Acrosomal Reaction of *Thyone* Sperm. I. Changes in the Sperm Head Visualized by High Resolution Video Microscopy

SHINYA INOUÉ and LEWIS G. TILNEY
Marine Biological Laboratory, Woods Hole, Massachusetts 02543; and the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT   Structural changes inside the head of *Thyone* sperm undergoing the acrosomal reaction were followed with a high-resolution, differential interference contrast (DIC) video microscope. The beating sperm, adhering by their midpiece to the cover slip of a wedge perfusion chamber, were activated by a calcium ionophore (20 μM A23187) suspended in sea water containing 50 mM excess $CaCl_2$.

Before activation of the sperm, the acrosomal region appears as a 1.1-μm diameter sphere, slightly less dense than the rest of the sperm head. Upon activation, the acrosome pops; the acrosomal region suddenly swells and its refractive index drops. After ~1 s, a crescent-shaped periacrosomal cup appears behind the acrosomal vacuole. In the next several seconds, the cup loses more refractive index and expands forward as the acrosomal process extends. The acrosomal vacuole becomes smaller, but without appreciable drop in refractive index. These observations, coupled with the behavior of the extending acrosomal process reported in the companion paper, and in electron microscopy (EM) and early physiological studies, suggest that the acrosomal process is extended by a combination of the explosive polymerization of actin and the osmotic swelling of the periacrosomal cup material.

In this paper, we also consider the meaning of the enhanced DIC image seen in the high-resolution video microscope, and discuss the reliability of measurements on small linear dimensions made with the DIC microscope.

For successful fertilization, a sperm encountering the egg must first traverse the egg's protective jelly and other coats. Within seconds after the arrival of sperm, a delicate thread is observed in the jelly of some echinoderm eggs, extending from the sperm head to the cytoplasmic surface of the egg. At the base of the thread, the surface of the egg may protrude, or display a "fertilization-" or "entrance-cone."

Some earlier observers believed that the fertilization cone, and the thread that was believed to be spun out of the cone, were the egg's way of lassoing the sperm into the ooplasm (4, 14). Others did not find the thread and also argued that the cone formed only after the sperm came into contact with the egg or its envelope (e.g., reference 27, p. 409).

In the 1950's, however, J. C. Dan (8) and the Colwins (5) showed decisively that the fine thread that had been seen in

the egg jelly was produced by the sperm rather than the egg. Even in the absence of the egg, they could induce the thread to grow from the sperm, specifically from its acrosome, a small bi- or tripartite body found at the apex of the sperm (1). The sperm was said to go through an acrosomal reaction, and the fine thread produced at the tip of the sperm was named the acrosomal process. Thus, in the normal process of fertilization, the sperm encountering the egg jelly undergoes an acrosomal reaction and the acrosomal process of the sperm extends and penetrates the jelly and vitelline envelope. After the sperm and egg plasma membrane have united at the tip of the acrosomal process, the nucleus and cytoplasm of the sperm are drawn into the egg.

In the acrosomal reaction, the sperm first reacts by a change in the cell membrane that lies in contact with the "acrosomal granule," or acrosomal vacuole (Fig. 1). In a reaction requiring the presence of Ca^{++}, the membranes of the acrosomal vacuole and the sperm fuse in a ring-shaped annulus at the apex of the

---

Dedicated to Jean Clark Dan, humanist, friend, pioneer in research on the acrosomal reaction.
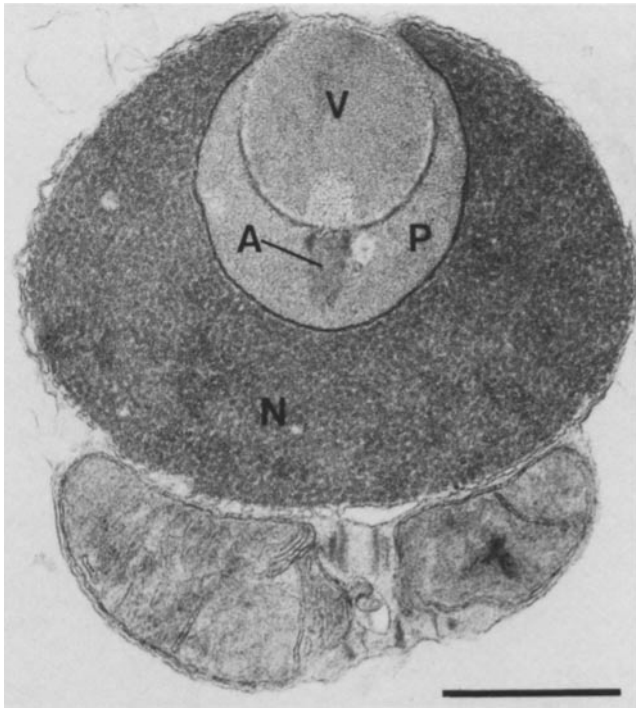
FIGURE 1   Thin section electron micrograph showing the head and midpiece of *Thyone briareus* sperm before acrosomal discharge. The acrosomal vacuole (*V*) is surrounded by the cup-shaped periacrosomal region (*P*) within the pocket of the sperm nucleus (*N*). The plasma membrane at the anterior of the sperm (top) and the vacuolar membrane are closely apposed and are still intact. The apposed membranes in this region fuse at their rim and are cast off when the acrosome pops. The anterior portion of the actomere (*A*), onto which the actin molecules polymerize, is seen within the periacrosomal cup. Electron microscope (EM) procedures used to obtain this micrograph can be found in Tilney and Inoué (25). Bar, 500 nm, × 49,000.

sperm head. The membrane permeability changes and, according to Dan et al. (10, 12), water enters the acrosome. Unless the pH of the medium is too low, the membrane bounded by the annulus is cast off (6, 11). At this time the outer face of the sperm membrane has become contiguous with the inner face of the membrane surrounding the acrosomal vacuole.

Meanwhile, actin that is contained in the cup-shaped cytoplasmic space, lateral to and immediately posterior to the acrosomal vacuole, starts to polymerize onto a rod-shaped organelle, the actomere, located at the base of the cytoplasmic cup (23). The newly forming filaments extend rapidly in parallel to form a thin axial bundle that is believed to provide the force for the extension of the acrosomal process. The axial filaments rapidly extend forward by addition of more actin subunits at the anterior tip of the growing acrosomal process (25, 26).

