THE POLYMERIZATION OF ACTIN

III. Aggregates of Nonfilamentous Actin and Its Associated Proteins: A Storage Form of Actin

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ABSTRACT

When echinoderm sperm are treated with the detergent Triton X-100 at pH 6.4 in 10 mM phosphate buffer, the membranes are solubilized, but the actin which is located in the periacrosomal region remains as a phase-dense cup. These cups can be isolated free from the flagella and chromatin and can be solubilized by increasing the pH to 8.0 and by changing the ionic strength and type of buffer used. Since the actin does not exist in the "F" state in unreacted sperm, and since the actin remains as a unit that does not diffuse away, it must be present in the mature sperm in a bound or storage state. The actin is, in fact, associated with a pair of proteins whose mol wt are 250,000 and 230,000. When the isolated cups are digested with trypsin, these high molecular weight proteins are digested, thereby liberating the actin. The actin will polymerize if heavy meromyosin or subfragment 1 is added to a preparation of isolated cups. Evidence is presented that this pair of high molecular weight proteins is similar in molecular weight and properties to erythrocyte spectrin. Attempts at transforming the storage form of actin in the cup into filaments were only moderately successful. The best conditions for filament formation involve incubating the cup in ATP and divalent salts. Careful examination of these cups reveals that the actin polymerizes preferentially on either end of oriented filaments that already exist in the cup, indicating that self-nucleation is inefficacious. I conclude that the actin can exist in the storage form by its association with spectrin-like molecules and that the actin in this state polymerizes preferentially onto existing filaments.

In the preceding paper in this series, I demonstrated that nonfilamentous actin appears to be associated with specialized regions of the nuclear envelope and the acrosomal vacuole membrane. Thus, when sperm are treated with solutions which solubilize or rupture the plasma membrane, such as hypotonic solutions, hexylene glycol, or the nonionic detergent, Triton X-100, the plasma membrane separates or is dissolved from the sperm, yet the periacrosomal material (profil. actin) remains bound to the specialized portions of the nuclear envelope and the acrosomal vacuole membrane. A careful examination of the sperm after plasma membrane removal reveals, furthermore, that the profil. actin not only remains associated with these membranes but also must adhere to itself. If not, with time the bulk of the profil. actin would diffuse away, but this does not
occur even if these preparations are kept at room temperature for prolonged periods.

Obviously, we would like to know more about how the nonfilamentous actin remains insoluble. Is it, for example, kept sequestered in the anterior end of the sperm by its association with other substances and, if so, what are these substances and how do they act? How stable is the aggregate of nonfilamentous actin? How can the actin be released from these substances and, once released, will the actin polymerize? Is this aggregate present only in sperm or is it present in many (all) nonmuscle cells as well?

In this report, I will attempt to answer some of these questions. More specifically, I will demonstrate that the aggregate of nonfilamentous actin is stable under certain conditions and will solubilize under other conditions. I will then show that the profil, actin consists of three proteins, actin and two high molecular weight proteins which are similar to spectrin, the major proteins of the erythrocyte ghost. Although attempts to polymerize the actin from isolated profil, actin were only moderately successful, these experiments indicate that the actin appears to nucleate poorly. Thus, the polymerization of actin seems to require the presence of existing filaments or ordered nuclei.

The importance of these findings in relation to the gelated cortex or plasmagel described by early light microscopists will be discussed.

MATERIALS AND METHODS

Obtaining Material

Sperm were collected from *Thyone briareus* as described in the preceding paper. The sperm were generally used the same day, although on rare occasions the sperm were kept as a pellet at 4°C until the following day. Sperm can be stored for several days in this state and when suspended in seawater will swim and fertilize eggs normally. Recently outdated (within a day or two) human whole blood was obtained from a local hospital and used immediately.

Preparation of Erythrocyte Ghosts

Ghosts were prepared after the method of Fairbanks et al. (4). The blood was washed four times with an equal volume of 0.15 M NaCl containing 5 mM phosphate buffer at pH 6.4 to remove platelets, then lysed with 5 mM phosphate buffer at pH 8.0. The released hemoglobin was removed by repeated washings and centrifugation.

Removal of Sperm Membranes

*Thyone* sperm were demembranated in 10 mM phosphate buffer at pH 6.4 which contained 1% Triton X-100 (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.). Small amounts of MgCl₂ were added to the preparation (0.5–1 mM) to inhibit uncoiling of the DNA.

