

Acrosomal Reaction of the *Thyone* Sperm.

III. The Relationship between Actin Assembly and Water Influx during the Extension of the Acrosomal Process

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ABSTRACT In an attempt to investigate the role of water influx in the extension of the acrosomal process of *Thyone* sperm, we induced the acrosomal reaction in sea water whose osmolarity varied from 50 to 150% of that of sea water. (a) Video sequences of the elongation of the acrosomal processes were made; plots of the length of the acrosomal process as a function of (time)^{1/2} produced a straight line except at the beginning of elongation and at the end in both hypotonic and hypertonic sea water (up to 1.33 times the osmolarity of sea water), although the rate of elongation was fastest in hypotonic sea water and was progressively slower as the tonicity was raised. (b) Close examination of the video sequences revealed that regardless of the tonicity of the sea water, the morphology of the acrosomal processes were similar. (c) From thin sections of fixed sperm, the amount of actin polymerization that takes place is roughly coupled to the length of the acrosomal process formed so that sperm with short processes only polymerize a portion of the actin that must be present in those sperm. From these facts we conclude that the influx of water and the release of actin monomers from their storage form in the profilactin (so that these monomers can polymerize) are coupled. The exact role of water influx, why it occurs, and whether it could contribute to the extension of the acrosomal process by a hydrostatic pressure mechanism is discussed.

When a *Thyone* sperm comes in contact with the outer surfaces of an egg, a series of reactions are elicited which culminate in the growth of a process which can form in less than 10 s, yet exceed 90 μm in length. In fact, the rate of elongation of this process, the acrosomal process, is comparable to the rate of contraction of some of the fastest skeletal muscles. Of the many questions that one could ask about this extraordinary motile event perhaps the most basic is, What generates the force required to drive the extension process? In earlier publications, we demonstrated that at all stages during the growth of the acrosomal process there is an explosive, yet controlled assembly of actin and concluded that the assembly per se provides the force for the elongation of the process. The assembly of actin is initiated by a rise in internal pH (20); this is followed by the nucleation of actin monomers on a cytoplasmic organelle, the actomere, which simultaneously nucleates and directs the assembly of filaments (19) to form a directed spear used to penetrate the jelly which surrounds the egg. Thus, it is clear that actin polymerization is involved

in the generation of the acrosomal extension, but there remains the mechanical issue of how a polymerization reaction can "push" a membrane protuberance in front of it, particularly as we know that the site of assembly of monomers to the elongating filaments occurs at the membrane protuberance (17, 18).

Beginning 20 years ago, Dan and her co-workers (4, 5) published two detailed fine structural studies on the early events in the acrosomal reaction. They demonstrated in sea urchin sperm that there is roughly a threefold increase in volume in the region which will be occupied by the actin filaments (5) and an even larger increase in volume in starfish sperm (4) and suggested that water influx may play the "major role in extending the process membrane" by an osmotic mechanism. More recently, we recorded and analyzed, through the use of high extinction video microscopy on living sperm, sequences in the initial formation (11) and elongation of the acrosomal process (18); we found that within 50 ms after induction, there is a roughly twofold increase in volume

in the acrosomal region and that within the next 1–2 s there is a precipitous drop in the refractive index of the periacrosomal region, the region from which actin monomers are released to polymerize into filaments. We observed, in association with this decrease in dry mass, a pronounced increase in volume of the periacrosomal region. Therefore, in vivo, the periacrosomal material imbibes water and the elongation of the acrosomal process is accompanied by a large increase in volume due to an influx of water into the cell.

Based on these results, Oster et al. (13) suggested that the entire “extension process may be osmotically driven and that actin polymerization merely ‘keeps up’ with the extension reinforcing it against bending moments.” Accordingly, they proposed a simplified mathematical model based upon hydrostatic pressure that was consistent with the kinetic data on the rate of elongation of the acrosomal process as previously measured (18). If hydrostatic pressure plays a role in the elongation of the acrosomal process, then one would predict that changes in the tonicity of the medium outside the sperm should greatly affect the rate and kinetics of elongation of the acrosomal process, parameters that we can accurately measure using the high extinction video microscopy techniques already mentioned.

In an earlier publication in this series (18), we demonstrated that a plot of the length of the acrosomal process as a function of $(\text{time})^{1/2}$ produces a straight line except at the beginning of elongation and at its completion. These observations are consistent with a model in which actin monomers, when released from their bound state adjacent to the nucleus, diffuse to the tip of the elongating acrosomal process where they assemble on the growing filaments, a model that is now confirmed by recent in vitro observations (17).

In this publication, we will show that we can markedly affect the rate of elongation of the acrosomal process by changing the osmolarity of the sea water surrounding the sperm, though plots of the length of the acrosomal process as a function of $(\text{time})^{1/2}$ show a straight line if one ignores the beginning and end of elongation. Thus, the rate of release of monomer from its bound state (so that it can migrate to the tip of the elongating process in order to polymerize) is tightly coupled, not only to the rate of elongation of the acrosomal process, but also to the tonicity of the medium or, in other words, to water entry. This surprising result reinforces the concept that water entry, perhaps hydration of the storage form of unpolymerized actin in the cell, may play a pivotal role in the elongation of the acrosomal process.

Such considerations as these on the relative contributions of osmotic forces and actin assembly on motile processes are, in fact, timely because several reports have appeared implicating that osmotically driven mechanisms may be operational in actin-related events such as blebbing (7), lamellipod extension (6), and the formation of sea urchin microvilli (1).

MATERIALS AND METHODS

Obtaining Sperm: *Thyone briareus* were collected by the Marine Resources Department, Marine Biological Laboratories, Woods Hole, MA. The testes were removed and minced in sea water. The suspension was filtered through cheese cloth and the supernate was 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.

Induction of the Acrosomal Reaction: *Thyone* sperm were suspended in sea water, introduced into the perfusion slide, and perfused for 1.5 min in sea water which contained mannitol or sucrose, or sea water which had been made hypotonic by the addition of distilled water. We added 50 mM

excess CaCl_2 to these perfusing solutions because we found earlier that ionophore-induced discharges were longer and more reproducible in excess CaCl_2 (18). After 1.5 min, we added to each milliliter of the perfusate 10 μl of a 1-mg/ml stock solution of the ionophore, A23187 (Calbiochem-Behring Corp., San Diego, CA), dissolved in dimethylsulfoxide.

Perfusion Chamber: The design of the chamber is described in detail in a previous article (11).

Video Observations and Analysis: Details of the video techniques and differential interference microscopy used here are described by Inoué (10) and Inoué and Tilney (11). For our kinetic analysis we rerecorded the video sequence on a video motion analyser (Sony SVM1010). We studied the 10-s sequence stored on the disk in the analyser either field by field or at various speeds backwards and forwards. The lengths of the acrosomal processes were determined from frozen images of the video tape record. X and Y video markers were positioned at the tip and the base of the acrosomal process and their coordinates recorded together with the time of the scene (Colorado Video No. 321). 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 sequence 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.

Electron Microscopy: *Thyone* sperm were suspended in sea water or sea water which contained 1 M sucrose or distilled water and excess CaCl_2 for 1.5 min; the acrosomal reaction was then induced with 10 μl of A23187 dissolved in dimethylsulfoxide per milliliter of sperm suspension. After 1.5 min, the sperm were fixed by the addition of sufficient glutaraldehyde (an 8% stock from Electron Microscope Sciences, Fort Washington, PA) to the solution 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 postfixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.2 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 rapidly dehydrated in acetone and embedded in Araldite 502.

