

Movement of the Actin Filament Bundle in *Mytilus* Sperm: A New Mechanism Is Proposed

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Abstract. An actin filament bundle $\sim 2\text{--}5\ \mu\text{m}$ in length is present in the sperm of the blue mussel, *Mytilus*. In unfired sperm this bundle extends from the midpiece through a canal in the center of the nucleus to terminate on the membrane limiting the inside of the cone-shaped acrosomal vacuole. The bundle is composed of 45–65 actin filaments which are hexagonally packed and regularly cross-bridged together to form an actin paracrystal so well ordered that it has six nearly equal faces. Upon induction of the acrosomal reaction, a needle-like process is formed in a few seconds. Within this process is the actin filament bundle which appears unchanged in filament number and packing as determined by optical diffrac-

tion methods. Using fluorescein-conjugated phalloidin we were able to establish that the bundle does not change length but instead is projected anteriorly out of the midpiece and nuclear canal like an arrow. Existing mechanisms to explain this extension cannot apply. Specifically, the bundle does not increase in length (no polymerization), does not change its organization (no change in actin twist), does not change filament number (no filament sliding), and cannot move by myosin (wrong polarity). Thus we are forced to look elsewhere for a mechanism and have postulated that at least a component of this movement, or cell elongation, is the interaction of the actin filament bundle with the plasma membrane.

DAN and Wada (4) were the first to investigate the acrosomal reaction of sperm from the bivalve molluscs. They demonstrated that if *Mytilus* sperm were placed in "egg water" or sea water to which a high concentration of calcium was added (12), the elongated acrosomal vacuole would break down and a process would be generated. When these sperm were examined by electron microscopy, Nijijima and Dan (11, 12) found an "axial rod" in both the newly formed acrosomal process and in the sperm before induction. As fixation methods improved, the axial rod was shown to consist of actin filaments (22) all aligned parallel to each other. What remained unclear was how, given this actin filament bundle, an acrosomal process is generated.

This became even more of a puzzle because, as we learned about the elongation of the acrosomal process in echinoderm and arthropod sperm (see 23 for references), it became increasingly apparent that the actin filament bundle in *Mytilus* is unique and generation of the acrosomal process can not be attributed either to actin polymerization (e.g., *Thyone* sperm) or to a change in twist of the component actin filaments (e.g., *Limulus* sperm). Furthermore, movement induced by myosin is not possible because the polarity of the actin filaments in *Mytilus* sperm is the reverse of that needed for myosin to actively elongate an acrosomal process. Since all three mechanisms seem inappropriate for providing the

extension of the acrosomal process in *Mytilus* sperm, we have been forced to look elsewhere.

Our search has been narrowed because during the course of this study we have determined what does happen during extension of the acrosomal process as well as what does not occur. What does occur is that the actin filament bundle is projected anteriorly like an arrow being shot forward. Our hypothetical arrow is a beautiful actin paracrystal which is unchanged during the acrosomal reaction. These and other observations lead us to postulate a mechanism for actin-induced motility that has not been considered in detail previously.

Materials and Methods

Obtaining Sperm

Large *Mytilus edulis*, the edible blue mussel, were obtained either from the Department of Marine Resources of the Marine Biological Laboratory, Woods Hole, MA, or from local fish markets. The animals are ripe in August through the fall, but a small number of spermatozoa can be obtained throughout the year from freshly collected animals. The animals are opened, and the gonad is removed from the males (white, not reddish) and cut into pieces in a small beaker of sea water. After ~ 5 min, the suspension is filtered through cheese cloth to remove the residual gonad, and the sperm are pelleted by centrifugation (1,000 g). The pellet of sperm is usually used the same day.

Observations on Living Sperm

Sperm were examined either by phase-contrast or differential interference contrast microscopy with a 100 \times oil immersion objective. For video recording we used the perfusion chamber and the high extinction video system outlined in Inoué and Tilney (10). The acrosomal reaction is not induced by ionophores such as A23187, nor reliably with high calcium as suggested by Nijima and Dan (12). However, if the sperm come from very ripe organisms (e.g., in late August) there are a larger number of inductions with high calcium than at other times of the year. Some sperm will undergo the acrosomal reaction spontaneously upon contact with the glass of the slide or coverslip. The ionophore, X537A, if added to sea water containing calcium, will induce the acrosomal reaction in \sim 20% of the sperm. The acrosomal vacuole is dramatically altered in all the rest as if a partial reaction is induced. Nigericin induces an incomplete reaction in all the sperm but few processes are seen.

Electron Microscope Procedures

Negative Staining. Drops of sperm which had been treated with high calcium in sea water or X537A were deposited on grids which had been coated with colloidal and stabilized with a thin film of carbon. Before negative staining with 1% uranyl acetate, the grids were rinsed with a solution containing 1% Triton X-100, 3 mM MgCl₂, and 30 mM Tris at pH 8.0. This detergent solution induces the acrosomal reaction and a few seconds later solubilizes the plasma membrane.

Fixation and Preparation of Thin Sections. A large number of different fixation protocols were attempted because the actin filament bundle in *Mytilus* sperm is difficult to preserve. What proved the most satisfactory was to suspend the sperm in sea water and to add to it an equal aliquot of a solution containing 2% OsO₄, 2% glutaraldehyde (from an 8% stock supplied by Electron Microscope Sciences, Fort Washington, PA) in 100 mM phosphate buffer at pH 6.2. Thus, the resultant fixative contains 1% OsO₄-1% glutaraldehyde in 50 mM phosphate buffer at pH 6.2 and 1/2 strength sea water. The aliquot containing the fixative was cooled to 4°C before addition to the suspension of sperm and was made up immediately before fixation. Fixation was for 30 min with the vial containing the sperm maintained in an ice bath. At the end of the 30-min period, the sperm were concentrated by centrifugation (750 g for 2 min), washed three times in water at 4°C, and en bloc stained with 0.5% uranyl acetate for 3 h. The pellet was then dehydrated rapidly in acetone and embedded in Epon. Thin sections were cut on a Sorvall Porter Blum ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and viewed with a Philips 200 electron microscope.

Decoration with Subfragment 1 of Myosin. *Mytilus* sperm in a pellet were first demembrated with a detergent containing 1% Triton X-100, 30 mM Tris, and 3 mM MgCl₂ at pH 8.0, pelleted by centrifugation (1,000 g), then suspended and incubated for 2–4 h on ice with subfragment 1 in 50 mM phosphate buffer containing 1 mM MgCl₂ at pH 7.0 at 4°C. The sperm were repelleted, washed in cold buffer, and fixed with 1% glutaraldehyde in 50 mM phosphate buffer at pH 7.0 at 4°C for 30 min, washed in phosphate buffer, and postfixed in 1% OsO₄ in 100 mM phosphate buffer at pH 6.2 for 30 min at 4°C, washed three times in cold water and en bloc stained with 0.5% uranyl acetate overnight. The next day they were dehydrated in acetone and embedded Epon 812.

The subfragment 1 was prepared from rabbit skeletal muscle myosin and stored at -20°C in glycerol.

Diffraction Pattern Analysis of the Actin Bundle from Electron Micrographs

Diffraction patterns were made from suitable regions of negatively stained preparations and thin sections. These patterns were either obtained optically using an optical bench set up (6) or produced digitally as outlined by DeRosier et al. (7).

Staining Sperm with Fluorescein-conjugated Phalloidin

A drop of *Mytilus* sperm suspended in sea water was overlain with a thin layer of agarose (30) on a slide, the excess fluid was removed, and a drop of staining solution containing 1% formalin, 2 μM fluorescein-conjugated phalloidin (Molecular Probes, Inc., Junction City, OR) in artificial sea water was added. The preparation was stained for 10 min at room temperature, then rinsed briefly in PBS (0.17 M NaCl, 3 mM KCl, 6 mM Na/K phosphate buffer at pH 7.4). The agarose was removed and the preparation mounted

with gelvatol. The preparation was examined in a Zeiss Photomicroscope III using a 63 \times oil immersion phase objective with an NA of 1.4. The same cell was examined and photographed with Kodak plus X film through the same objective using illumination for phase-contrast microscopy and reflected illumination for fluorescence microscopy. The film was developed in Diafine (Acufine Inc., Chicago, IL).

Results

Observations on Living Sperm

In *Mytilus* the anterior end of the sperm has a prominent, greatly elongated acrosomal vacuole somewhat longer than the combined length of the nucleus and midpiece (4). Induction of the acrosomal process is difficult in *Mytilus* sperm. At the peak of the season a small percentage can be induced with the ionophore, A23187. X537A is much more effective but still only 10–20% are induced. Interestingly 100% can be induced with Triton X-100 or digitonin in low ionic strength medium. From video sequences, detergent induction is exactly like induction by ionophores or spontaneous induction which occurs on glass. It is a true induction, not just membrane solubilization.