Preparation of Proteins

Subfragment 1 (S₁) was obtained from rabbit muscle myosin using the methods outlined by Tilney and Deters (24). It was stored in 50% glycerol at -20°C until use. Actomyosin was obtained from *Thyone* skeletal muscle. The muscles were dissected from the organism and homogenized in a Sorvall omnimixer (DuPont Instruments, Sorvall Operations, Newtown, Conn.) for 10 s at full speed in a standard salt solution (0.05 M KCl, 0.005 M MgCl₂, 0.006 M phosphate buffer at pH 7.0). The myofibrils were collected by centrifugation (10,000 g for 10 min) and then incubated in a solution containing 1% Triton X-100, 3 mM MgCl₂, 0.1 mM ethylenediaminetetraacetate (EDTA) and 30 mM Tris HCl at pH 8.0 to solubilize the membranes. They were washed twice in the above solution and then added to the SDS-gel mix. DNase and trypsin were obtained from Sigma Chemical Co., St. Louis, Missouri.

SDS-Gel Electrophoresis

Samples to be run by SDS-gel electrophoresis were boiled for 2 min in a solution containing 1% SDS, 2.5 mM Tris glycine buffer at pH 8.3, 1% mercaptoethanol and 10% glycerol. The samples were run on 5% polyacrylamide gels. Tris glycine (25 mM) at pH 8.3 was used as the running buffer. Bromphenol blue was used as the tracking dye.

Light Microscopy

To investigate the extent of solubilization of the periacrosomal material, its compactness or its purity, preparations of sperm were examined under oil immersion in a Zeiss phase-contrast microscope.

Electron Microscopy

After the termination of the various experimental treatments, the material was collected by centrifugation and resuspended in the last solution in which it was placed, to which 2% glutaraldehyde was added (Electron Microscope Services, Fort Washington, Pa.). The material was fixed for 1 h at room temperature. It was washed and postfixed in 1% OsO₄ in 0.1 M phosphate buffer at pH 6.8 or pH 6.0 at 4°C for 1 h, dehydrated rapidly in acetone, and embedded in Araldite. Sections were cut with a diamond knife on a Porter-Blum II ultramicrotome (Dupont Instruments, Sorvall Operations).
stained with uranyl acetate and lead citrate, and examined with a Philips 200 electron microscope.

RESULTS

The morphology of Thyone sperm has been extensively covered in earlier reports (21, 25), so I will not repeat the description here. Fig. 3 of the preceding paper illustrates the periacrosomal material or profil actin. This amorphous material appears finely granular at high magnification and assumes the form of a cup, limited on its basal surface by the nuclear envelope and on its apical surface by the nearly spherical acrosomal vacuole which forms the contents of the cup. The rim of the cup is in contact with the plasma membrane. It is important to bear in mind the overall shape and morphology of the periacrosomal material, in order to be able to identify it and to be sure that the consistency and, thus, the state of the actin is unchanged during the isolation procedure.

Treatment of the Sperm with Hexylene Glycol

In a typically innovative paper, Weisenberg (28) described a particulate form of tubulin in surf clam eggs. These eggs were homogenized in a solution of 1 M hexylene glycol at pH 6.2 (9, 10). Under these conditions, Weisenberg was able to isolate a particulate fraction which consisted of a small number of microtubules and a large amount of a granular matrix substance. As judged by the amount of colchicine binding in this fraction, the latter must have been composed of tubulin in some sort of “polymorphic aggregate.” From observations described in the preceding paper, it becomes obvious that actin in echinoderm sperm must also be bound in a nonfilamentous state, perhaps also forming a polymorphic aggregate. In order to define the composition of this aggregate of profil actin, I applied 1 M hexylene glycol in 10 mM phosphate buffer at pH 6.4 to Thyone sperm. Since the plasma membrane of the sperm, unlike that of the egg, cannot be removed by homogenization, I also added the detergent Triton X-100 (1%). Under these conditions, the periacrosomal material remains, but most of the cell membranes are solubilized; the nuclear envelope in the periacrosomal region and the basal portion of the acrosomal vacuole membrane remain (Fig. 15 of the preceding paper). Since the basal part of the acrosomal vacuole membrane is frequently broken in places and the plasma membrane is solubilized leaving the apicolateral surfaces of the cup (the rim) not membrane delimited, there is no reason why the profil actin should not gradually diffuse away. This does not occur; thus, this material must be bound to itself. These preparations are stable even overnight at room temperature.