It became apparent that although the above fixation procedure preserved actin filaments satisfactorily, it did not stop the swelling that occurs when the sperm suspension in 50% sea water remains in the ionophore-glutaraldehyde mixture. Therefore, towards the end of this study, we carried out a different protocol. Sperm were handled as before, however, 30 s after the addition of the ionophore to the suspension, we added an equal volume of fluid that contained two parts of what was in the suspension, one part of 4% OsO_4 in sea water, and one part of 4% glutaraldehyde. Thus, the final fixation solution contained 1% OsO_4 and 1% glutaraldehyde in sea water. The fixatives were made up just before use and fixation was carried out for 2 min at 0°C. After 2 min, the sperm were concentrated by centrifugation at 2,000 g for 2 min and the fixative was changed to 1% OsO_4 in 0.1 M phosphate buffer at pH 6.2. Fixation in this new medium was continued for 25 additional minutes. The sperm were then washed three times in cold water and en bloc-stained in 0.5% uranyl acetate for 3 h. They were rapidly dehydrated in acetone and embedded in Araldite 502.

RESULTS

The Kinetics of Elongation of the Acrosomal Process

As documented in an earlier publication (18), *Thyone* sperm were induced to undergo the acrosomal reaction with the ionophore, A23187, in high calcium sea water. As this solution is perfused past the sperm, there is an increased motility of the flagellum, the acrosome pops, and 1–2 s later the acrosomal process emerges. We measured the length of the acrosomal process under each experimental condition every 15 video fields or every 0.25 s. We selected those sperm in which the tip of the process and the head of the sperm remained in focus during the entire elongation procedure; this allowed for accurate measurements. In analysing the kinetics of elongation of the acrosomal process (18), we found it most convenient to plot the data as $(\text{length})^2$ vs. time, rather than as length vs. $(\text{time})^{1/2}$. This produces a straight line for all the sperm induced to discharge in sea water (18) except at the beginning of elongation of the acrosomal process and at the very end of extension. Two populations were encountered:

those that grew very fast ($850 \mu\text{m}^2/\text{s}$) and those that grew at approximately $1/2$ the maximal rate ($400 \mu\text{m}^2/\text{s}$).

All the biological recordings listed below were made during early July, 1982. Although the sperm were active, could be induced to undergo the acrosomal reaction with A23187, popped normally, and produced processes with linear kinetics when the data were plotted as $(\text{length})^2$ vs. time, the rate of elongation corresponded to that of the population of sperm in our earlier study that elongated at $1/2$ the maximal rate (e.g., $400 \mu\text{m}^2/\text{sec}$) (18). The absence of sperm that grew processes at the maximal rate seen in our earlier study (18) was perhaps due to the fact that the animals are maintained in an "over-ripe" condition by reducing the temperature of the sea water to 12°C because in nature gametes are released in early June and possibly due to the fact that the winter of 1982 was exceptionally mild leading to a severe underproduction of gametes. Nevertheless, because the 1982 population was consistent in firing and was normal in all other regards, including the formation of acrosomal processes that exceeded $90 \mu\text{m}$ in length, and because it corresponded to one population described in our earlier publications, we view the 1982 sample as completely normal.

Two sets of controls were run for the experiments that are outlined in this report. In the first, sperm were incubated in sea water to which excess Ca^{++} had been added and then 1.5 min later induced to undergo the acrosomal reaction by the addition of A23187 to the Ca^{++} sea water (Fig. 1a). Our second control was to perfuse sperm for 1.5 min with a solution that was isotonic with sea water, yet contained one part sea water and one part 1 M sucrose to which excess Ca^{++} had been added. After 1.5 min, A23187 was added to this perfusate (Fig. 1b). The latter is an important control because in the experiments that follow sucrose is added to sea water and it is important to establish that sucrose by itself does not change the kinetics of elongation. As seen in Fig. 1, plots of $(\text{length})^2$ vs. time are straight lines except at the beginning of elongation and at the completion of the reaction for both controls. This deviation at the beginning and end of elongation was also noted in our earlier publication (18). In Table I, the slopes of the plots, $(\text{length})^2$ vs. time, for the controls can be seen to be similar ranging from 188 – $455 \mu\text{m}^2/\text{s}$ with a mean of $343 \mu\text{m}^2/\text{s}$ for the sucrose control which compares favorably with the sea water control of $370 \mu\text{m}^2/\text{s}$.

Effect of Hypertonicity on the Kinetics of Extension of the Acrosomal Process

To study the effect of hypertonicity, we suspended sperm in sea water, then introduced them into the perfusion chamber, and perfused them with sea water containing varying concentrations of sucrose for 1.5 min; excess calcium (up to 50 mM) was added to the perfusate. The sperm were then induced to undergo the acrosomal reaction with A23187.

At tonicities of 1.15 osmol and 1.25 osmol plots of $(\text{length})^2$ vs. time produced mostly straight lines except at the beginning of elongation and at its completion (Figs. 2–4), as was seen in the controls. In some cases, e.g., the left curve of Fig. 3, the data might be fitted better with a continuously curved line of increasing slope rather than the straight line that is drawn. The significance of a deviation from a straight line such as seen in Fig. 3 and at the beginning and end of elongation will be discussed in more detail in the Discussion. In most cases, the rate of elongation of the acrosomal process was slower

TABLE I
Analysis of the Kinetics of Elongation of the Acrosomal Process

Sperm	Experimental regime	Rate of elongation $\mu\text{m}^2/\text{s}$	Average rate of elongation $\mu\text{m}^2/\text{s}$	Maximum length μm
<i>Control</i>				
A	S.W.*	370	370	43
B	Sucrose/S.W.	348		36.6
C		188	330	29.9
D		455		25.4+
<i>Hypertonic</i>				
L	1.15 osmol	102		26.3
M		145	216	29.6
N		400		19.2+
O	1.25 osmol	230		27
P		401	228	27.4+
Q		55		10.7
R	1.33 osmol	65	51	13
S	1.5 osmol	36		9.9
<i>Hypotonic</i>				
E	0.75 osmol	400		30.3
F		374	368	34+
G		420		27+
H		280		24.7
I	0.50 osmol	615	494	51.5
J		470		25
K		398		31.9

* S.W., sea water.

than that of the controls and in some cases was very much reduced. More specifically, as seen in Table I, at a tonicity of 1.15 osmol, the average rate of elongation was $216 \mu\text{m}^2/\text{s}$ with a range of 102 – $400 \mu\text{m}^2/\text{s}$. At a tonicity of 1.25 osmol, the range was 55 – $401 \mu\text{m}^2/\text{s}$ with a mean of $228 \mu\text{m}^2/\text{s}$ (Figs. 3 and 4).