The sequence illustrated in Fig. 1 is of a sperm that underwent the acrosomal reaction spontaneously. Fusion occurs during one frame of the video tape, \sim 20 ms (Fig. 1). Within the next three or four frames or 60–80 ms, the contents of the acrosomal vacuole disappears and by 200 ms after fusion the needle-shape of the acrosomal process is clearly visible (Fig. 1). Over the next second or seconds this needle-like process elongates somewhat. If Triton is the inducer, the plasma membrane solubilizes several seconds after completion of the acrosomal reaction. The final length of the acrosomal process varies widely from sperm to sperm. Nijima and Dan (11, 12) examined *Mytilus* sperm that had undergone the acrosomal reaction in response to *Mytilus* eggs, but had not fused with the egg and were detached. These sperm had acrosomal processes of 3 μm or less. Sperm that had been induced by high calcium had the longest acrosomal processes (Nijima and Dan [12] illustrate one 5- μm long and claim that they saw processes as long as 13 μm). In our hands the longest acrosomal processes that we have observed are \sim 5 μm in length with the bulk considerably shorter than this, \sim 3 μm long. We should mention that our measurements of the lengths of the acrosomal process, the acrosomal vacuole, the filament bundle, and the diameter of the sperm are all significantly smaller (e.g., 50% or more) than those reported by Nijima and Dan (12) and Dan and Wada (4). This may indicate differences due to different fixation techniques, microscope calibration variations and differences between Japanese and American *Mytilus edulis*. Furthermore, the sperm themselves vary significantly in size and thus in the length of the actin filament bundle in both undischarged and, as will be discussed, cells that have undergone the acrosomal reaction (see Fig. 8 a and b).

Fine Structure of Unreacted Sperm

From thin sections we now recognize that the acrosomal vacuole is in fact a hollow cone due to an invagination of its posterior side, its base forming a ring that rests against the anterior end of the nucleus (Fig. 2). The luminal space caused by the invagination of the acrosomal vacuole is con-

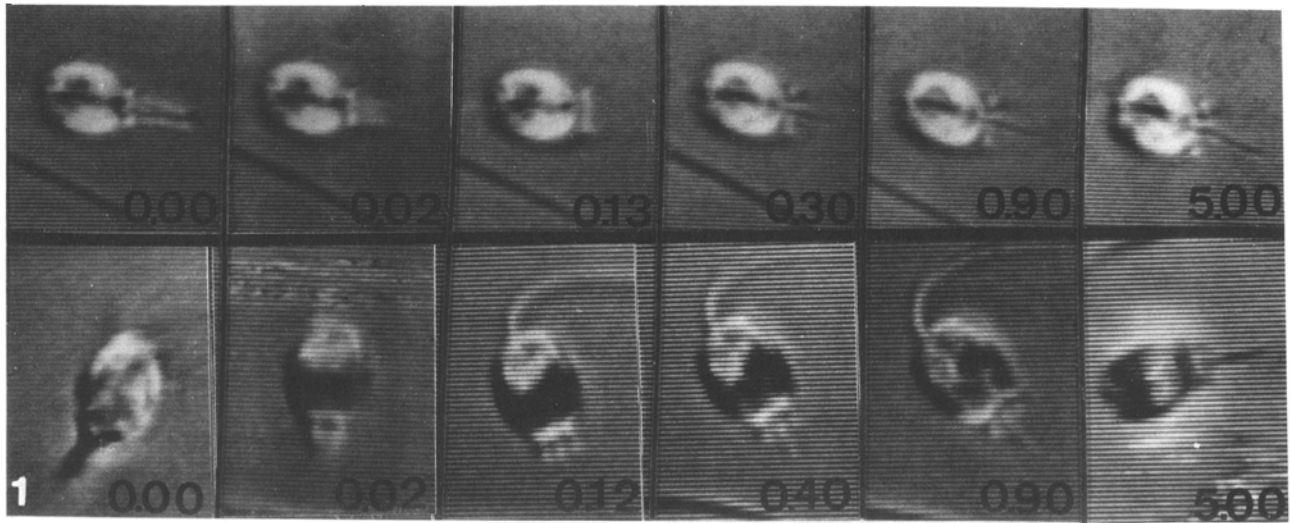


Figure 1. Frames from two video sequences showing the changes that occur in *Mytilus* sperm that are undergoing the acrosomal reaction. On each frame the time is indicated in seconds. These sequences were taken using a Leitz interference contrast system. Notice that fusion of the acrosomal vacuole with the cell surface occurs within a fraction of a second, whereas elongation of the acrosomal process is slow, requiring several seconds.

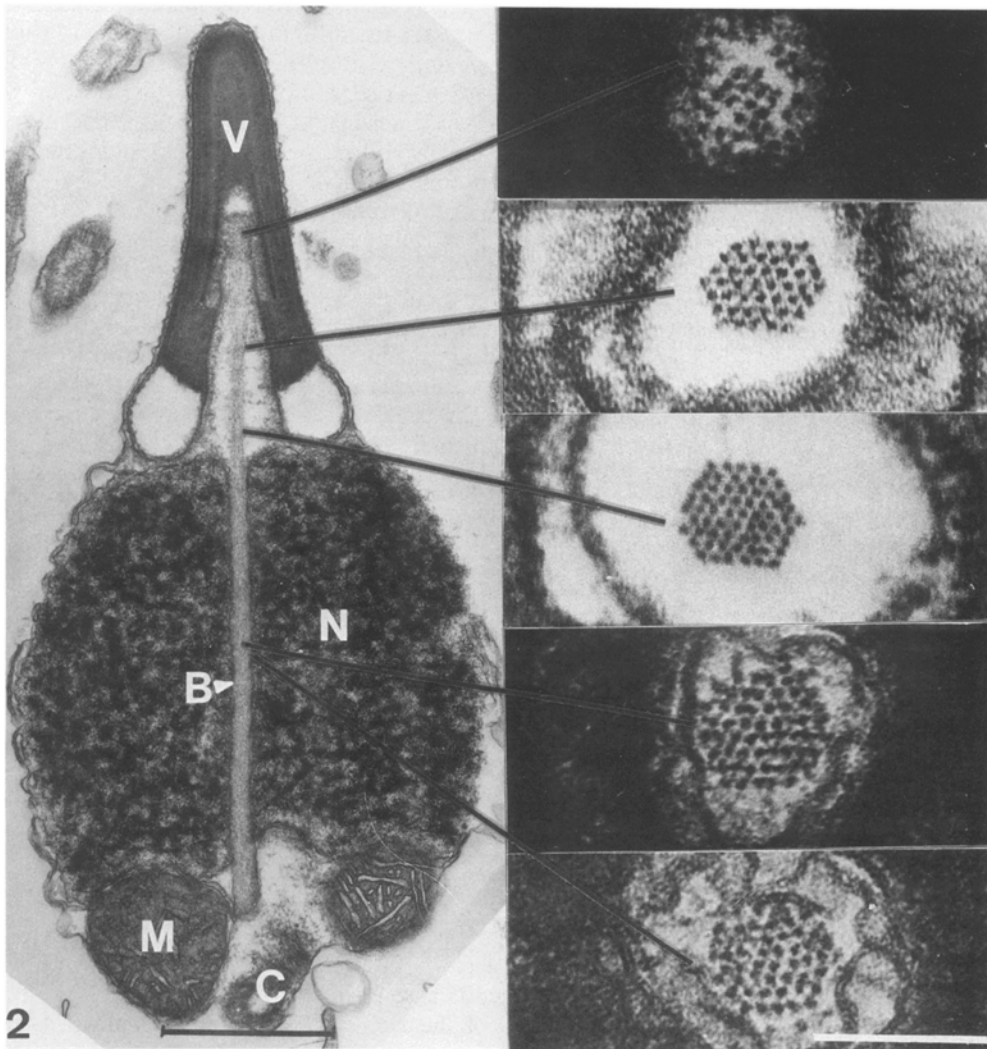
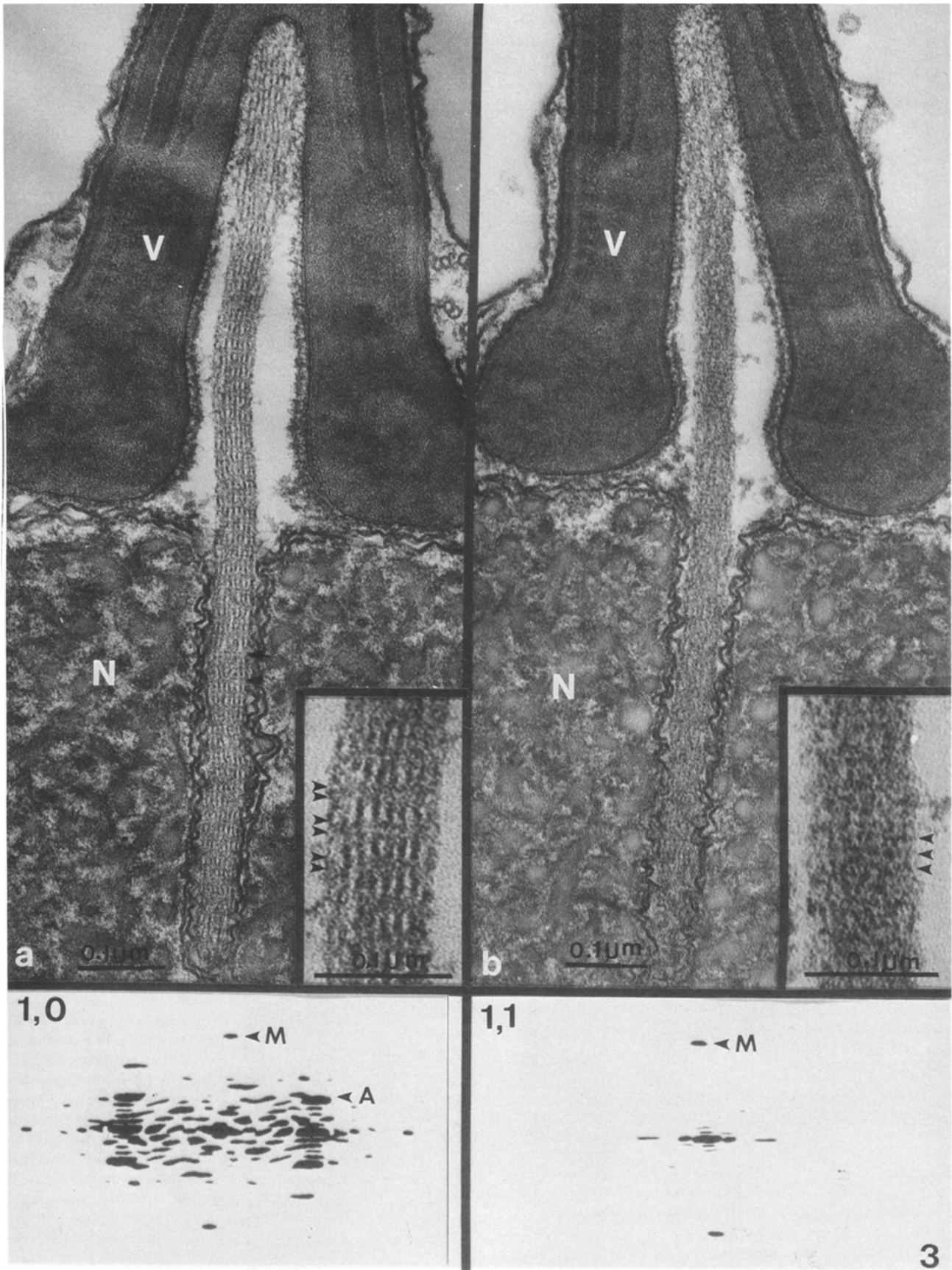


