Acrosomal Reaction of *Thyone* Sperm. II. The Kinetics and Possible Mechanism of Acrosomal Process Elongation

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ABSTRACT *Thyone* sperm were induced to undergo the acrosomal reaction with a calcium inophore A23187 in sea water containing 50 mM excess $CaCl_2$, and the extension of the acrosomal process was recorded with high-resolution, differential interference contrast video microscopy at 60 fields/sec. The length of the acrosomal process was measured at 0.25-s intervals on nine sperm. When the data were plotted as $(length)^2$ vs. time, the points fell exactly on a straight line except for the initial and very final stages of elongation. Cytochalasin B alters the rate of elongation of the acrosomal process in a dose-dependent way, inhibiting the elongation completely at high concentrations (20 μg/ml). However, no inhibition was observed unless excess Ca^{++} was added to sea water. The concentration of actin in the periacrosomal cup of the unreacted sperm is as high as 160 mg/ml; we calculate this concentration from the number and lengths of the actin filaments in a fully reacted sperm, and the volume of the periacrosomal cup in the unreacted sperm. These results are consistent with the hypothesis proposed earlier that monomers add to the ends of the actin filaments situated at the tip of the growing acrosomal process (the preferred end for monomer addition), and that the rate of elongation of the process is limited by diffusion of monomers from the sperm head (periacrosomal cup) to the tip of the elongating process.

During the extension of the acrosomal process, a few blebs distributed along its lengths move out with the process. These blebs maintain a constant distance from the tip of the growing process. At maximum length, the straight acrosomal process slackens into a bow, and numerous new blebs appear. A few seconds later, the process suddenly straightens out again and sometimes actually contracts. The behavior of the blebs indicates that membrane is inserted at the base of the growing acrosomal process, and that membrane assembly and water uptake must be coupled to actin assembly during elongation. We discuss how the dynamic balance of forces seems to determine the shape of the growing acrosomal process, and how actin assembly may be controlled during the acrosomal reaction.

One of the most fascinating biological processes yet described is the acrosomal reaction, a reaction the sperm undergoes when it comes in contact with the cellular material surrounding the egg. This reaction, which is particularly dramatic in invertebrate sperm, consists of the opening of the acrosomal vacuole, a reaction which can occur in less than 50 ms (7, 8, 15), followed by the formation of a process which can exceed 90 μm in length, yet forms in <10 s! The rate of elongation of this process is comparable to the rate of contraction of skeletal muscle. What is also appealing about this system is that this reaction occurs in a cell which is one of the simplest in the body and is designed to work just once. Thus the experimenter has some hope of really understanding what takes place, because not only can sperm be obtained in prodigious quantities, but they are so specialized that the reaction can easily be studied without the confusion of simultaneous reactions that occur in other cells.

According to earlier studies, the force needed to extend this process appears to reside in the explosive, yet controlled, assembly of actin that pushes out the acrosomal membrane (16, 19). The assembly of actin is initiated by a rise in the internal pH of the cell, followed by the formation of filaments that appear to be nucleated by a cytoplasmic organelle, the actomere (16, 20). In vitro and in vivo evidence suggests that

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the monomeric actin adds to the filaments at the tip of the elongating acrosomal process. Since a process 90-μm long can form in 10 s, the question immediately arises as to whether or not the monomer, liberated in the periacrosomal cup, has enough time to diffuse to the tip of the growing process. Using equations derived from Hermans (6), Tilney and Kallenbach (20) calculated that if the viscosity of the cytoplasm were that of water, then such a process could form in 12 s if the concentration of monomer in the sperm were 100 times that at the tip of the process. From Hermans' equations it is clear that if the elongation of the acrosomal process were limited by diffusion, then the length of the process plotted as a function of $(\text{time})^{1/2}$ would be a straight line, a prediction which we can now test by video recording the elongation of the acrosomal process.

Accordingly, we taped the elongation of the acrosomal process using differential interference contrast (DIC) optics and a video recording system that greatly increases the contrast and thus the visibility of this magnificent event (7, 8). When we plotted length vs. $(\text{time})^{1/2}$ from measurements of the video records, we found that indeed this plot gave a straight line. Addition of cytochalasin B markedly affected the rate of this extension and inhibited it altogether at high concentrations, as might be expected, since cytochalasin acts to cap actin filaments, thereby preventing further assembly (10). These results are consistent with the hypothesis that the elongation of the acrosomal process is a diffusion limited event.

In addition to these kinetic analyses, we observed the behavior of blebs that appear along the length of the acrosomal process. Based on these observations, we discuss (*a*) the site of insertion of membranes into the acrosomal process, and (*b*) the relative contributions of membrane addition, actin polymerization, and water influx, to the elongation of the acrosomal process.

MATERIALS AND METHODS

Details about procuring sperm from *Thyone briareus*, perfusion techniques, and the optical and video systems, are described in the preceding paper (8). We also describe there, how accurate measurements of the length of the growing acrosomal process, which is only 50-nm wide in places, can be made by video microscopy.

Induction of the Acrosomal Reaction

Thyone sperm were suspended in sea water containing 50 mM excess $CaCl_2$ (calcium sea water), placed on the perfusion slide, and perfused with calcium sea water containing the ionophore A23187 (Calbiochem, La Jolla, CA). 10 μl of a stock of 1 mg/ml A23187 in dimethyl sulfoxide was added to each 1 ml of calcium sea water.

For the experiments with cytochalasin B, we preincubated the sperm for 1 min in cytochalasin B (Calbiochem, La Jolla, CA) in calcium sea water, then induced the acrosomal reaction with the ionophore solution containing cytochalasin B. A stock of cytochalasin B (1 mg/ml) was prepared by dissolving it in dimethyl sulfoxide. We never added >20 μl of dimethyl sulfoxide to the sperm suspension, an amount that by itself had no effect on the sperm.

