MEMBRANE EVENTS IN THE ACROSOMAL REACTION OF *LIMULUS* SPERM

Membrane Fusion, Filament-Membrane Particle Attachment, and the Source and Formation of New Membrane Surface

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ABSTRACT

The membranes of *Limulus* (horseshoe crab) sperm were examined before and during the acrosomal reaction by using the technique of freeze-fracturing and thin sectioning. We focused on three areas. First, we examined stages in the fusion of the acrosomal vacuole with the cell surface. Fusion takes place in a particle-free zone which is surrounded by a circlet of particles on the P face of the plasma membrane and an underlying circlet of particles on the P face of the acrosomal vacuole membrane. These circlets of particles are present before induction. Up to nine focal points of fusion occur within the particle-free zone. Second, we describe a system of fine filaments, each 30 Å in diameter, which lies between the acrosomal vacuole and the plasma membrane. These filaments change their orientation as the vacuole opens, a process that takes place in <50 ms. Membrane particles seen on the P face of the acrosomal vacuole membrane change their orientation at the same time and in the same way as do the filaments, thus indicating that the membrane particles and filaments are probably connected. Third, we examined the source and the point of fusion of new membrane needed to cover the acrosomal process. This new membrane is almost certainly derived from the outer nuclear envelope and appears to insert into the plasma membrane in a particle-free area adjacent to an area rich in particles. The latter is the region where the particles are probably connected to the cytoplasmic filaments. The relevance of these observations in relation to the process of fertilization of this fantastic sperm is discussed.

KEY WORDS   membrane fusion · acrosomal reaction · filament-intramembrane particle association · new membrane source · exocytosis

The acrosomal reaction of *Limulus* (horseshoe crab) sperm is one of the most fascinating events ever recorded. It consists of the formation of a process 50-μm long in <10 s. What is particularly curious is that this process rotates as it elongates — the egg is literally screwed by the sperm (30). This extraordinary motion is brought about by an uncoiling of an actin filament bundle which, before the acrosomal reaction, is coiled up inside the sperm. As the process extends, this filament bundle untwists and, by passing up through a canal in

the nucleus, projects out through the opened anterior end of the sperm pushing the plasma membrane ahead of it. Recent studies have demonstrated that the most probable molecular mechanism for this motion is a change in the twist (pitch) of each actin helix in the bundle (10).

There is much interest currently among cell biologists in trying to define how actin filaments are attached to membranes, how fusion of two membranes occurs in processes such as exocytosis and cell fusion, and how and from where new membrane is added to or subtracted from existing membrane. Sperm, and more specifically *Limulus* sperm, are ideal systems with which to study these events, for five reasons. First, all of these events occur in *Limulus* sperm. Second, and more important, they occur at precisely defined positions on the membrane surfaces—a rare phenomenon in most cells. Third, they occur at times that can be easily defined and manipulated by the experimenter. Fourth, sperm cells contain minimal numbers of proteins and minimal numbers of simultaneously occurring events so that there is hope of ultimately characterizing the system completely. And fifth, *Limulus* sperm can be easily obtained in large numbers without contamination from other cells, facilitating biological characterization.

In this paper, we will present our data on how and where fusion of the acrosomal vacuole membrane and the plasma membrane occurs, how the acrosomal vacuole opens and discharges its contents, where the new membrane may come from to cover the acrosomal process, and how and where this new membrane intercalates into the existing plasma membrane. In the course of these studies, some unexpected results appeared. One of these is the presence of a population of membrane-associated filaments which not only change their packing during the opening of the acrosomal vacuole (which occurs in <50 ms), but also appear to restrict the mobility of particles that lie within the bilayer. Another is that the new plasma membrane appears to be derived from the nuclear envelope. This paper, by documenting some of the basic biology of this fascinating sperm, brings into focus questions to be explored in greater depth in the future. Because these events occur at prescribed positions in this cell, there is hope that by exposing what occurs in this system we will be able to unravel with greater ease the molecular components involved in exocytosis and understand how they work.

MATERIALS AND METHODS

Obtaining Sperm

Limulus polyphemus were collected by the supply department of the Marine Biological Laboratory, Woods Hole, Mass., and from Shark River Marine Lab, Wall, N. J. They were maintained in salt water aquaria at Woods Hole or in Instant Ocean Aquaria (Aquarium Systems, Inc., Eastlake, Ohio) in Philadelphia. The sperm were obtained by mechanical stimulation as described by Tilney (30).

Induction of the Acrosomal Reaction

Sperm were suspended in sea water which contained 50 mM $CaCl_2$ (natural sea water contains 10 mM $CaCl_2$). The ionophore X537A (courtesy of Hoffmann-La Roche, Inc., Nutley, N. J.) was added to the suspension of sperm (10 μl of a 1 mg/ml stock dissolved in ethanol was added to each ml of sperm suspension). Samples were fixed at intervals after induction by the addition of sufficient glutaraldehyde (obtained as an 8% stock from Electron Microscope Sciences, Fort Washington, Pa.) to make the suspension of sperm 1.25–2% glutaraldehyde.

Light Microscope Procedures

Sperm were examined under oil immersion using a Zeiss phase contrast microscope (Carl Zeiss, Inc., New York, N. Y.). Movies were taken with a 16-mm Bolex camera at 18 frames/s and processed commercially.

Electron Microscope Procedures

For thin sections, sperm were fixed in glutaraldehyde in sea water for 45 min at room temperature, washed briefly in sea water at room temperature, postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 6.0, at 0°C for 45 min, washed three times in distilled water, and en bloc stained in 0.5% uranyl acetate for 2–3 h at 0°C. Because of potential artefacts caused by glutaraldehyde fixation (15), sperm were also fixed in 1% OsO_4 in sea water at 0°C for 45 min without glutaraldehyde prefixation. Other sperm were fixed for 45 min in sea water containing both glutaraldehyde (1%) and OsO_4 (1%). These sperm were then washed in distilled water and en bloc stained. The sperm were then rapidly dehydrated in acetone and embedded in Epon or Araldite. Thin sections were cut with a diamond knife on a Sorvall Porter Blum II ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.), stained with uranyl acetate and lead citrate, and viewed with a Philips 200 electron microscope.

Negative Staining

A drop of whole sperm suspended in sea water was added to a carbon-coated grid, and within 30 s the excess sperm were washed off with sea water. Immediately thereafter, the grid was washed with 1% uranyl acetate.
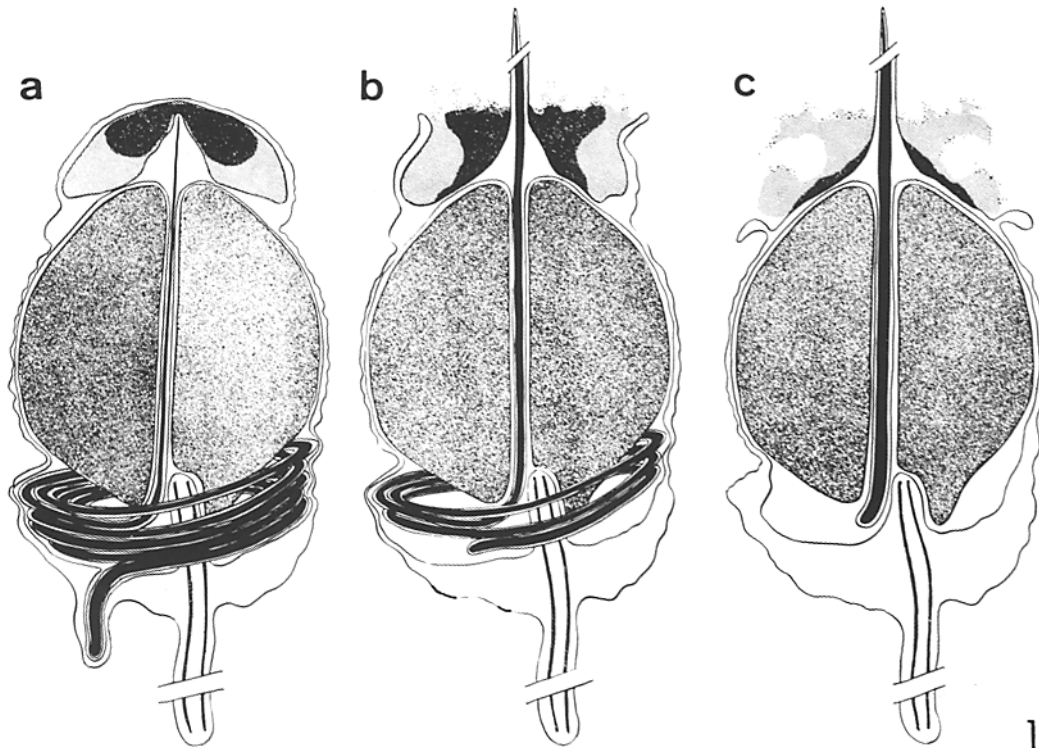
FIGURE 1   Drawing depicting the changes which occur in the membranes of the actin filament bundle during the acrosomal reaction. *a*, Before the acrosomal reaction is induced. *b*, During an early stage in the elongation of the acrosomal process. *c*, At the completion of the elongation of the acrosomal process.

Freeze-Fracture

For freeze-fracturing, we used the procedure of Tilney and Mooseker (32). Sperm were fixed by adding glutaraldehyde to the suspension at intervals after the addition of the ionophore. Specimens were fixed for 10–15 min, concentrated by centrifugation, and glycerinated in a graded series of glycerol solutions (10, 20, and 30%) at 4°C. The sperm were incubated for 15–30 min in each solution and pelletted after each step. The specimens were rapidly frozen in Freon 22, cooled with liquid nitrogen, and fractured and replicated in a Denton freeze-fracture apparatus (Denton Vacuum Inc., Cherry Hill, N. J.) at −115°C. The replicas were then digested with Chlorox (Proctor & Gambel Co., Cincinnati, Ohio), transferred to grids, and examined with a Philips 200 electron microscope.

RESULTS

Unreacted Sperm

THIN SECTIONS: Fig. 1 illustrates the relationship of the actin filament bundle to the membranes of *Limulus* sperm. Below we have listed the information necessary to follow the events

described in this paper. For details, we refer the reader to André (4), Fahrenbach (13), Shoger and Brown (29), and Tilney (30).