Figure 2. On the left of this figure is a longitudinal section cut through an unfired *Mytilus* sperm. Of interest is that the actin filament bundle (*B*) extends from the acrosomal vacuole (*V*) through a canal in the nucleus (*N*) into the midpiece between the mitochondria (*M*). The proximal centriole (*C*) is also present in this section. On the right hand side of the figure are transverse sections through the bundle at the positions indicated by the lines. (This low magnification first appeared in reference 18.) Bar, (left) 1.0 μm ; (right) 0.1 μm .



tinuous with another invagination, in this case a tube-like invagination in the nucleus. The nuclear invagination, which extends to the posterior margin of the nucleus (Fig. 2), is limited on its sides by the nuclear envelopes (Figs. 2 and 3). Lying within these two invaginations (11) is an ordered bundle of actin filaments (Figs. 2 and 3). In some sperm, the actin bundle and the invaginated part of the nuclear envelope extend into the midbody region which contains the five mitochondria and the basal body (Fig. 2). In others they do not extend as far as the basal end of the nucleus (Fig. 3). The average bundle is $\sim 3 \mu\text{m}$ in length but bundles up to $5 \mu\text{m}$ have been found.

We can determine the position of a transverse section of the actin filament bundle in the anterior half of the sperm (Fig. 2) because the acrosomal vacuole which surrounds it is in the form of a cone. Thus the amount of space surrounding the actin filament bundle identifies the level of the section; e.g., at the basal end there is the most "space" while at the most apical end where the filaments terminate on the membrane there is the least amount (Fig. 2). In that portion of the bundle that lies in the nuclear invagination we cannot accurately determine the position of the section.

What is striking in our transverse sections is that the filaments in the bundle are all hexagonally packed and so well ordered that a "crystal" with hexagonal faces is seen. This is true at any position in the bundle except at the anterior tip which is characteristically frayed. We counted the number of actin filaments making up the bundle, not only in the sections illustrated in Fig. 2, but in many more and found that they range from 43 to 65. Thus, unlike the situation in *Limulus* sperm (28), variations in filament number are not due to systematic increases and decreases in the number of filaments in different parts of the bundle but instead seem to be due to differences in the total number of filaments present in different sperm. This is confirmed by our longitudinal sections in which filaments appear to run from one end of the bundle to the other and "extra," short filaments are not seen at the periphery of the bundle proper (Fig. 3).

In longitudinal sections (Fig. 3) cut parallel to one of the three 1,0 planes (see Fig. 4 c) we see individual filaments separated by a small amount of space (Fig. 3 a). Careful examination of these bundles shows that the crossover points of adjacent filaments are in transverse register. We find other views in which we see very little or no space between the filaments (Fig. 3 b). These are cut near the 1,1 plane (see Fig. 4 d). Instead of the crossover points being visible, we see fine striations which cross the filaments with a period of 125 \AA . The patterns of these striations are different in the 1,0 and 1,1 view and are due to the cross-bridges that connect adjacent filaments.

To explain why the striations appear in the patterns that they do, why the filaments are separated by different amounts

of space in the 1,0 and 1,1 views, and what the diffraction patterns tell us, we have included Fig. 4 which shows computer drawings of hexagonally packed filament bundles. Beneath these drawings are how the diffraction patterns of these bundles should look. Fig. 4, a and b, shows top views of the bundle looking down the axis; one is rotated 30° relative to the other. The side views of these differently oriented bundles are depicted in Fig. 4, c and d, with perspective and Fig. 4, e and f, without perspective. In the 1,0 view (Fig. 4, c and e) we see the pattern corresponding to the micrograph in Fig. 3 a, and in the 1,1 view (Fig. 4, d and f) the pattern in Fig. 3 b. Fig. 4 f illustrates why we see 125 \AA striations due to the cross-bridges in Fig. 3 b; namely, the actin filament has an approximate repeat every 375 \AA . In that repeat distance, it is cross-bridged to its neighbors along the three hexagonal directions: at 0 \AA to the filaments at 0° and 180° , at 125 \AA further up to the pair at 60° and 240° , and at 250 \AA up to the pair at 120° and 300° . Then the pattern repeats. The pattern of cross-bridges (Fig. 4 a) can also be seen in Fig. 3 a (small arrows) but it is subtle and in thin sections from specimens that have not been detergent extracted it is often difficult to resolve. Below Fig. 3, a and b, are the diffraction patterns of these micrographs. Note the 125 \AA meridional spot due to the periodic nature of the cross-bridges. The appearance of a strong 125 \AA spot is diagnostic of a well ordered, hexagonally packed, highly cross-bridged actin bundle. The same spot is shown on the diffraction patterns corresponding to the models in Fig. 4. The spots on the layer line of $1/375 \text{ \AA}^{-1}$ are due to the twin stranded character of the actin filament (5).

The important point from the diffraction pattern analysis and from a careful consideration of the distribution of cross-bridges in the bundles viewed in the 1,0 and 1,1 views is that the actin bundle in unreacted *Mytilus* sperm is a paracrystal in which the actin filaments are maximally cross-bridged. (For references to other systems in which actin filaments are cross-bridged together see reference 5.)