In this manner, the posterior membrane of the acrosomal vacuole, now contiguous with the plasma membrane of the sperm, is everted and surrounds the growing axial filaments. The new acrosome membrane is in turn covered by the contents of the acrosomal vacuole which is believed to contain the lysins needed to penetrate the jelly coat and vitelline envelope (for review, see references 6, 9), and bindin, a protein thought to attach sperm to the egg (see references 19). The whole acrosomal reaction, including the extension of the process, is completed in a few seconds. Even in the sperm of the sea cucumber,

Thyone, that produces an acrosomal process 90 μm long, the reaction is completed in 10 s.

The acrosomal reaction can be artificially induced in echinoderm sperm with sea water that contains extracts of the egg jelly, or excess Ca^{++} or ammonia, or whose pH is raised to 9.5 (8). Sometimes the acrosomal process is produced spontaneously when the sperm lands on a solid substrate.

We studied the dynamic changes and kinetics of this rapid reaction of the sperm by recording and analyzing the events with a high-extinction video microscope. With this new instrument, we could obtain excellent quality, high-resolution, DIC images of *Thyone* sperm undergoing the acrosomal reaction. The recorded video image could be analyzed sequentially at time intervals as short as 17 ms (17).

In the first of these two reports, we discuss the methods devised for these studies, as well as describe the morphological and optical changes that take place inside the *Thyone* sperm head. The sperm were observed by video in a newly designed perfusion chamber, and triggered with a Ca^{++} ionophore in the presence of excess Ca^{++}. We conclude that the polymerization of actin and osmotic influx of water may both contribute to the extension of the acrosomal process.

In the second paper, we describe the kinetics of growth and the morphological changes of the growing acrosomal process. On the basis of these data, we analyze the diffusion and polymerization of actin within the acrosomal process as well as the addition of membrane to the process. We then discuss the balance of the various forces that must underly the production and transient maintenance of the extremely slender and long acrosomal process.

MATERIALS AND METHODS
Obtaining Sperm

Thyone briareus were collected by the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, MA. The testes were removed and minced in sea water. The suspension was filtered through cheese cloth and the supernate centrifuged at 5,000 *g* for 5 min to pellet the sperm. The pelleted sperm, stored in the refrigerator, were used either the same day or the next day.

Triggering of the Acrosomal Reaction

The acrosomal reaction of *Thyone* sperm can be triggered by adding an ionophore such as A23187 (CalBiochem, La Jolla, CA) to the sea water. Although the acrosomal processes produced are often shorter than normal (they must exceed 50 μm in length to penetrate the jelly layer which is that thick), all the sperm react. We found that the addition of excess Ca^{++} to the sea water induced longer processes, or processes of normal length. Accordingly, a small amount of the pellet of sperm was suspended in sea water containing an additional 50 mM $CaCl_2$. The dilute suspension of sperm, which was only slightly milky, was then added to the perfusion slide and within a minute high calcium sea water containing A23187 was perfused across the slide. A 1 mg/ml stock of A23187 was made by dissolving A23187 in dimethyl sulfoxide. Immediately before perfusion 10 μl of this stock was added to each ml of high calcium sea water. Thus, the final concentration of A23187 in sea water was ~20 μM.

Perfusion Chamber

The *Thyone* sperm were studied in a simple, metal-free perfusion chamber that we devised. In this chamber, the slide and cover slip form a wedge-shaped rather than a plane-parallel space (Fig. 2). The cells are trapped in the wedge and the flow is forced to spread out to provide a uniform exchange without stagnant zones (18). The chamber provides for thorough and rapid exchange of media, and permits high resolution observations of individual cells on an inverted microscope.

Diluted suspension of sperm is introduced into a clean chamber, and sperm that have attached to the cover slip and which are not drawn out by additional perfusion are used for observations. With slides and cover slips that were cleaned with neutral detergent and rinsed thoroughly according to our standard laboratory

procedures (15), healthy *Thyone* and *Arbacia* sperm become "attached" to the glass surface by their midpieces.

Video Observations and Analyses

Sperm in the perfusion chamber were observed with a 40/0.65 dry- or 100/ 1.30 oil immersion differential interference contrast (DIC objective lens of the Leitz Smith T-system, E. Leitz, Inc., Rockleigh, NJ). The DIC condenser and objective lenses were oriented to provide maximum extinction on a specially built inverted polarizing microscope (17). The specimen was illuminated with mono-chromatic green light of 546 nm by filtering the output of a 100 watt, concentrated mercury arc burner (III-100, Illumination Industries, Inc., Sunnyvale, CA or Osram HBO-100, Opto-Systems, Inc., Jenkintown, PA) through two layers of glass heat cut filters (Schott KG-1, Fish-Schurman Corp., New Rochelle, NY) and a high (>70%) transmission narrow bandpass multilayer interference filter (Baird Atomic, Bedford, MA). The deSénarmont compensator of the DIC condenser was oriented at between 10° and 20° off extinction to optimize the video image contrast of the acrosomal process or the contents of the sperm.

The microscope image was projected by the ocular onto the 1 inch Newvicon image tube in a video camera equipped with circuits that automatically adjust the gain and the pedestal level (model 65 II, Dage-MTI, Michigan City, IN). The signal from the video camera was looped through a sync stripper and video processor (nos. 302-2 and 604, Colorado Video, Inc., Boulder, CO) for manually optimizing the contrast and pedestal levels. After insertion of the time and date signals (Model ET 202, Cramer Video, Newton, MA), the video signal was stored on a ¾ inch cassette, time-lapse tape recorder (TVO-9000, Sony Corp., Long Island City, NY) recording at normal speed, then displayed on a 9 inch mono-chrome video monitor (Sanyo 4290, Compton, CA).

For analysis of morphological changes in the sperm head or of the acrosomal process, the video tape was played back at normal speed, or in the 96 h mode for study field-by-field, or re-recorded onto a video motion analyzer (Sony SVM 1010). We studied the 10-s sequence stored on the disk in the motion analyzer, either field-by-field or at various speeds, backwards and forwards.