The actin filament bundle is attached to the basal surface of the acrosomal vacuole membrane and extends posteriorly through a canal in the nucleus towards the basal end of the cell. While in the subacrosomal space, it is surrounded by an amorphous material (Figs. 2 and 3) whose nature is unknown. Small vesicles, generally of the coated variety, are common beyond the posteriolateral margins of the acrosomal vacuole, but are never present in the subacrosomal space. The nuclear envelope immediately below the subacrosomal space is specialized: the outer and inner nuclear membranes are tightly apposed and present a single dense profile termed the "subacrosomal plate" (13) (Fig. 2). Peripheral to this plate at the anteriolateral margin of the nucleus the outer nuclear envelope characteristically bulges out (Figs. 2 and 4), while the inner nuclear envelope remains tightly adherent to the chromatin. As illustrated in Figs. 1 and 2 and in Tilney (30), the
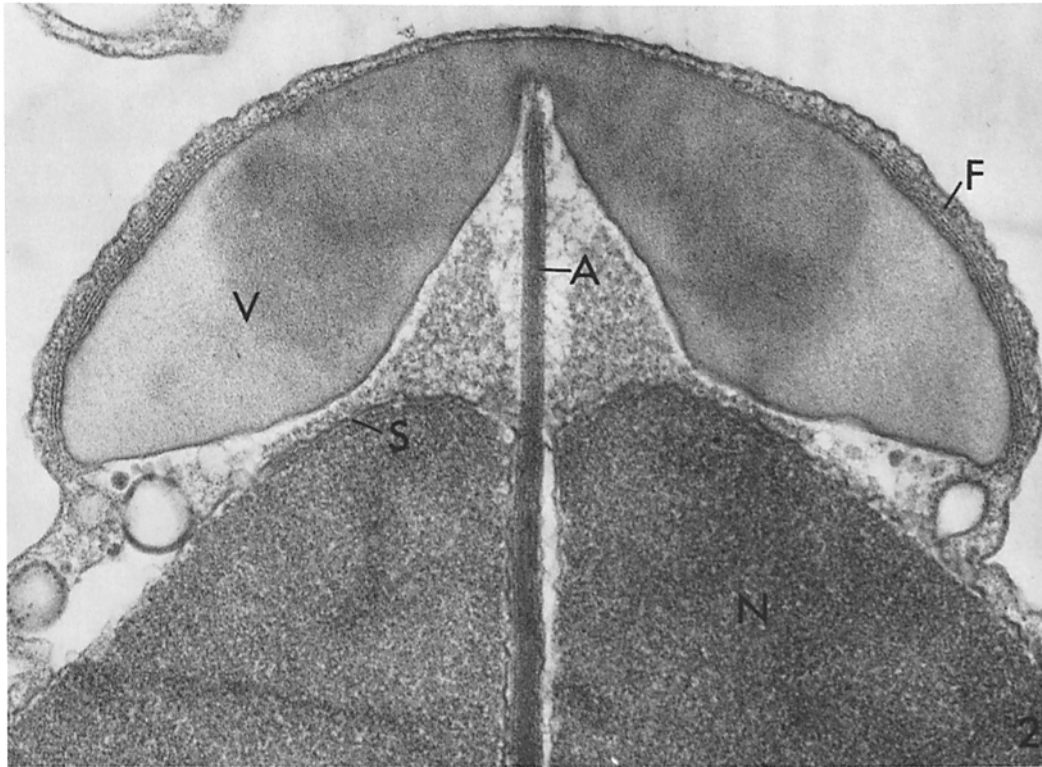
FIGURE 2 Thin section through the apical end of an unreacted *Limulus* sperm. The actin filament bundle (*A*) extends posteriorly from the acrosomal vacuole (*V*) through a canal in the center of the nucleus (*N*) where it is separated from the chromatin by the outer and inner nuclear envelopes. The outer nuclear envelope blebs outwards peripheral to the subacrosomal plate (*S*). Of interest are the fine filaments (*F*) located between the acrosomal vacuole and the plasma membrane. × 80,000.

filament bundle extends through a canal in the center of the nucleus where it is separated from the chromatin by both the inner and outer nuclear envelopes. At the basal margin of the nucleus the filament bundle, still covered by the outer nuclear envelope, pushes its way into the space limited by the posterior portion of the outer nuclear envelope. The easiest way to visualize this morphology is to reconstruct stages in spermiogenesis. The filament bundle begins to grow from the acrosomal vacuole; as it extends through the nucleus, it pushes both the outer and inner nuclear envelopes ahead of it. Upon reaching the posterior end of the nucleus, the inner nuclear envelope fuses with its posterior inner counterpart. The filament bundle, still covered by the outer nuclear envelope, pushes basally into a space between the posterior portion of the inner and outer nuclear envelopes. Then, as the filament bundle continues to elongate (and begins to coil), it is covered by two layers of

the outer nuclear envelope and by the plasma membrane (see Fig. 1 and reference 30).

The acrosomal vacuole assumes the form of a thick-walled, inverted bowl into whose open center the filament bundle inserts (Figs. 1 and 2). This vacuole is bounded by a membrane, 70 Å in thickness; the vacuole contents are not homogeneous. Between the apical surface of the vacuole and the plasma membrane is what appears to be a parallel array of fine filaments, each 30–35 Å in diameter, which run from the basolateral margins of the acrosomal vacuole towards the apex of the cell (Fig. 2). Instead of continuing across the most apical surface of the vacuole, they terminate, leaving a filament-free circular region (the base of the inverted bowl) ~1 μm in diameter. In this region the plasma membrane and the acrosomal vacuole membrane approach each other, but are separated by a minimum of 150 Å. The center-to-center spacing of adjacent filaments is ~85 Å;
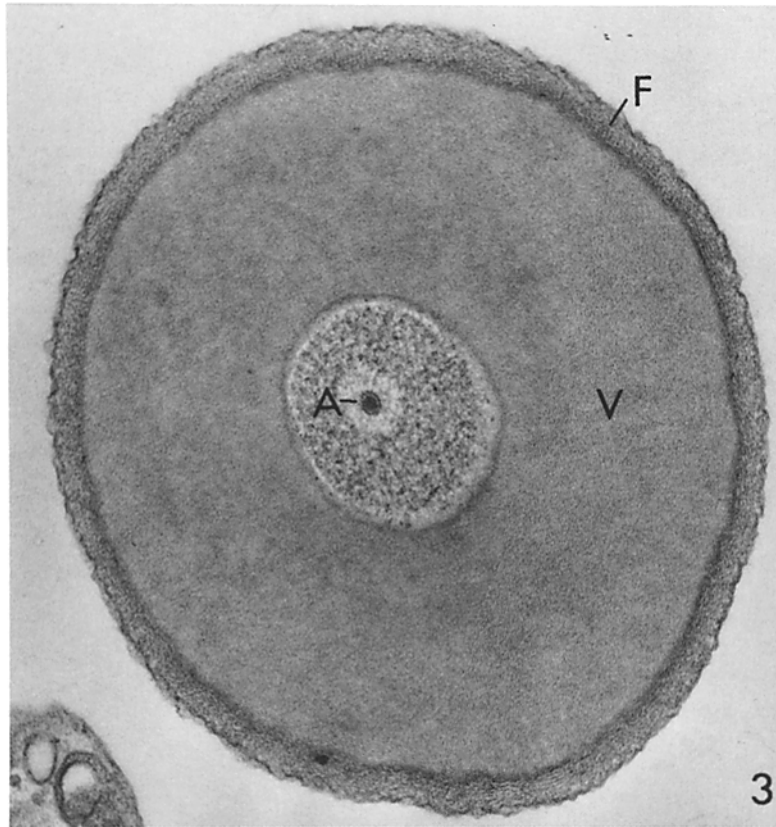
FIGURE 3 Transverse section through the apical end of an unreacted *Limulus* sperm. This section is cut perpendicular to Fig. 2 and passes approximately through the middle of the acrosomal vacuole (*V*) within whose center is the actin filament bundle (*A*). Of interest is the thin rim of cytoplasm between the plasma membrane and the acrosomal vacuole. This cytoplasm contains the fine filaments (*F*) cut in transverse and oblique section. × 58,000.
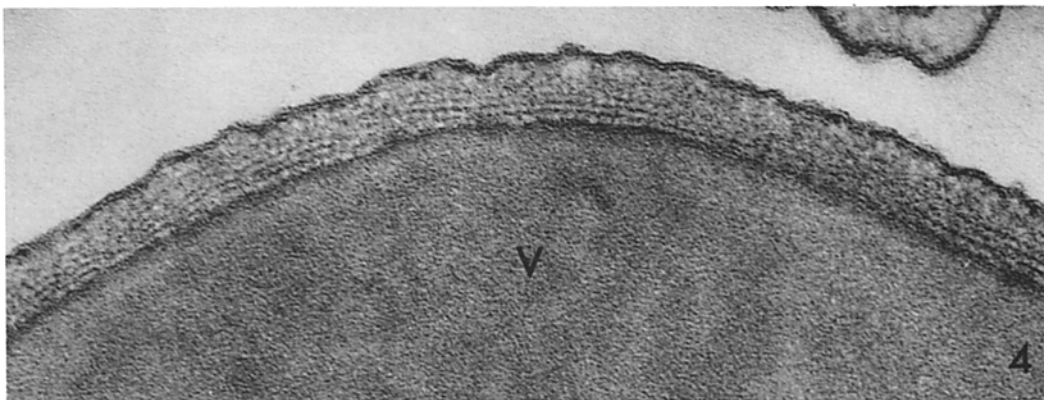

FIGURE 4 Thin section cut obliquely through a portion of the apical end of an unreacted *Limulus* sperm lateral to the midline. In this section the fine filaments lying between the plasma membrane and the acrosomal vacuole (*V*) can be seen to be interconnected. × 150,000.

maximally 4–5 filaments run parallel to each other in the space between the acrosomal vacuole membrane and the plasma membrane. In thin sections, individual filaments cannot be followed from their origin at the basolateral margins of the vacuole to the apical end. In some sections the filaments appear to terminate at various points along the acrosomal vacuole membrane, giving a shinglelike appearance; in other cases, we see breaks in individual filaments. Because the filaments are so closely spaced (85 Å) and so thin (30–35 Å in diameter), relative to our section thickness (300–400 Å), details about the exact arrangement of the filaments are difficult to resolve in thin sections because of superposition artefacts, particularly because the surface of the vacuole is a continuous curve. However, from micrographs such as Figs. 2, 3, and 4, we conclude that the filaments are most likely organized into a radial pattern extending from the basolateral margin of the acrosomal vacuole towards the apical surface. Thus, in sections cut near the lip of the bowl (Fig. 3), perpendicular to those just described (Fig. 2), we see dots (cross sections of filaments) in rows

which form a radial pattern as would be expected, fuzzy areas which are probably the filaments cut in oblique sections, and short segments of 4–5 filaments cut in longitudinal section. This image suggests that the filaments form clusters, a conclusion consistent with the negatively stained image of an entire sperm (Fig. 5). The filaments also seem to be cross-connected, most easily demonstrated by fortuitous oblique sections in which we see an ordered arrangement of matter resembling a wire screen (Fig. 4). The dots in this case are the filaments in transverse section, and the linear arrangement of these dots into lines is caused by the precise alignment of adjacent filaments. It is interesting that the filaments in adjacent layers are also in register. Grazing sections cut between the acrosomal vacuole and the plasma membrane also show this wire mesh appearance.

FREEZE FRACTURE: Particles 60–120 Å in diameter are found on the P face of the plasma membrane that limits the cell body and the flagellum. They are randomly arranged except at the flagellar necklace and over the center of the acrosomal vacuole where there is a patch, 0.4 μm
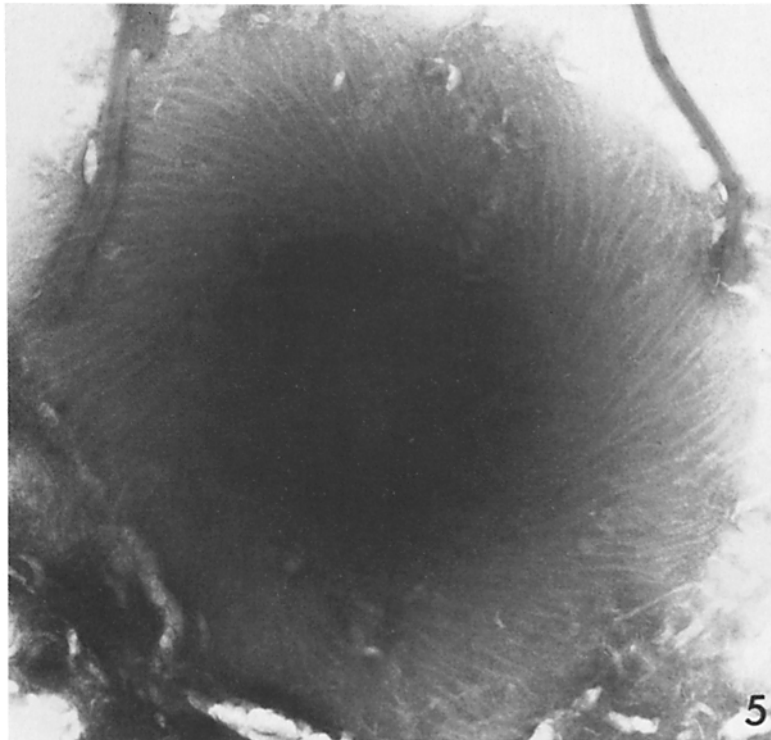

FIGURE 5 *Limulus* sperm were dried down on a grid and then negatively stained. In this preparation, we are looking down on the apical surface of the cell. × 50,000.

