies. *Cold Spring Harbor Symp. Quant. Biol.* **37**:319-330.
- LEHMAN, W., and A. G. SZENT-GYÖRGYI. 1972. Activation of the adenosine triphosphatase of *Limulus polyphemus* actomyosin by tropomyosin. *J. Gen. Physiol.* **59**:375-387.
- O'BRIEN, E. J., P. M. BENNETT, and J. HANSON. 1971. Optical diffraction studies of myofibrillar structure. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:201-208.
- POLLARD, T. D., and R. R. WEIHING. 1974. Actin and myosin in cell movements. *Crit. Rev. Biochem.* **2**:1-65.
- RENAUD, F. L., A. J. ROWE, and I. R. GIBBONS. 1968.

- Some properties of the protein forming the outer fibers of cilia. *J. Cell Biol.* **36**:79-90.
- SCHOLLMAYER, J. E., D. E. GOLL, R. M. ROBSON, and M. H. STROMER. 1973. Localization of  $\alpha$ -actinin and tropomyosin in different muscles. *J. Cell Biol.* **59**(2, Pt. 2):306 a. (Abstr.).
- SCHOLLMAYER, J. E., D. E. GOLL, L. TILNEY, M. MOOSEKER, R. ROBSON, and M. STROMER. 1974. Localization of  $\alpha$ -actinin in nonmuscle material. *J. Cell Biol.* **63**(2, Pt. 2):304 a (Abstr.).
- SCHRANK, W. W., R. L. SHOGER, L. M. SCHECHTMAN, and D. W. BISHOP. 1967. Electrically induced spawning in the male and female horseshoe crab, *Limulus polyphemus*. *Biol. Bull. (Woods Hole)*. **133**:453.
- SHOGER, R. L., and D. W. BISHOP. 1967. Sperm activation and fertilization in *Limulus polyphemus*. *Biol. Bull. (Woods Hole)*. **133**:485.
- SHOGER, R. L., and G. G. BROWN. 1970. Ultrastructural study of sperm-egg interaction of the horseshoe crab, *Limulus polyphemus* L. (Merostomata: Xiphosura). *J. Submicrosc. Cytol.* **2**:167-179.
- STEPHENS, R. E. 1968. Reassociation of microtubule protein. *J. Mol. Biol.* **33**:517-519.
- STEPHENS, R. E. 1970. Thermal fractionation of outer fiber doublet microtubules into A and B subfiber components: A and B tubulin. *J. Mol. Biol.* **147**:353-363.
- TANAKA, A., and F. OOSAWA. 1971. The effect of temperature on the interaction between F-actin and tropomyosin. *Biochim. Biophys. Acta.* **253**:274-283.
- TILNEY, L. G. 1973. Alterations in the packing of actin filaments giving rise to motion elicited by the acrosomal reaction in *Limulus* sperm. *J. Cell Biol.* **59**(2, Pt. 2):346 a. (Abstr.).
- TILNEY, L. G., S. HATANO, H. ISHIKAWA, and M. S. MOOSEKER. 1973. The polymerization of actin. Its role in the generation of the acrosomal process of certain echinoderm sperm. *J. Cell Biol.* **59**:109-126.
- WEBER, K., and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406-4412.