There is a thin layer of amorphous material lining the cytoplasmic surface of the acrosomal vacuole membrane within the invagination of the acrosomal vacuole. This layer is also seen lining the basal end of the acrosomal vacuole (Fig. 3) but this material is not present in the nuclear invagination.

Decoration of the Actin Filaments with Subfragment 1 of Myosin

Mytilus sperm were detergent extracted and then decorated with subfragment 1 of myosin. In favorable regions the arrowheads (see Fig. 5) can be seen to be pointing exclusively toward the midpiece of the sperm. This polarity is invariant from sperm to sperm.

Figure 3. Longitudinal sections through the actin filament bundle, the basal end of the acrosomal vacuole (V) and the nucleus (N) of two unfired *Mytilus* sperm. (a) The actin filament bundle is displayed in the 1,0 view. The small arrows indicate striations due to the cross-bridges. These are particularly obvious in the inset. Notice that one sees a pair of bridges separated by a space and then another pair of bridges. Below this electron micrograph is a diffraction pattern of the bundle. Of interest is the density on the meridian (M) which is diagnostic of a hexagonally packed bundle of filaments held together by cross-bridging macromolecules. The density on the first layer line (A) ($1/375 \text{ \AA}^{-1}$) is due to the inherent nature of actin. (b) In this electron micrograph the actin filament bundle is cut so that we are close to the 1,1 view of the bundle. Notice that in this view the filaments appear very close together. The arrows indicate striations due to the cross-bridges. These can be most easily visualized by looking at the bundle from its side. Below this micrograph is a diffraction pattern of the 1,1 view. Of interest is the meridional (M) reflection due to the cross-bridges.

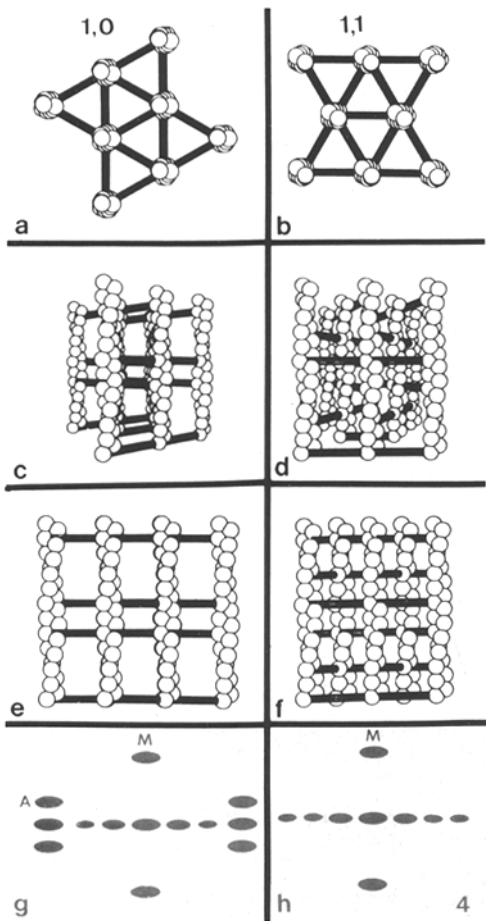
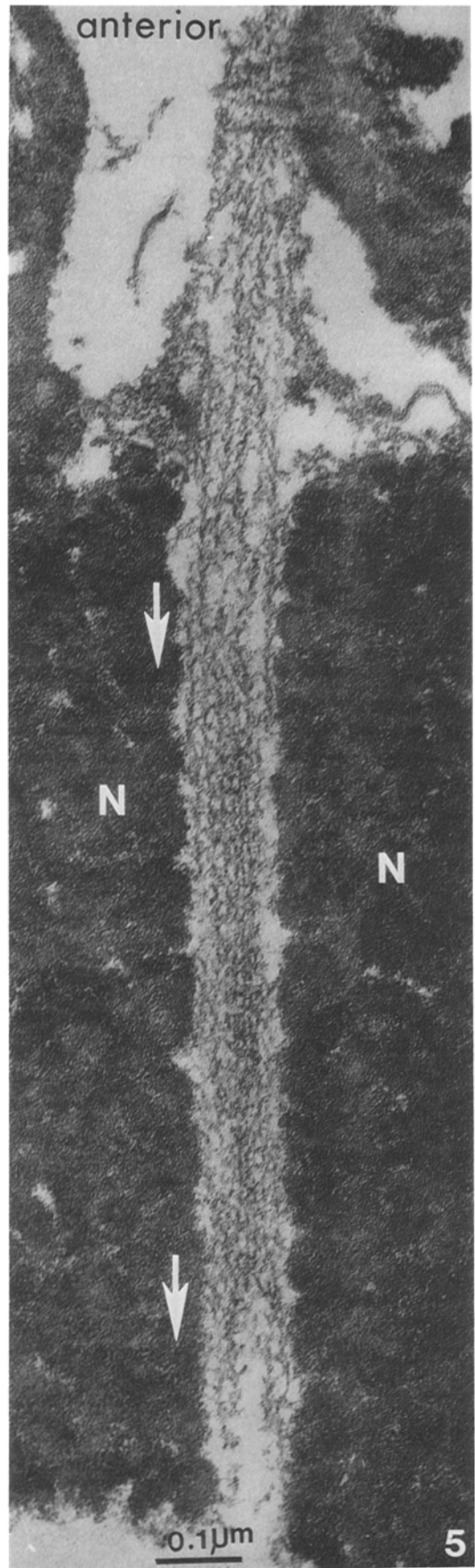


Figure 4. Models of actin bundles and model diffraction patterns of these bundles. (*a* and *b*) Top view of the model in the 1,0 and 1,1 orientations, respectively. The model in *b* is rotated 30° clockwise. (*c* and *d*) *c* is a side view of the bundle in *a*, the 1,0 view. *d* illustrates the side view of the bundle in *b*, the 1,1 view. (*e* and *f*) 1,0 and 1,1 views without perspective. Note that in *f* there are three sets of cross-bridges per crossover of actin. These cross-bridges occur every 125 Å. In *e* one set of cross-bridges points directly toward the viewer and is eclipsed by the filaments in front of it. Thus, while there are still cross-bridges every 125 Å, one set seems to disappear. (*g* and *h*) Transforms of the 1,0 and 1,1 views. The horizontal line of reflections at the center, known as the equator, is common to both. The transform of the 1,1 view has in addition to the equator a pair of reflections lying at $1/125 \text{ \AA}^{-1}$ above and below the equator, upon the meridian (*M*). This pair of spots arises from the cross-bridges. If the bundle contained only actin or if the bundle had cross-bridges but were not hexagonal, the spot would not appear. In addition to the equator and the meridional spot, the 1,0 view has additional spots (*A*) at $1/375 \text{ \AA}^{-1}$ above and below the equator. This arises from the hexagonal packing of the actin filaments. If the cross-bridging subunits were removed but the filaments left in place, the meridional spot would disappear but the $1/375 \text{ \AA}^{-1}$ spots would remain. Thus the transform provides evidence for the presence of cross-bridging subunits and for the hexagonal packing of the filaments.

Figure 5. Longitudinal section through the actin filament bundle in a *Mytilus* sperm that had been detergent extracted and then decorated with subfragment 1 of myosin. The arrow indicates the polarity of the actin filaments. On either side of the bundle in this section is a portion of the nucleus (*N*).