FIGURE 6 Freeze-fracture preparation of the *P* face of the plasma membrane that covers the apical surface of the acrosomal vacuole in an unreacted *Limulus* sperm. Worthy of note is that there is a flattened particle-free area on this membrane. This area is located in thin sections at the center of the apical surface of the sperm directly above the actin filament bundle and the nuclear canal. × 70,000.

in diameter, almost completely devoid of particles. This patch overlies the region devoid of meshwork filaments which is between the acrosomal vacuole and the plasma membrane. At the edges of this particle-free patch are large numbers of particles most of which are 100–120 Å in diameter (Fig. 6). In fact, these larger particles appear to be concentrated here. The *E* face of the plasma membrane, on the other hand, is nearly particle free; but, as with the *P* face, a central patch can be distinguished with small numbers of particles limiting its perimeter.

The *P* face of the acrosomal vacuole membrane immediately beneath the particle-free patch on the plasma membrane is also differentiated by a

circlet of particles which limits a particle-free, or nearly particle-free, zone (Figs. 7 and 8). As will be demonstrated shortly, the point of fusion of these two membranes occurs in the center of these two circlets of particles. The *P* face of the acrosomal vacuole membrane is further differentiated with particles arranged into nearly radial rows (Fig. 8) corresponding to the orientation of the filament clusters located between the acrosomal vacuole membrane and the plasma membrane. The *E* face of the acrosomal vacuole membrane in the region of the meshwork filaments is almost devoid of particles; sometimes, we see ridges organized with the same radial pattern as the particles on the *P* face. Presumably, these ridges
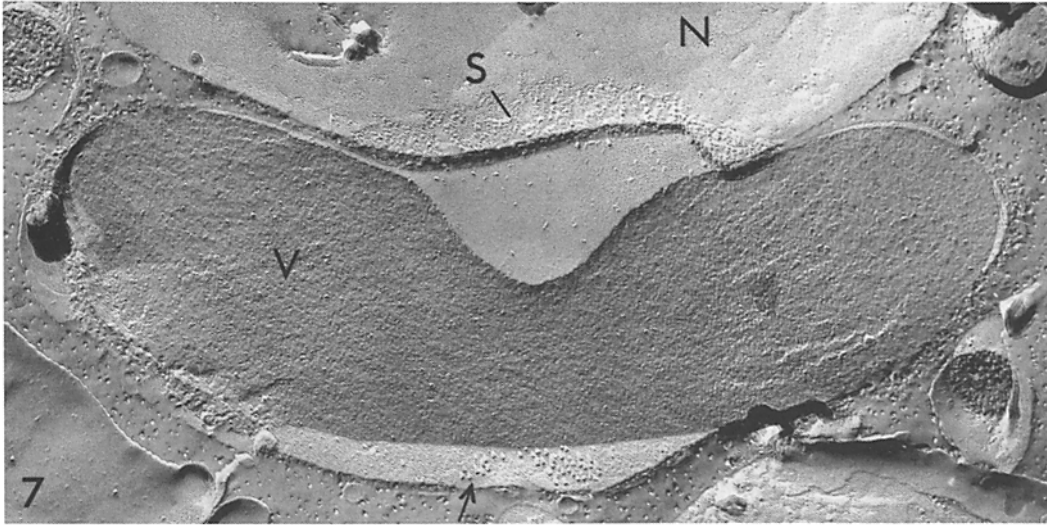
FIGURE 7 Freeze-fracture preparation of an unreacted *Limulus* sperm. The fracture plane (viewed from the top to the bottom of this micrograph) passes through the outer nuclear envelope (*N*), jumps down to the *E* face of the acrosomal vacuole, then passes through the contents of the acrosomal vacuole (*V*), and then for a brief period passes along the *P* face of the acrosomal vacuole membrane. Of interest is the particle-rich area in the outer nuclear envelope at the subacrosomal plate (*S*) and a portion of the circlet of particles (arrow) on the *P* face of the acrosomal vacuole membrane that lies directly beneath the circlet of particles on the plasma membrane illustrated in Fig. 6. × 47,000.

are related to the filament clusters.

On both the *P* and *E* faces of the outer nuclear envelope, the particles are sparse except on that portion which corresponds to the subacrosomal plate. The *P* face of the outer nuclear envelope in this region is studded with particles, 75 Å in diameter (Fig. 9), which frequently appear as small clusters. We should repeat that this portion of the nuclear envelope is very stable and does not bleb outwards, although that part immediately peripheral to this subacrosomal plate does. Nuclear pores are found on only a small part of the posterior region of the nucleus immediately adjacent to the basal body of the flagellum. The *P* and *E* faces of both outer nuclear envelopes that surround the coiled actin filament bundle are completely particle free.

Reacted Sperm

LIGHT MICROSCOPY: *Limulus* sperm undergo the acrosomal reaction when they make contact with the extracellular coats which surround the egg. Unfortunately, events occur too rapidly under these conditions to be suitable for filming, so we have induced the acrosomal reaction either by the addition of high concentrations of calcium (50 mM) to sperm (29, 30) or by the addition of ionophores such as A23187 or X537A in high external calcium. With calcium alone, ~10% of the sperm undergo the acrosomal reaction in 90 s; with the ionophore and calcium, >90% of the sperm undergo the acrosomal reaction in this time.

Successive frames of a movie sequence in which sperm are induced to undergo the acrosomal reaction with calcium are depicted in Fig. 10. The first visible change is an alteration in the refractive index of the acrosomal vacuole including a slight increase in its volume. In the next frame, the acrosomal vacuole is fully open, a process that takes place in <50 ms. This event always occurs in one frame of every movie sequence we have examined. The opened vacuole has a collarlike shape; the rim of the collar is made up of the cytoplasm which was apical to the vacuole in unreacted sperm (cf. Figs. 1 and 2). The acrosomal filament bundle does not extend forward until the next frame of the movie.

In an earlier paper (30), it was shown that the acrosomal process rotates as it moves forward, yet the cell body remains stationary. We have evidence that the membrane that limits the acrosomal

process also rotates along with the acrosomal filament bundle. This evidence is based upon the motion of processes with kinks or processes in which the filament bundle has broken; as these processes extend, the broken part or the kink will spin around at an oblique angle to the process. We have also examined the motion of processes whose tips stick to the substrate; as elongation
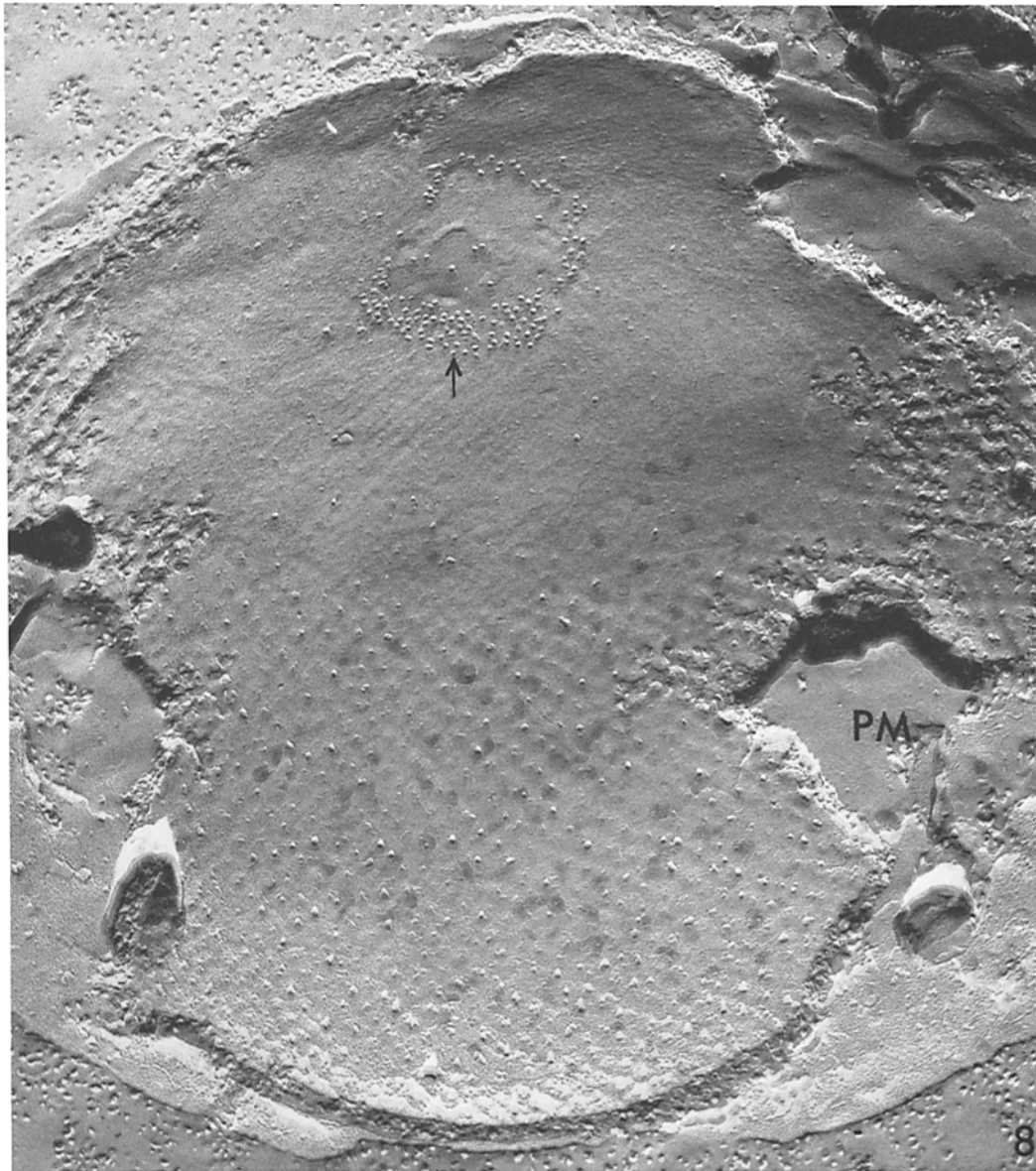

FIGURE 8   Freeze-fracture preparation through a portion of the P face of the acrosomal vacuole membrane of an unreacted sperm. The arrow indicates the circlet of particles located at the most apical point on this vacuole. This circlet lies directly beneath the circlet of particles on the P face of the plasma membrane. PM indicates the E face of the plasma membrane which lies beneath the acrosomal vacuole membrane. Also of interest are the lines of particles which extend over the apicolateral surface of this vacuole. These lines correspond to the long dimensions of the cytoplasmic filaments which lie in the cytoplasm just beneath this membrane. × 71,000.
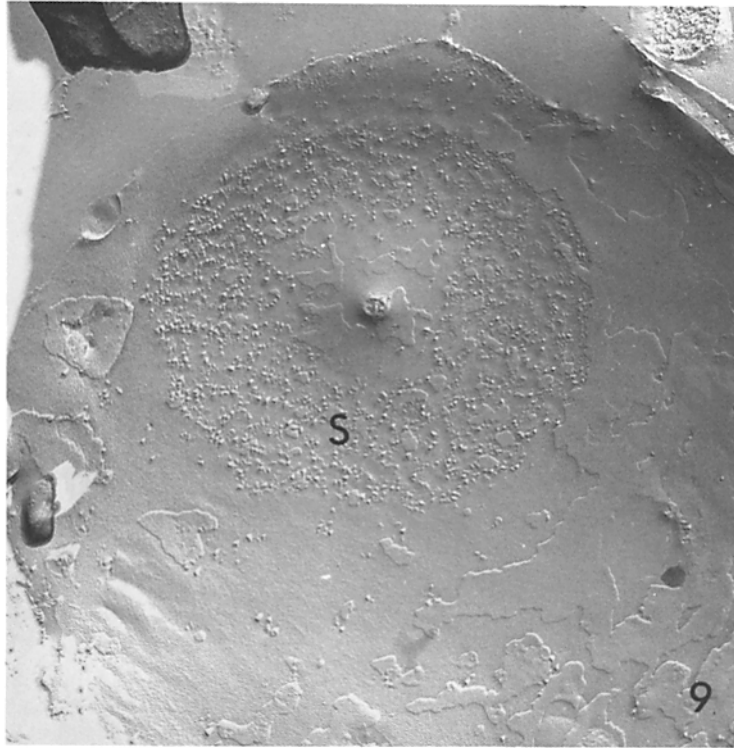
FIGURE 9 Freeze-fracture preparation through a portion of the P face of the outer nuclear envelope in an unreacted sperm. Of interest is the particle-rich area which corresponds to the subacrosomal plate (S) seen in thin sections. Extending through the center of the subacrosomal plate is the actin filament bundle. Small areas of the E face of the inner nuclear envelope can also be seen. × 65,000.
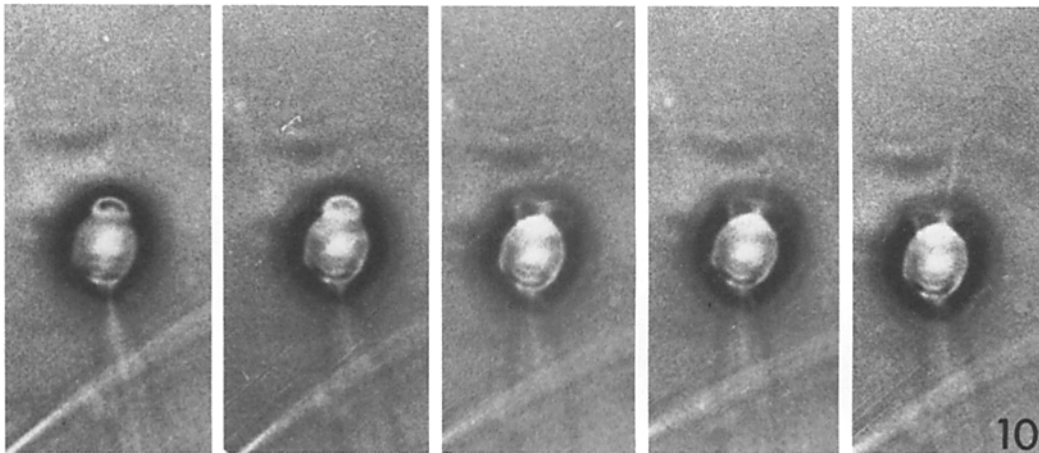