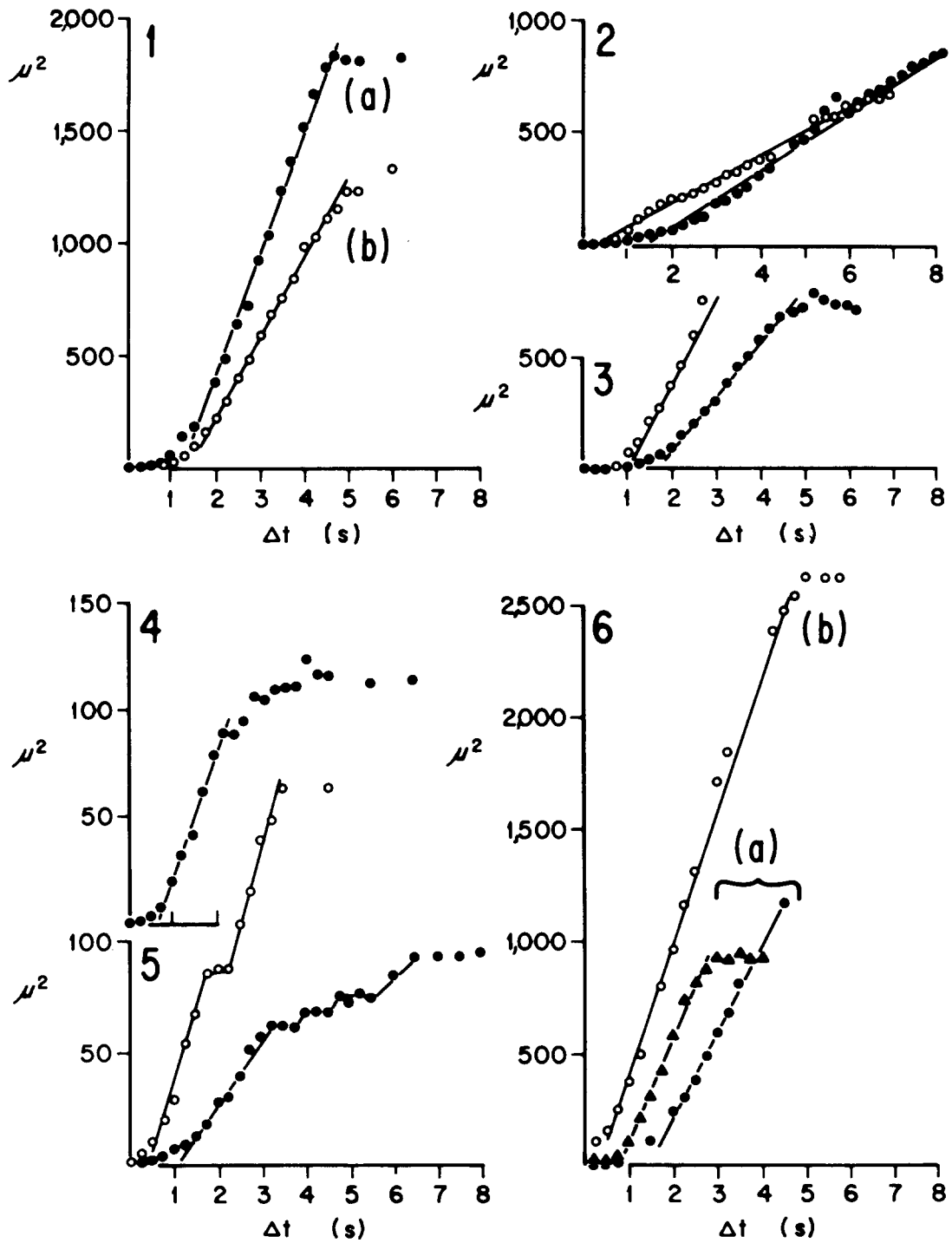
Interestingly, the overall length of the acrosomal process tends to be significantly shorter than that in the controls with an average length of only about 20 – $25 \mu\text{m}$ compared with the control curves in which the average length was $\sim 37 \mu\text{m}$; this is nearly double that seen in the samples whose tonicities were 1.15 or 1.25 osmol (see Table I).

At a tonicity of 1.33 osmol there was a dramatic change not only in the kinetics of elongation, but also in the extent. Some sperm produced only short blebs. Others produced short processes such as illustrated in Fig. 5 in which the extent of elongation is from 10 to $13 \mu\text{m}$. In no instances did we get processes that exceeded $15 \mu\text{m}$ in length. Plots of $(\text{length})^2$ vs. time show irregular curves with some linear regions followed by regions that slow down, etc. Measurements of the slopes of the linear segments reveal values of 36 – $65 \mu\text{m}^2/\text{s}$ or a mean of $50 \mu\text{m}^2/\text{s}$.

At a tonicity of 1.5 osmol, the acrosomal vacuole popped, but processes did not extend.

Effect of Hypotonicity on the Kinetics of Extension of the Acrosomal Process

To study the effect of hypotonicity, we suspended sperm in sea water, introduced them into the perfusion chamber, and then perfused them with sea water that had been diluted with



distilled water. Excess CaCl_2 (50 mM) was added after perfusion for 1.5 min. Sperm were then induced to undergo the acrosomal reaction with A23187.

At a tonicity of 0.75 osmol, plots of $(\text{length})^2$ vs. time were linear except at the beginning and end of the reaction with slopes ranging from 280 to 400 $\mu\text{m}^2/\text{s}$ with a mean of 368 $\mu\text{m}^2/\text{s}$ (Fig. 6a and Table I). The processes generated were 25 μm to well over 35 μm in length and in two instances were still elongating although the overall extent could not be measured because the processes extended out of the field.

At a tonicity of 0.50 osmol, plots of $(\text{length})^2$ vs. time were also linear with slopes ranging from 398 to 615 $\mu\text{m}^2/\text{s}$ with a mean of 494 (Table I). The lengths of the processes generated were up to 51.5 μm (Fig. 6b).

The Relationship between the Rate of Elongation of the Acrosomal Process ($[\text{length}]^2$ vs. time) and the Osmolarity of the Medium

A plot of the rate of elongation of the acrosomal process, $(\text{length})^2$ vs. time, as a function of the osmolarity of the medium is depicted in Fig. 7. In this figure, it is obvious that there is considerable variation from sperm to sperm but, nevertheless, what we see is that at low osmolarity, the sperm, in fact, elongate processes at higher rates than in sea water alone, and also that as the osmolarity increases, the rate of elongation decreases dramatically, ceasing altogether at osmolarities of 1,500 mosmol.

Observations on the Surface Morphology of the Acrosomal Process during Elongation at Varying Osmolarities

In an earlier publication (18), we demonstrated that during the early stages of growth the acrosomal process extended

very slowly, and was fat and non-uniform in diameter, but as the rate of elongation accelerated, the process became slender and uniform in diameter except at its bulbous tip and at a few locations along the length, marked by "blebs." In this section, we compare these earlier results with the morphology of an acrosomal process extending under hyper- and hypo-osmotic conditions, in particular concentrating on the tips of the acrosomal processes and the blebs.

Of particular interest is that under both hyper- (Fig. 8) and hypo- (Fig. 9) osmotic conditions, the tips of the acrosomal processes are invariably bulbous or tear-shaped as in the controls. They never appear to be needle-like or sharp. An occasional bleb is found along the length of the elongating acrosomal process.