Electron Microscopy

Thyone sperm were suspended in calcium sea water and 10 μl of a 1 mg/ml stock of A23187 was added to each 1 ml of sea water. 1.5 min later the sperm were fixed by the addition of sufficient glutaraldehyde (an 8% stock from Electron Microscope Sciences, Fort Washington, PA) to the sea water to make the solution 1% glutaraldehyde. The sperm were fixed at room temperature for 30 min, concentrated by centrifugation, washed briefly in sea water, and post fixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.0 for 30 min at 0°C. The fixed sperm were washed three times in cold water and en bloc stained with 0.5% uranyl acetate overnight. They were then rapidly dehydrated in acetone and embedded in Epon 812 (Ernst Fullam, Inc., Burlington, VT). Thin sections were cut with a Sorvall Porter Blum II ultramicrotome (DuPont Co., Newtown, CT), stained with uranyl acetate and lead citrate, and examined in a Philips 200 electron microscope.

RESULTS

Induction of the Acrosomal Reaction

Thyone sperm were selected because they produce the longest (up to 90 μm) processes on record (4, 15) greatly exceeding those of starfish sperm (20 μm in length) or sea urchin sperm (<1 μm in length).

The ideal way to induce the acrosomal reaction would be to use natural stimuli such as eggs or the jelly coats which surround the eggs. Unfortunately, ripe eggs of *Thyone* are difficult to obtain. Thus it is necessary to induce the acrosomal reaction by artificial means such as with an ionophore. Induction of the acrosomal reaction of *Thyone* sperm with the ionophore A23187 in calcium sea water produces many processes 90 μm in length, most exceeding 50 μm, the length needed in nature for fertilization. Thus we believe we are recording a natural event.

Kinetics of Elongation of the Acrosomal Process

As we perfuse calcium sea water containing the ionophore past the sperm the first observable reaction is an increased motility of the flagellum of the sperm. Shortly thereafter, the acrosome pops; there is a rapid and dramatic change in the refractive index of the acrosomal region (see the accompanying paper [8]). ~1–2 s later the acrosomal process is detectable.

We taped the events of acrosomal process elongation in about 50 sperm and examined them qualitatively. Of these, we analyzed nine quantitatively, measuring the length of the acrosomal process every 15 video fields, or every 0.25 s. These sperm were selected because the tip of the process remained in focus so that accurate measurements could be made. An example of the type of image we measured is illustrated in Fig. 1. The montage was assembled from images of single fields of the video monitor photographed every 0.75 s.

In analyzing the kinetics of elongation of the acrosomal process, we found it more convenient to plot our data as $(\text{length})^2$ vs. time, rather than as length vs. $(\text{time})^{1/2}$, which is the immediate form expressing the diffusion limited relationship in Eq. 1 (see Discussion). The data from six sperm are illustrated in Fig. 2. What is amazing, is that, except for the early stages in induction or the very latest stages when the extension is essentially complete, all the values lie on a straight line consistent with the view that the extension of this process is diffusion limited. This is true for all nine sperm. To be sure that $(\text{length})^2$ vs time is really a straight line, we also plotted the data with length as a linear function of time. The points did not fall on a straight line, rather giving a curve with a sigmoid shape.

In six out of the nine sperm, the slopes of the plot, $(\text{length})^2$ vs time, were similar, ranging from 790 $\mu m^2/s$ to 960 $\mu m^2/s$ (Table 1) with a mean of 850 $\mu m^2/sec$. Four of these are illustrated in Fig. 2. Of the three remaining sperm, two produced only short processes (one is illustrated in Fig. 2—sperm *D*) and one produced a process 33 μm long (Fig. 2, sperm *F*). The slopes of these three sperm were about one half the slopes of the other six (Table 1).

Effect of Cytochalasin B on the Kinetics of Extension of the Acrosomal Process

For studying the effect of cytochalasin B, sperm were suspended in calcium sea water containing cytochalasin B and immediately introduced into the perfusion chamber. 1 min