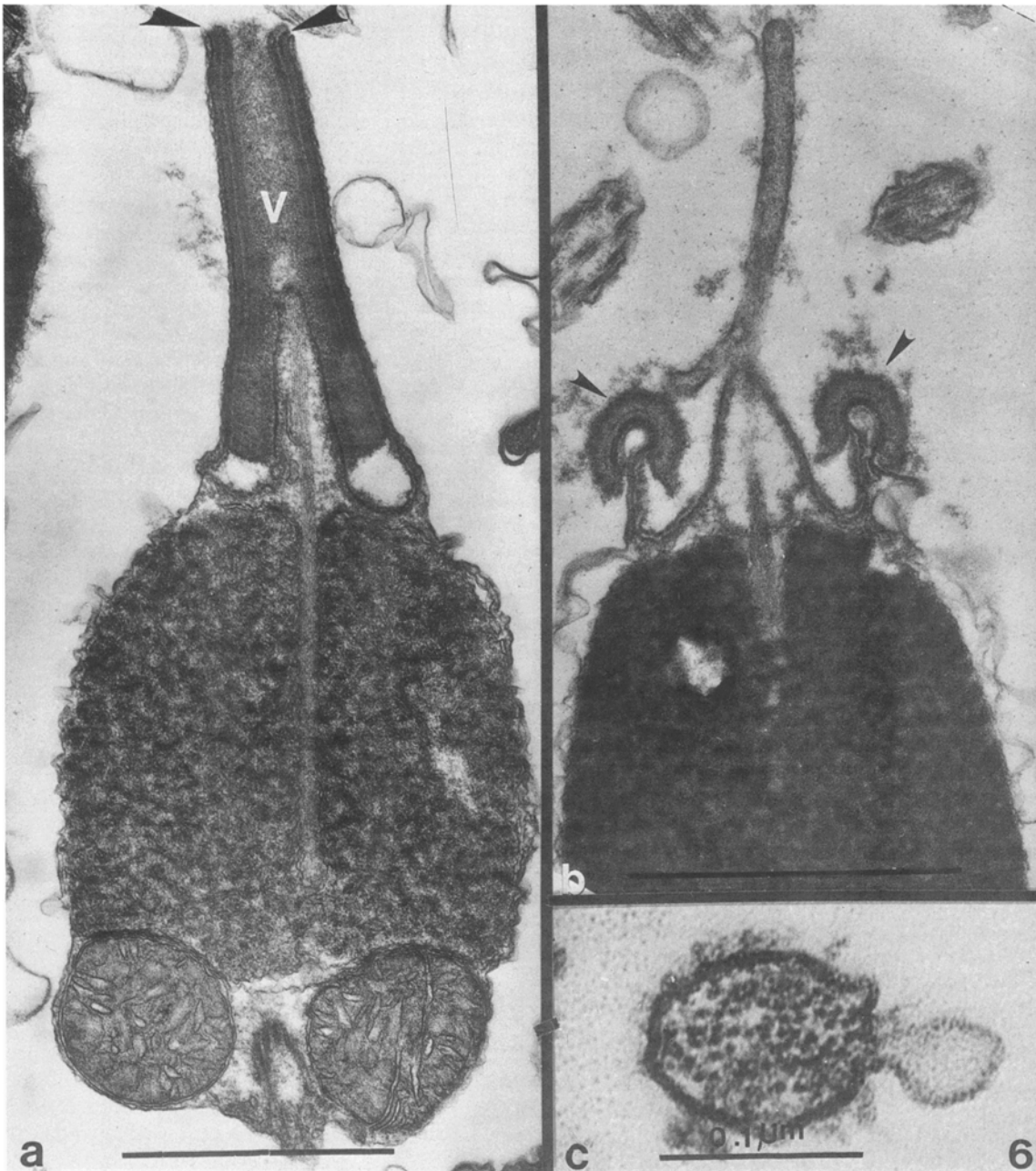
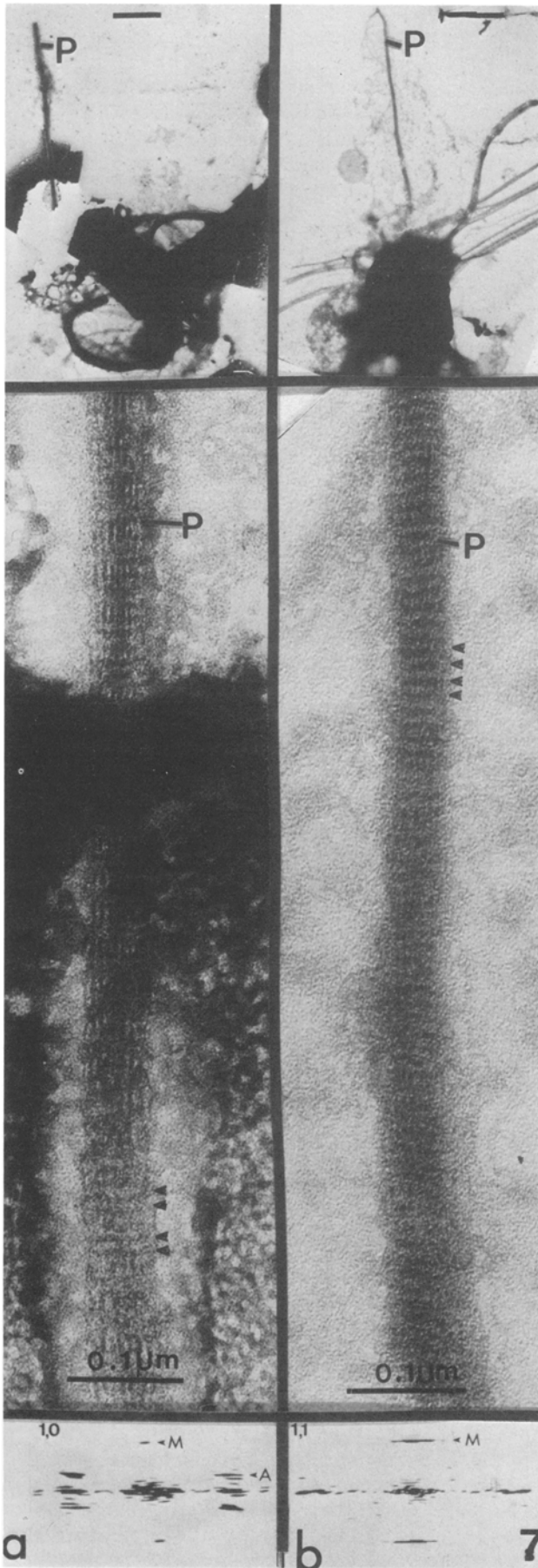


Figure 6. Longitudinal section through a *Mytilus* sperm which had been fixed immediately after the induction of the acrosomal reaction. The first step is a fusion of the membrane that limits the tip of the acrosomal vacuole with the plasma membrane in this region (*arrowheads*). This externalizes the contents of the acrosomal vacuole (*V*). (*b*) Longitudinal section through a *Mytilus* sperm which was fixed after the initiation of the acrosomal reaction. This is probably an example of a "partially" reacted sperm. Note that an acrosomal process has formed. The membrane limiting this process snugly covers the anterior end of the actin filament bundle. The two arrows indicate all that is left of the acrosomal vacuole that has peeled away from the forming process like the skin of a banana. (*c*) Cross section through an acrosomal process. Within the plasma membrane are a number of dots which are the actin filaments cut in transverse section. There are 55–65 actin filaments in this section. Bars, (*a* and *b*) 1.0 μm ; (*c*) 0.1 μm .

Fine Structure of Reacting and Reacted Sperm

The first step in the acrosomal reaction is a fusion of the acrosomal vacuole with the plasma membrane. This occurs at the tip of the acrosomal vacuole (Fig. 6 *a*), thereby exposing the contents of the acrosomal vacuole to the outside world. The next step is the appearance of a needle-like acrosomal process which occurs by the dissolution of the contents of the vacuole and a retraction of the cone of mem-

brane which is a combination of the plasma membrane and the membrane that formerly covered the outer surface of the acrosomal vacuole (Fig. 6 *b*). At the same time as the cone of membrane is retracting the membrane covering the actin bundle in the acrosomal process, formerly the acrosomal vacuole membrane, becomes plastered tightly against the actin bundle. Cross sections of these processes were not often encountered because it is difficult to induce the acrosomal



reaction in *Mytilus* sperm except by treatment with detergents, but in our best micrograph (Fig. 6 c) of sperm induced by ionophores or high calcium we count 55–65 dots indicative of the number of actin filaments present. In this micrograph the filaments are poorly ordered, but there is a tendency for them to appear in nearly parallel rows. The amorphous material which was applied to the surface of the membrane limiting the inner surface of the acrosomal vacuole in unfired sperm is not found in these cross sections of reacted sperm.

It is difficult to determine from the few cross sections we have whether the packing of the filaments in these processes is disordered or if this section was cut through a portion of the bundle that is disordered. More specifically, in cross sections through the anterior tip of the actin filament bundle in sperm fixed before induction (Fig. 2, top) the filaments are not well ordered and it is possible that this section from an induced sperm corresponds to that same part of the bundle in an uninduced sperm. As will be discussed below, in our negatively stained images of reacted sperm we know that the filaments are as hexagonally packed and as maximally cross-bridged as in unreacted sperm so it seems likely that the lack of order in our few thin sections is the result either of the section being cut near the tip of the process or of improper fixation. The important point is, however, that the total number of filaments in these sections through the acrosomal process is the same as in unfired sperm.