Fine Structural Observations on Sperm Induced to Undergo the Acrosomal Reaction under Hyper- and Hypo-osmotic Conditions

From the preceding sections we have learned that the rate of elongation of the acrosomal process is correlated with the osmolarity of the sea water. At this point we wondered if these differences in rate were related to the amount of actin assembled (perhaps by the osmolarity affecting Z in Hermans' equation [8]) or if all the actin assembles under all conditions and differences in rate are related to some other factor. To distinguish between these two possibilities, sperm were incubated in hyperosmotic (1.33 or 1.25 osmol), iso-osmotic, and hypo-osmotic (50% sea water) conditions, the acrosomal reaction was then induced with A23187, and 1.5 min later the sperm were fixed by the addition of glutaraldehyde to the solution.

Thin sections of sperm fixed under hyperosmotic conditions such as 1.33 osmol showed us that in most instances the membrane that limits the acrosomal vacuole had fused or

FIGURES 1-6 (Fig. 1a) Plot of (length of the acrosomal process)² in μm^2 as a function of time (in seconds) after the acrosomal process first becomes visible. Of note is that except for the beginning and end of the reaction, the points fall on a straight line. This sperm was induced by the addition of A23187 to sea water. Sperm A. (Fig. 1b) Plot of (length of the acrosomal process)² in μm^2 as a function of time which serves as a control. In this experiment, one part of sea water was added to one part of a 1-M sucrose solution so that the tonicity remains that of sea water. Note that, as in Fig. 1a, all the points except those at the beginning and end of the reaction fall on a straight line. Sperm B. (Fig. 2) Plot of (length of the acrosomal process)² in μm^2 as a function of time for two sperm, L (○) and M (●), which were induced to undergo the acrosomal reaction in sea water whose osmolarity was 1.15 that of natural sea water or ~1,150 mosmol. Note that all the points except those at the beginning and end of the reaction tend to fall on a straight line. (Fig. 3) Plot of (length of the acrosomal process)² in μm^2 as a function of time for two sperm, P (○) and O (●), which were induced to undergo the acrosomal reaction in sea water whose osmolarity was 1.25 that of natural sea water or ~1,250 mosmol. Note that all the points except those at the beginning and end of the reaction tend to fall on a straight line. (Fig. 4) Plot of (length of the acrosomal process)² in μm^2 as a function of time for sperm Q which was induced to undergo the acrosomal reaction in sea water whose osmolarity was 1.25 that of natural sea water. Notice that the scale on the Y axis of this figure is one-tenth that of Fig. 3; the process formed is very short. Even so, except at the beginning and end of the reaction the points all fall on a straight line. (Fig. 5) Plot of (length of the acrosomal process)² in μm^2 as a function of time for two sperm, R (○) and S (●), which were induced to undergo the acrosomal reaction in sea water whose tonicity was 1.33 that of natural sea water or an osmolarity of ~1,330 mosmol. Two points should be noted. First, the scale on the Y axis indicates that the processes that develop are very short, and second, although many of the points fall on a straight line, there are clearly sharp bends where the sperm stop elongating, only to begin to elongate again after 0.75 s. (Fig. 6a) Plot of (length of the acrosomal process)² in μm^2 as a function of time for two sperm, E (▲) and F (●), which were induced to undergo the acrosomal reaction in sea water whose tonicity was 75% that of natural sea water by being diluted with distilled water. Of interest is that all the points except at the beginning and end of the reaction fall on a straight line. In the case of sperm F the process was still elongating after 4.5 s, but unfortunately, the tip of the acrosomal process disappeared from view by extending out of the video field selected. (Fig. 6b) Plot of (length of the acrosomal process)² in μm^2 as a function of time for sperm I which was induced to undergo the acrosomal reaction in sea water whose tonicity was 50% that of natural sea water by being diluted with distilled water. Note that the resulting process is long, extends rapidly, and except at the beginning and end of the reaction all the points tend to fall on a straight line.

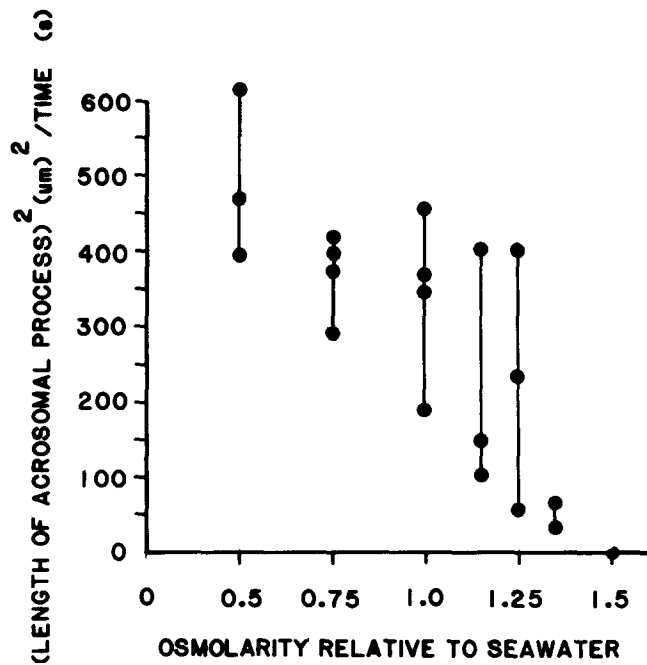


FIGURE 7 Plot of the rate of elongation of the acrosomal process expressed in $\mu\text{m}^2/\text{s}$ as a function of the osmolarity of the sea water in which the sperm were induced to discharge. Of interest is that under hypo-osmotic conditions the processes elongate rapidly, whereas under hyperosmotic conditions the rate of the reaction is reduced such that at a toxicity of 1.5 that of sea water or $\sim 1,500$ mosmol no processes extend at all. However, it can be seen that there is a large variation in the rate of the reaction from sperm to sperm treated under the same conditions, as illustrated by the separate points indicated for each tonicity.

partially fused with the plasma membrane lying immediately above it. Accordingly, the contents of the acrosomal vacuole were less dense and the vacuole had swollen dramatically. In general, the profilactin region was intact and very dense. Some short processes could be found extending directly from the center of the profilactin cup. Elsewhere the profilactin looked unaltered except in certain regions where dense clumps of material were visible. Within the processes were actin filaments which extended from the actomere. We estimated that there were ~ 50 – 100 actin filaments in the processes. Thus, at a tonicity of 1.33 osmol, only a portion of the actin seems competent to assemble.

At a tonicity of 1.25 osmol, more processes were present. As before, the filaments in the processes, which we estimate to be about 50, extended from the actomere. The bulk of the profilactin, except in the vicinity of the actomere, was dense and homogeneous (Fig. 10).

In contrast to the situation in hypertonic sea water, sperm induced to undergo the acrosomal reaction in sea water had long processes and little of their profilactin remained. The preservation of the filaments in these processes seemed excellent, although the sperm membrane which surrounded the sperm head often appeared to be distended away from the surface of the cell. In some instances, the flagellar axoneme had been retracted and was wrapped around the nucleus.