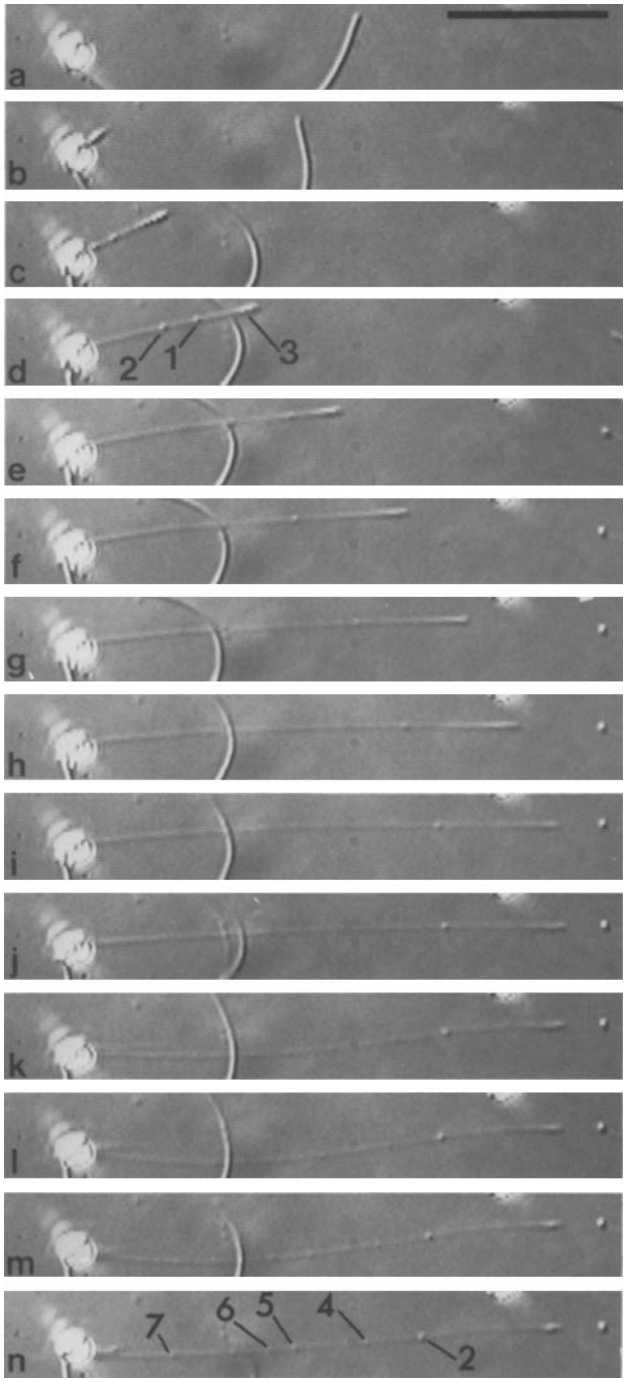


FIGURE 1 Stages in the elongation of the acrosomal reaction. Single fields of sperm C were photographed off the TV monitor every 0.75 s. Frame *a* was taken 1.95 s after the acrosome had popped. In frame *a*, the acrosomal vacuole has opened and just the beginning of a process can be seen. By frame *j*, the process has reached its maximum length. In *k*, the process begins to bow and blebs appear throughout the length. In *d*, the numbers indicate the three blebs whose motion relative to the tip is illustrated in Fig. 5 *a*, and in *n* the numbers indicate blebs whose motion relative to the tip is illustrated in Fig. 5 *b*. The jaggedness of the early, thick process seen in the frames *b*, *c*, and *d* result from the discrete steps of video scan that affect the edges that are oriented diagonally. Much of the jaggedness, therefore, does not represent real structural features. The distinct arc to the right of the sperm head is a portion of the sperm tail which is twitching as the process elongates. Bar, 20 μm. × 1,100.

later calcium sea water containing A23187 and cytochalasin B was perfused. The sperm reacted to the ionophore within a minute. With this cytochalasin B treatment, the maximum length attained by the acrosomal process, and the rate of elongation of the process, were markedly reduced as shown in Fig. 3. The effect of cytochalasin B concentration on the maximum length attained by the process is plotted in Fig. 4.

In these sperm, the $(\text{length})^2$ vs. time plot no longer fell on a straight line (Fig. 3), in marked contrast to sperm activated in the absence of cytochalasin B (Fig. 2). When length, rather than $(\text{length})^2$, was plotted against time, some of the data fit more closely to a straight line, but in almost all cases there were breaks in the curve where the rate of elongation was clearly changing. This was particularly clear in sperm undergoing the acrosomal reaction in low concentrations of cytochalasin B. Measurements of the rates of elongation show that, at low concentrations of cytochalasin B (i.e., 2 μg/ml), the maximum rate is similar to that of the slowly growing processes of untreated cells, i.e. 240–300 $\mu m^2/s$, but at higher concentrations of cytochalasin B (i.e., 3.3 or 5 μg/ml), the elongation rate was only 67–88 $\mu m^2/s$. When sperm were preincubated for as long as 3 min in calcium sea water containing 10 μg/ml cytochalasin B, no acrosomal process was formed when we perfused calcium sea water containing A23187 and cytochalasin B. Since this suggests that the cytochalasin B effect is limited by its permeability, we also tested the effect of applying cytochalasin B at the same time as A23187 in calcium sea water. In this case the acrosomal process grew longer and faster than when the sperm were preincubated with the same concentration of cytochalasin B. However, the $(\text{length})^2$ vs. time curves were irregular. For example, if 10 μg/ml of cytochalasin B were added at the same time as A23187 in calcium sea water, slopes of between 53 $\mu m^2/s$ and 23 $\mu m^2/s$ were recorded. Thus, the effect of cytochalasin is not instantaneous and it appears that time is needed for its penetration.

When excess calcium was not supplied, cytochalasin B had no effect on the acrosomal process elongation. When *Thyone* sperm, preincubated with cytochalasin B in normal sea water, were treated with cytochalasin B and A23187 in normal sea water, the acrosomal process grew to their normal lengths, even though the cells were preincubated in cytochalasin B for periods of up to 10 min. They also grew at the normal rates, even though we used concentrations of cytochalasin B as high as 10 μg/ml. In other words, it is necessary to have Ca^{++} in excess of the concentration present in sea water for cytochalasin B to exert its effect on the elongation of the acrosomal process.

Changes in the Surface Morphology of the Acrosomal Process during Elongation

In the early stages of growth, the acrosomal process extends slowly, is fat and nonuniform in diameter (Fig. 1 *b*). As the process grows, the rate of elongation accelerates. The process becomes slender and more uniform in diameter except at its somewhat bulbous, spear-shaped tip, and in a few locations along the length marked by spherical "blebs" (Fig. 1 *d*). Once in the rapid growth phase, the contour of the slender process is nearly straight, or barely curved.

