THE POLYMERIZATION OF ACTIN: ITS ROLE IN THE GENERATION OF THE ACROSOMAL PROCESS OF CERTAIN ECHINODERM SPERM

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ABSTRACT

When *Asterias* or *Thyone* sperm come in contact with egg jelly, a long process which in *Thyone* measures up to 90 μm in length is formed from the acrosomal region. This process can be generated in less than 30 s. Within this process is a bundle of microfilaments. Water extracts prepared from acetone powders of *Asterias* sperm contain a protein which binds rabbit skeletal muscle myosin forming a complex whose viscosity is reduced by ATP. Within this extract is a protein with the same molecular weight as muscle actin. It can be purified either by collecting the pellet produced after the addition of Mg^{++} or by reextracting an acetone powder of actomyosin prepared by the addition of highly purified muscle myosin to the extract. The sperm actin can be polymerized and by electron microscopy the polymer is indistinguishable from muscle F-actin. The sperm actin was shown to be localized in the microfilaments in the acrosomal processes by: (*a*) heavy meromyosin binding *in situ*, (*b*) sodium dodecyl sulfate (SDS) gel electrophoresis of the isolated acrosomal processes and a comparison to gels of flagella which contain no band corresponding to the molecular weight of actin, and (*c*) SDS gel electrophoresis of the extract from isolated acrosomal caps. Since the precursor for the microfilaments in the unreacted sperm appears amorphous, we suspected that the force for the generation of the acrosomal process is brought about by the *polymerization* of the sperm actin. This supposition was confirmed, for when unreacted sperm were lysed with the detergent Triton X-100 and the state of the actin in the sperm extract was analyzed by centrifugation, we determined that at least 80% of the actin in the unreacted sperm was in the monomeric state.

INTRODUCTION

Microfilaments, approximately 50 Å in diameter, have now been described in a wide variety of nonmuscle cells. By their number and location these filaments have been implicated in certain types of primitive motility, such as cytokinesis, neurulation, blastulation, shortening of processes which occur in development, clot retraction, pulsation of processes, and cytoplasmic streaming.

There is now a wealth of literature beginning with the work of Hatano and Oosawa (1966) which demonstrates that actin and myosin are present in a wide variety of nonmuscle cells (Hatano and Tazawa, 1968; Hatano and Ohnuma, 1970; Adelman and Taylor, 1969 *a*, *b*; Weihing and Korn, 1971; Pollard et al., 1970; Zucker-Franklin and Grusky, 1972; Adelstein et al., 1971; Tilney and Mooseker, 1971; Nachmias et al., 1970; Miki-Noumura and Oosawa, 1969; Nachmias, 1972; Fine and Bray, 1971). Equally important is the contribution of Ishikawa et al. (1969) who first demonstrated that the 50 Å microfilaments in nonmuscle cells would bind heavy meromyosin (HMM) *in situ*. With this technique it became possible to identify these filaments as actin and to determine their location in the cytoplasm. This has now been carried out for many of the examples listed above.

Unlike those in skeletal muscle cells, however, the actin filaments in nonmuscle cells usually have two quite different properties. First, they are often situated only in a localized region of the cell as, for example, in the cleavage furrow; secondly, they are often transient structures, appearing at certain times during the cell cycle only to disappear at other times. Thus, unlike the actin in skeletal muscle which appears to be rather permanent, the actin in nonmuscle cells must be able to come and go under the appropriate signal, whatever that may be. It seems likely, therefore, that much of the control of the motile processes in nonmuscle cells may be at the level of the polymerization of actin, a secondary control perhaps occurring as well at the level of filament interaction as is the case in muscle cells.

We looked for a model system in which the spatial and temporal control of the appearance of actin filaments could be easily studied. We chose as our experimental system the acrosomal reaction of certain echinoderm sperm. In many marine invertebrate sperm the rapid production of a process, often many microns in length, is generated when the sperm come in contact with the jelly of an egg (see the recent review of Dan, 1970, for references). This reaction is remarkable since an acrosomal process of more than 90 μm in length (in *Thyone*) can be generated within only a few seconds. Within these processes, which usually measure less than 0.1 μm in diameter, is a fibrous material which is thought to polymerize during the acrosomal reaction (Dan, 1960; Dan and Hagiwara, 1967; Colwin and Colwin, 1963). Even more convenient is the fact that echinoderm sperm can be induced to form their acrosomal processes in the absence of eggs by the use of egg water (Dan, 1954) or alkaline sea water (Colwin and Colwin, 1956).

We suspected that the fibrils described by Dan and Hagiwara (1967) might be actin and, therefore, that this system might be an ideal one with which to investigate the factors which control the distribution of actin since, (*a*) sperm can be easily collected in large quantities, (*b*) the acrosomal region occupies a large percent of the total cell volume so that if actin is present, it must be one of the most abundant proteins, (*c*) the formation of the fibrils in the processes must be generated explosively, (*d*) the sperm have lost their ability to synthesize proteins, and (*e*) the number of proteins in these cells is minimal.

In this report we will demonstrate that the microfilaments in the acrosomal process are indeed actin and conclude that elongation of the process is due to the polymerization of this actin. This will be the first in a series of reports concerning the control of the spatial and temporal location of actin in nonmuscle systems.

MATERIALS AND METHODS

Collection of Sperm

Testes were removed from *Asterias amurensis*, *A. forbesi*, *Asterina pectinifera*, and *Thyone briareus*. The sperm which oozed out of the testes were washed in sea water, strained through cheese cloth, and centrifuged to concentrate. The concentrated sperm were covered and stored in the "dry" state at 4°C until use. They can be stored for several days without a decrease in activity or in ability to fertilize eggs. However, most of the sperm were used the same day.

Formation of the Acrosomal Process

The acrosomal reaction was brought about using either egg water (Dan and Hagiwara, 1967) or sea water to which ammonium hydroxide was added to bring the pH to 9.4 (alkaline sea water) (Colwin and Colwin, 1956).

Preparation of Heads, Tails, and Acrosomal Processes

Fresh sperm were homogenized in sea water in a Sorvall omnimixer (1–2 s at maximum speed) (Ivan Sorvall, Inc., Newtown, Conn.) or in a motor driven Teflon homogenizer (30 s at moderate speed).

The heads were collected by mild centrifugation (1,000 *g* for 3 min); the tails were pelleted from the supernate at 10,000 *g* for 10 min and washed several times with sea water. Isolated heads were induced to undergo the acrosomal reaction with alkaline sea water. The acrosomal processes were then isolated by homogenization in the omnimixer (1–2 s at maximum speed) and the heads separated as before by centrifugation. The isolated acrosomal processes were collected (10,000 *g* for 10 min).

Protein Preparations

Acetone powder of whole sperm was prepared by adding acetone to dry sperm, washing three times with fresh acetone, and air drying. 1 g of acetone powder was extracted for 30 min at 4°C in 7 ml of distilled water or distilled water which contained 5 mM TrisHCl at pH 8.2. The extract was then centrifuged at 10,000 *g* and the supernate filtered through a Buchner funnel by suction. The protein concentration of the extract was estimated using the biuret reaction (Gornall et al., 1949), using the absorbance of 0.068 for 1 mg/ml (Hatano et al., 1967). Aliquots were used for sodium dodecyl sulfate (SDS) gel electrophoresis, viscosity determination, or for further purification.

Myosin was prepared from rabbit skeletal muscle after the method of Perry (1955). We modified his procedure slightly. The high salt extract of minced muscle was precipitated in low salt for only 1 h, collected, and resuspended in phosphate buffer at pH 6.7 to which sufficient 3 M KCl was added to give a final concentration of 0.5 M KCl. The actomyosin was removed by centrifugation for 1 h at 45,000 *g* after the salt concentration was reduced to 0.3 M. The myosin was reprecipitated by reducing the salt concentration to 0.04 M, collected by centrifugation, and resuspended in enough 3 M KCl to make the ionic strength of the solution 0.5 M. Undissolved myosin was removed by centrifugation at 77,000 *g* for 3 h. Purity was determined by SDS gel electrophoresis. No actin appeared to contaminate the myosin. HMM was prepared after the procedure of Szent-Györgyi (1953). The HMM was freeze-dried in sucrose and stored until use in a desiccator at 4°C. The activity of the HMM in the frozen-dried state did not deteriorate with time. For binding *in situ*, immediately before use the HMM was weighed and dissolved in the standard salt solution used by Ishikawa et al. (1969).