Treatment of the Sperm with 1% Triton X-100 without Hexylene Glycol

Since profil actin was stable under conditions of acid pH and hexylene glycol, I tried treating the Thyone sperm with 1% Triton X-100 at acid pH (10 mM phosphate buffer at pH 6.4; if no Mg++ is added, the DNA tends to form a gel in the test tube, so, in general, 0.5–1 mM MgCl₂ was added to the preparation). When these sperm are examined by phase-contrast microscopy, a phase-dense cup which corresponds in position and shape to profil actin (Fig. 1) is easily recognizable; beneath it is the swollen nucleus. The mitochondrion and the acrosomal vacuole have disappeared. The flagellar axonemes are stable but generally float free from the nucleus. When the preparation is examined in thin sections, it is clear that the detergent has solubilized all the membranes. Yet, the morphology of the profil actin

FIGURE 1 Phase-contrast micrograph of Thyone sperm which have been demembranated with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4. Attached to the nucleus is a phase-dense cup. This cup corresponds to the profil actin seen in electron micrographs. × 3,000.
actin is unchanged from that of profil. actin in untreated sperm (cf. Fig. 2 with Fig. 3 of the preceding paper). This material still appears amorphous and still preserves its cuplike shape. Some of the chromatin sticks to it.

Isolation of the “Cups” of Profil. Actin

Since these cups are stable at room temperature for long periods, I tried to isolate them free from the chromatin and the flagellar axonemes as a prelude to characterizing their chemical composition. Sperm chromatin is remarkably DNase insensitive, presumably due to the protamines. In fact, in order to get any effect of the DNase at all, one has first to form a DNA gel. This can be done most conveniently by removing much of the Mg ++ either by treating the sperm with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4 with no additional Mg ++ or by washing detergent-treated sperm with 10 mM phosphate buffer. When DNase is now added (50 𝜇 of a stock solution of 1 mg/ml of DNase is added to a test tube containing 3 ml of a demembranated suspension of sperm prepared with no added Mg ++ ), the DNA gel solubilizes. The cups are released in 1-2 min (Fig. 3) and undergo rapid Brownian motion. All that remains then is to isolate the cups free from the flagellar axonemes and the contaminating undigested DNA. The easiest way to remove the flagellar axonemes is to deflagellate the sperm before detergent treatment. Large lumps of chromatin can be removed by centrifuging the preparation at low speed (700 g for 5 min). The cups can then be collected by centrifugation (10,000 g for 10 min). Unfortunately, some chromatin always con-
taminates this preparation (Fig. 4). I have tried to remove the contaminating chromatin by using a sucrose gradient. This procedure is only moderately successful as, within about 10 min after the DNase is added, the remaining chromatin becomes sticky and binds to the cups or to contaminating flagellar axonemes. Thus, even though I can greatly enrich the preparation for cups which contain the profil actin there is invariably some chromatin contamination. These preparations can, however, be analysed for their chemical composition using techniques such as SDS-gel electrophoresis as will be demonstrated.

Solubilization of the Cups

We demonstrated in our first paper on echinoderm sperm (25) that when sperm are treated with 1% Triton X-100 in 30 mM Tris HCl, 3 mM MgCl₂ and 0.1 mM EDTA at pH 8.0, the material in the periacrosomal region is solubilized.
Since this material is stable at low-ionic-strength phosphate buffer at acid pH, I took a preparation of sperm that had been demembranated at pH 6.4 or a preparation of isolated cups and placed them into the above solution (30 mM Tris HCl, 3 mM MgCl₂, 0.1 mM EDTA at pH 8.0) which lacked detergent. The phase-dense cups almost immediately disappeared, leaving only a thin, faint, phase-dense ring (Fig. 5). In the electron microscope, it is clear that the bulk of the profil. actin has disappeared (Fig. 6); all that remains is a thin layer. The chromatin is quite condensed under these conditions, due to the addition of MgCl₂. The flagellar axonemes remain unchanged.

A careful examination of the center of the cup in the periacrosomal region in untreated sperm reveals that there are a few filaments (about 500-1,000 Å in length) present which run from the base of the acrosomal vacuole towards the nuclear envelope. They are embedded in the amorphous profil. actin. This bundle of short filaments has been previously described (3, 21). These filaments can be recognized in demembranated sperm as well. When sperm are demembranated in phosphate buffer at acid pH, then transferred to Tris at alkaline pH which contains Mg²⁺, much of the profil. actin material in the cup is solubilized. That which remains becomes compact and phase dense.

**FIGURE 5** Phase-contrast micrograph of a sperm which has been first treated with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4, then washed in buffer, and finally treated with 30 mM Tris HCl at pH 8.0 containing 3 mM MgCl₂ and 0.1 mM EDTA. Most of the phase-dense cups have disappeared. Only a thin rim of the cup can be detected (see arrow). × 3,000.