As the acrosomal process elongates, the blebs move forward with the process. During the earlier phases of process elongation, the distances between the tip of the process and the blebs, and in between adjoining blebs, increase somewhat (Fig. 1 *c–e*). Some of blebs also disappear, leaving a smooth uniform

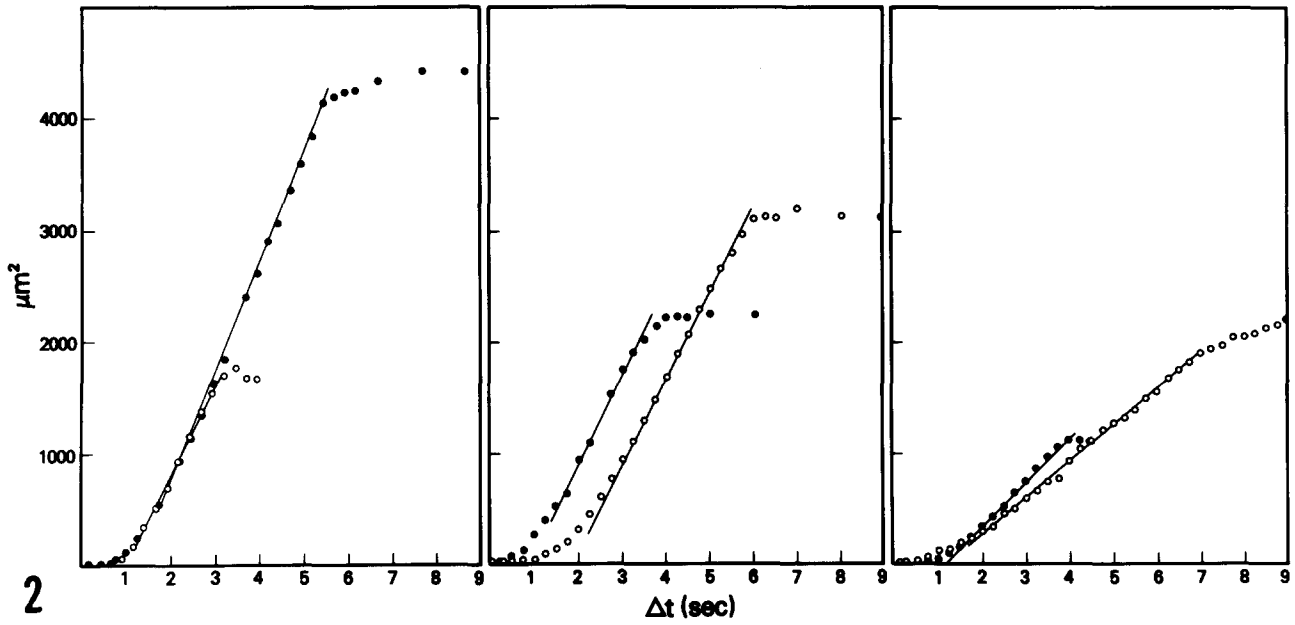


FIGURE 2 Plots of (length of the acrosomal process)^2 in square micrometers as a function of time in seconds after the process first becomes visible. Of note is that, except for the beginning and very end of the reaction, the points fall exactly on a straight line. In the left hand curve sperm B (closed rcircles) and J (open circles) are illustrated; in the middle, we see sperm D (closed circles) and C (open circles); and in the right hand graph, sperm F (closed circles) and A (open circles) are seen.

TABLE I

Analysis of the Kinetics of Elongation of the Acrosomal Process

Sperm	Rate of elongation	Maximum length
	$\mu m^2/s$	μm
B	960	67
E	950	62.5
D	810	47
J	800	41
H	800	38
C	790	56.5
G	420	28
F	400	33
A	330	47

contour as though the bleb were incorporated into the growing acrosomal process (Fig. 1 *d–f*); these blebs seem to flow forward and disappear at the same time. However, once in the rapid growth phase, the distance between the tip of the process and the persisting blebs remains unchanged (Figs. 1 *e–i*, and 5 *a*).

Eventually, the process stops elongating (Fig. 1 *j*), and about a second later tends to slacken into a bow (Fig. 1 *k*). As the acrosomal process starts to bow, new blebs become visible along the length of the process (Fig. 1 *k*).

Many of these blebs glide backwards towards the sperm cell body. While all of these blebs tend to move backwards, they move at different times and at different rates. Thus, the distances between some blebs increase while others decrease (Fig. 5 *b*). The fully bowed process is covered by many blebs interspersed with slender regions whose thickness appears to be fairly uniform (Fig. 1 *l*).

Finally, ~10 to 12 s after the acrosome had popped, the process abruptly (in ~50 ms) straightens out as though contracting (Fig. 1 *n*); in some cases the process in fact shortens somewhat. All of these changes in the structure of the acrosomal process are observed whether or not the process sticks to the cover glass or is freely waving in the solution.

Concentration of actin in the Sperm Before Activation

Since we can determine (*a*) the volume occupied by the unpolymerized actin before activation, (*b*) the number of actin

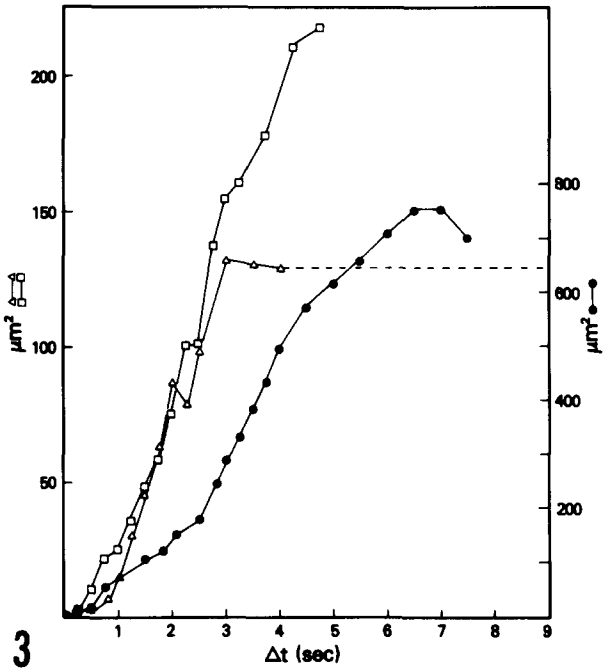


FIGURE 3 The (length of the acrosomal process)^2 in square micrometers plotted as a function of time (as in Fig. 2) for *Thyone* sperm treated with cytochalasin B. Sperm were treated in calcium sea water containing cytochalasin B for 1.5 min, and then acrosomal reaction was induced with A23187 in the presence of cytochalasin B. 2 μg/ml of cytochalasin B (closed circle), 3.3 μg/ml (squares), 5.0 μg/ml (triangles).

filaments in the acrosomal process, and (*c*) the final length of the acrosomal process, we can calculate the approximate concentration of actin in the periacrosomal cup before activation of the sperm. The unpolymerized actin is contained in the periacrosomal cup, in the form of profilactin, before activation of the sperm (15).