Viscosity

Viscosity was measured by Ostwald-type capillary viscometers at 22 ± 0.1°C. The flow times for buffer solutions were about 35 s.

SDS Gel Electrophoresis

5% polyacrylamide gels containing 0.1% SDS were used (Weber and Osborn, 1969). Muscle actin, myosin, and bovine serum albumin served as protein standards. Bromphenol blue was used as the tracking dye and the gels were stained with Coomassie blue. Protein standards, extracts from acetone powders, or portions of sperm such as flagella, flagellar axonemes, or acrosomal processes, or even whole sperm heads were dissolved in 1% SDS in 0.1 M phosphate buffer at pH 6.8 which contained 1% mercaptoethanol and then boiled for 2 min.

Light Microscopy

Whole sperm, sperm heads, flagella, and sperm heads which had undergone the acrosomal reaction were observed with phase-contrast microscopy using Leitz or Zeiss optics. The best observations were made with 100 × oil planachro Zeiss phase objective and Zeiss condenser.

Electron Microscopy

NEGATIVE STAINING: Sperm heads which had been induced to undergo the acrosomal reaction with alkaline sea water were dried on a grid and stained with 1% uranyl acetate. To observe F-actin a drop of polymerized and purified sperm actin was added to a carbon-coated grid and negative stain (1% uranyl acetate) was added. In some cases the solution of sperm actin had to be diluted ten times.

FIXATION AND EMBEDDING: Whole sperm or sperm heads before or after induction of the acrosomal reaction were fixed for 1 h in 2% glutaraldehyde in sea water at room temperature. The sperm were washed briefly in sea water and postfixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 7.0. They were dehydrated in acetone and embedded in Epon 812.

PREPARATION OF THIN SECTIONS: Thin sections were cut on a Sorvall Porter-Blum II ultramicrotome and stained with uranyl acetate and lead citrate. They were viewed with an Hitachi II or Philips 200 electron microscope. The microscope was calibrated using a replica grating (Ernest F. Fullam, Inc., Schenectady, N. Y.).

HMM BINDING IN SITU: We followed the procedure of Ishikawa et al. (1969). The formation of the acrosomal processes was first induced with egg water or alkaline sea water and then glycerinated.

RESULTS

Morphology

Light Microscopy

Earlier investigations on unreacted and reacted sperm by Dan (1954) and Colwin and Colwin

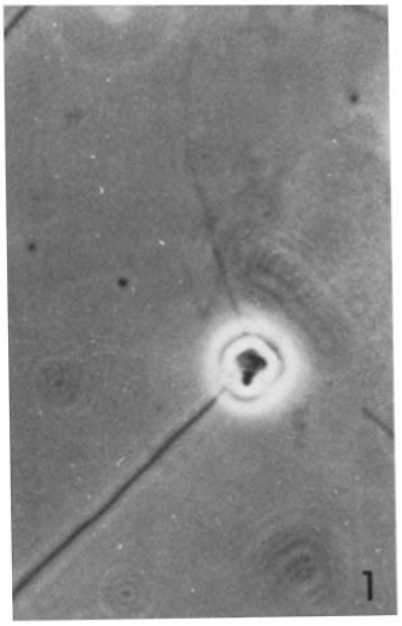

FIGURE 1 *Thyone* sperm induced to undergo the acrosomal reaction by sea water to which ammonium hydroxide had been added. The acrosomal process extends vertically from the head. Note thatthe acrosomal process is less than one-half the diameter of the flagellum. Phase-contrast microscopy. × 2,500.

(1956) make it unnecessary here to describe the sperm again. We will mention only those features directly pertaining to subsequent discussion.

The refractile acrosome in the unreacted sperm of *Thyone* or *Asterias* is cone shaped with its apex pointing toward the midpiece, which appears to give the anterior surface its characteristic flattened appearance. The head measures 2–3 μm in diameter and the tails 50–60 μm in length. Upon the addition of egg water or alkaline sea water, the acrosomal processes form from about 25 to 50% of the sperm (Fig. 1). Reacted sperm can be identified easily, as the flagella now project laterally from the midpiece, an observation initially made by Chambers (1930) and confirmed by Dan (1954) and Colwin and Colwin (1955, 1956). The anterior surface of the head no longer appears flattened, but is rounded. Extending from it is the acrosomal process which measures about 0.1 μm in diameter, less than one-half the diameter of a flagellum (Fig. 1). This process, which is difficult to resolve unless it lies in one plane, i e., perpendicular to the objective lens, is variable in length. In *Thyone* (Colwin and Colwin, 1956) it can measure maximally 90 μm and in *Asterias* (Dan, 1954) about 25 μm.

ELECTRON MICROSCOPY

UNREACTED SPERM: The acrosome of *Asterias* or *Thyone* consists of two parts, an acrosomal vesicle and an amorphous material which surrounds it (Figs. 2 and 3). The acrosome itself is bounded anteriorly by the plasma membrane and posterolaterally by the nuclear envelope. The vesicle is membrane limited and shows apical, lateral, and basal specializations. These have been described in detail by Hagiwara et al. (1967) and Dan (1960) for *Asterias* and by Summers et al. (1971) for *Thyone* and *Ctenodiscus*. The amorphous material is not membrane bound, but seems to be contained in the anterior portion of the sperm only by the close apposition of the plasmalemma and the nuclear envelope. Fig. 3 illustrates this very well as the membranes have pulled away from the sperm itself due to imperfect fixation. The importance of this observation will be presented in the Discussion. For further discussion it is convenient to subdivide the periacrosomal amorphous material into three regions. Region *a*, located posterior to the acrosomal vesicle in an indentation in the nuclear envelope, consists of two components, a central dense homogeneous material surrounded by a less dense amorphous material (Fig. 2). It is difficult to define the latter precisely, but in most micrographs it looks "cotton-like." Similar material is present in regions *b* and *c* as well.

The chromatin in the nucleus of *Asterias* sperm is in the form of long rods which measure about 300 Å in diameter (Fig. 3). The midpiece contains a mitochondrion, a centriole, and the basal body of the flagellum (Fig. 2). Sperm with more than one flagellum are not uncommon.

REACTED SPERM: One of the first events in the acrosomal reaction is the fusion of the membrane surrounding the acrosomal vesicle with the plasma membrane, thus liberating the contents of the acrosomal vesicle into the sea water. (See Dan, 1960; Dan and Hagiwara, 1967; Summers et al., 1971.) Stages fixed during formation of the acrosomal process have been described by Dan and Hagiwara (1967). Incredible though it may seem, within a couple of seconds after activation, elongation of the process begins. The reaction is complete within 60 s, with most of the elongation occurring within the first 10 s.