Examination of thin sections of sperm induced to undergo the acrosomal reaction in hypo-osmotic conditions, e.g. 50% sea water, then fixed in glutaraldehyde, reveals that all the profilactin had largely disappeared and in its place were actin

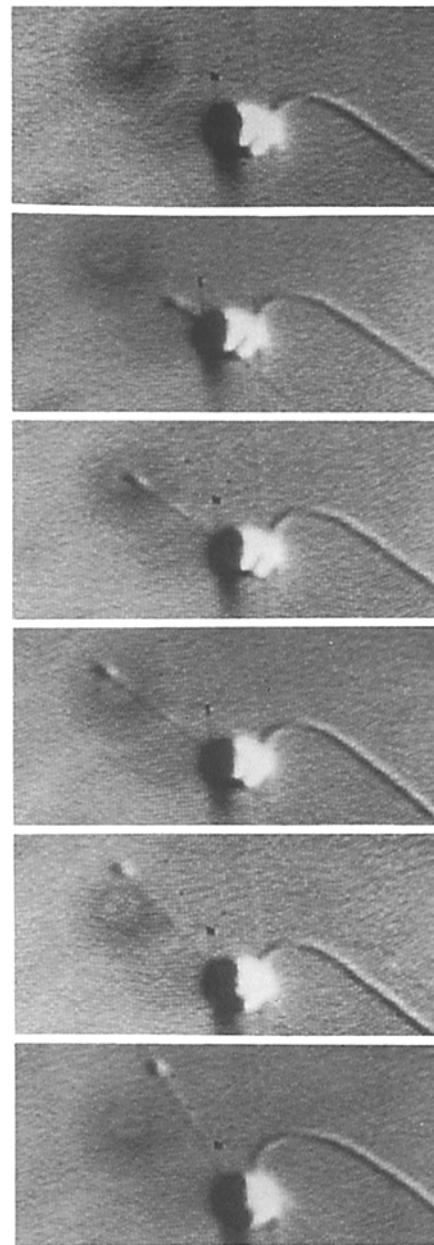


FIGURE 8 Stages in the elongation of the acrosomal process for sperm Q which was induced to undergo the acrosomal reaction at a tonicity that was 1.25 that of natural sea water or an osmolarity of $\sim 1,250$ mosmol. Single fields of this sperm were photographed off the TV monitor every 1.0 s. Of interest is that the tip of the acrosomal process is bulbous at all stages; it does not taper to a sharp point. $\times 1,800$.

filaments. However, the fixation of these sperm was very poor. The sperm tended to bloat or increase in diameter caused in part by the retraction of the flagellar axoneme during the fixation period. Thus, the morphology of the sperm is not instantly frozen by the addition of glutaraldehyde; further changes occur. We found that by fixing the sperm briefly in a glutaraldehyde-osmium mixture followed by osmium fixation, we could reduce the amount of bloating that occurs during the fixation period. However, this combination fixative reacts strongly to the substances released from the acrosomal vacuole so that the glutaraldehyde-osmium mixture was changed 4 min after the immersion of sperm in it.

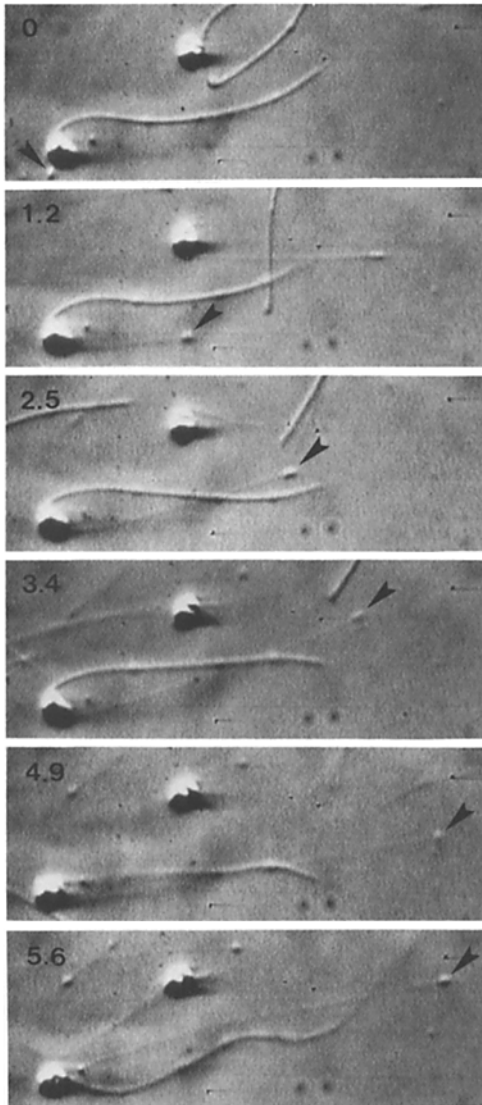


FIGURE 9 Stages in the elongation of the acrosomal process of sperm I which was induced to undergo the acrosomal reaction in sea water whose tonicity was 50% that of natural sea water by being diluted with distilled water. Single fields of this sperm were photographed off the TV monitor at the time intervals (in seconds) indicated on each frame. In frame 1 the process is still short and extends to the left, whereas in frames 2–6 the sperm has rotated so that the process is extending towards the right. Note that at all stages, as in the control, the tip of the acrosomal process is bulbous or tear-shaped; it does not taper to a sharp point. $\times 1,000$.

Examination of thin sections revealed that the profilactin almost completely disappeared and the processes that form are filled with actin filaments aligned parallel to one another (Fig. 11). Some of the contents formerly in the acrosomal vacuole remain attached to these processes.

Thus, the amount of actin polymerization is roughly correlated with the osmolarity of the sea water with little assembly occurring under hyperosmotic conditions.

The Effect of Salt on the Profilactin Region of Detergent-extracted Sperm

It has been demonstrated that, prior to induction, the actin remains not only unpolymerized, but if the sperm are deter-

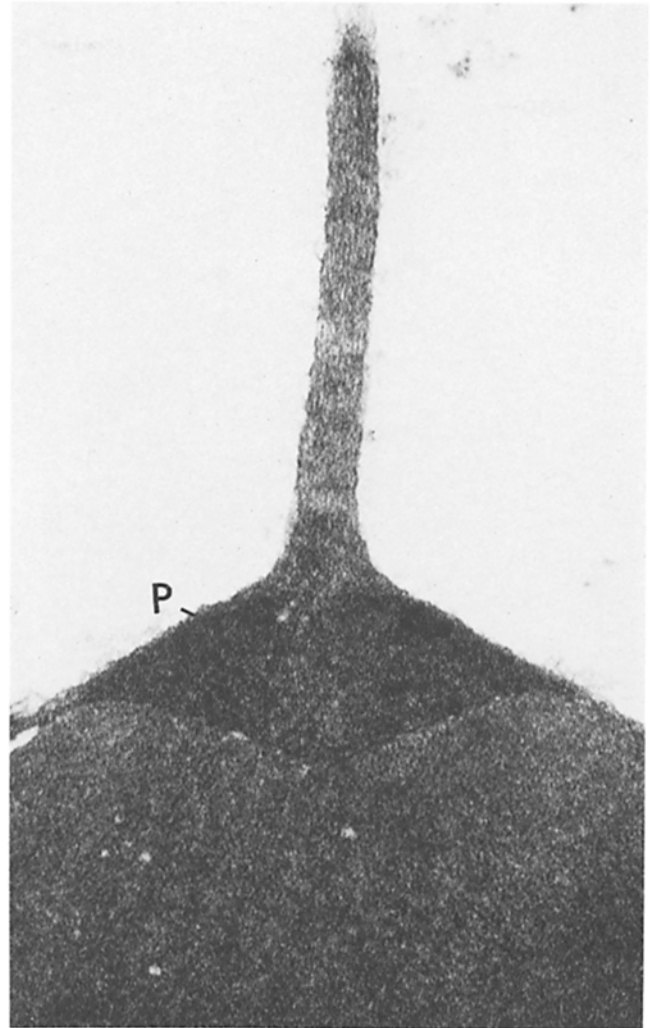


FIGURE 10 Thin section through the apical end of *Thyone* sperm which was induced to undergo the acrosomal reaction in sea water whose osmolarity was increased to 1,250 mosmol with sucrose. Under these hyperosmotic conditions the sperm becomes very dense. Of interest is the acrosomal process within which is a parallel array of filaments. At the base of the acrosomal process is the profilactin region (*P*). This material has not assembled into filaments. $\times 69,000$.

gent-extracted at low pH (a pH thought to be present in vivo) the actin remains bound or insoluble (20). In this section, we present some observations on sperm which were extracted with detergent under a variety of salt conditions to see what most closely approximates the in vivo state prior to induction. These observations, then, are an attempt to understand what might be the salt conditions that exist in unreacted sperm.