FIGURE 10 Successive frames (18 frames/s) of a movie sequence showing the early events in the acrosomal reaction induced with high calcium. Note that the first observable change is an alteration in the refractive index of the acrosomal vacuole. × 1,600.

proceeds, the process coils around itself with a series of hairpin turns. In all these cases, the membrane that limits the acrosomal process ap- pears to rotate along with the filament bundle, yet the membrane around the cell body remains sta- tionary. Thus, it is likely that the point of shear

between the cell body and the process exists somewhere within this collar.

ELECTRON MICROSCOPY: Although all the images displayed in this report are from sperm induced to undergo the acrosomal reaction with ionophores, we recently examined thin sections of sperm which had reacted to eggs. Comparisons of naturally reacted sperm with those induced by ionophores show, in all cases, identical results. What we are describing, therefore, are the natural changes which occur.

The first observable change in sperm fixed at early stages after induction, as seen in freeze-fractures through the plasma membrane overlying the acrosomal vacuole, is the appearance of 6–9 small, round depressions in the particle-free zone enclosed by the particle circlets (Fig. 11). These depressions are presumed to be the point of fusion of the acrosomal vacuole membrane with the free surface of the plasma membrane (Fig. 11). In thin sections through sperm fixed with glutaraldehyde after half the sperm had undergone the acrosomal reaction, we see two types of images that could be interpreted as intermediates in the fusion process (Fig. 12). The more common image is that occur-
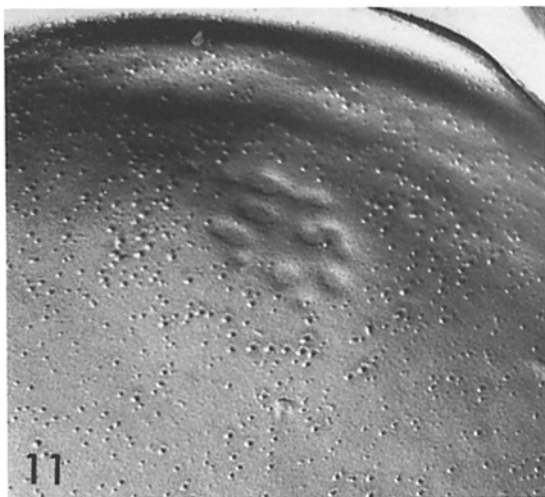

FIGURE 11   Freeze-fracture preparation of a portion of a sperm plasma membrane fixed shortly after the sperm had been induced to undergo the acrosomal reaction with the ionophore X537A. The portion of the plasma membrane (P face) illustrated here is the same as that shown in Fig. 6. This image illustrates the earliest events in the fusion of the acrosomal vacuole with the plasma membrane. The first event seems to be the appearance of dimples in the particle-free zone enclosed by the circlet of particles. × 65,000.

ring when the acrosomal vacuole membrane and the plasma membrane have come sufficiently close together in the central, particle-free zone to have formed a "pentalaminar" structure (Fig. 12a). The second type of image has been seen only a few times and consists of what appears to be a trilaminar fusion point (Fig. 12b). The latter is difficult to document conclusively, as the acrosomal vacuole membrane invariably stains less effectively than the plasma membrane.

We have tried unsuccessfully to find stages in the opening of the acrosomal vacuole which must immediately follow membrane fusion, presumably because this rapid process (<50 ms) cannot be arrested by fixation once opening has started. We have, however, found stages in the elongation of the acrosomal process. Thin sections through sperm which have completed the opening of the acrosomal vacuole reveal a vaselike collar within whose center is the lengthening acrosomal process (Figs. 1 and 13). Both of these structures, collar and process, are covered by the plasma membrane which is continuous over the rest of the cell body and flagellum. The collar is derived from the cytoplasm that was between the anterior surface of the acrosomal vacuole membrane and the plasma membrane in untreated sperm (cf. Figs. 1, 2, and 13). Within the collar is material that was formerly part of the contents of the acrosomal vacuole.

Because a small aperture is rapidly transformed into a large hole during the opening of the acrosomal vacuole, it is important to investigate carefully any changes in the packing of the filamentous network, as these filaments will now be located in the cytoplasm of the collar. When sections are cut parallel to the longitudinal axis but not through the center of the sperm (Fig. 14), so that we get a grazing section through the collar, we see a series of parallel bars which are actually clusters of filaments (6–14 in each); these clusters tend to run nearly parallel to the surface of the collar. Thus, in an oblique section through the center of the collar, the filaments will be cut in transverse section on one side of the collar (Figs. 15 and 16), and oriented at an oblique angle on the other side (Fig. 15). Therefore, during induction the filamentous meshwork has been transformed into clusters of filaments which now run at a shallower angle up the side of the collar. This change in orientation of the filaments appears to be related to the fact that the filaments must move laterally to allow the diameter of the aperture to increase.
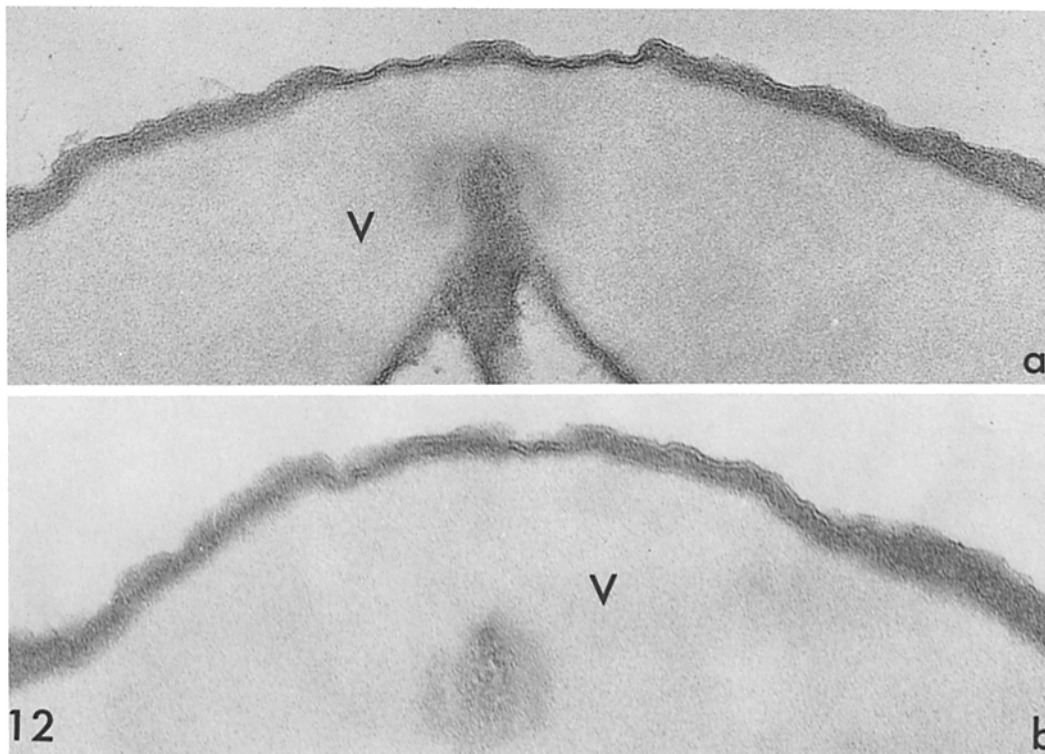
FIGURE 12 Thin sections through the apical surface of *Limulus* sperm fixed in an early stage of the acrosomal reaction. Of interest is that the membrane limiting the acrosomal vacuole (*V*) has formed (*a*) a pentalaminar, and (*b*) a trilaminar junction with the plasma membrane at the same position as we see the dimples in the freeze-fracture image. × 95,000.

This can best be visualized by comparing it to an iris in a camera (Fig. 17). As the iris opens, the plates making up the iris move laterally. If the leaves are oriented radially in the nearly closed iris, each leaf would lie at 90° to its original position when the iris is opened. When the iris is opened only halfway, the leaves would lie at 45°, etc. In the same way the meshwork filaments assume a nearly radial orientation in unreacted sperm, but after induction have moved so that they now lie at a shallow angle to the surface of the collar. This means that there must be a breakage and reformation of the bonds between adjacent filaments.

The membrane limiting the inside of the collar (formerly the apical surface of the acrosomal vacuole membrane) presents an undulatory profile (Fig. 16) as if it were tightly associated with the filament clusters. Freeze-fractures through the *P* face of this membrane reveal that the particles are organized into nearly parallel rows (Fig. 18).

These rows of particles, like the distribution of filament clusters, are aligned at a shallow angle to the surface of the collar. This membrane surface consists of a series of raised ridges as one would expect from the undulatory profile seen in thin sections (Fig. 16). Of particular interest is that the particles are located on the crest of each ridge and thus could, and probably do, attach to the filaments in each cluster. Examination of the *E* face of the membrane that limits the inside of the collar reveals that, although there are very few particles on this fracture face, the existence of a series of ridges and valleys is still evident (Fig. 19). In contrast to the arrangement of the particles on the inside of the collar, the particles on the *P* face of the plasma membrane that limits the outside of the collar (Fig. 19) are randomly distributed, as is true over the rest of the cell body.