Negatively stained images of reacted sperm with their diffraction patterns are shown in Fig. 7. The sperm were first treated with ionophore to induce the acrosomal reaction, then allowed to adhere to the grid. To get better images of the actin filament bundle, the grid was rinsed in the detergent, Triton X-100 (in 3 mM MgCl₂, 30 mM Tris at pH 8.0), and negatively stained. What was not realized at the time was that the detergent solution induces the acrosomal reaction in unfired sperm even if they are stuck on the grid. Since nearly all of the sperm on the grid had acrosomal processes and only a relatively low percentage are induced by ionophores, the bulk of the images we see are ones induced by the detergent solutions. What is important is that our video sequences of sperm induced to undergo the acrosomal reaction by detergents (either Triton or digitonin) are identical to those in which the sperm were induced with excess calcium or with

Figure 7. Negatively stained preparation of the actin filament bundle in the acrosomal process of a *Mytilus* sperm that has undergone the acrosomal reaction. After the sperm head attached to the grid, the grid was rinsed briefly in a solution containing 1% Triton X-100 in 3 mM MgCl₂ and 30 mM Tris at pH 8.0 to remove the membrane, then negatively stained with 1% uranyl acetate. The inserts show a low magnification image of the whole sperm; the filament bundle in the acrosomal process is indicated (P). (a) 1,0 view of the bundle. The arrows indicate the pair of stripes separated by a space, then a pair of stripes, etc. These stripes are due to the cross-bridges between filaments. Below this bundle is its diffraction pattern. Of interest is the meridional reflection (M) due to the cross-bridges in this hexagonally packed bundle and the 1/375 Å⁻¹ reflections (A) due to the inherent structure of actin. (b) 1,1 view of the bundle. The arrows indicate the 125 Å striping due to the cross-bridges. Beneath this is a diffraction pattern of this bundle. Notice the prominent meridional reflection (M) at 1/125 Å⁻¹. This indicates that the bundle remains hexagonally packed and cross-bridged after the reaction.

ionophores. It is a true acrosomal reaction, not just a solubilization of the sperm membranes which allows the filament bundle to float free. By studying video sequences of sperm perfused with the Triton solution or lower concentrations of Triton, we now know that the key for induction of the acrosomal reaction with Triton is for the Triton to be ~ 0.1 – 0.5% in buffer at low ionic strength when it hits the sperm; the original sea water is gradually diluted by the Triton solution. If, on the other hand, a pellet of sperm is solubilized in an excess of Triton (1%), the membranes become solubilized before the acrosomal reaction is "induced." Thus the filament bundle remains in the nuclear canal and can be subsequently decorated with S1 (Fig. 5). Digitonin, which also induces an acrosomal reaction in all the sperm, is an even better inducer of the acrosomal reaction because how it is applied to sperm is not important, nor is the tonicity of the medium it is placed in. We presume this is because it only pokes small holes in the membrane, not drastically solubilizing the membrane as the Triton does.

If we examine the bundle of actin filaments in the acro-

somal process by negative staining, the transverse bands indicative of the distribution of the cross-bridges referred to in Fig. 4 are apparent (Fig. 7). From these images and the diffraction patterns of these bundles, we see the 125 \AA meridional reflection diagnostic of a hexagonally packed, cross-bridged bundle. Thus the actin filaments in the acrosomal process are packed in the same way as those in uninduced sperm. The lengths of these are 3.5 – 4 \mu m .

Staining of the Sperm with Fluorescein-conjugated Phalloidin

When unreacted *Mytilus* sperm are stained with fluorescein-conjugated phalloidin, the filament bundle becomes brightly fluorescent (Fig. 8, *a* and *b*). The overall length of the actin filament bundle varies from sperm to sperm; some are only 3 \mu m , others 4 \mu m , and at least one exceeded 5 \mu m in length. Interestingly at the point of contact between the acrosomal vacuole and the anterior end of the nucleus we also see a spot of fluorescence. It is as if the fluorescent actin bundle is sur-

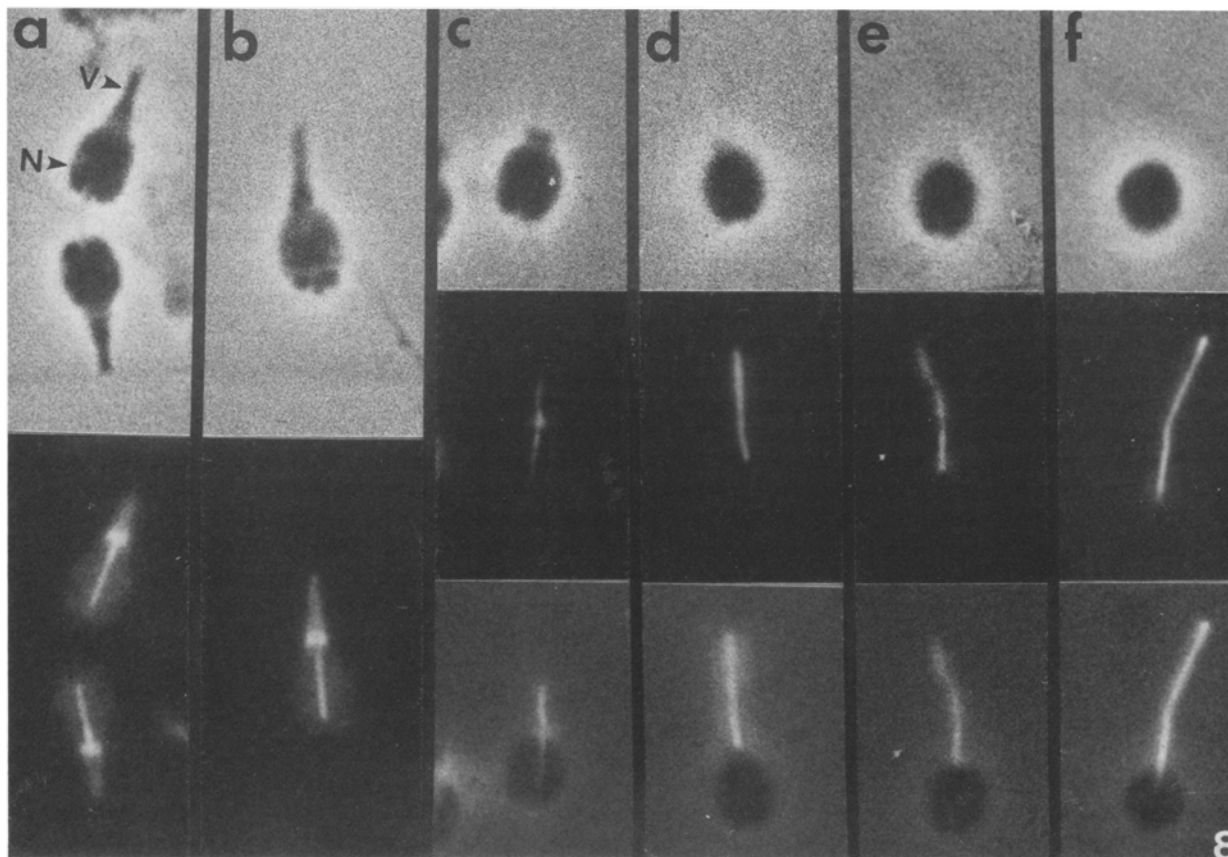


Figure 8 Unfired and fired *Mytilus* sperm were flattened with an agarose strip, then fixed and incubated in fluorescein-conjugated phalloidin. After washing, selected fields of sperm were photographed through the same objective using phase-contrast microscopy (transmitted illumination) and fluorescence microscopy (epiillumination). The phase image of a particular sperm is illustrated at the top of each sequence. Below this image is the fluorescent image of the same sperm. Below the sperm cell in *c*–*f* we have superimposed the phase and fluorescent images by first exposing the photographic paper to the phase image and then exposing the same paper to the fluorescent image. (*a* and *b*) Unfired sperm. Notice that there is a fluorescent bar that extends from the nuclear region (*N*) into the acrosomal vacuole (*V*). At the junction between these two regions is a bright fluorescent blob through which the bar extends. Notice that the sperm are different in size. This illustrates the biological variation from sperm to sperm, not a flattening artefact. (*c*) *Mytilus* sperm that has undergone a "partial" activation such that the acrosomal vacuole has broken down but at least half of the bundle remains in the nuclear region. The fluorescent spot remains at the anterior end of the nuclear region. When the bundle moves forward as it may have done to some extent in the figure, it moves through the spot of fluorescent material. (*d*–*f*) Fully reacted sperm. Notice that the bundle has moved forward so that little or none remains in the nuclear region.

rounded by a ring of fluorescent material just at this location. The only candidate for this spot of fluorescent material in our electron micrographs is the ring of amorphous material which, because it is being looked at edge-on when the sperm is lying flat in front of us on a slide, now will become quite apparent. It is unlikely that this fluorescent material is unpolymerized actin because phalloidin only binds to the polymeric form, not the monomeric form (1).