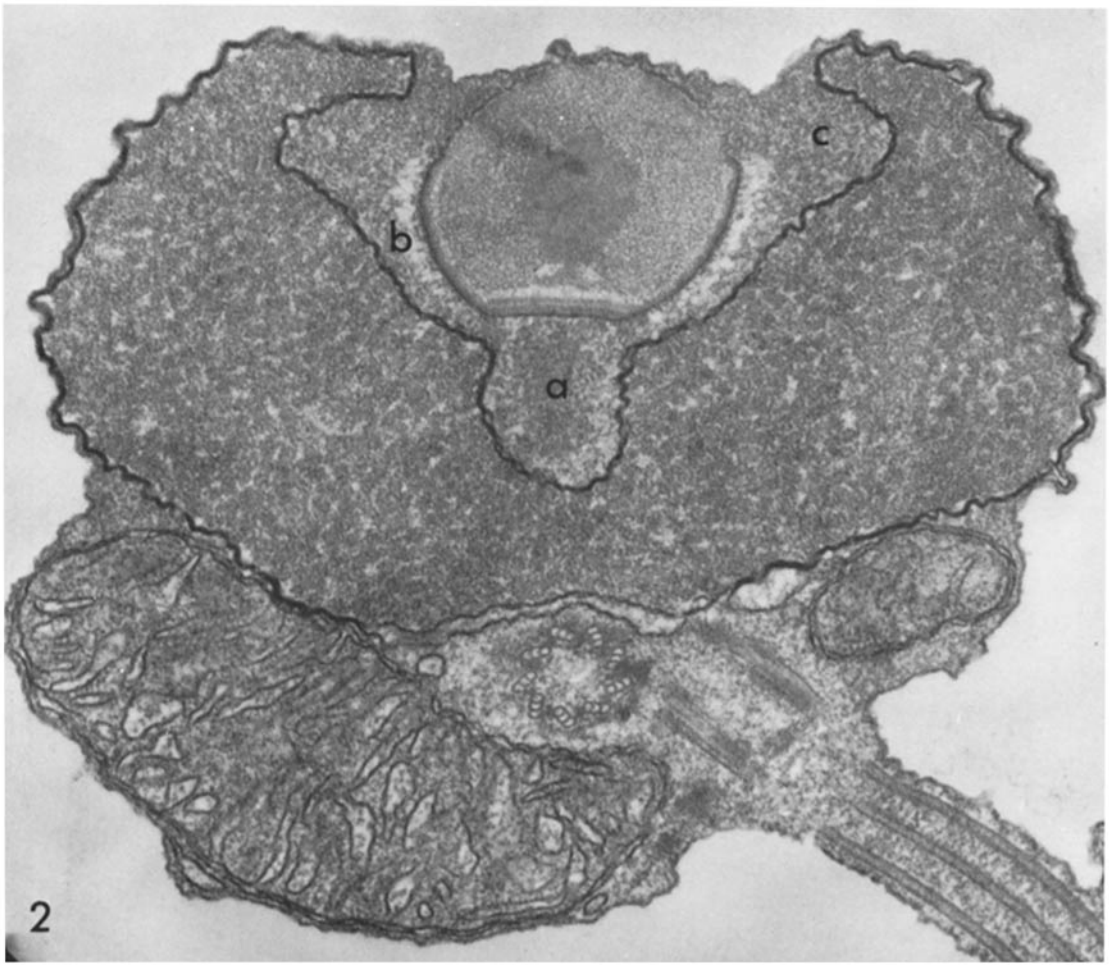

FIGURE 2 Electron micrograph of a section of the sperm of *A. amurensis*. Located within an indentation in the nucleus is the acrosomal region. It consists of a vesicle and surrounding it is an amorphous material which is labeled *a*, *b*, or *c*. The flagellum of the sperm extends to the lower right. × 73,000.

The acrosomal process is readily identified in thin sections through reacted sperm (Fig. 4). Within each process is a core of densely packed microfilaments. The microfilaments measure 50 Å in diameter (Figs. 4 and 5). We have counted up to 140 filaments in a single acrosomal process and as few as 35. We do not know how constant the filament number is at varying distances along a single process as serial sections were not cut, but clearly the number varies from process to process.

As already discussed in detail by Dan and Hagiwara (1967) regions *a* and *b* appear to contribute to the filamentous core in the acrosomal process. Region *c*, however, adheres closely to the anterolateral surfaces of the nucleus (see Fig. 4). In some reacted sperm, a remnant of the dense material present in region *a* can still be identified.

Evidence for the Presence of Actin in Sperm

MYOSIN OR HMM BINDING TO A PROTEIN IN AN EXTRACT FROM AN ACETONE POWDER OF WHOLE SPERM

When a water extract from acetone-dried sperm was mixed with a solution of myosin in 0.5 M KCl, buffered to pH 6.5 with 10 mM Tris maleate or, in another group of experiments, with HMM in 0.1 M KCl at 22°C, a marked increase in viscosity took place (Fig. 6). Generally there was a lag of 1–3 h between the time of the addition of

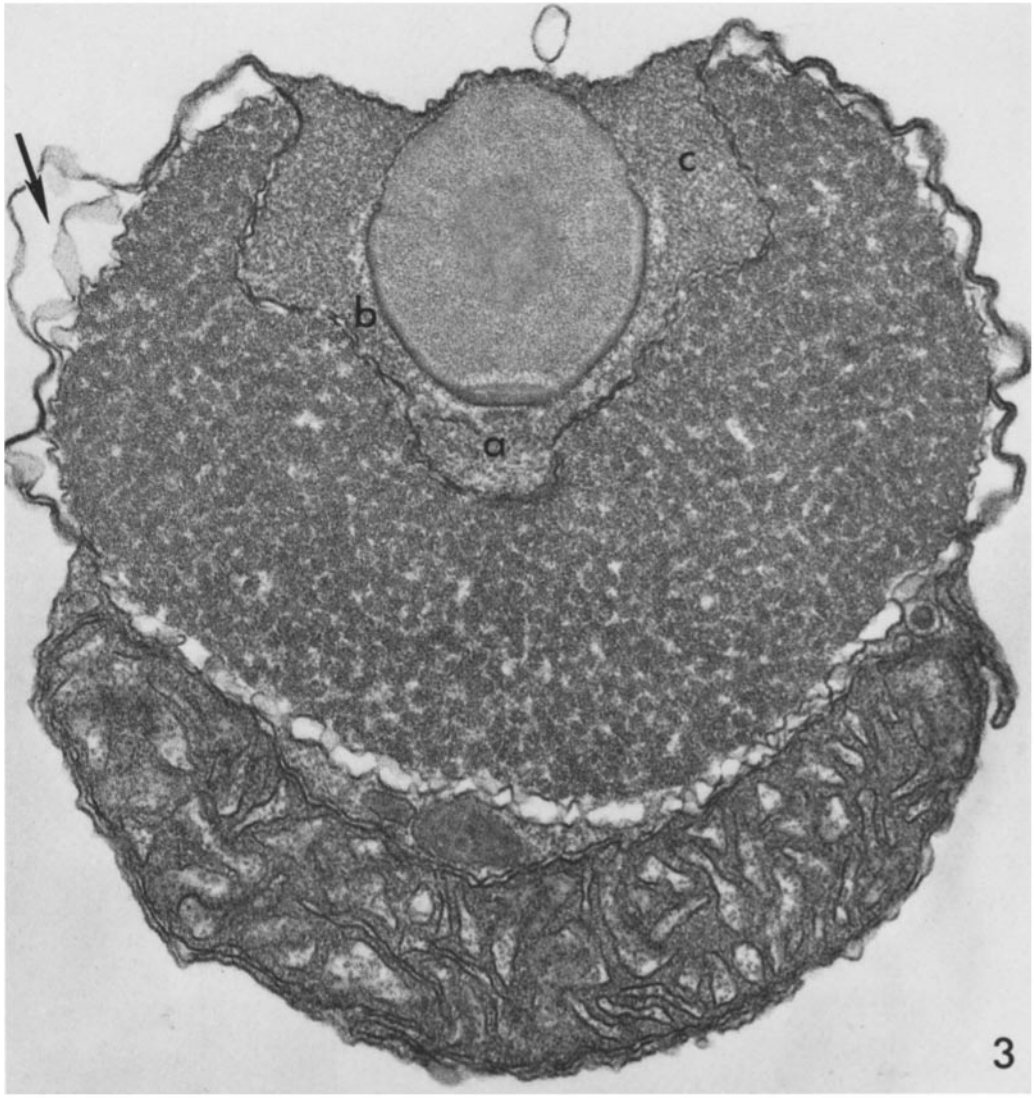

FIGURE 3 Electron micrograph of a section through a sperm of *A. amurensis*. The close apposition between the nuclear membrane and the plasma membrane can be clearly seen in this micrograph. This connection appears to enclose the amorphous material depicted by the letters *a*, *b*, and *c* in the anterior end of the sperm. The arrow indicates a region where the limiting membrane is separated from the nuclear envelope. × 73,000.

the myosin or HMM and a change in viscosity. This lag was dependent upon the batch of acetone powder and presumably demonstrates the amount of ATP extracted from the powder. In fact, when the extract was treated with a small amount of Dowex 1 Cl (about ¼) to remove ATP, myosin bound to the actin in the extract immediately after the addition of myosin. The increase in viscosity of the mixture (Fig. 6) dropped to the baseline level upon the addition of 0.5 mM ATP. When the myosin hydrolyzed the ATP, the viscosity rose again (Fig. 6). If ATP were added a second time, the viscosity again fell to the baseline. As before, when it was hydrolyzed, the viscosity increased again, and so forth. This drop in viscosity due to ATP has been referred to as "ATP sensitivity" (see Miki-Noumura and Oosawa, 1969) and varied from 46 to 55% de-