The *P* face of the plasma membrane that limits the apical end of the cell within the confines of the collar (formerly the acrosomal vacuole mem-

brane) and the acrosomal process membrane are almost completely devoid of particles (Fig. 20). This is true irrespective of the length of the acrosomal process or the degree of elongation of the process before fixation has arrested further extension.
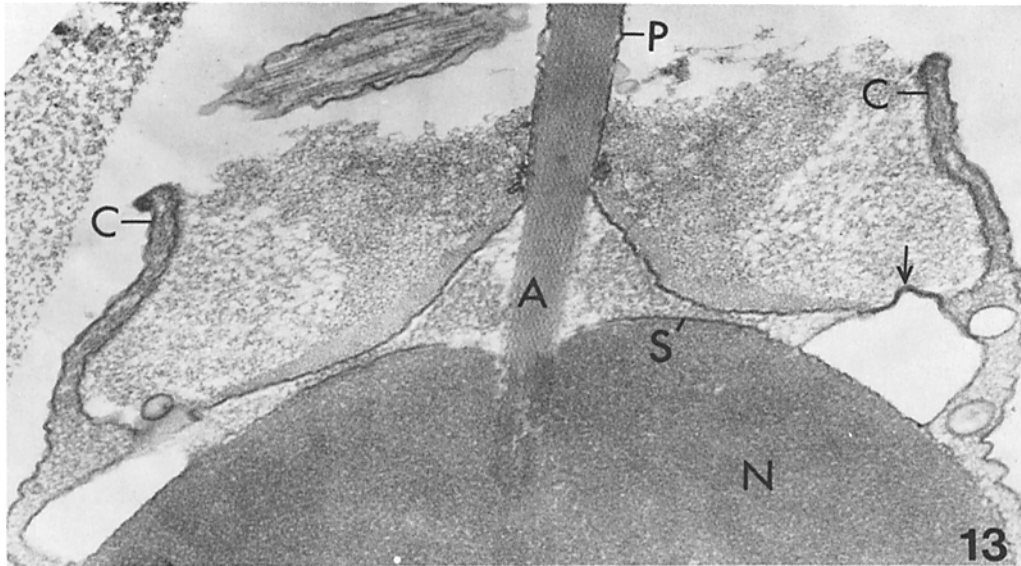

FIGURE 13 Thin longitudinal section through the apical end of a sperm fixed after the induction of the acrosomal reaction. Extending anteriorly from the nucleus (N) is the actin filament bundle (A) which extends out through the acrosomal process (P). The collar (C) is cut in transverse section. Just lateral to the subacrosomal plate (S), the nuclear envelope blebs outward and forms a pentalaminar structure with the plasma membrane inside the collar (see arrow). × 53,000.
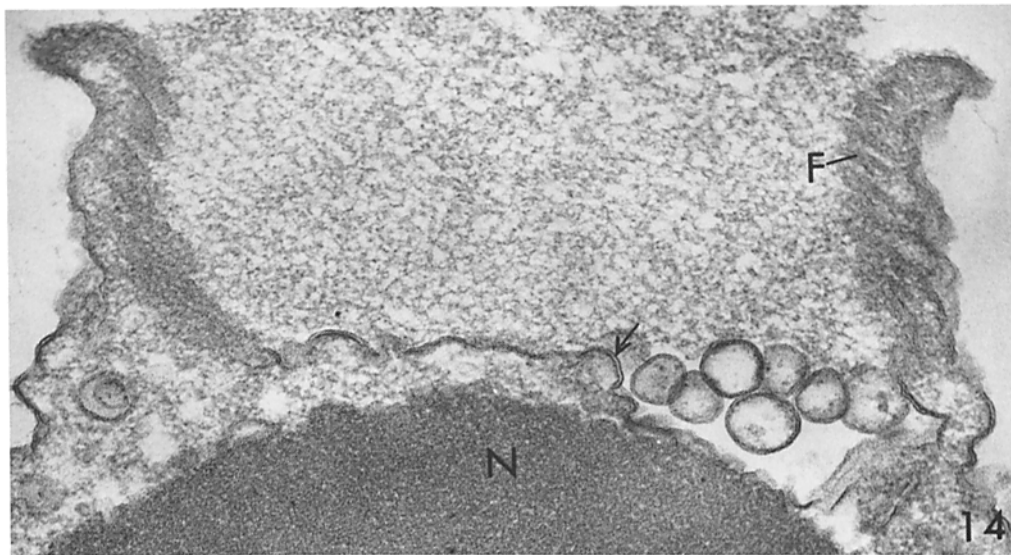

FIGURE 14 Thin section through the apical end of a sperm fixed during the early phases of the acrosomal reaction. Although this section is a longitudinal section through the sperm, it is not positioned down the midline, but instead near the edge of the collar. Of interest are the bars in the collar (F) which are composed of filaments. The arrow indicates a point of continuity between the plasma membrane and the outer nuclear envelope. N, nucleus. × 75,000.
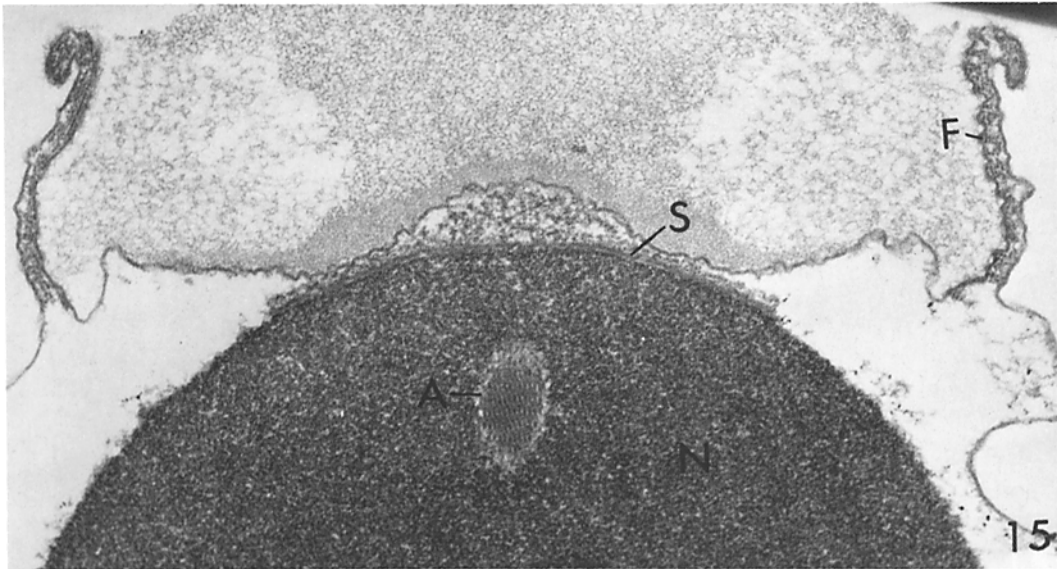
FIGURE 15 Thin section through the apical end of a reacted sperm. This section is cut at an oblique angle to the longitudinal axis of the sperm. Of interest are the bundles of filaments (*F*) in the collar and the fact that the outer nuclear envelope peripheral to the subacrosomal plate (*S*) has disappeared. The base of the actin filament bundle (*A*) remains in the canal in the center of the nucleus (*N*). × 46,000.
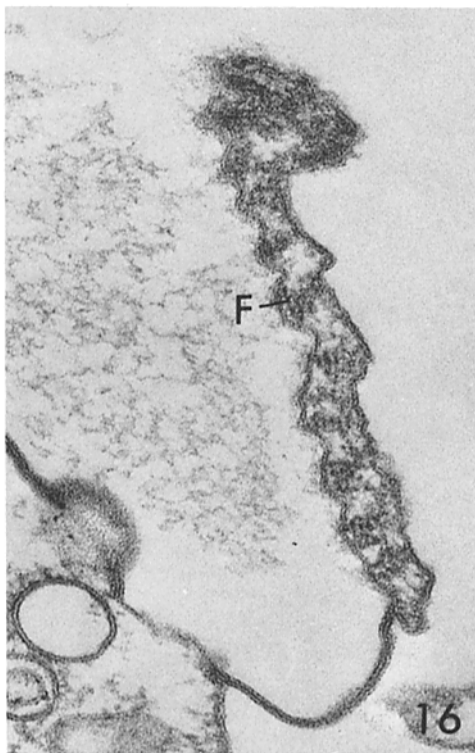

FIGURE 16 Thin oblique section through a collar of a reacted *Limulus* sperm. Of interest are the bundles of filaments (*F*) that are cut in transverse section. × 96,000.
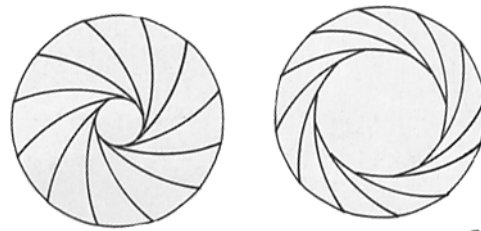

FIGURE 17 Drawing depicting the opening of an iris such as in a camera. As the iris opens, the plates move laterally. In the same way, the filament bundles, which assume an almost radial orientation in unreacted sperm, move laterally to lie within the collar.

Of interest is that the plasma membrane just medial to the collar often bubbles outwards, forming what looks like numerous bulges (Fig. 20). These bulges appear to be caused by blebs or vacuoles derived from the nuclear envelope (see Fig. 13). In some instances these blebs or vacuoles appear to be actually fusing with the cell surface (Fig. 22, arrow). The blebbing of the outer nuclear envelope occurs just laterally to the subacrosomal plate as can be seen in thin sections (Figs. 13 and 14) and in freeze-fractures (Fig. 20). On numerous occasions the blebbing portion of the outer nuclear envelope is seen in close contact with the plasma membrane forming the so-called pentalaminar structure (Fig. 13), a situation in
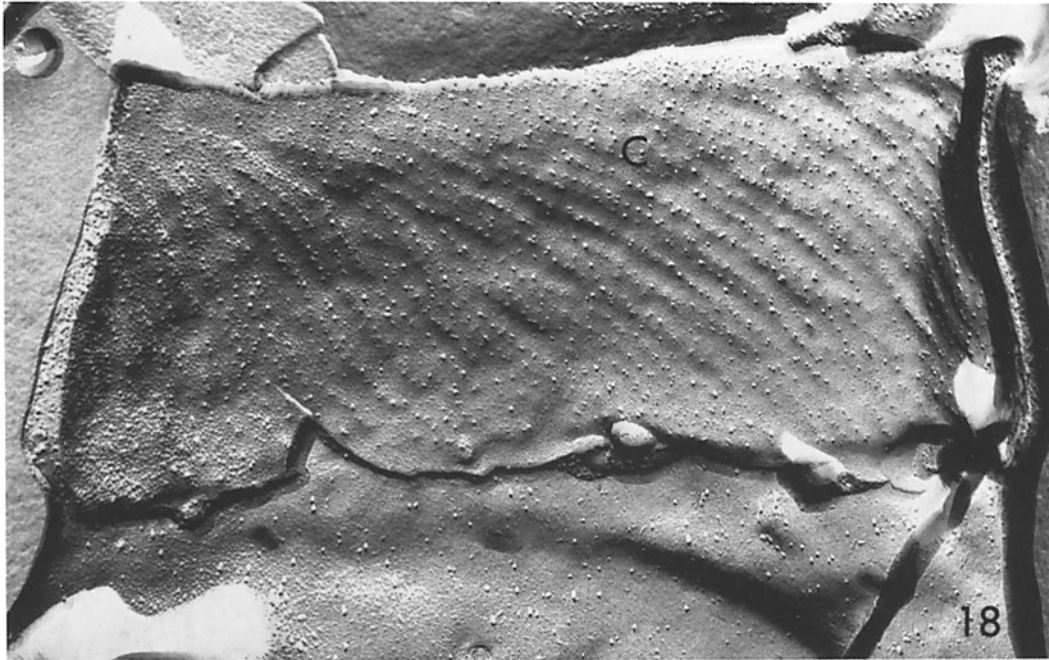
FIGURE 18 Freeze-fracture preparation through the P face of the plasma membrane that lines the inside of the collar (C) of a reacted *Limulus* sperm. On this membrane, we see that the particles are aligned into rows that are situated on the crests of small ridges. These ridges lie directly above the filament clusters seen in Fig. 16. Near the bottom of this figure the fracture plane jumps to the plasma membrane limiting the surface of the cell just beneath the collar. We are looking at the E face of this membrane. × 58,000.