In some sperm we also see a spot of fluorescence along the basolateral surface. This is a variable feature and we do not know what it corresponds to in our electron micrographs.

Sperm which had undergone the acrosomal reaction were also examined with this fluorescent technique. In the simplest cases (Fig. 8, *d-f*) we see only a fluorescent acrosomal process; no fluorescence remains in the nuclear region. Measurements of the processes reveal that they are 3–5 μm long, the length of the original filament bundle. What seems to have happened is that the bundle has moved forward out of the nuclear invagination to appear in the acrosomal process. In one case we found a fluorescent process 5 μm long (Fig. 8 *f*). The amount of fluorescence was comparable along its entire length although the process was somewhat bent. In all these cases the only fluorescence that we see is in the filament bundle; there is no spot of fluorescence remaining at the anterior end of the nucleus.

We have found reacted sperm in which the bundle remains in the nuclear canal. In these sperm only that part of the bundle which was formerly in the invagination of the acrosomal vacuole is now present in the acrosomal process. In these sperm the fluorescent spot remains at the apical end of the nucleus or the basal end of the acrosomal process (Fig. 8 *c*). We assume that these are examples of the "partially reacted sperm" described by Nijijima and Dan (12). They probably correspond in fine structure to the sperm illustrated in Fig. 6 *b*. In these, the contents of the acrosomal vacuole have dissolved, but the bundle has not appreciably changed its position, unlike the situation described in the previous paragraph in which the bundle has moved forward out of the nucleus.

The bulk of reacted sperm appears to fall in a category between these two extremes. The bundles have moved forward to varying degrees out of the nuclear invagination. Accordingly, the lengths of the acrosomal processes are between 1 and 5 μm . What is interesting is that the bundle seems to pass through the spot of fluorescent material as it moves anteriorly out of the nuclear invagination.

Discussion

The actin filaments in the bundle in uninduced *Mytilus* sperm are hexagonally packed and aligned with their crossover points in register, being held together by cross-bridges placed every 125 Å. If the filaments are decorated with S1, the pointed ends uniformly point away from the acrosomal vacuole. After induction of the acrosomal process, the structure of the filament bundle is unchanged: in particular, the filaments remain hexagonally packed and maximally cross-bridged and the number of filaments in cross section does not appear to be altered, nor does the length of the filaments change. Instead what happens is that the bundle, which can be thought of as an actin paracrystal, moves forward. These observations allow us to rule out five possible mechanisms for motility as outlined below.

First, in the sperm of the echinoderm, *Thyone*, the acrosomal process is generated by the polymerization of actin monomers into filaments (23). The actomere, a short actin filament bundle, serves as a nucleating site for filament formation. In the unfired sperm, the actomere sits atop the nucleus surrounded by a granular mass of unpolymerized actin. After the generation of the acrosomal process, the actomere is still in the same position, the granular mass is gone, and a 90- μm actin filament bundle has been generated at the anterior end of the actomere. The results of our study of the acrosomal reaction in *Mytilus* sperm clearly rule out this model as the main source of elongation. In *Mytilus* sperm, the preformed actin filament bundle moves anteriorly out of the nuclear canal, an event not seen in *Thyone* sperm. Furthermore, there is no mass of unpolymerized actin in the sperm before the induction of the acrosomal reaction. While this paper was in the process of review and revision a paper appeared on the sperm of the abalone, a relative of *Mytilus* (17). Although these workers concluded that the bundle elongates by actin assembly like the situation with *Thyone* sperm, what their data show is that the bundle moves forward out of the nuclear canal as is demonstrated here for *Mytilus* sperm.

Second, in *Limulus* sperm, the generation of the acrosomal process is the result of the extension of a long actin bundle which is coiled about the base of the nucleus (7). The tight coiling is achieved by the regular placement of bends or elbows in the bundle such that all the elbows lie almost in the same plane. Extension is caused by a change in twist of the bundle (i.e., in its helical symmetry). The change in twist causes the planes of successive elbows to change from nearly coplanar to 60°. The bundle under these conditions extends to a longer pitch structure in much the same way as the extension of a compressed spring. Our studies of the *Mytilus* sperm rule out this mechanism as well because the bundle in *Mytilus* is not coiled about the base of the nucleus. It is already fully extended and has no elbows built into it.

Third, in enzymatically treated eukaryotic flagella, there is an extension of the microtubular doublets caused by the sliding of adjacent doublets. Such a mechanism adapted to actin filaments could generate a process. In the treated flagella, the elongation of the bundle occurs with a concomitant decrease in the number of doublets appearing in any transverse section across the elongated bundle (21). The data we have accumulated on *Mytilus* sperm do not support this mechanism because there is no substantial change in either the length of the bundle or in the number of filaments in cross section. Moreover, such a mechanism would not result in movement of the bundle out of the nuclear canal.

Fourth, the motion of the bundle could be generated by the translation of the bundle by myosin. In this mechanism, myosin molecules attached to a fixed point in the cell would behave as a ratchet to propel the bundle out of the nuclear canal. Here again there is clear evidence contradicting this mechanism. The difficulty lies in the polarity of the bundle which is such that myosin could only translate the bundle out the posterior end of the sperm, opposite the observed motion of the *Mytilus* sperm bundle which moves out the anterior end. Turning the myosin molecules around would not lead to motion in the opposite direction because myosin molecules cannot interact with actin in this orientation.

And finally, there is one other possibility which was first

addressed by Otto and Bryan (13) in filopodial extensions in sea urchin coelomocytes. They suggested that rather than the bundle extending, the membrane retracts down around the bundle so that a filopodium ends up sticking out of the cell. This clearly cannot be the case in *Mytilus* sperm because our video sequences show that the process extends rather than the membrane retracting, the opposite of what is thought to happen in coelomocytes. Furthermore, the sperm has so little cytoplasm that there is no space in the sperm for a retracting membrane.

Therefore, the most likely mechanisms described above for actin-based motility are inappropriate for explaining the elongation of the acrosomal process in *Mytilus* sperm. Before we suggest how the movement of this bundle might occur, we should reemphasize four observations that have been documented in the Results.

First, what exists in the sperm before discharge is an almost perfect crystal of actin that is not changed during and after the acrosomal reaction except that it moves forward (Fig. 9). Second, although fusion of the acrosomal vacuole with the cell surface is rapid (20 ms) elongation of the acrosomal process occurs very slowly taking several seconds. This observation was also made by Nijima and Dan (12) in which sperm fixed only 1 s after induction had undergone exocytosis, but elongation of the process was seen only in sperm fixed a number of seconds after induction. Third, once fusion of the acrosomal vacuole membrane with the plasma membrane occurs, over the next 200 ms that portion of the actin filament bundle that formerly lay within the invagination of the acrosomal vacuole attaches laterally to the plasma membrane which formerly lined the inside of the acrosomal vacuole. At the end of this period (200 ms) in our video sequences we see a tiny needle-like process and in our thin sections we see the membrane tightly enclosing the actin filament bundle. This is surprising because the membrane that limits the inside of the acrosomal vacuole, membrane that is

destined to become the plasma membrane lining the acrosomal process, does not adhere to the actin filament bundle before induction yet after induction it adheres tightly as if bound to it. Over the next few seconds, the acrosomal process elongates by the forward movement of the actin filament bundle which is closely applied to the plasma membrane as it moves forward. Evidence in favor of actin filament bundle attachment to the membrane as the bundle moves forward comes not only from our video sequences but also from the fact that in our thin sections through reacted sperm and in those of Nijima and Dan (11), regardless of the length of the acrosomal process, the plasma membrane covers the bundle tightly, without blebs or sags. And fourth, during the acrosomal reaction the ring of phalloidin staining at the junction of the acrosomal vacuole and the nucleus disappears. This coincides with the disappearance of the amorphous material which occupies that space. As a result of the phalloidin binding, it is plausible to assume that the material is actin and therefore, upon the reaction, it also is moved or altered. The correlation of the disappearance of this material with the motion of the bundle suggests that the two may be coupled.