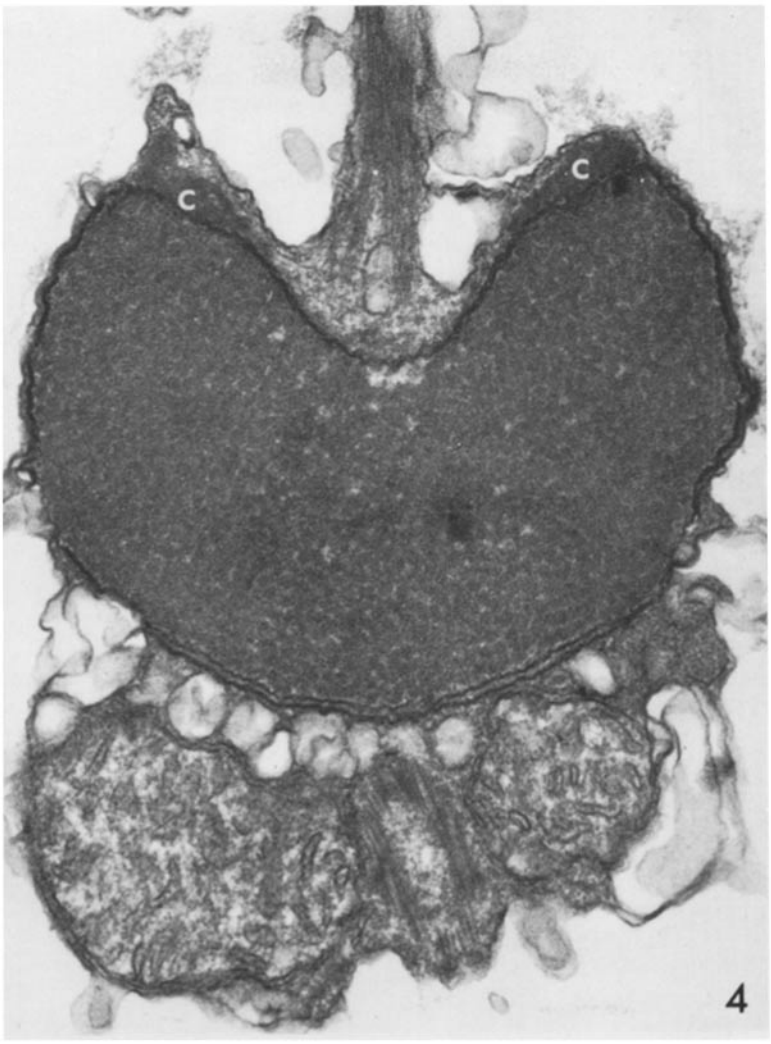

FIGURE 4 Electron micrograph of a section of a sperm of *A. amurensis* which has undergone the acrosomal reaction. The lower portion of the acrosomal process is present with its core of microfilaments. Region *c* corresponds to region *c* seen in the preceding micrograph. The flagellum is cut obliquely. × 62,000.

pending upon the preparation. Controls were run to demonstrate that muscle myosin was not contaminated with muscle actin. For example, no change in the viscosity of a solution of myosin in the absence of the extract could be seen even when ATP was added to this solution of myosin.

SDS Gel Electrophoresis

When a water extract from an acetone powder of sperm was analyzed by SDS gel electrophoresis, two major bands and a number of minor bands could be seen (Fig. 7). One of the major bands had a molecular weight indistinguishable from that of muscle actin; the other had a molecular weight indistinguishable from that of tubulin.

If myosin was added to the water extract in 0.5 M KCl at 20°C and association of the actin and myosin allowed to proceed, and then the KCl concentration reduced with distilled water to 0.05 M, precipitation was initiated. The precipitate was collected by centrifugation, washed with 0.05 M KCl, extracted with acetone, and air dried. This acetone powder was reextracted with water and the extract run on SDS gel electropho-

resis (Fig. 8). The gels contained a major band whose molecular weight was indistinguishable from that of skeletal muscle actin. This purification method eliminates contamination by tubulin and other proteins. Yet present in these gels were bands corresponding in molecular weight to the small subunits of myosin. When the myosin itself was run on an SDS gel, we could not demonstrate a band whose molecular weight corresponded to that of actin. Only the light and heavy chains of myosin were present. Thus, our myosin did not appear to be contaminated with muscle actin.

Purification of the "sperm actin" could also be achieved if magnesium ($MgCl_2$ at 2 mM) was added to the water extract from an acetone powder, and the solution spun at 80,000 *g* for 3 h. When the pellet was run on the SDS gel, only a single band could be demonstrated. This band had a molecular weight indistinguishable from that of actin. We have no data as to the yield of this actin; that is, how much still remained in the supernate. Our water extract contained about 7 mg/ml of protein. From our gels and from the amount of the protein in the extract that can be precipitated with myosin, we estimated that less than 20% of the total soluble protein was actin. From these estimates we calculate that our extracts contain about 1 mg/ml of actin. Thus there should be more than sufficient actin to exceed the "critical" concentration necessary for actin polymerization.

G to F Transformation

Unlike water extracts from acetone powders of skeletal muscle, those from sperm showed a very small change in viscosity upon the addition of 0.1 M KCl or 2 mM $MgCl_2$. Nevertheless, if a drop of this solution were negatively stained, 50 Å filaments indistinguishable from those formed from skeletal muscle actin could be seen (identical to those illustrated in Fig. 9).

A marked increase in the number of filaments and in the viscosity could be achieved by combining the sperm actin with purified muscle myosin, isolating the actomyosin, acetone drying, and reextracting the acetone powder (Hatano and Oosawa, 1966). The extract was concentrated and dialyzed against a solution of 3 mM cysteine and 0.05 mM ATP at pH 8.0 for 2 h. It was further purified by isoelectric precipitation at pH 4.7.

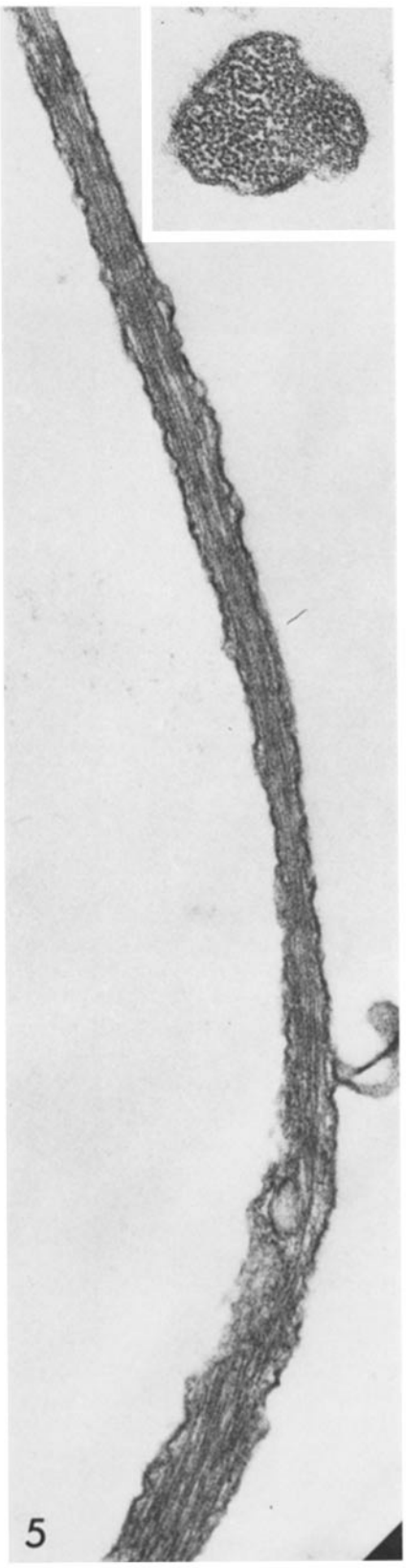

FIGURE 5 Longitudinal section through an acrosomal process. × 72,000. *Inset*: Transverse section through an acrosomal process. × 120,000.