The volume of the periacrosomal cup is calculated as follows. From longitudinal sections through a sperm head of *Thyone* such as is illustrated in Fig. 2 of Tilney (15), and Fig. 1 of the accompanying paper (8), we see that the acrosomal region, i.e., the space occupied by the acrosomal vacuole and the periacrosomal cup, is nearly spherical. By subtracting the volume occupied by the spherical acrosomal vacuole from the total volume of the acrosomal region, we can determine the volume of the periacrosomal cup, or the space occupied by the profilactin. To be more specific, we determined from electron micrographs that the diameter of the vacuole was 0.49 μm, and the diameter of the acrosomal region including the vacuole was 0.76 μm. Therefore, the volume occupied by the profilactin is 1.68×10^{-13} cm^3. This value is actually too small because we know that the tissue shrinks during dehydration and embedding for electron microscopy. The amount of shrinkage depends on a number of factors, but by comparing recent optical and x-ray diffraction patterns of unfixed and dehydrated actin paracrystals (18) with optical diffraction patterns of electron microscope (EM) thin sections, we know that there is a 20 to 40% (linear) shrinkage in the thin sections. Shrinkage can also be calculated by comparing electron micrographs with light micrographs of living sperm. In the living sperm, the unpopped acrosomal region measures 1.1 μm in diameter in the differential interference contrast image (see Fig. 3, and Discussion in the accompanying paper [8]). Therefore, we calculate a shrinkage of 30% ([1.1 − 0.76]/1.1). This is in agreement with the value determined from the diffraction studies. If we take this 30% shrinkage in diameter into account, the volume occupied by the profilactin region in the living sperm should be 5×10^{-13} cm^3.

Since we have been unable to determine the exact amount of actin per sperm chemically or from serial sections, we

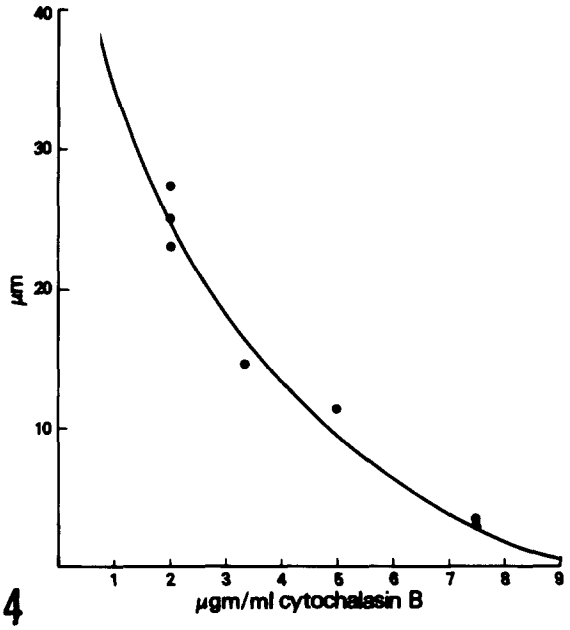
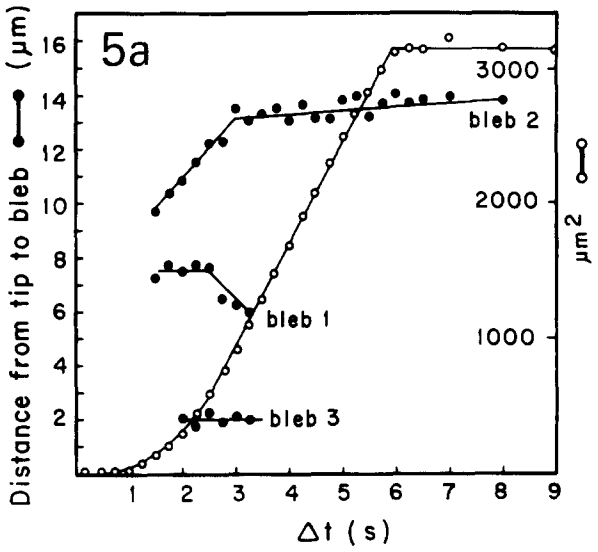


FIGURE 4 The final length of the acrosomal process in micrometers as a function of the concentration of cytochalasin B in μg/ml.