The lengths of the acrosomal process were determined from frozen images of the video tape record. X- and Y- video markers generated by a video analyzer (Colorado Video No. 321) were positioned onto the tip and base of the acrosomal process, and their coordinates recorded together with the time of the scene. The length of the process was calculated from the coordinates which had been calibrated with images of a stage micrometer.

The exact time of a particular scene was determined by counting the number of video fields elapsed after the seconds digit had last advanced. This method provides the time point with a 16.7 ms precision since each second is represented by exactly 60 fields in standard United States video equipment.

Changes in the dimensions of complex structures were measured from pho-tographed scenes of the monitor, calibrated with scenes of the stage micrometer. The monitor was photographed on Kodak Plus-X or Agfa Super-Pan film with a Nikon FM, single lens reflex camera, equipped with a Micro-Nikkor 55 mm f/ 3.5 macro lens. Some scenes (e.g., Fig. 3) were photographed through a 50-lines-per-inch Ronchi grating (Rolyn Optical Co., Arcadia, CA) to suppress the intrusive video scan lines (17). The film was developed according to Kodak specifications in Microdol-X diluted 1:1. Further details of the video equipment and the theory of video enhancement for polarized light and DIC microscopy are described elsewhere (17; also see reference 3).

RESULTS

Thyone sperm, suspended in sea water and introduced into the perfusion chamber, attach in large numbers to the clean sur-faces of the slide and cover slip. While the attachment is secure enough so that most sperm, once attached, are not dislodged by additional perfusion, the tail and head, nevertheless, appear to remain free. The tail of the attached sperm can beat at its normal frequency, and the tail and head are often seen swing-ing around the sperm as though pivoted at the attachment. Sperm of *Arbacia* clearly pivot around the midpiece, while *Thyone* sperm appear to pivot either around the midpiece, or the junction of the midpiece and the sperm head. In some preparations the sperm tail also stuck to the slide, but with no apparent effect on the acrosomal reaction.

In an unreacted sperm, the acrosomal region (i.e., the acro-somal vacuole plus the periacrosomal cup) is visible as a small sphere of somewhat lower refractive index than the rest of the sperm head (Fig. 3A). The acrosomal region in the living, unreacted sperm measures ~1.1 μm in diameter.
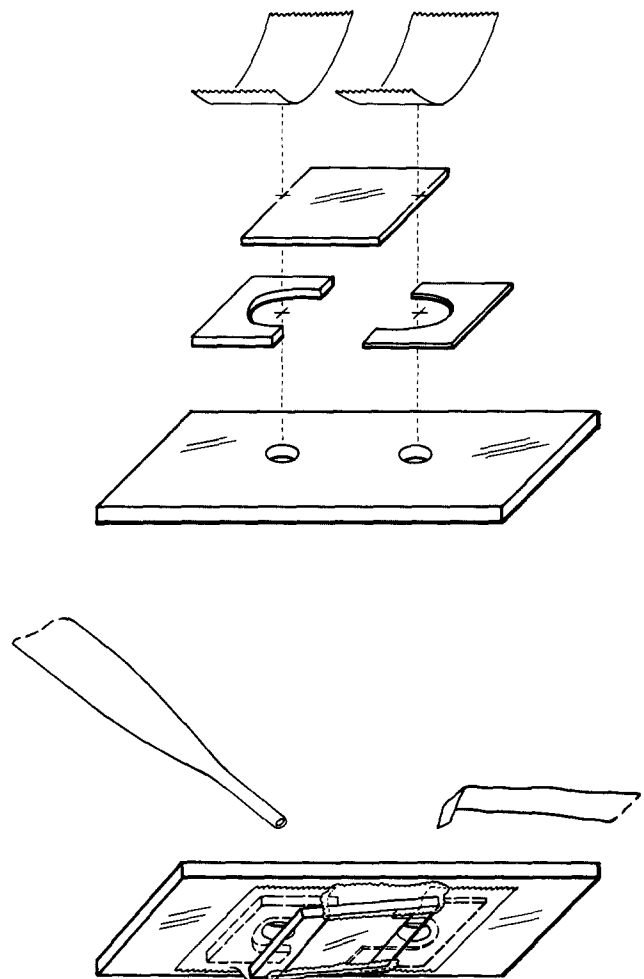

FIGURE 2 Design of the wedge perfusion chamber for use on an inverted microscope. Top: In the process of assembly. Bottom: The inverted, assembled chamber as seen in perspective from below. Two holes, 3-4 mm in diameter, are drilled in stress-free, standard size (1″ × 3″ × 1.0-1.2 mm thick) microscope slides, with their centers spaced apart by 18 mm, the width of the cover slip. On a clean perforated slide we place 2 U-shaped plastic spacers, with the open ends of the U's clearing the holes in the slide, and facing each other. One is ~12-μm thick (cut out from DuPont Mylar 48-S sheet), and the other is ~100-μm thick (DuPont Mylar 400-S sheet). A clean 18 × 18 mm cover slip is placed on top of the two spacers, spanning the two holes in the slide. The cover slip and spacers are taped onto the slide with two 1″ long pieces of ½″ wide Scotch tape. The tape seals the spacers, and the entrance and exit sides of the cover slip, leaving the middle 80% of the cover slip clear. The remaining exposed sides of the cover slip and the small recess under the Scotch tape are sealed with molten Valap-B, a 1:1:1 simmered mixture of vaseline, lanolin, and bees wax. The assembled slide is inverted, and a 5-6 mm diameter circle is drawn with a china-marking wax pencil around the hole above the thicker spacer. The wax pencil circle indicates the entrance hole, and also prevents the perfusion solutions from flooding over the slide. The other hole receives the filter paper wick that draws the solution out from the perfusion chamber. The tapered piece of filter paper is replaced every few minutes, generally more frequently when fresh solutions are added to the entrance port. The angle of taper of the filter paper, and the frequency of its replacement, control the rate of perfusion.