When sperm are extracted in 1% Triton X-100 in 0.1 M KCl or 0.25 M KCl which contains 10 mM phosphate buffer at pH 6.4, the cup of profilactin remains intact and of comparable density and size to that in unextracted sperm. If the pH is raised to 8.0, the profilactin disappears almost completely in 0.25 M salt, and less so at 0.1 M salt. Some of the liberated actin assembles on the actomere. On the other hand, if sperm are extracted with 1% Triton X-100 in 0.5 M KCl in 10 mM phosphate buffer at pH 6.4, the cup of profilactin is reduced in amount and often we see assembly on the actomere (Fig. 12). Thus, if the inside of the sperm contains KCl under conditions which maintain the actin unpolymer-

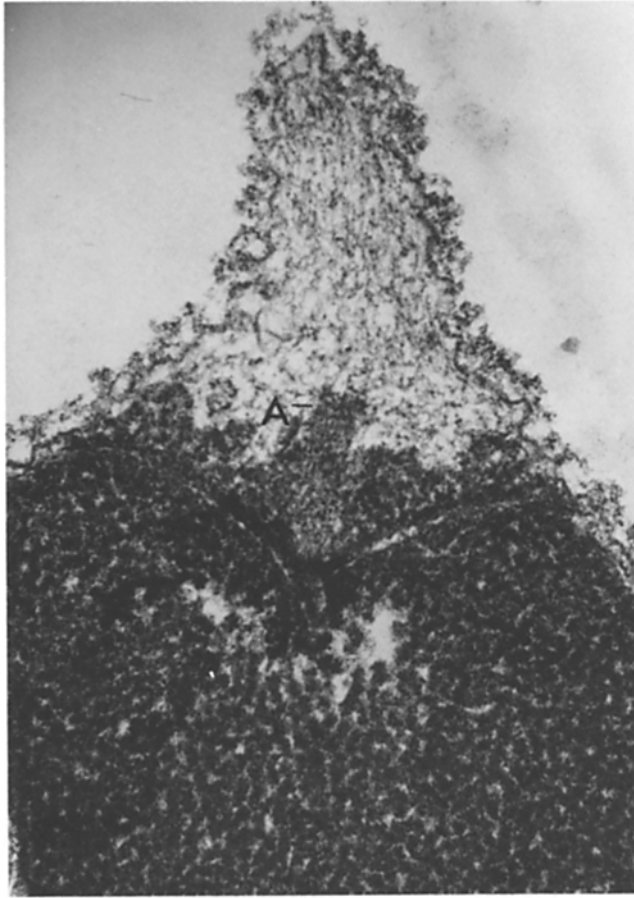


FIGURE 11 Thin section through the apical end of a *Thyone* sperm which was induced to undergo the acrosomal reaction in sea water which had been diluted with distilled water so that the final osmolarity of the sea water was ~500 mosmol or 50% sea water. The actomere (A) is indicated and filaments at the basal end of the acrosomal process can be seen. $\times 69,000$.

ized and insoluble, the concentration of KCl must be 0.25 M KCl or lower, not 0.5 M KCl, a concentration that would be iso-osmotic with sea water.

Intracellularly, most cells are low in chloride ions but high in potassium ions, so we suspected that if we detergent-extracted sperm in phosphate buffer at pH 6.4 in 0.5 M potassium aspartate, a solution iso-osmotic with sea water, perhaps the profilactin would remain intact. Interestingly, in 0.5 M potassium aspartate, the profilactin indeed remains intact and there was no assembly from the actomere (Fig. 13). Thus, the profilactin seems completely stable at 0.5 M potassium aspartate, a concentration that is iso-osmotic with sea water and has the added benefit of being low in chloride yet high in potassium ions that are likely to be present in sperm. However, when the profilactin cups are extracted with Triton X-100 in 0.1 M or 0.25 M potassium aspartate at pH 6.4, they are somewhat reduced in size and some of the liberated actin are assembled from the actomere. The chromatin also begins to uncoil. Thus, it is likely that in unreacted sperm the ionic conditions would mimic 0.5 M K aspartate, not 0.5 M KCl.

Obviously, these observations are only morphological and on the small number of sperm that are included in a thin section (~100). Perhaps it would have been more convincing if we had examined these samples by SDS gel electrophoresis

because the sample size would be larger; but these observations and the light microscope observations that preceded them allow us to at least know which direction to proceed, e.g., examine extracted sperm cells *not* in iso-osmotic KCl but in iso-osmotic solutions such as K aspartate.

DISCUSSION

The Site of Actin Assembly

In our earlier study on the kinetics of elongation of the acrosomal process (18), we demonstrated by high resolution video microscopy that plots of the length of the acrosomal process as a function of $(\text{time})^{1/2}$ produced straight lines except at the beginning and at the very end of the reaction. Such a relationship was consistent with the hypothesis suggested earlier by Tilney and Kallenbach (19) that elongation of the acrosomal process is limited by the diffusion of the actin from the sperm proper (the profilactin region) to the tip of the elongating process where they assemble on the tips of the extending filaments. In fact, our results could be quantitatively related to the mathematical relationship first described by Hermans (8), namely that the distance monomers must travel (δ) before being trapped or assembled should be proportional to time (t), the diffusion constant (D), and Z (which relates to the concentration of the diffusing substance at a particular point and at the source). Therefore: $\delta = 2Z (D \times t)^{1/2}$.