FIGURE 19 Freeze-fracture preparation through the collar of a reacted *Limulus* sperm. The fracture plane (looking from left to right) first goes through the P face of the plasma membrane that limits the outside of the collar (M), then jumps to the E face of the plasma membrane that limits the inside of the collar, and then jumps back to the P face of the plasma membrane (M) that limits the outside of the collar. Of interest is that the E face of the collar membrane has ridges on it with the same angle as the filaments seen in Fig. 14. × 54,000.

which the outer dense line of the nuclear envelope membrane "fuses" with the inner dense line of the plasma membrane. Sometimes we see instances where the plasma membrane and the outer nuclear envelope are continuous (Fig. 14). In contrast to the blebbing of the outer nuclear envelope
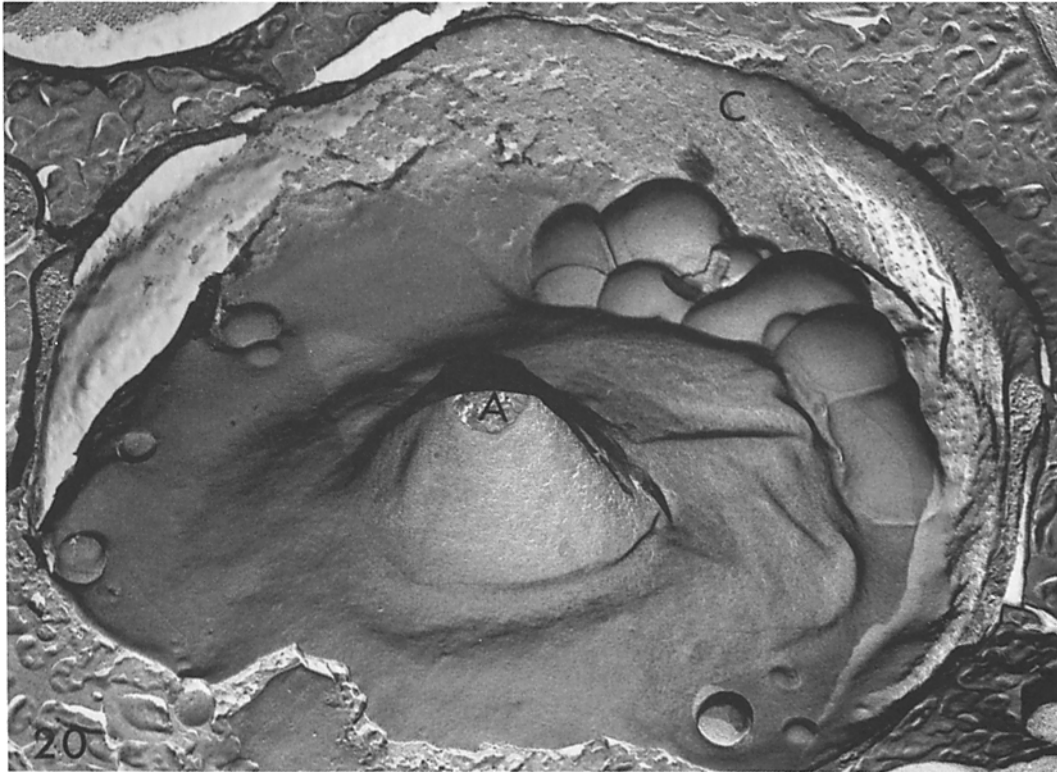
FIGURE 20   Freeze-fracture preparation through the apical end of the *P* face of a *Limulus* sperm which has just completed the acrosomal reaction. On that portion of the membrane that limits the inside of the collar (*C*) there are particles arranged in oblique rows, yet no particles are present on that portion of the membrane that limits the acrosomal process (*A*) and the apical end of the cell. Of interest are the blebs present on the apical surface of the membrane just medial to the collar. This irregularity of the surface is caused by a blebbing of the nuclear envelope in this region just below. × 43,000.

at the apicolateral margin of the nucleus, the nuclear envelope at the subacrosomal plate never blebs. The lack of blebbing in this area correlates with the distribution of numerous particles on the *P* face of the outer nuclear envelope at the subacrosomal plate (Fig. 9). The plasma membrane which covers the lateral and basal surfaces of the sperm posterior to the collar does not bulge or balloon out.

From our micrographs it seems reasonable to conclude that the increase in surface of the plasma membrane that must occur upon the elongation of the acrosomal process (an increase of 15–20 μm^2) is brought about by the addition of the outer nuclear envelope to the plasma membrane. The pentalaminar structures may be intermediate stages. Of interest is that the ballooning out and vesiculation of the outer nuclear envelope is so severe that in many sperm only remnants of the

outer nuclear envelope are left in the apical region (Fig. 15). The chromatin then is covered either by only the inner nuclear envelope or by patches of both nuclear envelopes. This does not appear to be a fixation artefact, because in the same section uninduced sperm adjacent to sperm devoid of the outer nuclear envelope have perfectly normal nuclear envelopes. Also the plasma membrane is intact, even in sperm in which the outer nuclear envelope is totally gone, as demonstrated with experiments in which cationized ferritin is used (unpublished observations); the ferritin does not penetrate the cell. From these observations it appears as if the outer nuclear envelope was fusing with the plasma membrane in this region to provide new membrane surface. This conclusion is consistent with the fact that the outer nuclear envelope, which formerly covered the actin filament bundle when it was coiled at the base of the

nucleus, disappears at the same time as the process elongates.

It is possible that the blebbing and bubbling in this system might be a fixation artefact induced by glutaraldehyde fixation similar to what has been demonstrated by Hasty and Hay (15) in corneal fibroblasts. Hasty and Hay (15) showed that these artefacts did not appear in corneal cells fixed in OsO_4 alone or in a solution of OsO_4 and glutaraldehyde together. Accordingly, we fixed reacted *Limulus* sperm in OsO_4 without prior fixation in glutaraldehyde or in a solution of OsO_4 and glutaraldehyde together (Fig. 21). In both cases, we saw blebbing and bubbling of the outer nuclear envelope beneath the collar and extensive invaginations just inside the collar. Thus, these other fixation methods confirm our observations using glutaraldehyde fixation followed by or without osmication, and demonstrate that what we are seeing probably is not an artefact.

Variations in the Acrosomal Reaction Induced by Ionophores

There is considerable variation in the height of the collar, even in sperm which have been fixed

shortly after they have reacted (i.e., 25 s after the ionophore was added to the sperm suspension). In sperm where the collar is low, the diagonal rows of particles on the crests of ridges which would normally be present in the inside of the collar now can be seen spreading over the rim of the collar (Fig. 22). It is as if the collar had flattened but the filament cluster within had remained intact, as can be demonstrated by thin sections through a collarless sperm (Fig. 23). Freeze-fracture preparations of these collarless reacted sperm (Fig. 22) show curved rows of ridges with particles on the crests. As before, both the P face of the plasma membrane that limits the acrosomal process and the plasma membrane medial to the rows of particles are completely devoid of particles. In our thin sections (Fig. 23), we see filament clusters beneath the particle-rich zone (collar). A blebbing or bubbling of the plasma membrane can be seen at the interface between the ridges with the particles and the particle-free area. This corresponds to the area in the "collared" sperm just medial to the collar. There is evidence of a fusion of the vesicles underlying the plasma membrane in this region (Fig. 22, arrow). The significant observa-
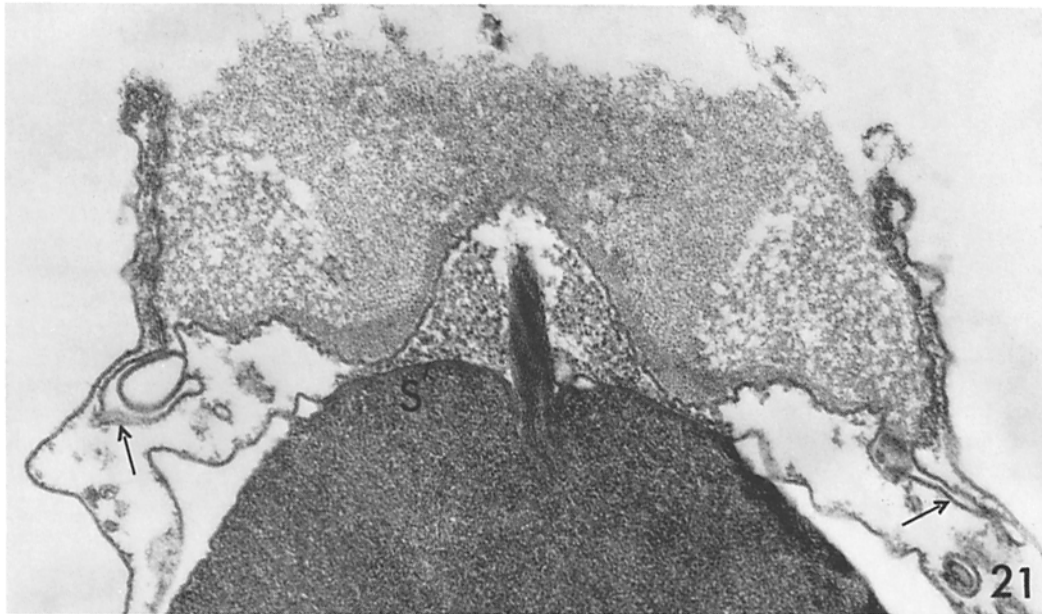

FIGURE 21 Thin section through the apical end of a *Limulus* sperm fixed shortly after the induction of the acrosomal reaction. The sperm were fixed by adding a mixture of glutaraldehyde and osmium tetroxide to the reacted sperm. Of interest is that, even with this method of fixation, the outer nuclear envelope bulges out just peripheral to the subacrosomal plate (*S*) and the plasma membrane just outside the collar has deep invaginations into the apical cytoplasm of the sperm (see arrows). The latter is the region that blebs in the freeze-fracture images (see Fig. 20). × 40,000.
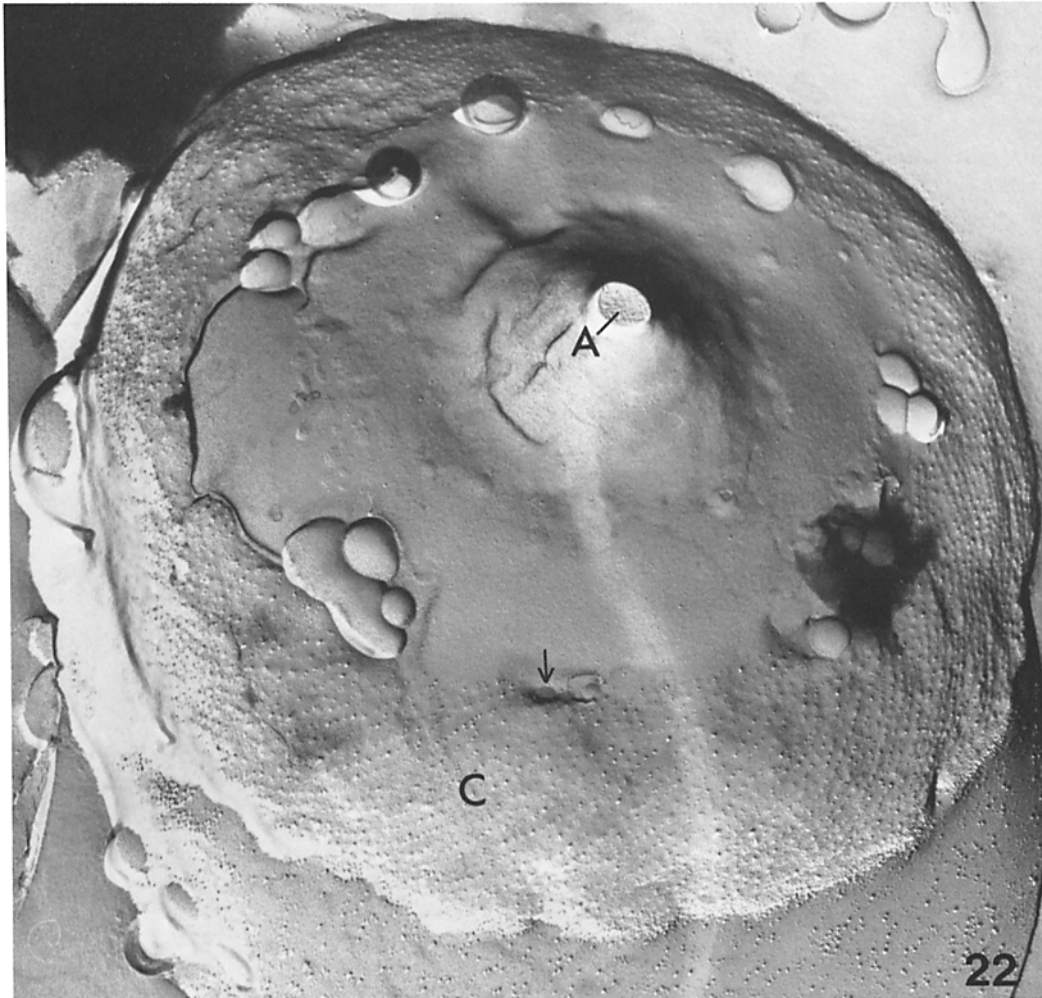
FIGURE 22 Freeze-fracture preparation through the *P* face of the plasma membrane covering the apical end of a *Limulus* sperm fixed after induction of the acrosomal reaction. In this sperm the collar failed to form, but on the surface which would have contained the collar (*C*) we see rows of particles. Beneath this particle-rich zone we see a portion of the *P* face of the plasma membrane that covers the lateral surface of the sperm. The membrane is particle free medial to the area demarcated by the rows of particles. The acrosomal process (*A*) extends out of the center of this particle-free area. Of interest is that there is extensive blebbing in the particle-free portion of the plasma membrane just medial to the rows of particles. The arrow indicates a point of fusion of an underlying vacuole, presumably derived from the outer nuclear envelope, with the plasma membrane in this region. × 45,000.

tions from these cases are: (*a*) the rows of particles invariably have beneath them the filament clusters (note that the particles are on the crests of the ridges, not in the valleys), and (*b*) the point of membrane blebbing and fusion invariably occurs in a particle-free area just medial to the organized rows of particles.