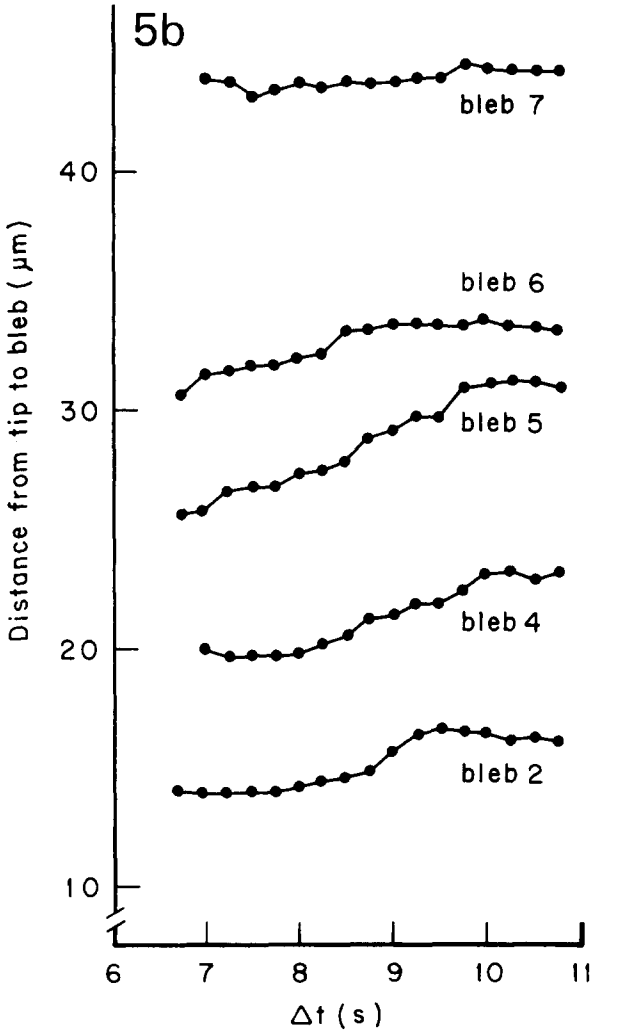


FIGURE 5 (*a*) The distance of the blebs from the tip of the process (μm) plotted as a function of time after the process first becomes visible (closed circles). The blebs considered here are seen in Fig. 1 and are indicated in Fig. 1 *d*. This is sperm C. The (length of the process)^2 as a function of time is illustrated in this figure (opened circles) as well as in Fig. 2. (*b*) Continuation of *a* showing the behavior of blebs after the acrosomal process has maximally elongated. Blebs 4 through 7 first become visible at ~7 s after the acrosomal process first appeared.

approached the measurement in the following way. The number of actin filaments were counted in electron micrographs of transverse sections cut through the acrosomal process. While a large number of sections were examined, most could not be used for counting since they were not cut exactly normal to the filament axis. Fig. 6 shows the sections that were used; we counted 18, 30, 47, 50, 58, 90, and 150 filaments in these sections. The transverse sections with the largest number of filaments are located near the sperm nucleus, or the basal portions of the acrosomal process. Sometimes it is difficult to judge whether a bit of density is a filament or not, particularly if it is not cut perfectly normal to its axis, but we believe that our values are no more than 20% off the real value. Taking these factors into consideration, we picked an average number of 60 actin filaments per process. The average lengths of the processes were estimated from living sperm to be ~50 μm. Accounting for the variation in filament lengths, we judged that the total length of actin filaments per process is ~50 μm $\times$ 60 = 3,000 μm. Since each micrometer of an actin filament contains ~370 monomeric units (1), the total number of actin monomers polymerized in a sperm would be 1.1×10^6.

From the volume of the periacrosomal cup (5×10^{-13} cm^3), the number of actin monomers (1.1×10^6), and the molecular weight of actin (43,000), we calculate that the concentration of actin in the periacrosomal cup of unreacted sperm is 160 mg/ml.

In addition to actin, SDS polyacrylamide gels of the periacrosomal cup material show that there are four other proteins present (16). These are: a 16,000-dalton protein in a 1:1 stoichiometry with actin, 250,000- and 220,000-dalton proteins each in a 1:12 stoichiometry with actin, and a 25,000-dalton protein in a 1:4 stoichiometry with actin (unpublished observations and reference 16). On a weight basis, these proteins would contribute 210 mg/ml. Thus we calculate that the total protein concentration, i.e., the concentration of profilactin, in the periacrosomal cup is 370 mg/ml.

It is interesting to compare these values, calculated for the concentrations of actin and profilactin in the undischarged periacrosomal cup, with the value that one would calculate for actin molecules that were packed tightly together. Monomeric actin is considered to have the following dimensions: $5.5 \times 4.0 \times 3.3$ nm^3 (reference 1). Taking Matthew's (9) value of 43% for the volume occupied by solvent, each molecule would occupy a volume of 1.27×10^{-19} ml; or 7.87×10^{18} actin molecules would fit into 1 cm^3. This gives a concentration of 564 mg/ml. Therefore, the concentration of 370 mg/ml that we calculate for profilactin, although seemingly high, is plausible.

DISCUSSION

Kinetics of Elongation of the Acrosomal Process is Consistent with the Diffusion of Actin to, and Their Assembly on Filaments at, the Tip of the Process

According to Hermans (6), the linear diffusion of particles that are trapped, and eliminated from the pool, produces a sharp moving boundary beyond which no particles are found. In such a system, he calculates that the distance traveled (ξ) should be proportional to (time)$^{1/2}$. Thus,

$$\xi = 2 \times Z \times (D \times t)^{1/2} \qquad \text{(Eq.1)}$$

where t is time.

D is the diffusion constant, and Z is a constant that relates to concentration ratios of the diffusing substance at a particular point and at the source, and to the "error function" (18).

Hermans (6) verified his theoretical calculations with the following experiment. He immersed a glass capillary, filled with iodine bound to starch and dispersed in a 10% gel of gelatine, into a solution of thiosulphate. The penetration of the thiosulphate, which combines with and bleaches the iodine, is indicated by a sharp boundary that he measured with a microscope. A plot of the boundary vs. (time)$^{1/2}$ "proved to be perfectly linear." Similarly, if the elongation of the acrosomal process is limited by the diffusion of actin to the tip of the process, a plot of the length of the acrosomal as a function of $t^{1/2}$, should also produce a straight line.

As reported in Results, we found that the data do indeed fall on a straight line, except at the beginning and at the very end of the reaction. These data, then, are consistent with the hypothesis suggested by Tilney and Kallenbach (20) that the reaction is diffusion-limited—limited by the diffusion of actin monomers from the sperm proper (the periacrosomal cup) to the tip of the elongating process where they assemble on the tip of the extending filaments.