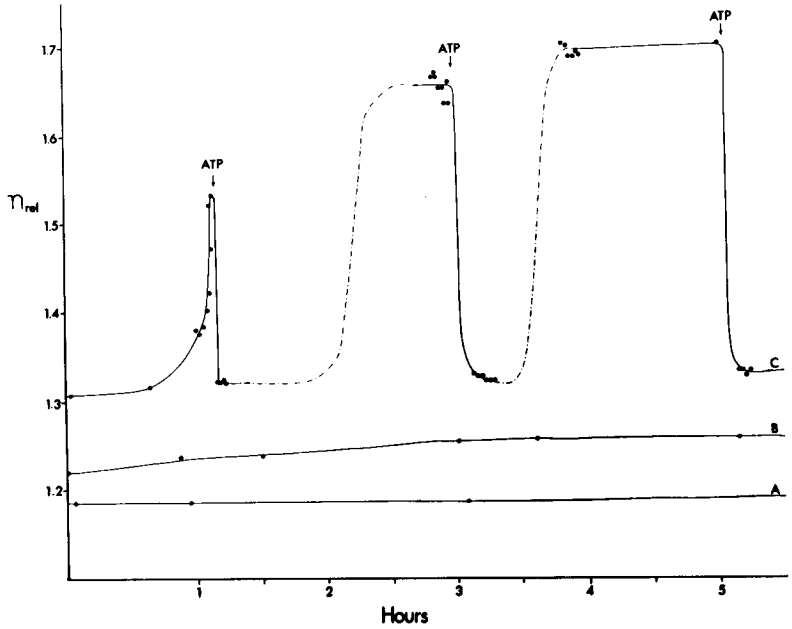

FIGURE 6 The relative viscosity of a solution is plotted against time. (*a*) Skeletal muscle myosin (1 mg/ml) in 0.5 M KCl and 10 mM Tris maleate at pH 7.0. (*b*) Extract from an acetone powder of sperm (5 mg/ml protein) in 10 mM Tris maleate at pH 7.0 and containing 0.5 M KCl. (*c*) Extract from an acetone powder of sperm (5 mg/ml protein) to which myosin (1 mg/ml), KCl (0.5 M), and Tris maleate buffer (10 mM) at pH 7.0 had been added. At the intervals indicated, ATP (0.5 mM) neutralized to pH 7.0 was added. All measurements were made at 22 ± 0.1°C.

Salting out with ammonium sulfate was found to be impossible because the sperm G-actin thus obtained is easily denatured. This partially purified G-actin solution has some viscosity (a relative viscosity of 1.6). However, when 0.1 M KCl was added, the viscosity of the G-actin increased, indicating polymerization to F-actin. A drop of this solution diluted ten times and negatively stained showed the presence of large numbers of filaments (Fig. 9). The specific viscosity was estimated to be 3 dl/g which is a little smaller than that of plasmodium F-actin (7 dl/g) and of skeletal muscle F-actin (10 dl/g) (Hatano et al., 1969).

Localization of Sperm Actin

Sperm Head or Flagellum

We separated the flagella from their heads by homogenization and centrifugation (Fig. 10). The flagella or the isolated flagellar axoneme were analyzed by SDS gel electrophoresis to determine the molecular weights of the component proteins (Fig. 11, gel *a*). Although tubulin and the dynein bands (note arrow in Fig. 11; for a detailed presentation of the proteins present in cilia, or flagella, see Linck, 1970, and Stephens, 1971) were present, no band whose molecular weight corresponded to that of actin could be found. Yet gels run of whole sperm or sperm heads after homogenization, DNase digestion, and SDS solubilization showed a prominent band whose molecular weight was indistinguishable from that of muscle actin. Alternatively, sperm heads could be treated with Triton X-100 (Fig. 12) and the soluble proteins precipitated with TCA. This precipitate was acetone extracted and air dried. The resulting acetone powder was extracted with water and the extract run on gels. In this case, only a single band was present (Fig. 11, gel *c*) its molecular weight indistinguishable from that of muscle actin (Fig. 11, gel *b*).

The Location of Actin in the Isolated Acrosomal Processes

We induced the acrosomal reaction in isolated sperm heads of *Thyone* with alkaline sea water (Fig. 13). The acrosomal processes were then isolated by homogenization and run on SDS gels.

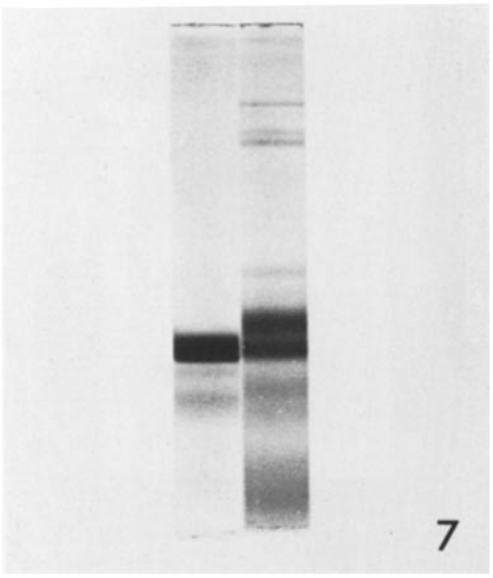

FIGURE 7 SDS gel electrophoresis. In the right-hand gel an extract from an acetone powder of sperm is shown; in the left-hand gel actin prepared from rabbit skeletal muscle is depicted.

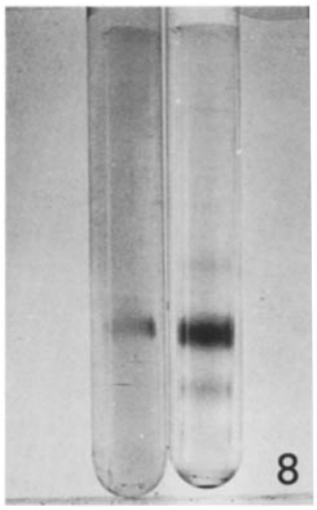

FIGURE 8 SDS gel electrophoresis. Myosin was added to a water extract of an acetone powder of *Asterias* sperm, the actomyosin isolated, acetone extracted, dried, and reextracted with water. This extract was treated with SDS and run on the gel on the right. Rabbit actin was run as a standard on the gel on the left.

The most prominent band had the same molecular weight as that of actin (Fig. 11, gel *d*). A small amount of tubulin contaminated the preparation. This contamination appeared to be due to tiny stubs of flagella that remained after the first homogenization. The other bands on the gel were presumably membrane proteins.

HMM Binding In Situ

We carried out two separate experiments designed to indicate the intracellular distribution of actin in sperm, one on the sperm of *A. amurensis*, the other on the sperm of *A. pectinifera*. We treated the former with egg water, the latter with alkaline sea water. Only about 20% of the sperm underwent the acrosomal reaction with either technique, possibly because the season was nearly over for the former and just beginning for the latter. Yet thin sections of glycerinated sperm which had been treated with HMM revealed that nearly all the sperm had filaments extending from the anterior end of the nucleus, the position from which the acrosomal process develops. The acrosomal vesicles could not be identified in these glycerinated sperm but, presumably due to breakage of the membranes, the anterior end of the sperm could be readily identified by the indentation in the nucleus (Figs. 14 and 15) and the presence of the flagellum beneath the nucleus (Fig. 14). The filaments, nevertheless, all appeared decorated with HMM.

We attempted to determine the polarity of the filaments as judged by the direction of the HMM arrowheads. Preliminary observations on thin sections indicate that, (*a*) the arrowheads point in the same direction in a given filament and (*b*) adjacent filaments making up an acrosomal bundle appear to have the same polarity; the arrowheads seem to point towards the nucleus (Fig. 15). We are currently trying to repeat this experiment by negative staining, as superposition of filaments and arrowheads in sections makes firm statements about polarity impossible so that the above observations should be viewed with caution at this time.

On rare occasions filaments could be seen lying parallel to the axoneme. Here, unlike the situation mentioned above, the polarity of adjacent filaments was not unidirectional. As will be mentioned in the Discussion, the location of decorated filaments near the axoneme is best interpreted as caused by diffusion of the monomer into the flagella during glycerination.