There is one mechanism which accounts for all four of these observations. Let us start by assuming that the phalloidin-staining material is indeed actin and that it is bound to membranes as is suggested by its close association with them as seen in the electron micrographs. At an early stage in the generation of the acrosomal process there appears to be an association of the membrane-associated actin with the filament bundle. This brings the membrane of the vacuole into a tight sheath around the bundle. If the bundle moves forward slightly out of the nuclear canal, the membrane-associated actin will bind to the exposed part of the bundle and in this way hold the bundle so that it does not retract back. The slight motion of the bundle out of the nuclear canal might be due to thermally induced motion (e.g., Brownian motion), hydrostatic pressure caused by the swelling of the nucleus

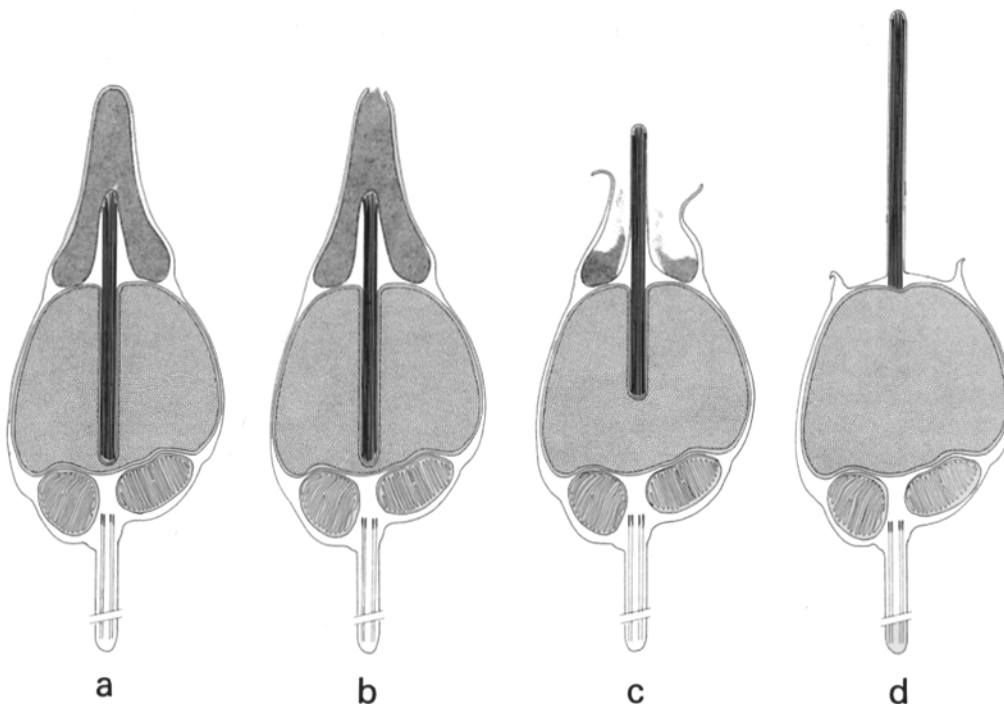


Figure 9. Illustration of the steps that occur during the formation and extension of the acrosomal process.

such as occurs in *Thyone* sperm during the acrosomal reaction (25), elastic forces stored in the deformed nucleus, or even forces coming from the membrane-associated actin. Irrespective of the actual mechanism of this movement, the forward motion of the bundle, once it has occurred, is "locked in" by the actin filament bundle being attached to the membrane. In fact, if the bundle is not bound in the nuclear canal, the act of attachment of the bundle to the membrane may provide the force to move the bundle forward.

We next wondered if the rate of diffusion for the bundle from the nuclear canal is equal to the rate of extension of the acrosomal process. A simple calculation shows that this is true as will be developed below. It should be remembered, however, that such a calculation does not prove that extension takes place by the mechanism already mentioned, but only that it is slow enough to be compatible with such a mechanism. More specifically, we assumed that the motion is a biased one-dimensional stochastic walk introduced by assuming that membrane attaches to the bundle as it emerges permitting only forward but not backward motion of the bundle. The average mean square distance is given by the following: $\langle x^2 \rangle = 4Dt$, where D = diffusion coefficient and t = time. The factor of 4 replaces the usual factor of 2 since the random walk is biased to forward steps only. The diffusion coefficient for a prolate ellipsoid moving parallel to its long axis can be approximated by the diffusion coefficient averaged over all three directions:

$$D = \frac{kt}{\frac{[f]}{[fo]} 6\pi\eta a}$$

where f/fo is the friction ratio for a prolate ellipsoid, η is the viscosity of water, and a is the radius of a sphere of the same volume as the bundle. The bundle is 2.7 μm long by 0.042 μm in radius. Its volume is, therefore, $1.50 \times 10^{-14} \text{ cm}^3$. A sphere of the same volume would have a radius of $1.53 \times 10^{-5} \text{ cm}$. The friction ratio, f/fo , for a prolate ellipsoid 2.7 $\mu\text{m} \times 0.084 \mu\text{m}$ is 2.43 calculated from Gosting (9). D is then $5.78 \times 10^{-9} \text{ cm}^2/\text{s}$. To find t , we assume that the bundle diffuses by one bundle length ($2.7 \times 10^{-4} \text{ cm}$). Thus t is about 3 s, which is of the same order (i.e., a few seconds) as the time for bundle extension. We should reiterate that the calculation does not prove that extension takes place by such a mechanism, but suggests that it is slow enough to be compatible with such a mechanism.

One concern might be that our model does not define a mechanism of extension, rather one of stabilization. In a recent paper on the elongation of the acrosomal process of *Thyone* sperm, Tilney and Inoué (25) suggested that elongation is a coupled reaction involving actin assembly and hydrostatic pressure. The former is there mainly to provide direction and inhibit retraction of the process as it elongates whereas the latter is there to allow space for addition of more monomers to the existing filaments. Trinkaus (29) has suggested a similar mechanism. Likewise in *Mytilus* sperm what we are proposing is that movement forward of the filament bundle is a coupled reaction where progressive attachment of the bundle to the plasma membrane provides direction and inhibits backwards movement while osmotic pressure, thermal energy, or Brownian motion, etc., will induce forward movements which are "stabilized" or "locked in" by the actin

membrane attachments. Thus, in our thinking, elongation of a cell or portion of a cell is produced by physical phenomena such as osmotic pressure, thermal energy, etc., which become directional because they are coupled to structures that prevent movement backwards to the resting level.

If our proposed mechanism is correct, we might expect the amount of membrane covering the inside of the acrosomal vacuole to equal the amount of membrane surrounding the fully extended bundle. The bottom side of the acrosomal vacuole has a rather peculiar shape. The upper 0.72 μm has the shape of a cone while the bottom part has the shape of the inner surface of a toroid whose outer radius is 0.48 μm and whose inner radius is 0.012 μm . The combined area for such a surface is $\sim 0.84 \mu\text{m}^2$. We can now compare this surface area with the surface area of the actin filament bundle present in the acrosomal process. It has a height of 2.7 μm and a radius of 0.042 μm giving rise to a total surface area of $1.13 \mu\text{m}^2$. Thus these two areas seem approximately equal as predicted.

What is interesting about our study of *Mytilus* sperm is that it may well provide a model for the extension of filopodia and microvilli in all cells for the following two reasons. First, in *Mytilus* sperm the acrosomal reaction occurs at a precisely defined site in the cell. As such it can be more easily dissected than other systems which generate filopodia or microvilli at random positions on the cell surface. For example, the elongation of microvilli on the surface of sea urchin eggs (2, 14, 16, 26), or in filopodial extension in coelomocytes (8, 19) occurs at random positions. In other systems such as intestinal epithelial cells (3, 20) or hair cells of the inner ear (24, 27) the microvilli (or in the terminology of the inner ear, stereocilia) elongate only from the apical surface of the cell yet still not at precisely defined points as in *Mytilus* sperm. Second, in all the systems mentioned above not only are the actin filaments being attached to their requisite plasma membranes, but also at the same time the actin filaments are increasing in length by actin polymerization (13-15, 18) as well as the component filaments increasing their interconnections with each other (13) so that the mechanism for the extension is complex, involving several interrelated mechanisms. On the other hand, in *Mytilus* sperm there is no change in the length of the actin bundle nor are the lateral interactions of adjacent filaments changing. Furthermore there is no room in this tiny cell for stored bundle precursors, nor are there any ribosomes so no new precursors can be synthesized. Thus we are forced to concentrate on other features such as the association of the bundle with the plasma membrane. We should not lose sight, however, of the fact that in more complex systems such as the elongation of filopodia or microvilli neither actin assembly nor zippering of adjacent actin filaments to each other can cause elongation of a cell extension in the absence of points of attachment of the bundle to the plasma membrane. If lateral interactions from the bundle to the plasma membrane do not occur, all we would expect is a bulge in the plasma membrane, not microspikes, filopodia, or microvilli. Thus our study of the elongation of the acrosomal process in *Mytilus* sperm, which is a system that has simplified the question down to its barest elements, is telling us that in the future we should be examining the frequency, the process of attachment, and the biochemistry of these actin filament-membrane interactions if we want to understand motile mechanisms that involve elongation.

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