Soon after high-calcium ionophore sea water is introduced into the perfusion chamber, the sperm tail starts to beat more vigorously as though stimulated by a signal. Then, the acro-
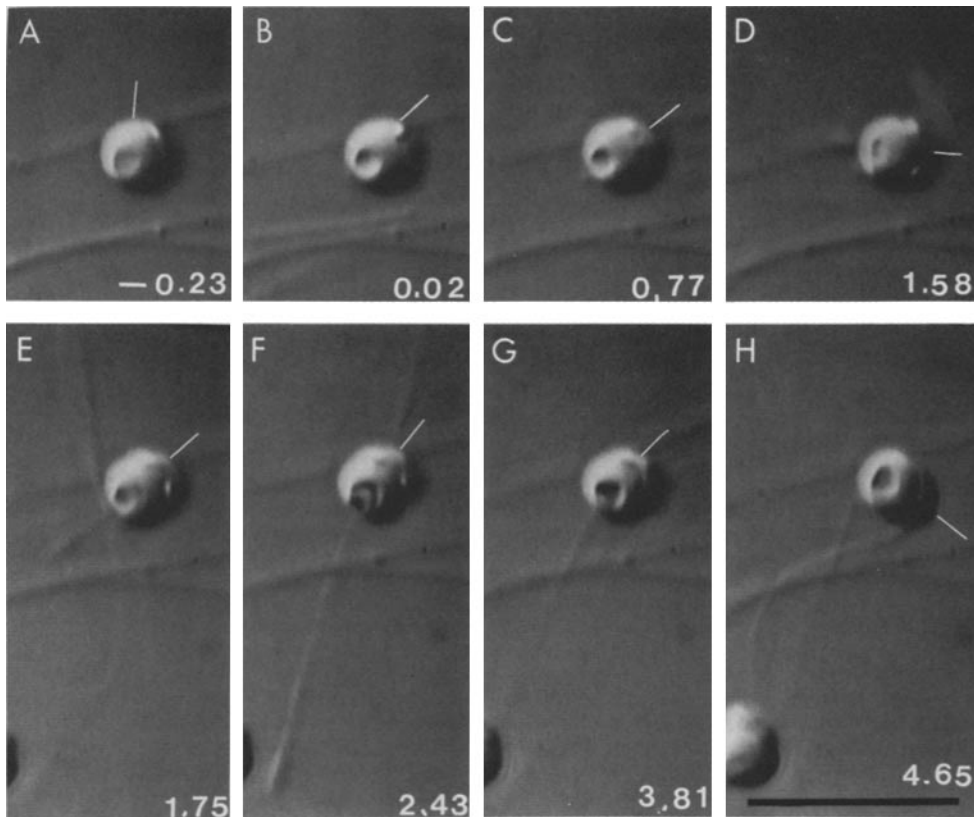
FIGURE 3 High magnification video sequence of changes taking place in the *Thyone* sperm head during the acrosomal reaction. White lines indicate the location and orientation of the sperm tail that would be seen if the tail were to project straight back from the sperm, which it does not. These pictures were taken through a Leitz 100/1.30 Smith T-system DIC objective, with the condenser oil immersed and used at an N.A. of 0.91. The video monitor was photographed through a Ronchi grating to reduce the prominence of the scan lines (for detail of the method, see reference 17). Time shown is in seconds after the acrosome has popped. × 2,800. *A*: 0.23 s before the acrosome pops, the refractive index of the acrosomal region is somewhat less than the remainder of the sperm head. *B*: After the acrosome has popped, the acrosomal region is slightly larger and shows more contrast; its refractive index has dropped. *C*: The acrosomal process has still not started to elongate. *D*: The sperm head has turned and the process is just starting to elongate (to the left). The bundle of actin filaments is visible in the vacuole. The periacrosomal cup is just becoming discernible. *E*: The sperm is turning again. The process is now about as long as the sperm head is wide. Two compartments, the acrosomal vacuole and the periacrosomal cup, are now clearly visible. *F*: The acrosomal process has grown to about four times the diameter of the sperm head. The spear-shaped tip of the acrosomal process has reached the lower left corner of the scene. Some blebs are detectable on the process, which is somewhat out of focus. The refractive index of the periacrosomal cup has dropped dramatically. The acrosomal vacuole is moving forward, but without appreciable loss of refractive index. The tail of the sperm has come into focus at the upper right. *G*: The periacrosomal cup has swelled considerably and appears heart shaped. The remmant of the acrosomal vacuole is now considerably smaller. *H*: The sperm head has turned again and presents a different view of the periacrosomal cup. Within it, the actomere and/or the actin filament bundle is visible. The sperm tail is now whipping down below, and in this scene appears S-shaped, crossing the thin, acrosomal process.

some "pops." That is, all of a sudden, the acrosomal region becomes more prominent, in part due to a drop in its refractive index, and in part due to a slight increase in diameter of ~25% (Fig. 3 *A* and *B*). The sudden drop in refractive index and increase in diameter are completed in three to four video fields, or in 50–70 ms. At the same time that the acrosomal region becomes more prominent, the tip of the acrosome bursts open into a faint inverted funnel, and the acrosome spits forward a fine mist. Sometimes what appears to be a very pale vacuole, ~0.5 μm in diameter, floats down stream from the popped acrosome, but the image of the pale structure is too faint to permit a more definite description.

With the exceptionally high contrast provided by the video DIC image, some of these changes were quite obvious, while we became aware of other features only after repeated viewing of the recorded video sequence.

One to two seconds after the acrosome pops, the acrosomal process comes into sight at the tip of the sperm (Fig. 3 *D*). As detailed in the next paper (25), the process grows rapidly into a slender thread many tens of micrometers long over the next several seconds.

Early during the growth of the acrosomal process, two compartments become visible in the acrosomal region (Fig. 3 *D* and *E*). The anterior part is a sphere with a diameter of ~1.2 μm. It is located at the apex of the sperm head, at the base of the acrosomal process. The posterior part is shaped as a cup that partly surrounds the anterior sphere.

The location and size of the anterior sphere clearly identifies it as the "acrosomal granule," or acrosomal vacuole, seen in electron micrographs (Fig. 1). The cup-shaped compartment with a somewhat cone-shaped posterior contour corresponds, in shape and location, to the periacrosomal cup seated in the anterior depression of the nucleus. In favorable video frames a refractile rodlet, presumably the actomere, is detected in that region.

In the first few seconds after the acrosomal process comes into sight, the cup-shaped periacrosomal compartment expands forward. At the same time, its refractive index drops sharply (Fig. 3 *E-G*). The posterior contour of the cup becomes increasingly clear, but is unchanged in shape. Only its anterior contour, i.e., the boundary with the sphere, moves forward so that the cup expands into a heart-shaped chamber.