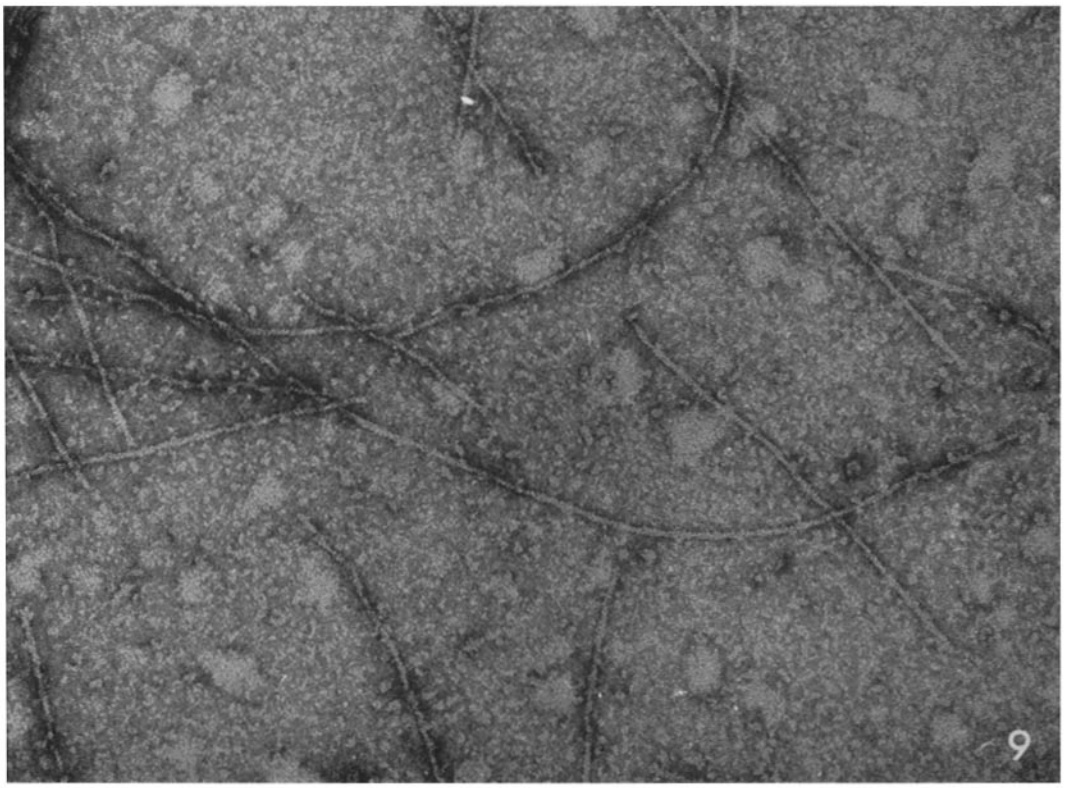

FIG. 9 A water extract from an acetone powder of *Asterias* sperm was precipitated at its isoelectric point, redissolved at neutral pH, and negatively stained. The filaments present show the chraracteristic double helical structure of F-actin. × 110,000.

Evidence That the Actin in the Unreacted Sperm is in the Monomeric State Rather Than as F-Actin or as a Flexible Polymer

Freshly collected sperm of *Thyone* were washed in a large volume of 1 M sucrose which contained 20 mM EDTA at pH 7.0 at 0°C. They were collected by centrifugation (5,000 *g* for 5 min). Since the sucrose and EDTA solution is isotonic, the sperm are not damaged by washing to remove sea water salts. The pellet was resuspended in 10 vol of 0.1% Triton X-100, 5 mM Tris HCl at pH 7.5, and 20 mM EDTA for 20 min. This procedure and subsequent ones were all carried out on ice. Within a few seconds after the addition of the detergent solution the membranes in the sperm were broken down. The suspension was then spun at low speed for 10 min (10,000 *g*) to remove the nuclei which in a subsequent step would form a DNA gel if not removed. The supernate was then spun at 80,000 *g* for 3 h. The protein in the high speed supernate was precipitated in cold TCA and collected at 1,000 *g* for 5 min. This precipitate was dissolved in 1% mercaptoethanol, 1% SDS in 0.1 M phosphate buffer, and boiled for 2 min. The high speed pellet was dissolved in an equal volume of mercaptoethanol, SDS, and buffer, and then boiled. Since the protein in the high speed supernate and the protein in the high speed pellet were dissolved to an equal volume of SDS-mercaptoethanol-buffer, the amount of actin in these two samples could be compared quantitatively by SDS gel electrophoresis. The gels of the high speed pellet and supernate are depicted in Fig. 16. As can easily be seen, most of the actin is present in the supernate. By densitometry we determined that at least 80% of the actin is present in the supernate. Thus the actin must exist in the monomeric form in the sperm.

To prove that the actin did not depolymerize to any appreciable extent during treatment with the detergent solution containing EDTA or during the

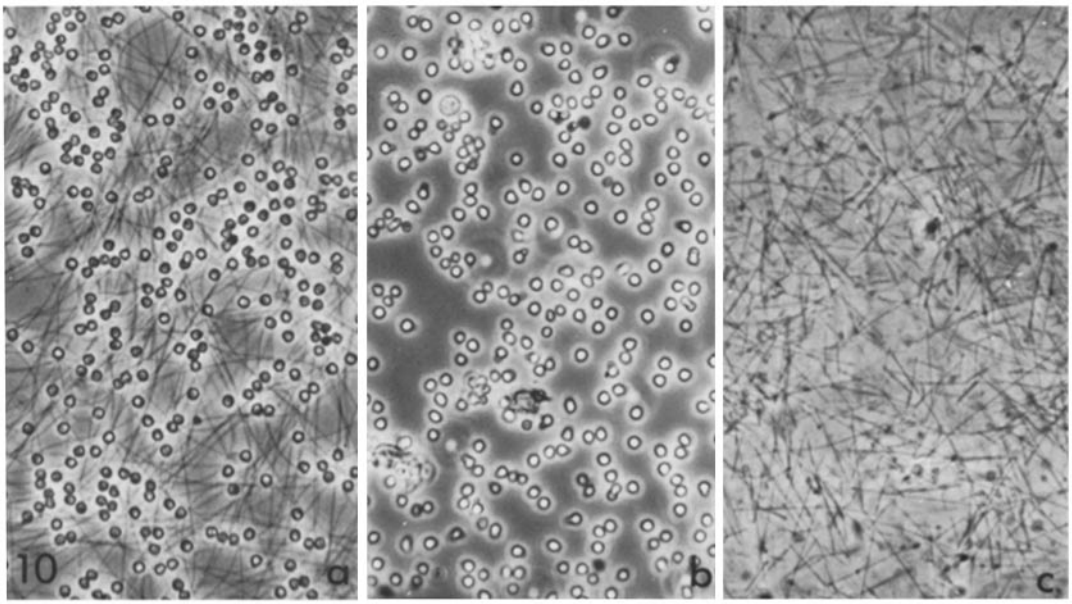

FIGURE 10 (*a*) *Thyone* sperm. (*b*) Isolated heads of *Thyone* sperm. (*c*) Isolated flagella of *Thyone* sperm. × 660.

high speed centrifugation and thus give us a false idea of the state of actin in the sperm, we extracted acetone powders of *Thyone* sperm and acetone powders of rabbit skeletal muscle with water, polymerized the actin with 2 mM $MgCl_2$ and 0.1 M KCl, collected the F-actin by centrifugation at 80,000 *g* for 2 h, and then resuspended the F-actin pellet in 0.1% Triton X-100, 5 mM Tris HCl at pH 7.5, and 20 mM EDTA, then centrifuged at 80,000 *g* for 3 h. We processed the supernate and pellets from the second high speed spin as before and analyzed them by SDS gel electrophoresis. About 90% of the actin in these cases was in the pellet, which proves that the actin does not *depolymerize* to any appreciable extent during our procedure. These experiments demonstrate that the actin in the sperm is neither in the F state nor in an aggregate such as a flexible polymer, but rather in the G state.

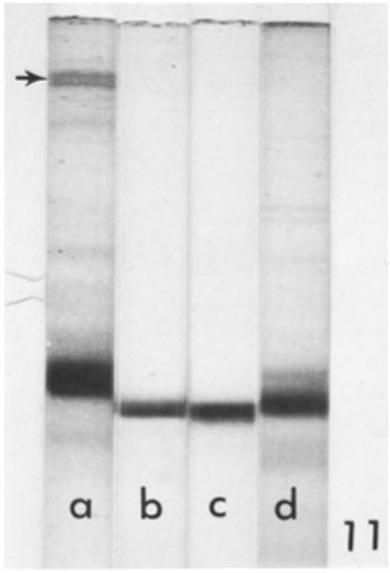

FIGURE 11 SDS gel electrophoresis. (*a*) Isolated *Thyone* flagella. The arrow indicates dynein bands. (*b*) Rabbit actin as a standard. (*c*) Isolated heads of *Thyone* sperm were treated with Triton X-100 and the proteins in the supernate precipitated with TCA, acetone extracted, air dried, and a water extract run on this gel. (*d*) Isolated acrosomal process of *Thyone* sperm.