If, on the other hand, the actin monomers were uniformly

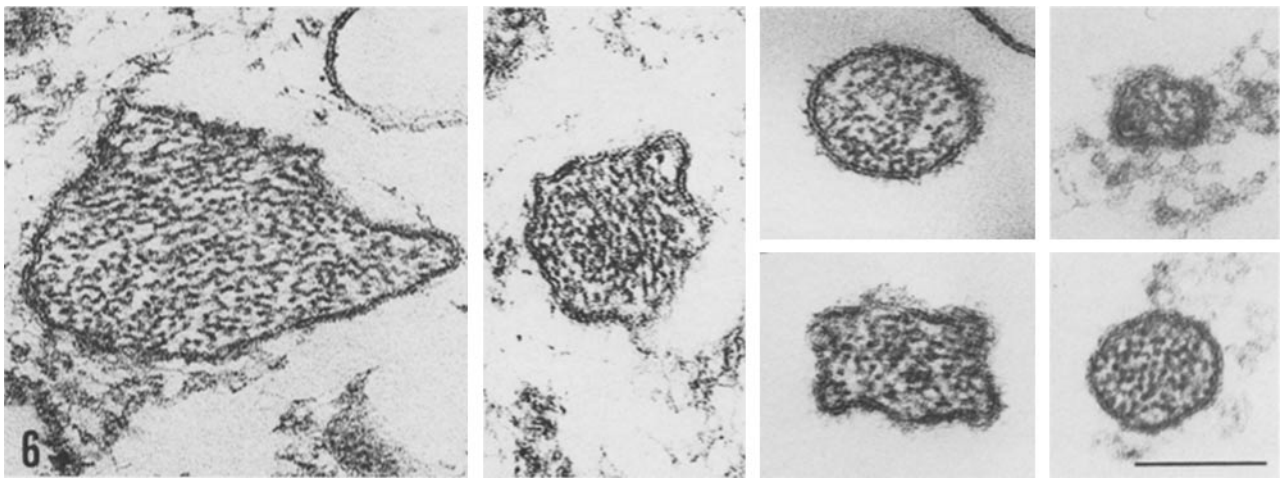


FIGURE 6 Electron micrographs of transverse sections cut through the acrosomal process of *Thyone* sperm that had been induced to undergo the acrosomal reaction with A23187 in Ca-sea water. The dots within the membrane are cross sections of actin filaments. Bar, 100 nm. × 175,000.

distributed throughout the acrosomal process, one would expect the rate of actin assembly to follow a pseudo-first order reaction that reflects the reduced concentration of monomer with time, as in fact is observed for actin polymerization in vitro (5). Our data plotted in the same manner as Cooke, i.e., as log (L_{max} − L) vs. time, where L is length of the acrosomal process, is "parabolic" and not even close to a straight line.

It is remarkable that the data should fall so precisely on a straight line that fits the relationship specified in Eq. 1. This implies that D and Z in fact do remain unchanged. This is a somewhat surprising conclusion, since the constancy of Z depends on the concentration of the diffusable actin in the periacrosomal cup to remain unchanged. Upon reflection, however, it is entirely possible that the water and ion influx into the periacrosomal cup (8, 21) modifies the actin-associated proteins, resulting in the release of an approximately constant concentration of diffusable actin (see Discussion on control of actin assembly).

Consistent with the interpretation that monomers assemble on the elongating tips of the actin filaments, is our experiment with cytochalasin B. Several investigators have reported that this drug acts by capping the actin filaments, or more specifically, the "preferred end" for addition of monomers to actin filaments, thus preventing further elongation (10). This would explain why the elongation of the acrosomal process is inhibited in a dose dependent way by cytochalasin B.

It is interesting that cytochalasin B without excess calcium has no effect on the elongation of the acrosomal process discharging in natural sea water. This result is consistent with the observations of Sanger and Sanger (13). However, if we supply additional calcium to the sea water, cytochalasin B is now effective at slowing the rate, decreasing the length, and at high concentrations entirely inhibiting the elongation, of the acrosomal process. Since extra calcium in the sea water increases the length of the process in sperm induced to discharge with an ionophore, and in fact causes some sperm to spontaneously fire, we presume that the extra calcium may render the sperm sensitive to cytochalasin B by making them more permeable to the drug.

Addition of New Membrane Appears to Occur at the Base of the Elongation Process

In an earlier publication, Sardet and Tilney (14) demonstrated that an enormous amount of "new" plasma membrane must appear during the acrosomal reaction: the membrane covering the head of the sperm must increase by 140%. More specifically, these investigators calculated that there is a 16 μm^2 of membrane surface in the unreacted *Thyone* sperm head, which must increase to 38 μm^2 at the completion of acrosomal process elongation (17).

In the present study, blebs were encountered along the elongating acrosomal process. A bleb appears to be a ballooning of the plasma membrane that surrounds a puddle of cytoplasm (4). Consistent with this interpretation is our observation that blebs spontaneously appear and are reabsorbed during elongation (also see next section of Discussion).

Of particular interest to this report is that the distance from the persistent blebs to the tip of the process essentially remains constant during the major period of process elongation. Since we believe that the axial filaments are growing by addition at their tips, the blebs must be sliding forward over the filaments at the same rate that the tip is growing. If the bleb remained attached to the filament core, or if insertion of membrane occurred at the tip of the process, the distance between the bleb and the tip of the process should increase. In other words, the blebs should remain stationary relative to the sperm head rather than traveling forward with the growing tip of the acrosomal process. The most straightforward interpretation of this observation is that cytoplasm and new membrane are added at the base of the acrosomal process. Thus, even though actin seems to assemble at the growing tip, new membrane seems to be inserted at the base of the growing process.