As the posterior chamber expands forward, the anterior sphere gradually becomes smaller and perhaps somewhat flattened. However, the refractive index of the "sphere" does not appreciably change (Fig. 3 *E-G*). Meanwhile, the refractive

index of the posterior chamber continues to drop so that the boundary between the two chambers becomes increasingly more pronounced (Fig. 3 D–H).

The rate of expansion of the posterior chamber correlated closely with the growth of the acrosomal process. The height of the periacrosomal cup, i.e., the gap between the base of the recess in the nucleus and the posterior margin of the acrosomal vacuole (Fig. 3), is plotted in Fig. 4 against the length of the acrosomal process. As the acrosomal process becomes extended tens of micrometers, the gap expands from ~0.4 to 1.0 μm.

When the size of the gap is plotted against time in seconds after the acrosome has popped, the gap is seen to expand linearly for the first few seconds (Fig. 5). While the acrosomal process and the gap do not become visible until over a full second after the acrosome has popped, the size of the expanding gap interestingly extrapolates to zero just when the acrosome pops.

DISCUSSION

Interpretation of the Image

The application of high-extinction, differential interference contrast video microscopy has allowed us to visualize detailed structures within living sperm with unprecedented clarity. Video recordings of the *Thyone* sperm, triggered by the Ca^{++} ionophore in an appropriate perfusion chamber, has allowed us to analyze rapid, fractional-second events that take place within an individual sperm undergoing the acrosomal reaction.

While these observations serve to illustrate the power of modern video microscopy, and provide the data needed for analyzing the kinetics of acrosomal process growth (25), some of the optical and morphological changes that we found within the *Thyone* sperm were also quite unexpected.

Before considering these biological findings, however, we shall first examine the nature and reliability of the highly magnified video images that are produced by differential interference contrast microscopy (see references 16 and 21 for studies on the nature of DIC images). Much of our observations on the structural changes, and distribution of refractive indexes, in the reacting *Thyone* sperm were made on live and recorded video displays. Just how reliable are the enhanced and recorded video displays, and what can we deduce about the morpholog-
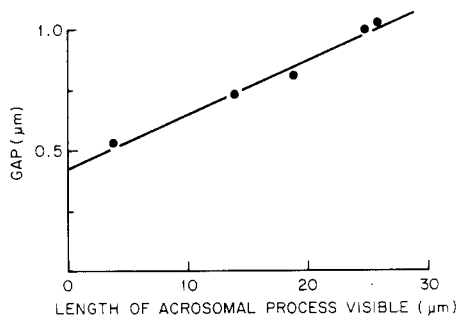

FIGURE 4 Height of the periacrosomal cup (i.e., the gap between the base of the recess in the nucleus and the posterior margin of the acrosomal vacuole) vs. length of the acrosomal process. The data were taken from the sperm illustrated in Fig. 3.
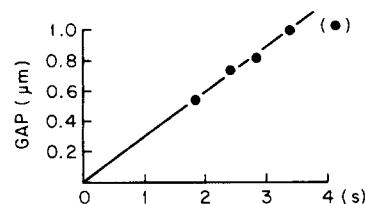

FIGURE 5 Height of periacrosomal cup (gap) plotted against time, in seconds, after popping. Data taken from the sperm shown in Fig. 3.

ical details of the specimen? How reliably can we deduce the distribution of refractive indexes from the differential interference contrast microscope images?

With video images in general, the distribution of image brightnesses seen by the video camera across a contrasting, or sharply bounded, edge may or may not be maintained in the image displayed on the monitor. The electronic frequency response characteristics of the video camera, recorder, and monitor can all modify the appearance of an edge, depending on the degree of contrast at that edge, the direction of the edge relative to the scan lines, the absolute brightness, and the electrical signal level. Edge sharpening, or compensating, circuits are commonly built into video equipment, especially the monitor, to sharpen up the edges and provide a crisper looking image.

In view of these characteristics of video systems, the interpretation of the image detail on the video monitor needs to be approached with some caution. We have examined the overall system performance of our microscope-optics-and-video-combination by examining the images of sharp edges of thin metal films deposited on microscope slides. Abbe test plates and some stage micrometers (e.g., those made by Bausch and Lomb Optical Company, Rochester, NY) are made in this fashion and provide cover slip thicknesses that match the optical corrections demanded by the objective lenses.

We find that with careful alignment of the light source and the optical axes of the condenser and objective lenses, and by eliminating the flare from various sources in the microscope (especially that arising from between the eyepiece and the video camera image tube), that we can obtain images that are reasonably reliable. For the video train, it was also necessary to keep the video signal at each stage within certain bounds, and to refrain from raising the monitor gain (contrast) too high. With too strong a video signal, horizontal bands of altered intensities appeared and, in the extreme, even distorted the vertical outlines of the image into wavy patterns. With too high a gain for the monitor, an electrical overshoot results in a spurious bright line following (i.e., in going from left to right on a normal monitor) a sharp transition from a dark to bright area in the image.

Once these distortions and spurious contrast arising from the instruments are reduced to an acceptable level, what information can we glean from the differential interference contrast video image?

In differential interference, contrast is provided by the gradient of optical path differences experienced in the specimen by two beams, or wave fronts, sheared laterally by slightly less than the minimum distance resolvable by the particular objective lens (20). Since the Optical Path = (Refractive Index) × (Thickness), the contrast depends on the cross-sectional shape of the edges or boundaries, and the gradients of refractive indexes in the specimen. The contrast in a differential interference microscope image also depends on the setting of the compensator and the direction of the boundaries, the contrast being maximum when the optical shear direction runs perpendicular to the boundary (2, 16, 21).

Once the intensity distribution in the image is provided by differential interference contrast, a psychological factor also enters into the viewing of the image (21). A circular grey disk with a light crescent on top and dark crescent below is interpreted as though it were a white sphere in a dark room illuminated from above. A white sphere illuminated from below, and the corresponding disk with a dark crescent on top,

are interpreted as hollow bowls in the absence of additional cues. The differential interference contrast image gives a similar sense of three-dimensional relief.