DISCUSSION

We have demonstrated that there is actin in echinoderm sperm and that this actin is localized in the numerous microfilaments which are present within the acrosomal processes. Before the acrosomal reaction the sperm actin is present as an amorphous material in the periacrosomal region

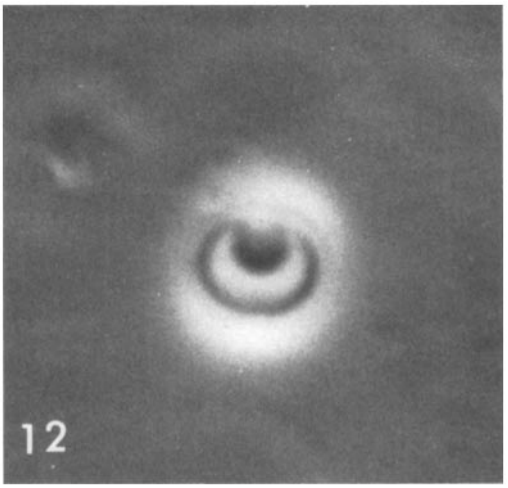

FIGURE 12 The isolated sperm heads of *Thyone* after treatment with Triton X-100. × 3,500.

beneath and lateral to the acrosomal vesicle (regions *a* and *b* of Fig. 2). During the reaction this material seems to change rapidly from its amorphous condition into a series of linear filaments.

It seems reasonable to interpret this morphological change from the amorphous state to a filamentous state as the result of the polymerization of actin or, in the terminology of muscle biochemists, as a G to F transformation. Since the limiting membrane does not display rigidity in any other system and seems to be unable to influence the asymmetry of a cell in the absence of an intracellular core of supporting elements (see Tilney, 1968, or Tilney and Cardell, 1970), it seems reasonable to postulate that the force for the rapid formation of this process is provided by the polymerization of actin. One possibility still remains and that is that the amorphous material is in reality a series of extremely flexible filaments, all wound around each other, which undergo a striking conformational change during the acrosomal reaction. To rule out this possibility, we lysed the sperm in the absence of Mg^{++} so that polymerization would not take place and then spun the lysate at high speed to analyze how much of the actin was in the pellet, i.e., existing as F-actin or as a flexible polymer, and how much was in the supernate indicating the monomeric or G state. About 80% of the actin appeared in the supernate. Thus the actin must exist in the monomeric state in the unreacted sperm and the change to the F form appears to give rise to the rapid elongation of the acrosomal process.

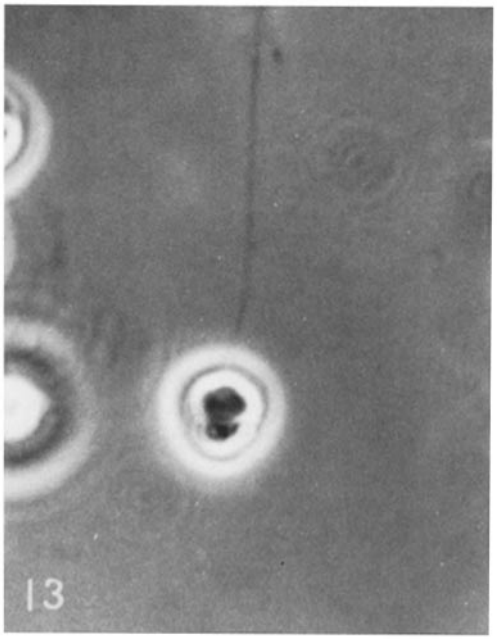

FIGURE 13 An isolated head of *Thyone* was induced to undergo the acrosomal reaction with sea water to which ammonium hydroxide had been added. × 3,000.

Our experiments are also consistent with other observations which suggest that the actin in the sperm cannot exist as a flexible polymer. These observations are discussed below as they bring out several interesting facts not only about this model system but about why other investigators have incorrectly concluded that there is actin in flagella. First, when an acetone powder of sperm is extracted and the actin polymerized into filaments, these filaments appear straight or slightly curved by negative staining, never do they make angular bends. A similar situation exists when actin is isolated from either nonmuscle cells (Tilney and Mooseker, 1971) or skeletal or smooth muscle cells, and polymerized. This is also true when thin sections of cells are examined. We should mention that there are invertebrate sperm which contain, before the acrosomal reaction, linear filaments of the same dimensions and morphology as those described in this report (for example, in *Nereis*, or *Mytilis*, see Dan, 1970, for references). These filaments are stored in the sperm as straight rods. Thus there seems to be no precedent in the literature for actin filaments of extreme flexibility; instead both in vivo and in vitro studies of actin show the filaments as predominantly straight.

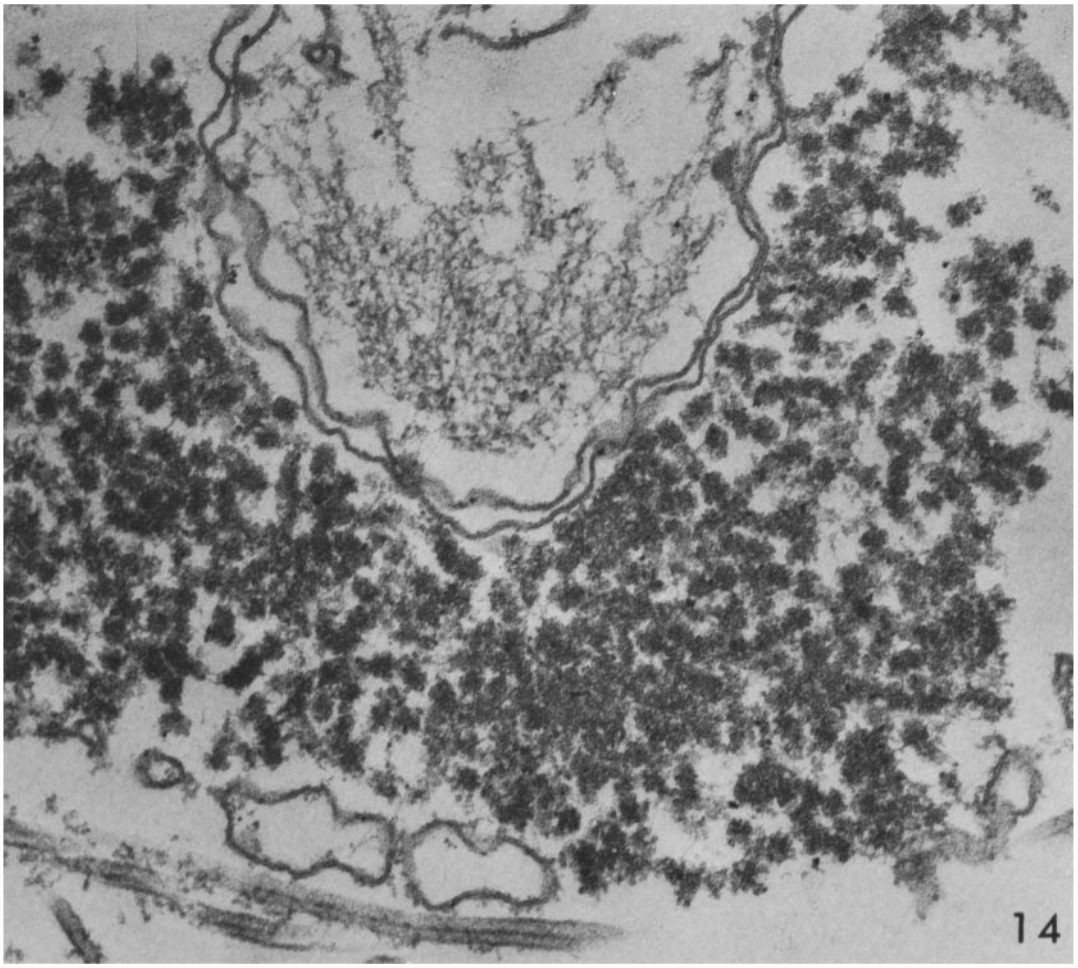

FIGURE 14 Electron micrograph illustrating HMM binding to glycerinated sperm of *A. amurensis* which had been previously induced to form their acrosomal processes. Decorated filaments are present extending from the anterior end of the sperm. A portion of a flagellum is seen beneath the nuclear region. × 125,000.