Balance of Forces That Appear to Determine the Shape of the Growing Acrosomal Process

In a fascinating classical discourse on soap bubbles, Boys discusses the influence of surface tension on the shape of fluid cylinders (2). He points out that a thin spider web or quartz fiber that is coated with oil or other liquids, initially acquires a thin smooth coat of the liquid, but that the liquid quickly transforms into a series of small beads interspersed by thinner cylinders (reference 2, Fig. 39). Boys points out that the initial coat is unstable and cannot remain a cylinder, and therefore breaks up into beads. This is explained by the fact that a liquid cylinder, bounded by surface tension, is unstable when the length of the cylinder is greater than π times its diameter.

We propose that this same explanation accounts for the appearance of blebs on the acrosomal process. When the process first appears, it grows slowly (Fig. 2), is fat and club-shaped, and is covered with many blebs. (The still pictures in Figs. 1 *b* and *c*, show some of the early blebs, but more blebs are visible on the monitor when the video tape is running.) As the process grows more rapidly, several of the blebs elongate forward and their contours become indistinguishable from the contour of the thin, smooth region of the process. That would be expected if the growing axial filament bundle were drawing the cytoplasm and membrane forward. Shortly after the process stops elongating, and bows, many blebs appear along the whole length of the process.

We interpret these observations in the following manner. The slender, smooth contour that is seen during rapid elongation of the acrosomal process reflects the uniform, thin layer of acrosomal cytoplasm and fluid plasma membrane that are being drawn forward by the growing tip of the axial filaments, sliding over the basal parts of the filaments. When the cytoplasm and membrane are supplied more rapidly than they are drawn out by the growing tip of the filament bundle, beads appear as the fluid membrane seeks a more stable, surface configuration.

Translating this explanation to the early stages of acrosomal process growth, the volume of the acrosomal cytoplasm increases (due to the influx of water into the periacrosomal cup [8]), as the axial filaments polymerize and extend. The acrosomal process grows as a fat rod, since the rate of water influx exceeds the rate of filament extension. Therefore, the filaments are coated by an excess of cytoplasm and membrane which form blebs.

As the rate of filament growth accelerates, the cytoplasm and membrane are added to the process at just below the rate determined by the growing axial filaments. The filaments are thus coated by a smooth, uniform layer of cytoplasm and membrane, and even some of the existing blebs are drawn out.

When the filaments stop extending, the cytoplasm and membrane cease to be drawn out over the axial filaments and the membrane apparently shifts towards its minimum surface energy configuration; many blebs appear concurrently with the

bowing of the acrosomal process. It is during this process that the blebs glide a short way back towards the cell body.

As described in Results (Fig. 5 *b*), different blebs on the same acrosomal process glide at various rates, to various extents, and at slightly different times. Thus, some blebs move farther apart and others move closer together until they finally end up in stable positions. This dynamic behavior clearly shows that the blebs are not clumps of cytoplasm that are fixed onto the axial filaments.

About the time the blebs have become stabilized, we have seen a sudden shortening, or straightening, of some acrosomal processes. While the degree of shortening is not great, the speed reminds one of a muscle twitch. We have no idea what mechanism could account for this contraction.

In summary, we propose that the shape of the slender acrosomal process is governed by a dynamic equilibrium between: (*a*) the extending filaments of actin that are polymerizing at the tip of the process; (*b*) the surface tension of the fluid plasma membrane that is being replenished at the base of the extending acrosomal process; (*c*) the drawing out of the acrosomal cytoplasm by the tip of the growing axial bundle; and (*d*) the influx of water. The rate of water influx and consequent swelling of the contents of the periacrosomal cup, described in the accompanying paper (8), presumably govern the rate of supply of the cytoplasm into the acrosomal process.

How Might the Assembly of Actin be Controlled during the Acrosomal Reaction

In an earlier publication, Tilney et al. (21) presented evidence that a change in the internal pH was necessary for actin assembly. The rise in pH presumably releases the actin from its storage form so that it can assemble into filaments. In the present study, we demonstrated that the concentration of diffusable actin at the base of the elongating process is enormous, being at least 160 mg/ml. At these concentrations, the actin would nucleate spontaneously unless some controls are present. Thus monomeric actin would never be able to diffuse to the tip of the process; instead the monomers would be assembled directly into filaments inside the periacrosomal cup. Since that does not happen, the cell must control two events: (*a*) The release of monomeric actin from its storage form, and (*b*) inhibition of spontaneous nucleation of the released actin.

Tilney earlier showed that the cup of profilactin contains, besides actin, four additional proteins whose molecular weights are 16,000, 25,000, 230,000, and 250,000 (16). Preliminary evidence indicates that the 16,000-dalton protein resembles the profilin described by Carlsson et al. (3), Reichstein and Korn (11), and Runge et al. (12). Although not precisely the same molcular weight as profilin in amoebae (see Runge et al.), it lowers the rate of actin assembly in vitro, without affecting the critical concentration for actin assembly. It also occurs in a 1:1 stoichiometry with actin. One hypothesis then is that the 16,000-dalton protein in *Thyone* sperm decreases the rate of nucleation of actin and, at the same time, being small, would not appreciably affect the diffusability of actin. The small size is important, as a larger molecule or molecules could drastically change D in Eq. 1. The other proteins present may singly or collectively form an insoluble complex with actin, sequestering it in the unreacted sperm.

Thus our scheme for filament formation requires a two-step reaction. The first step releases the actin from an insoluble complex. The actin, still bound to an inhibitor of nucleation, then diffuses to the tip of the elongating process. There it assembles onto the extending filaments. As discussed in the previous section, a delicate balance between the extending actin filaments, membrane addition, and water influx, appears to govern the shape of the growing acrosomal process.

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