For interpreting the changes we see in the activated sperm head, we know e.g., that (a) the sperm head and tail have refractive indexes greater than that of the surrounding sea water, and (b) both are more or less cylindrically symmetric around the long axis of the sperm. Starting with these familiar relationships we can interpret the contrast distribution of other regions of the sperm seen in differential interference contrast with reasonable certainty.

Even before the sperm is exposed to the ionophore, the acrosomal region shows a faint relief, with the shadowing reversed from the sperm head and tail. Therefore, the refractive index of the unreacted acrosome is somewhat less than the index of the rest of the sperm head.

When the acrosome "pops", the shading of that region becomes more pronounced. Since the direction of the shading remains the same as with the unreacted acrosome, the popped acrosome must also have a refractive index below the surrounding parts of the sperm head, but with even a greater difference of refractive index. The refractive index of an organelle above that of the solvent ($\simeq$ water) is proportional to the solute concentration (13). Therefore, the contents of the early acrosome must have been diluted or swollen at popping.

Similar arguments hold for the periacrosomal compartment and its relation to the sperm nucleus, as well as to the forward sphere, or acrosomal vacuole.

In our observations, we do not think there is any ambiguity in the deductions we have made regarding the concentrations of material in various parts of the acrosomal region save one. That exception is just at the base of the extending acrosomal process, at the anterior tip of the acrosomal vacuole (Fig. 3 F). The region looks as though it were a "hole" that opens anteriorly, although that is probably not the correct interpretation. However, the shading and curvature of this particular minute region are so complex that the structure of the region cannot be interpreted unambiguously.

While we feel comfortable about the qualitative interpretations of the morphological and refractive index changes, we are not so sanguine about the quantitative measurements of the absolute sizes of the compartments in the sperm.

The location of sharp lines, or the distance between sharp boundaries, can generally be determined with a precision considerably greater than the diffraction limited optical resolution of the microscope. On the other hand, the boundaries we deal with in differential interference microscopy are graded and not sharply discontinuous (16). Enhancement with video could make the edges appear sharp and discontinuous, but the level of grey scale that gives rise to the discontinuity can be set arbitrarily. Even without such extreme enhancement, where do we define the edges of a sphere with greater or less refraction than its surroundings?

A simple experiment points up the difficulty and subjective nature of the criteria used for defining the edges. With a pair of precision micrometer calipers we measured the diameter or height of the various acrosomal regions of interest. Repeated measurements, e.g., of photographs shown in Fig. 3, generally agreed to within 2% of each other. That is so until the photographs are turned upside down and new measurements are made. Again, the repeated values agreed to about 2%, but not with the measurements made with the picture turned the other way around. Those two sets of values were easily off by 10 to 15%!

Therefore the quantitative statements made in this paper regarding the absolute dimensions inside the sperm head or acrosomal parts have less precision and are more meaningful in terms of relative changes. The 10 to 15% uncertainty experienced here, however, may not compare unfavorably with the errors encountered in determining absolute dimensions by conventional cytological studies and electron microscopy where errors can be introduced by fixation, dehydration, knife compression, and lens hysteresis.

Popping

In all of the many *Thyone* sperm that responded to the ionophore sea water and produced an acrosomal process, the acrosome popped, consistently and explosively, about 1–2 s before the acrosomal process came into sight. The behavior and duration of excitement of the sperm before popping and process extension varied somewhat. Therefore, it was generally not possible to predict beforehand exactly when a sperm would pop. While we often missed these events at first viewing, reviewing the video tape allowed us to study these early changes.

At first sight, the sudden pop, the bursting open into an inverted funnel, and associated forward spitting of the fine mist, might be taken to mean that the contents of the acrosomal vacuole are suddenly expelled at the start of the acrosomal reaction. Such a view would be consistent with the loss of the membranes at the anterior tips of the sperm and the acrosomal vacuole, as well as the loss of the acrosomal contents inferred from electron microscopy (e.g., reference 7, Fig. 6; reference 24, Fig. 6).

According to our present observations on living sperm, however, the initial drop of refractive index of the whole acrosomal region is rather slight, and can probably be accounted for by a roughly twofold increase in volume (the diameter increases by ~25%). It is not until a second or two later, and then only in the periacrosomal chamber, that a major drop in refractive index is observed. Furthermore, the spherical forward compartment of the acrosome, or the acrosomal vacuole, does not become appreciably less refractive after its initial change.

We therefore interpret the popping to indicate an initial slight swelling of the whole acrosomal region, including the periacrosomal cup. The bulk of the contents of the acrosomal vacuole are not expelled. The mist, the faint inverted funnel, and the pale minute vesicle that are sometimes seen, are possibly the membrane released from the acrosomal vacuole and the apex of the sperm when they fuse and dehiss (to use the Colwins' 1961 expression).

The Periacrosomal Cup

Earlier, Tilney demonstrated that the periacrosomal cup containing the profilamentous actin can be isolated as a compact structure seated in the pocket of the nucleus (22). Our present observation on living sperm shows clearly that the material in that region swells dramatically as the acrosomal process extends. The refractive index of the cup drops precipitously as the periacrosomal cup swells into a heart-shaped cavity. Therefore, in vivo, the periacrosomal material imbibes water rapidly during the acrosomal reaction. Within the expanding compartment, what appears to be the actomere and the base of the polymerizing actin filament bundle that polymerizes onto the actomere, are seen running through the compartment in favorable video frames (Fig. 3 D, H).

The Acrosomal Vacuole

As the acrosomal process extends and the periacrosomal chamber swells, the acrosomal vacuole, while becoming smaller, persists in front of the swelling periacrosomal chamber. Furthermore, the bulk of the material making up the vacuolar contents is not, as suggested by electron micrographs, expelled at the time of popping. Instead, the material is gradually lost without appreciable decrease in concentration after the initial influx of water at popping.

Interpretation of Structural Changes

Our observations on living sperm by video microscopy, coupled with the EM and physiological studies of earlier workers, lead us to the following schematic (Fig. 6) of the events that take place within the acrosomal region of an activated sperm.

(a) Associated with a change in the permeability of the sperm and acrosomal vacuolar membrane, the whole acrosomal region imbibes some water (as suggested by Dan et al., 10, 12) over an initial reaction period of 50 to 70 ms (Fig. 6 A and B).