Secondly, from our own observations, although they are still tentative, the filaments in the acrosomal process appear to have unidirectional polarity as determined by the direction of "arrowheads" when HMM is added. If a conformational change were to take place, the filaments, in order to achieve the requisite polarity within a few seconds, would have to be initially attached to a particular region or show some preexisting organization. Neither has been observed. On the other hand, if polymerization were to take place, polarity could be established by nucleation, as seems to be the case during the formation of microvilli (Tilney and Cardell, 1970). Finally, diffusion of the actin to the tail during glycerination which precedes HMM binding would probably occur only if the actin existed in the monomeric form. To be more specific, we demonstrated biochemically that there is no actin in the flagellum, but decorated filaments can occasionally be found in the flagellum after glycerination and HMM addition. We conclude that these are derived from the periacrosomal material (regions *a*, *b*, and *c* of Fig. 2). Forer and Behnke (1972) recently concluded that HMM induces the polymerization of actin in glycerinated cells. Since the actin before the acrosomal reaction is not enclosed in a vesicle but trapped between the nuclear en-

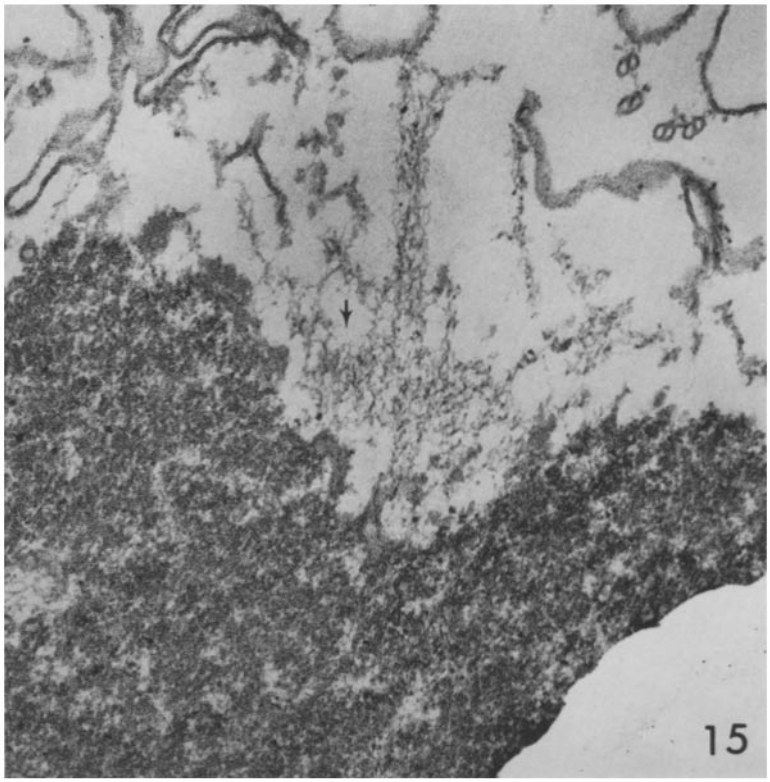

FIGURE 15 Electron micrograph of a section of a sperm head of *A. pectinifera* which had been induced to undergo the acrosomal reaction. The sperm was then glycerinated and HMM was added. Note the decorated filaments in the acrosomal region. The HMM arrowheads appear to point towards the nucleus as indicated by the arrow. × 100,000.

velope and the limiting plasmalemma, it could diffuse from the head to the tail, provided the limiting plasma membrane is removed or "loosened" as during glycerination (see Fig. 3). The possibility of a filament diffusing to the flagellum is extremely unlikely. Actin in the monomeric state, however, could diffuse to the flagellum. This argument for the route of transport of the actin to the flagellum is strengthened by observations of the Colwins (Colwin and Colwin, 1961) during fertilization in *Hydroides*. After the acrosomal process has fused with the egg plasma membrane, egg cytoplasm may flow to at least the basal portion of the flagellum using the same route as outlined above for monomeric actin. Surprising though it may seem, the periacrosomal material is kept in the anterior end of the sperm only because of the tight juxtaposition of the nuclear and plasma membranes. How the G-actin is sequestered in the anterior end of the sperm during spermiogenesis is an intriguing problem which cannot be answered at this time.

We should comment further on reports in the literature which state that actin is present in sperm flagella. These claims, in light of our observations mentioned above, probably result from contamination of the actin from the acrosomal region. In our study we demonstrated by SDS gel electrophoresis on isolated flagella that there is no band of comparable molecular weight to actin, rather the actin is located in the acrosomal region. Consistent with our observations, Miki-Noumura and Mohri (personal communications) have shown that when myosin is added to the axoneme of sea urchin flagella, there is some increase in viscosity, but no ATP sensitivity. These observations lead us to conclude that actin is not present in flagella. The claims of actin in the flagella of *Asterias* (Nelson, 1966) and in the flagella of *Nephrotoma* (Behnke et al., 1971) are better interpreted as

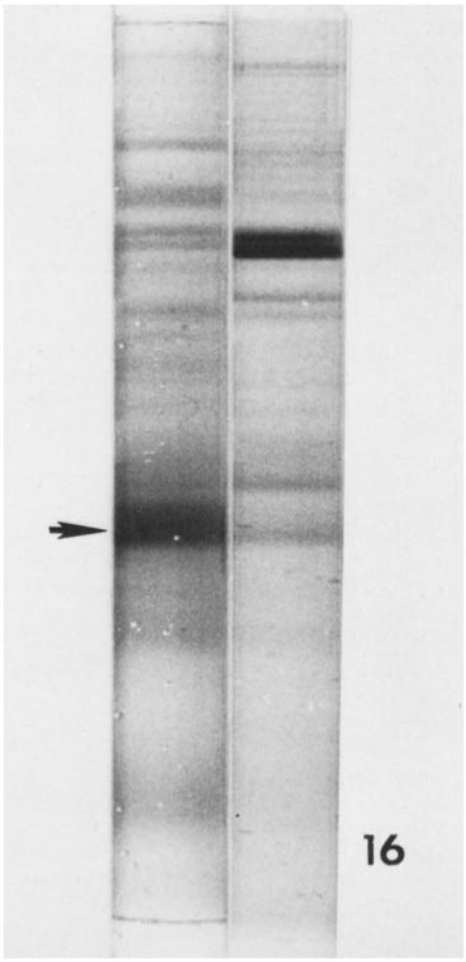

FIGURE 16 SDS gel electrophoresis. Sperm were washed in 1 M sucrose which contained 20 mM EDTA and 5 mM Tris HCl at pH 7.5, then lysed with 0.1% Triton X-100, 20 mM EDTA, and 5 mM Tris HCl at 0°C. After removal of the heads the supernate was spun at 80,000 *g* for 3 h and the amount of actin (see arrow) present in the supernate (left gel) was compared quantitatively to that in the pellet (right gel). Over 80% of the actin is present in the supernate showing that in the sperm the actin exists in the monomeric or G state.

contamination of actin from the acrosomal region during sonication or glycerination.

Our study has left us with many unanswered questions, most of which are concerned with the control of the polymerization of actin and how the actin might function during fertilization, questions which are basic to an understanding of actin associated motility in nonmuscle cells. Foremost in our minds is the fact that actin, whether derived from skeletal muscle or nonmuscle cells, will explosively undergo a G to F transformation in vitro in the presence of K^+ and/or Mg^{++}. These ions must be present in sperm as they are essential for flagellar movement (Gibbons and Fronk, 1972). The spatial and temporal control of the disposition of actin filaments is of considerable importance in nonmuscle cells because filaments appear in specific regions in the cell at certain times; at succeeding stages they disappear, only to reappear at another place. Thus there must be a mechanism in sperm and in other cells as well which keeps the actin in the unpolymerized state. What this mechanism is must await further experimentation now in progress.

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