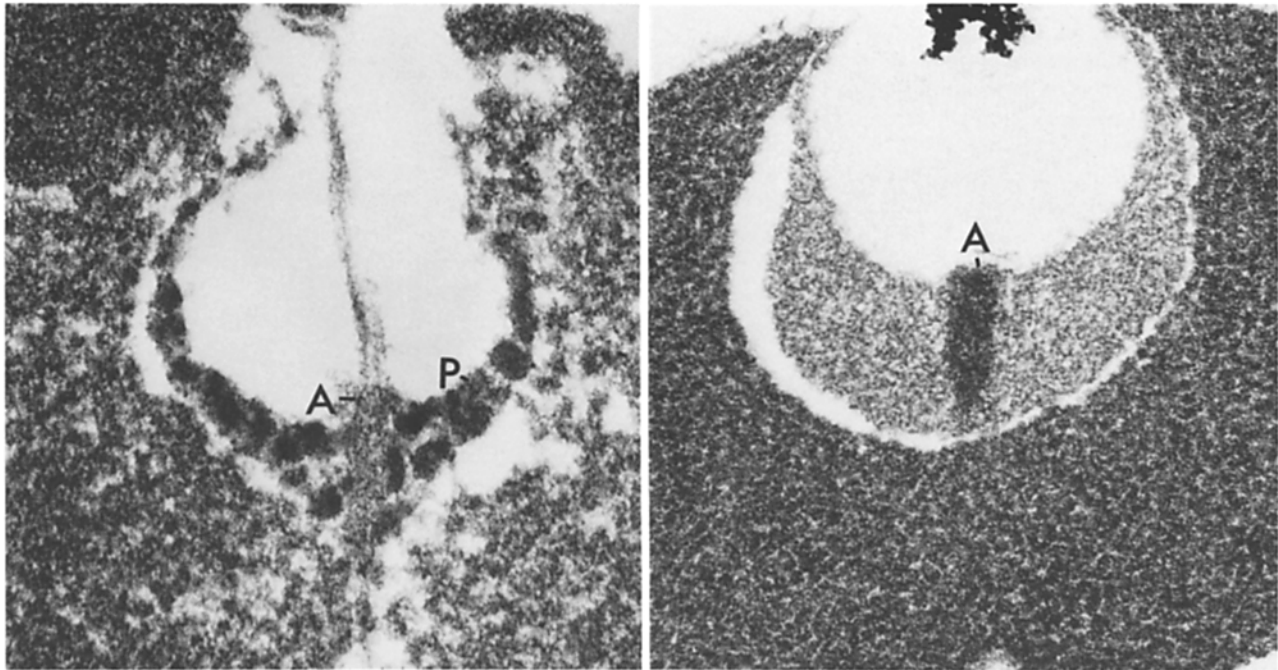
Subsequent to these publications, the protein profilin was isolated from sperm and shown to function in two ways (17). First, it inhibits spontaneous nucleation of the actin so that filament elongation would proceed from preformed actin filaments, e.g., in the actomere. Second, and more relevant to this report, by binding to the actin monomers, profilin forces the assembly of monomers to take place only at the "barbed end" of the elongating actin filaments; this end is located at the tip of the acrosomal process. Thus, it must be the actin-profilin complex that migrates to the tip of the acrosomal process; once there, the profilin dissociates from the actin monomers releasing them to polymerize on to the elongating actin filaments (14).

While these observations and the agreement of the data with Hermans' equation (8) confirm the original hypothesis of Tilney and Kallenbach (19), we left open the question as to whether the growth of actin filaments alone is responsible for extending the acrosomal process or whether the influx of water into the sperm also contributes to the growth of the acrosomal process.

The Assembly of Actin Is Sensitive to the Osmolarity of the Medium Which Surrounds the Sperm

For this report, we incubated *Thyone* sperm in media of different osmolarities, then induced the acrosomal reaction. Interestingly, if the osmolarity of the sea water is increased, the rate of elongation of the acrosomal process gradually decreases until at a tonicity of 1.33 times that of sea water, many of the sperm fail to form acrosomal processes; others form only short ones, and at 1.5 osmol no processes at all are formed (Fig. 7). On the other hand, if the osmolarity of the sea water is decreased by the addition of an equal volume of distilled water, the rate of elongation is increased.

The first point to be made from our observations is that



FIGURES 12 and 13 (Fig. 12) Thin section through the profilactin region of a sperm which had been extracted with 1% Triton X-100 at pH 6.4 containing 0.5 M KCl. Of interest is that the bulk of the profilactin (P) has been solubilized. Some of the actin in the profilactin region has assembled on the actomere (A). $\times 86,000$. (Fig. 13) Thin section through the profilactin region of a sperm which had been extracted with 1% Triton X-100 at pH 6.4 containing 0.5 M potassium aspartate. Note that the profilactin is intact. In the center of this material is the actomere. $\times 86,000$.

plots of the length of the acrosomal process vs. $(\text{time})^{1/2}$, or more conveniently, $(\text{length})^2$ vs. time, except for under the most hyperosmotic conditions, are linear except at the very beginning of the reaction and at the completion. It is remarkable and interesting that the data from a variety of osmolarities should fall on a straight line and maintain the relationship specified in Hermans' equation (8) because the slopes of the lines that define the rates of extension change by over an order of magnitude. What this tells us is that Z cannot be a constant as should be expected because Z depends on the ratio of actin monomer concentration at the tip of the sperm and at its base. This ratio must be changed by modifying the proteins associated with actin; actin monomers must be released from its storage state in the profilactin at a rate that is proportional to ion movements and water influx. More specifically, we know that it is a change in internal pH which releases the actin from its bound form to its diffusible and presumably polymerizable state as an actin-profilin complex (see references 17 and 18), a state which occurs because protons are released from the sperm (3, 15, 16, 20).

However, as pointed out by one of the reviewers of this paper, a careful examination of both the control and the experimental curves in the graphs reveals that not only is there deviation from the straight line at the beginning and end of elongation, but, in fact, there are experimental points that deviate from linearity. For example, in Fig. 3 (left curve), a better fit of the points might be a smooth curve of increasing upward slope rather than a straight line. This would allow one to also include the beginning and end of the curve. We believe that this is a very important point. One explanation for deviations from linearity is that the elongation of the acrosomal process consists of several interrelated events. First exocytosis has to occur, and then the elongating process must push its way through the contents of the acrosomal vacuole.

One might suspect that this elongation through the contents of the acrosomal vacuole might slow down the initial phase of the reaction, particularly as during this period water inrush is beginning. Second, as the process elongates not only are actin monomers diffusing to the tip of the process where they will assemble on the existing actin filaments, but new membrane must be inserted at the base of the acrosomal process (18) to allow elongation to occur. Again, one might suspect that at the beginning and end of elongation these two events might not be perfectly coupled, thus slowing down elongation. To us it seems remarkable that this straight line relationship holds as well as it does for most of the elongation process as there are several events occurring at once (17, 18) and it is this rather than slight deviations from linearity that must be explained. We suspect, therefore, that deviations from linearity are telling us that either additional variables are exerting an influence or, under very hyperosmotic conditions such as depicted in Figs. 3 and 5, water influx and actin assembly are no longer perfectly coupled as they appear to be "most of the time." Thus, we recognize that our interpretation using Hermans' relationship may not hold true at the beginning and end of elongation and at extreme hyperosmotic conditions, but the important experimental fact is that plots of the length of the acrosomal process vs. $(\text{time})^{1/2}$ are remarkably linear which is indicative of the diffusion-limited reaction described by Hermans. Significantly, recent biochemical evidence on profilin, one of the actin-binding proteins in *Thyone*, supports a relationship between Hermans' equation (8) and the experimentally determined kinetics of elongation (17).

Second, careful observations on the morphology of sperm induced to undergo the acrosomal reaction at various osmolarities show that the processes do not bleb excessively (which would indicate a net inrush of water), nor does the shape of the tips of the acrosomal processes differ from those induced

under isotonic conditions; they remain broad and bulbous, not needle-like. Our morphological observations indicate, therefore, that the mechanism for elongation of the acrosomal process remains the same, although the rate varies with varying tonicities.

Finally, an examination of thin sections of sperm fixed at various tonicities revealed that at a tonicity of 1.33 osmol little actin assembly takes place, whereas in 50% sea water all the actin assembles. Thus actin assembly seems crudely correlated with the extent and rate of elongation of the acrosomal process.