(b) Early during this initial imbibition of water, the anterior portions of the sperm plasma membrane and vacuolar membrane dehiss (6), and the membrane is cast off explosively within a few milliseconds (Fig. 6 B). However, the contents of the acrosomal vacuole remain undischarged.

(c) As profilamentous actin is solubilized and released in the periacrosomal compartment, actin filaments polymerize onto the actomere (23) anchored to the base of the pocket in the nucleus (Fig. 6 B). The growing actin filament bundle pushes into, and deforms, the posterior surface of the acrosomal vacuole (Fig. 6 C).

(d) The posterior membrane of the vacuole is now contiguous at its anterior margin with the exterior plasma membrane of the sperm (Figs. 6 B and C, as emphasized by Colwin et al. [7] Fig. 1). The posterior vacuolar membrane is indented as a cap that covers the tip of the growing actin filament bundle and a thin layer of surrounding cytoplasm (Fig. 6 C).

(e) The periacrosomal material continues to imbibe water, pushing the acrosomal vacuole forward (Figs. 6 D and E).
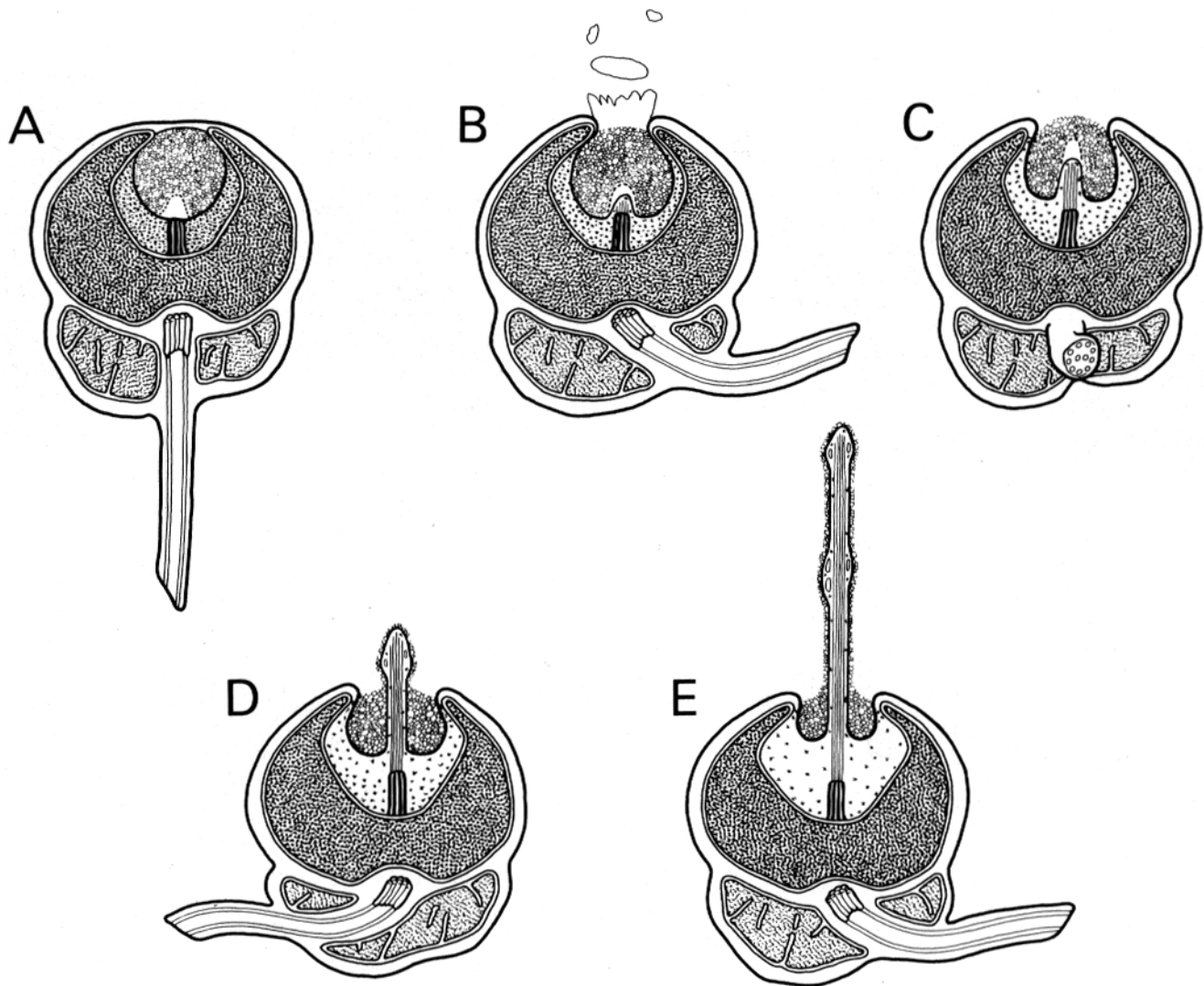

FIGURE 6 Schematics of structural changes in *Thyone* sperm undergoing the acrosomal reaction. The schematics were derived from our observation on living sperm by DIC video microscopy, combined with published EM and physiological accounts of the processes.

(*f*) Simultaneously with the extension of the actin filaments, the influx of water contributes to the growth of the acrosomal process through the donut-shaped acrosomal vacuole (Fig. 6 *D*).

(*g*) As the acrosomal process continues to extend, the external face of its membrane (the inverted membrane from the posterior part of the vacuolar wall) is coated with a thin layer of material arising from inside the acrosomal vacuole (Fig. 6 *E*).

The intermediate stages (Figs. 6 *B* through *E*) have not been seen by electron microscopy of sectioned sperm, even though our Fig. 6 *C*, for example, is superficially similar to Figs. 8–11 in Tilney (23). Those figures show actin filaments within a membrane bound tunnel piercing the acrosomal vacuole. Tilney's electron micrographs, however, show sections of *Thyone* sperm fixed 1.5 min after the addition of ionophore to sea water at pH 7.0. At this low a pH, the apical plasma membrane of the sperm and the vacuolar membrane do not dehiss but remain intact, and prevent the axial filament from extending beyond the apex of the sperm. Under this condition, the tip of the actin filament bundle is surrounded by three membranes: the sperm plasma membrane, the anterior, and basal parts of the acrosomal vacuole; the innermost of which is the inverted, extended membrane from the basal part of the vacuole. In this case, the contents of the vacuole naturally remain trapped in place.