Which Ions Must Enter the Cell to Induce Fusion and the Reorganization of the Filamentous Meshwork?

To determine which ions had to be carried into the cell from the external media to induce the acrosomal reaction, we performed a series of
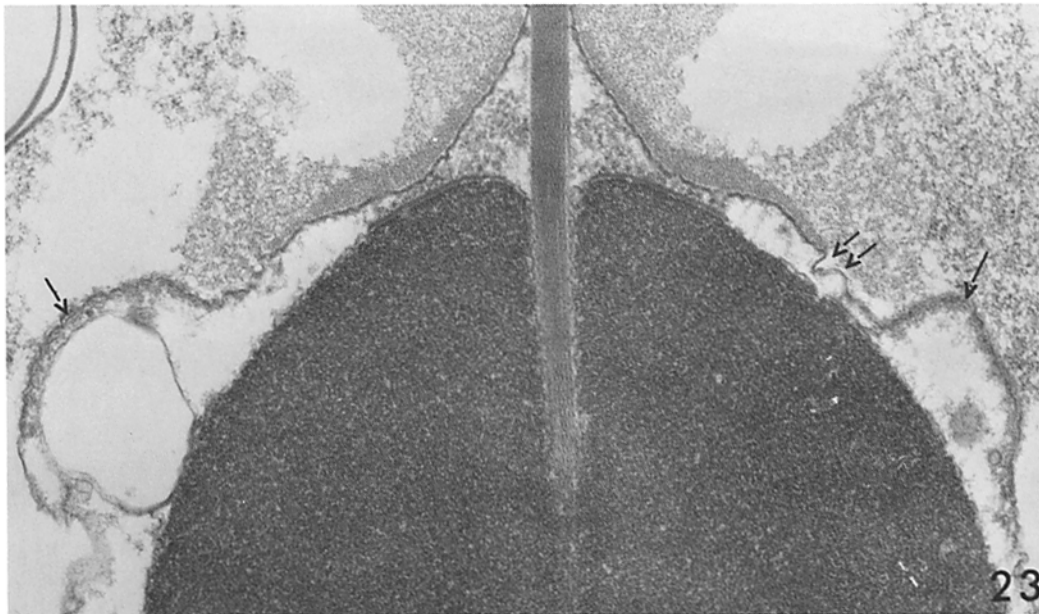
FIGURE 23 Thin section through the apical portion of a sperm that has undergone the acrosomal reaction. This sperm has a collar of very reduced height and corresponds to the freeze-fracture image illustrated in Fig. 22. Beneath the collar indicated by the arrows are bundles of fine filaments. The double arrow indicates a site where vesicles would be likely to fuse with the plasma membrane. This region would lie just within the collar. × 50,000.

experiments in which we suspended *Limulus* sperm in a variety of external media and then added the ionophore. We found that calcium was an absolute requirement, but apparently no other ions were necessary. For example, vacuole fusion does not occur when sperm are suspended in isotonic NaCl, KCl (0.5 M), or sucrose (1 M) and the ionophore added in the absence of calcium, even if the above solutions contain $MgCl_2$ or if the tonicity is doubled. But when 50 mM $CaCl_2$ is added to any of the above solutions in the presence of the ionophore, fusion can be induced in >90% of the sperm. In all cases, provided calcium is present, the acrosomal vacuole opens normally, the filamentous meshwork is transformed into clusters of filaments with a different orientation than that in the untreated sperm, and an acrosomal process is generated.

DISCUSSION

Fusion of the Acrosomal Vacuole with the Cell Surface

The acrosomal vacuole membrane fuses with the plasma membrane at a prescribed site which is defined by a circlet of particles on the *P* faces of both these membranes. The exact point of fusion within these circlets, however, is particle free (see Fig. 24). Replicas of the point of fusion of two membranes have now been examined in a variety of other systems, i.e., the fusion of mucocysts and trichocysts in ciliates (27, 28), synaptic vesicle fusion in neurons (16, 17, 18), mast-cell granule exocytosis (8, 9, 19), fusion of the contractile vacuole of *Chlamydomonas* (34), cell fusion during mating (35), lysosomal granule release (22), myoblast fusion (19), pancreatic exocytosis (24), the acrosomal granule fusion in mammalian sperm (14) and in invertebrate sperm (*Thyone* and *Mytilus*, unpublished observations), and others. A comparison of the results from all these reports demonstrates two obvious discrepancies. First, in some cases the point of fusion is predetermined as in *Limulus* sperm, while in other systems fusion can apparently take place at any position on the surface of the cell and thus a redistribution of material within or beneath the membrane must occur before the fusion event. Second, there is considerable debate in the literature as to whether or not particles indicating aggregates of protein
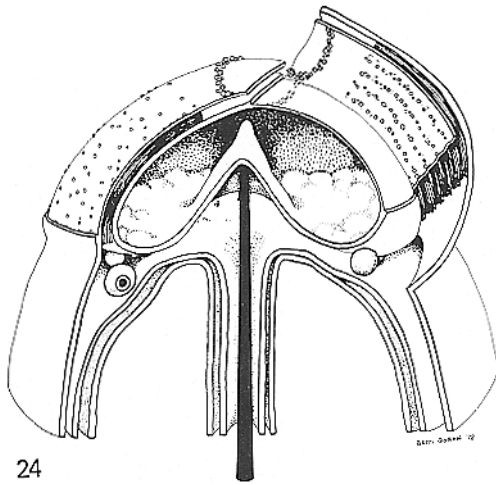
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FIGURE 24 Drawing of the apical end of a *Limulus* sperm. On the right hand side of this drawing the *P* face of the acrosomal vacuole membrane and the filaments beneath are folded back. Of interest is that: (*a*) fusion of the acrosomal vacuole membrane and the plasma membrane will occur within a particle-free area in the center of two superimposed rings of intramembrane particles located in each of these membranes, and (*b*) there are radial rows of intramembrane particles on the *P* face of the acrosomal vacuole membrane and directly beneath these particles are clusters of fine filaments oriented in the same way as the particle rows.

molecules which span the bilayer are actually involved in the point of fusion. In mucocyst fusion (28) and in mating in *Chlamydomonas* (35), it has been suggested that particles are involved in fusion, whereas most of the other studies indicate that fusion occurs between membranes which are free of particles, although particles are nearby, in some cases having been cleared from the area.

On the basis of a number of in vitro studies which have been undertaken on membrane fusion in artificial lipid systems (5, 21, 33), we can think of the process of fusion as involving two events. First, the two surfaces to be fused must come very close together; and second, there must be an increased instability in the lipids at the point of contact allowing the formerly buried hydrophobic parts of the lipid molecules to become more exposed and thus able to form hydrophobic interactions with lipids in the adjacent membranes. This concept is supported by the fact that artificial fusion can be induced either by raising the temperature to approach the phase transition temperature of the lipids, or by the addition of a variety of fat soluble compounds (2), lysolecithin (5), iono-

phores (1), glycerol (3), microneedles (11, 12), viruses, etc., all of which act to destabilize the lipids. From these studies and from what we already know by using freeze-fracture techniques on biological systems, it seems likely that once the membranes have come sufficiently close to one another to interact, proteins lying within the bilayer (intrinsic proteins) or beneath the bilayer (peripheral proteins) must be moved out of the way because they tend to stabilize the lipids in the bilayer. Thus, fusion should occur in an area sparse of membrane particles.

If fusion of two membranes must occur rapidly and at a precisely predetermined position, then these two membrane systems not only must be anchored relative to each other, but they must be able to rapidly approach each other without the necessity of moving intrinsic and extrinsic proteins out of the way. Our observations on *Limulus* sperm are consistent with these speculations. Thus, the overlying circlets of particles on both the plasma membrane and on the acrosomal vacuole membrane could be important in anchoring these membranes relative to one another. In addition, these particle circlets would probably prevent other particles or protein molecules situated within or associated with the membranes from entering the particle-free zone. We conclude, however, that these rings of particles could and probably do play a more direct role in membrane fusion based upon two lines of evidence. First, in all cases of fusion of biological membranes of which we know, fusion occurs near areas where particles are common, but generally in an area that is totally devoid of particles or in which the particle number is reduced (35). Second, Van der Bosch and McConnell (33) have shown in vitro that concanavalin A interacts with dipalmitylphosphatidyl choline vesicles and induces fusion. These workers conclude that a pure phospholipid bilayer will not fuse with another because the lipid would not be destabilized locally; however, if proteins are bound to membranes, the halo of lipid molecules peripheral to the proteins may be perturbed and thereby stabilize "intermediate lipid conformations that facilitate fusion." (Of course, experimentally we know that phospholipid vesicles will not easily fuse with one another, otherwise liposomes of defined size could not be made.) Thus, the membrane proteins could interconnect membranes and facilitate fusion of the lipid in the "halo" near the protein. In *Limulus* sperm, then, the ring of particles (proteins) could destabilize

the lipids within the ring so that membrane fusion will occur in this area when excess calcium enters the system.

From our freeze-fracture images of cells fixed in glutaraldehyde and glycerinated shortly after induction, we see within the ring of membrane particles a series of dimples. At what we interpret as a slightly later stage, these dimples become deeper and are candidates for tiny foci of fusion. In thin sections of sperm fixed during the same period, we see, in the same location, regions where the fusing membranes associate to form pentalaminar and trilaminar (bilayer diaphragm) profiles. These images (both freeze-fracture and thin sections) are reminiscent of the thin-sectioned images of Pinto da Silva and Nogueira (26). Palade and Bruns (25) were the first to suggest that the initial step in membrane fusion was the formation of a "fusion complex" or pentalaminar structure (20, 25, 26). This structure then gives rise to a trilaminar structure or a "membrane diaphragm." Pinto da Silva and Nogueira (26) recently correlated the images seen in freeze-fractures (membrane diaphragm) with the thin section images of a trilaminar structure. We do not know if the dimples we see in the freeze-fracture image should be correlated with the pentalaminar or trilaminar images seen, or, in fact, if many of these images (multiple dimples indicating multiple fusion sites and pentalaminar and trilaminar complexes) are not induced by the technique of fixation and/or glycerination necessary for conventional freeze-fracturing and thin sectioning. We think, in fact, that many of these images may be artefactual, based on several observations. First, pentalaminar structures, as viewed in thin sections of fusion of the outer leaflets of adjacent membranes, occur naturally in a number of systems in which true membrane fusion does not occur or occurs at a later time. Examples include fusion of the outer and inner nuclear envelope in a variety of invertebrate sperm (see reference 31 for references), of the plasma membrane in myelin formation, and in goblet cells (see reference 23 for references). Secondly, Heuser et al. (16, 18) have not been able to find any intermediates in membrane fusion such as a membrane diaphragm by using methods of rapid freezing (5 ms) without glycerol pretreatment. Because all other methods rely on chemical fixation, which Heuser et al. (18) have demonstrated cannot arrest the events of fusion of synaptic vesicles with the plasma membrane, the question becomes one of trying to distinguish whether the intermediates that we and others see are real, or whether they might be extensions of intermediates caused by chemical fixation and glycerination. We suggest that perhaps a pentalaminar-to-trilaminar transition does occur, but in the normal situation this intermediate is only present for a very short period, i.e., 1 ms. Chemical fixation may induce the formation of large areas of this intermediate if present at the appropriate time. Likewise, multiple sites of fusion such as implicated here by the multiple dimples in our freeze-fracture images may also be artefactual, as fusion of cortical granules with the cell surface in sea urchin eggs does not show multiple sites of fusion using the rapid freezing techniques (Heuser, personal communication).