Thus, even though the rate of extension of the acrosomal process changes by more than an order of magnitude, as does its final length, the assembly of actin seems to remain correlated with ion and water movements.

What Causes the Entry of Water into the Sperm during the Acrosomal Reaction and Where Does This Water Influx Occur?

Studies from several labs have now established that within seconds after the sperm have been induced to undergo the acrosomal reaction either naturally by encountering "egg jelly" or artificially by means of ammonia, uncouplers, or ionophores, Ca^{++} , Na^+ , and Cl^- enter the sperm and H^+ and K^+ leave it. At the same time that this is occurring in sea urchin sperm, there is approximately a 30-mV depolarization in resting potential in naturally induced sperm as measured by an efflux in [^3H]tetraphenylphosphonium ions or an uptake of (^{14}C)-labeled thiocyanate (SCN^-) (15). From this fact plus the facts that Na^+ , Cl^- , and Ca^{++} all enter the sperm during induction (2), it seems likely that the change in resting potential is the result of the opening of Na^+ and possibly Ca^{++} channels followed by K^+ efflux and Cl^- influx. From what we know from neurophysiology, this movement of ions may at first seem trivial since the number of ions involved in the depolarization of an axon by 30 mV would be small until we take into account that the sperm cell, unlike the squid axon, occupies a very small volume, much of which is packed with molecules that take up space such as chromatin and profilactin. The net result of these ion movements, all following electrical and concentration gradients, would be to increase the number of osmotically active particles within the cell, a situation which will osmotically pull water from outside into the sperm cell. In fact, it has been shown experimentally (2) that there is indeed a net movement of ions into the sperm cell during induction.

While this change in resting potential is occurring, we know that actin is released from its bound state in the profilactin. The rise in solute concentration in turn will osmotically drag in even more water. More specifically, we know that profilactin consists of actin, profilin, and two high molecular weight (230,000 and 250,000) proteins, all of which are bound at a pH of 6.5. At pH 8.0, however, the actin-profilin becomes separated from the other components so that it will polymerize on the actomere, and thereby increase the number of osmotically active particles. It is informative to compare these observations with the situation that exists in the exocrine pancreas. In the pancreas, the zymogen granules can be isolated and stored in media of different osmolarities in the absence of ATP. The granules do not rupture nor do they take up or lose an appreciable amount of water. The explanation for this behavior apparently resides in the fact that in

the granules the largely anionic charge of the pancreatic enzymes is effectively neutralized by small sulfated cations which interact with the anions to form a complex that is essentially charge-free; this complex is a precipitate. It is only if these compounds become dissociated by changing the ionic strength or pH that the components separate and form hydrated species (see reference 12). The beauty of this concept is that proteins can be stored at high concentrations, yet be osmotically inactive until the components become separated; the net charge is no longer masked. We imagine that a similar phenomenon exists in the unreacted sperm. Net charge is eliminated by the interaction of the various components in the profilactin leading to an osmotically inert material, a kind of precipitate. Water fails to hydrate the components until a change in the pH occurs so that the components effect a new conformation, separate, and accordingly hydrate abruptly, a process that pulls in water from outside the sperm. Thus, a small change in pH will cause a dramatic hydration of this region of the cell. In fact, we can see this effect in our thin sections.

There is at least one further reason why water will tend to enter the sperm cell. This will occur when the actin monomers assemble on the end of the elongating filaments located at the tip of the acrosomal process. As assembly occurs profilin will be released effectively, thus increasing the number of osmotically active particles. One could argue that the release of profilin will be exactly matched by a decrease in monomers and therefore no net water movement will take place. This is doubtful because assembly of actin, as is true of many self-assembly proteins such as tubulin and tobacco mosaic virus protein, results in an increase in volume (9) and a concomitant increase in entropy. These changes can be easily reconciled if polymerization results in hydrophobic interactions of monomers, thereby leaving their hydrophilic portion still hydrated. Thus, as profilin comes off the actin monomers, presumably more osmotically active particles are released.

In summary, we have three separate reasons for water entry into the sperm cell: (a) Water will enter because of the depolarization of the membrane with a concomitant entry of Na^+ and Cl^- ; (b) as protons are eliminated, the actin becomes liberated from its binding proteins which will cause an increase in the number of osmotically active particles and an increase in their hydration; and (c) water will enter as profilin is released from the actin-profilin complex. In the first two instances water will enter the head of the sperm cell since in both cases these events precede the elongation of the acrosomal process. In the third case water will enter the tip of the acrosomal process as it is here that the actin monomers will assemble to the tips of existing actin filaments. This may explain why the tip of the acrosomal process is morphologically bulbous rather than pointed.

Preliminary Observations on the Ionic Composition of a Sperm Cell

Measurements on the species and concentration of ions in unreacted sea urchin sperm have recently been made by Cantino et al. (2) using X-ray microanalysis. There are several problems with these measurements. First, the values for the concentrations of ions vary from sperm to sperm presumably because it is difficult to wash the salts present in sea water away from the outside of the sperm. However, there are significant differences in the concentrations of K^+ , Na^+ , and

Cl⁻ before and after induction of the acrosomal reaction even though there is an enormous range in the values of these ions in the two states. Second, because the periacrosomal region is so small in sea urchin sperm and at the same time the probe size is so large, what is measured is the ionic consistency of the nucleus or the mitochondrion. What is germane here is not these two membrane-limited compartments, but rather the cytoplasm of the periacrosomal region. Nevertheless what is clear from the measurements of Cantino et al. (2) is that in the sperm nucleus of an unreacted cell there is a high internal concentration of K⁺ and a low concentration of Na⁺ and Cl⁻, as is the case in other marine cells. These observations are consistent with our observations that profilactin dissolves in 0.5 M KCl even at pH 6.2, yet the profilactin is stable at pH 6.2 in compounds such as 0.5 M potassium aspartate, potassium acetate, or potassium glycinate. Interestingly, if the pH is raised to 8.0, the actin-profilin becomes immediately released from the other binding components in the profilactin so that the actin can polymerize on the actomere. It is interesting that amino acids such as these are present in high concentration in marine eggs, and thus are appropriate candidates for the ionic species present in sperm.

Conclusions

(a) Plots of the length of the acrosomal process vs. (time)^{1/2} tend to produce a straight line relationship except at the beginning of elongation and at its completion even though the tonicity of the sea water surrounding the sperm is profoundly changed.

(b) An examination of the video sequences reveals that in all sperm, regardless of the tonicity of the external medium, the tips of the acrosomal processes are similar, presenting a bulbous profile.

(c) From thin sections of fixed sperm the degree of conversion of the profilactin into filaments is roughly coupled to the length of the acrosomal process that is formed. Thus, a sperm that only produces a short process only polymerizes a portion of the actin present in that sperm.

(d) The fact that the rate of elongation of the acrosomal process is inversely correlated with the osmolarity of the medium suggests that water is playing a role in the extension of the process. On the other hand, the fact that Hermans' relationship seems to be maintained over a 10-fold increase in rate of elongation (except at the beginning and end of elongation) suggests that water is not simply acting as an osmotic pump, but rather that the influx of water and the release of actin monomers seem to be coupled in such a way

that the osmotic work and growth of the actin filaments both contribute to the extension of the acrosomal process in a coordinated manner.

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