Our observations on living sperm require that the contents of the acrosomal vacuole not be expelled as the acrosome pops. Rather, even after the vacuolar contents become exposed to sea water, they must remain in place for a few seconds until the vacuolar contents are drawn out and coat the growing acrosomal process.

The stages shown in Figs. 6 *B–E* may not have been seen by electron microscopy because: (*a*) these dynamic changes, which do not occur synchronously in different sperm, last for only a few seconds; (*b*) the contents of the acrosomal vacuole are not bounded anteriorly by a membrane. The fixative, or dehydrating, or other reagents may well extract and not preserve the exposed contents of the vacuole. Since no image corresponding to our video observations were found in EM thin sections deliberately prepared after the video observations, we feel that the second alternative is the more likely. We are now making attempts to capture these transient intermediate events by alternative methods of specimen preparation for electron microscopy.

We thank Ed Horn of the University of Pennsylvania for making the perforated slides used in the wedge perfusion chamber. We also thank

REFERENCES

1. Afzelius, B., and A. Murray. 1957. The acrosome reaction of spermatozoa during fertilization or treatment with egg water. *Exp. Cell Res.* 12:325–337.
2. Allen, R. D., G. B. David, and G. Nomarski. 1969. The Zeiss-Nomarski differential interference equipment for transmitted-light microscopy. *Z. Wiss. Mikrosk. Mikrosk. Tech.* 69:193–221.
3. Allen, R. D., N. S. Allen, and J. L. Travis. 1981. Video-enhanced contrast, differential interference contrast (AVEC-DIC) microscopy: a new method capable of analyzing microtubule-related motility in the reticulopodial network of *Allogromia laticollaris*. *Cell Motil.* 1:291–302.
4. Chambers, R. 1923. The mechanism of the entrance of sperm into the starfish egg. *J. Gen. Physiol.* 5:821–829.
5. Colwin, A. L., and L. H. Colwin. 1955. Sperm entry and the acrosome filament (*Holothuria atria* and *Asterias amurensis*). *J. Morphol.* 97:543–567.
6. Colwin, L. H., and A. L. Colwin. 1961. Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida) I. Passage of the acrosomal region through the vitelline membrane. *J. Biophys. Biochem. Cytol.* 10:231–254.
7. Colwin, A. L., L. H. Colwin, and R. G. Summers. 1975. The acrosomal region and the beginning of fertilization in the holothurian, *Thyone briareus*. *In:* The Functional Anatomy of the Spermatozoan. B. A. Afzelius, editor. Pergamon Press, New York. 27–38.
8. Dan, J. C. 1954. Studies on the acrosome II. Acrosome reaction in starfish spermatozoa. *Biol. Bull.* 107:203–218.
9. Dan, J. C. 1967. Acrosome reaction and Lysins. *In:* Fertilization I. C. Metz and A. Monroy, editors. Academic Press, New York. 237–293.
10. Dan, J. C., and Y. Hagiwara. 1967. Studies on the acrosome IX. Course of acrosome reaction in the starfish. *J. Ultrastruct. Res.* 18:562–579.
11. Dan, J. C., Y. Kakizawa, H. Kushida, and K. Fujita. 1972. Acrosomal triggers. *Exp. Cell Res.* 72:60–68.
12. Dan, J. C., Y. Ohori, and H. Kushida. 1964. Studies on the acrosome VII. Formation of the acrosomal process in sea urchin spermatozoa. *J. Ultrastruct. Res.* 11:508–524.
13. Davies, H. G. 1958. The determination of mass and concentration by microscope interferometry. *In:* General Cytochemical Methods. Vol. I. J. F. Danelli, editor. Academic Press, Inc., New York. 55–161.
14. Fol, H. 1879. Recherches sur la fécondation et le commencement de l'hénogénue chez divers animaux. *Mem. Soc. Phys. Hist. Nat. Genève* 26:89–397.
15. Fuseler, J. W. 1975. Mitosis in *Tilia americana* endosperm. *J. Cell Biol.* 64:159–171.
16. Galbraith, W., and G. B. David. 1976. An aid to understanding differential interference contrast microscopy: computer simulation. *J. Microsc.* 108:147–176.
17. Inoué, S. 1981. Video image processing greatly enhances contrast, quality, and speed in polarization-based microscopy. *J. Cell Biol.* 89:346–356.
18. Inoué, S., G. B. Borisy, and D. P. Kiehart. 1974. Growth and lability of *Chaetopterus* oocyte mitotic spindle isolated in the presence of porcine brain tubulin. *J. Cell Biol.* 62:175–184.
19. Moy, G. W., and V. D. Vacquier. 1979. Immunoperoxidase localization of bindin during adhesion of sperm to sea urchin eggs. *Curr. Top. Dev. Biol.* 13:31–44.
20. Nomarski, G. 1955. Microinterféromètre différential à ondes polarisées. *J. Phys. Radium* 16:9–13.
21. Padawer, J. 1968. The Nomarski interference-contrast microscope. An experimental basis for image interpretation. *J. R. Microsc. Soc.* 83:305–349.
22. Tilney, L. G. 1976. The polymerization of actin III. Aggregates of nonfilamentous actin and its associated proteins: a storage form of actin. *J. Cell Biol.* 69:73–89.
23. Tilney, L. G. 1978. The polymerization of actin V. A new organelle, the actomere, that initiates the assembly of actin filaments in *Thyone* sperm. *J. Cell Biol.* 77:551–564.
24. Tilney, L. G. 1979. Actin, motility, and membranes. *In:* Membrane Transduction Mechanisms. R. A. Cone and J. E. Dowling, editors. Raven Press, New York. 163–186.
25. Tilney, L. G., and S. Inoué. 1981. The acrosome reaction of *Thyone* sperm II. The kinetics and possible mechanism of acrosomal process elongation. *J. Cell Biol.* 93:820–827.
26. Tilney, L. G., and N. Kallenbach. 1979. Polymerization of actin VI. The polarity of actin filaments in the acrosomal process and how it might be generated. *J. Cell Biol.* 81:608–623.
27. Wilson, E. B. 1928. The Cell in Development and Heredity. *MacMillan, New York.*