Thus, until the rapid freezing technique is applied to the acrosomal reaction of *Limulus* sperm, it is unproductive to present any hypothesis of what the true intermediates of fusion are. The important observation, however, is that fusion occurs within a particle-free zone surrounded by a circlet of particles.

The Importance of the Attachment of Membrane Particles to the Underlying Filament Clusters

In unreacted sperm there is a meshwork of fine interconnected filaments lying between the acrosomal vacuole and the plasma membrane. In freeze-fracture preparations through the *P* face of the acrosomal vacuole membrane, we find that particles are aligned into rows and that these rows have the same orientation as the filaments (Fig. 24). In reacted sperm the filaments form clusters within the collar; each cluster lies at a shallow angle to the surface of the collar, a much shallower angle than in unreacted sperm. Rows of particles are present inside the collar as they were on the acrosomal vacuole membrane, and these also lie with the same orientation as the filaments in the collar (Fig. 25). We demonstrated that the plasma membrane inside the collar has an undulatory profile, with the particles invariably located on the crests of the undulations, thus indicating a direct connection to the filament bundles because the filaments lie directly beneath the crests. Because there are rarely any particles on the *P* face of the plasma membrane medial to the collar, which of course includes the membrane that limits the acrosomal process, the particles are restricted in their distribution, thus implying that they must be
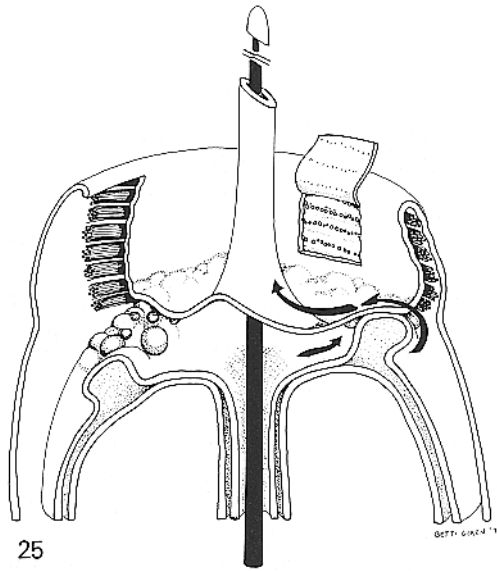
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FIGURE 25 Drawing of the apical end of a reacted *Limulus* sperm. The filament bundles that lie in the collar are indicated, and a small flap of the *E* face of the plasma membrane lining the inside of the collar is lifted up to illustrate the distribution of intramembrane particles that lie beneath. These particles lie just anterior to the filament bundles. The arrows indicate the position of fusion of the outer nuclear envelope with the plasma membrane that lies directly above.

Let us now direct our attention to the question of the significance of the presumed attachment of filaments to membrane particles. First, to determine the exact point of fusion of the acrosomal vacuole with the cell surface, the membranes involved have to be kept separated except at the future point of fusion. Thus, the vacuole must be precisely positioned and then held in that position. The filaments must, at least in part, be responsible here; to effectively do their job, they have to be securely anchored to the acrosomal vacuole membrane. This could be accomplished by their connection to intrinsic membrane proteins, i.e., the particles seen on the *P* face of the acrosomal vacuole membrane. Second, we know that the reacted sperm is attached to the egg surface by material retained within the collar after release from the acrosomal vacuole, a point which will be further discussed in the last subheading. The rigidity of the collar is presumably maintained by the filament bundles which must also be anchored to the plasma membrane (formerly the acrosomal vacuole membrane), which in turn must be anchored to the egg surface. This attachment must be secure because the acrosomal process rotates as it extends, thus tending to push the sperm away from the egg. In other words, the sperm is held on the egg surface by the collar which is attached to the intrinsic membrane proteins which in turn are immobilized by the filamentous meshwork. Third, and perhaps more important, the attachment of the filaments to the particles defines a transition zone between a particle-rich region and a particle-free zone which then may define the point at which vesicles (derived from the outer nuclear envelope) can fuse with the cell surface forming the plasma membrane of the acrosomal process.

bound to each other by some invisible long-range force or, more logically, to the underlying filamentous meshwork.

Many fanciful drawings have been published in the literature over the past few years that illustrate the attachment of cytoplasmic filaments to membrane particles. These drawings have been made largely to explain how membrane proteins may be redistributed during such processes as capping, blebbing, and the like, but as yet evidence in favor of these speculations has been very meager or nonexistent. Our observations are consistent with such a concept. However, we should point out that the filaments illustrated here are very thin, 30–35 Å in diameter (and incidentally are not actin filaments, as demonstrated by their failure to decorate with subfragment 1 of myosin even though cells known to contain actin, when added to the same solution, did decorate [unpublished observations]), so that individual filaments would be difficult to distinguish in a conventional thin section (200–300 Å thick) as they would be obscured by the cytoplasmic ground substance.

Changes in the Orientation of the Filaments During Induction: Do the Filaments Play an Active or Passive Role in the Opening of the Acrosomal Vacuole?

From our movie sequences and from thin sections, we know the following facts. First, the acrosomal vacuole opens in <50 ms because opening occurs in one frame of the sequence. Second, we know that one of the earliest changes is a small swelling of the acrosomal vacuole; thus, at least part of the force for the opening of the vacuole could be the result of a swelling of the vacuole contents. Third, the formation and elongation of the acrosomal process cannot be in-

volved in this rapid opening of the vacuole be-
cause the opening is achieved before the process
extends. And fourth, from our thin sections we
know that there is a dramatic change in the
meshwork filaments at the time of opening.

The important question to consider is whether
or not the opening of the acrosomal vacuole is
caused entirely by the release of pressure pro-
duced by a swelling of the contents of the vacuole
or whether the filaments play an active role as
well. Unfortunately, we cannot unequivocally dis-
tinguish between an active or passive role for the
filaments because they must change their distri-
bution regardless of the mechanism. However,
because, speaking teleologically, there is a biolog-
ical necessity for a rapid and complete opening (to
glue the sperm to the egg before the process
comes shooting out, thus, by Newton's laws, push-
ing the sperm backwards), and because the fila-
ments rearrange rather than dissolve, we suggest
that the filaments probably play an active role.

The Role of Calcium in Triggering the Acrosomal Reaction

We demonstrated that external Ca^{++} is essential
for the acrosomal reaction; Mg^{++} or monovalent
cations or anions will not substitute, as demon-
strated by the addition of ionophores to sperm
suspended in a variety of media. Also, when we
incubated sperm in sea water or isotonic saline
containing five times the concentration of Ca^{++}
present in normal sea water, the acrosomal reac-
tion was elicited in ~10% of the sperm without
the use of ionophores or eggs. Thus, calcium
seems to enter the sperm from the external me-
dium and induce the reaction, but exactly what
the calcium does remains mysterious. This is an
area that we are currently pursuing.

Increase in Membrane Surface: Evidence that the Nuclear Envelope Gives Rise to the Plasma Membrane at a Prescribed Site

The work of Fahrenbach (13) and Tilney (30)
demonstrated that the actin filament bundle,
coiled below the nucleus before the acrosomal
reaction, is enclosed by two layers of outer nuclear
envelope; yet after the reaction this extra mem-
brane cannot be found in the basal or apical end
of the sperm. From this observation, Fahrenbach
(13) suggested that the outer nuclear envelope
gives rise directly to the additional plasma mem-
brane needed to cover the actin filament bundle

in the acrosomal process, a contention Tilney (30)
refuted because he could not find a direct connec-
tion between the plasma membrane of reacted
sperm and the outer nuclear envelope. In this
study, however, we noted that at the apicolateral
surface of the nucleus the outer nuclear envelope
tends to bleb and vesiculate even in untreated
sperm. Large numbers of vacuoles and vesicles
are found in this region in partially reacted sperm,
and in our freeze-fractures we consistently find
evidence of fusion of these vacuoles with the
plasma membrane just within the base of the
collar (see Figs. 20 and 22). Also, we routinely
see in thin sections of partially reacted sperm that
the membrane of these vesicles or vacuoles comes
into close association with the plasma membrane
at the inside base of the collar and forms a
pentalaminar (Fig. 13) and, on occasion, a trilam-
inar profile. Sometimes, we see what appears to
be a direct connection between the plasma mem-
brane and the outer nuclear envelope (Fig. 14).
Furthermore, in some sperm activated by the
ionophore in the presence of high Ca^{++}, the outer
nuclear envelope seems to disappear completely,
except in the region of the subacrosomal plate.
Blebbing of the plasma membrane within the
collar is common in these reacted sperm, yet
infrequent on the lateral surfaces of the cell body.
And, finally, the outer nuclear membrane of
unreacted sperm, except in the subacrosomal
plate, is essentially particle free, as is the mem-
brane that covers the acrosomal process and the
basal region within the collar. From all these data,
we conclude that the outer nuclear envelope does
indeed give rise to the 15 μm^2 of new membrane
needed to cover the acrosomal process, and that
fusion of these membranes occurs at a prescribed
position just within the collar. As one might
predict from the discussion in a preceding sub-
heading, this point of fusion is between an area of
the membrane which is particle rich and an area
that is devoid of particles.

How Our Observations Relate to Fertilization of Limulus Eggs

The surface of the Limulus egg is covered by an
outer basement lamina, 5 μm thick, and an inner
vitelline layer, 35 μm thick. There are many pores
in the outer lamina, but none in the inner layer
(7). The apical end of the sperm attaches to the
surface of the egg (6); a short while later, the
acrosomal reaction is induced. Brown and Hum-

phreys (7) and Brown (6) demonstrated that the acrosomal process apparently passes through one of the pores in the basement lamina and bores a hole through the 35-μm vitelline layer, ultimately coming into contact with the plasma membrane limiting the egg. Because the process must find a small aperture in the basement lamina and because the sperm must be held at a precise angle (90°) relative to the egg so that the process can reach the plasma membrane (after all, the process is only 50 μm long, yet the extracellular layers of the egg exceed 40 μm in thickness), the point of fusion of the vacuole with the cell surface must be precisely determined. Because the process screws as it elongates (30), the sperm cell body must be strongly attached to the basement lamina of the egg via the collar by interaction of material which was formerly contained within the acrosomal vacuole. Because the sperm cell body does not rotate, yet the filament bundle and most likely the membrane surrounding the acrosomal process do, the point of shear between the stationary plasma membrane surrounding the cell body and the rotating acrosomal process must be at the base of the process, an area which we know from our freeze-fractures is free of particles. Yet we also know that as the acrosomal process elongates, more membrane needs to be added to it. A logical place for insertion of new membrane is at the base of the process, for if it were to be added lateral or posterior to the collar, it would be difficult to imagine how the sperm cell body could remain immobile. Our observations show that the new membrane, indeed, appears to be added just medial to the collar. Because the acrosomal process begins to extend out of the sperm within 100 ms after the sperm has been induced to discharge, it is important biologically for the acrosomal vacuole to open as rapidly as possible for the collar to be bound securely to the egg and for the point of shear to be defined relative to new membrane insertion. We demonstrated that the vacuole, indeed, opens rapidly and that the collar forms in <50 ms before the extension of the acrosomal process. Thus, the collar appears to serve several purposes: it is important in attaching the sperm to the egg, it defines the interface between a rotating membrane and the insertion of new membrane, and it has sufficient organization to define where new membrane will be added. This organization presumably involves the necessity of maintaining a particle-rich area separate from a particle-free zone.

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