**FIGURE 6** Thin section through a sperm which first has been demembranated with Triton X-100, then washed twice in phosphate buffer at pH 6.4, then treated with 30 mM Tris HCl, 3 mM MgCl₂, and 0.1 mM EDTA at pH 8.0. Note that the profil. actin has been largely solubilized. Only a thin rim of profil. actin (P) remains. × 70,000.
FIGURE 7 Thin section through a sperm treated as in Fig. 6. Within the center of the periacrosomal region is a bundle of filaments indicated by the arrow. These filaments are present in untreated sperm as well. × 75,000.

In these preparations, this bundle of short filaments projects up and above this compacted material (Fig. 7). Since this bundle is not present at the base of the acrosomal process in reacted sperm (25), I presume that during discharge it will ultimately become located at the tip of the process.

I tried a number of experiments designed to find out under what conditions the cups will solubilize. Do the cups, for example, solubilize due to the change in pH or due to the slight increase in ionic strength or due to the type of buffer used? It turns out that all of these conditions help to solubilize the cup. Certainly, the most important factor is the change in pH, and a lesser important factor is the change in type of buffer from phosphate to Tris. Large numbers of experiments to determine under what conditions the cups will solubilize are easily carried out because the method of assaying the disappearance or reduction in the amount of material in the cup is light microscopy (phase-contrast microscope). If the sperm are demembranated in 5-10 mM phosphate buffer at pH 6.4 and kept at pH 6.4, the addition of salt, 0.075-0.6 M KCl, reduces the size of the cup only slightly. Similarly, if sperm are demembranated in a solution containing 20 mM Tris HCl at pH 6.6 and 1% Triton X-100, the cups are only slightly smaller than those examined after demembranation in 10 mM phosphate buffer at pH 6.6 and 1% Triton X-100. However, if the demembranation is carried out in 20 mM Tris HCl and 1% Triton X-100 at pH 8.0, very small cups are seen. Alternatively, if demembranation is carried out in 10 mM phosphate buffer at pH 8.0, the cups are almost comparable (slightly smaller) in size to those demembranated in Tris HCl at pH 6.6. Thus, solubilization of the cups is enhanced by alkaline pH, by incubation in Tris buffer rather than phosphate buffer and, to a certain extent, by increasing the ionic strength.

To test the importance of hydrophobic interactions in maintaining the profil actin, I subjected demembranated sperm to 0.5% Sarkosyl (Geigy Chemical Corp., Ardsley, N.Y.) or 1% deoxycho-
late or 1.5 M sodium azide, in 10 mM phosphate buffer at pH 6.4. Each of these reagents solubilizes the cups. The DNA also unwinds, giving rise to a DNA gel. The addition of hexylene glycol to a solution of azide or Sarkosyl protects the integrity of the cups to a limited extent.

To test whether or not disulfide bonds are involved in maintaining the actin in the cups, the reducing agent dithiothreitol (1 mM) was added to a preparation of demembranated sperm or to a preparation of isolated cups. No effect could be seen, indicating that disulfide links are probably not important.

Changes in the Compactness or Density of the Cup

In an attempt to determine the conditions necessary for transforming the cup into filaments in vitro, I added a number of reagents to demembranated sperm, hoping to induce this transformation. In the process of this study, which I will describe in more detail later, I noticed that divalent cations had a profound effect on the compactness and, thus, the density of the profil actin. If, for example, either 3 mM Ca++ or 3 mM Mg++ are added to the demembranated sperm, the cups become smaller and more electron dense (Fig. 8). This effect could be inhibited by the addition of an appropriate concentration of EDTA or Ethylene-glycolbis[β-aminoethyl-ether]N,N'-tetraacetic acid (EGTA). Alternatively, if ATP is added to the Triton solution during demembranation or after demembranation, the cups tend to swell and increase in size while their density is reduced. In the experiments reported in the preceding section in which the cups are solubilized by incubating demembranated sperm in Tris at alkaline pH, I pointed out that there is a residual layer of very dense material in the cup that is not solubilized (Figs. 6, 7, and 14). Part of the explanation for the density of the residual layer may be related to the Mg++ that is present in the solution.

The Chemical Composition of the Cup

Thyone sperm, with and without flagella, were first treated with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4 containing 1.5 mM MgCl₂. The demembranated sperm or the sperm

![Figure 8](image-url)

**Figure 8** Thin section through a sperm which has been treated with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4 containing 2.5 mM MgCl₂. Of particular interest is that the profil actin (P) becomes highly condensed under these conditions. × 60,000.
heads were then pelleted (2,000 g for 5 min) and washed twice in 10 mM phosphate buffer at pH 6.4 which contained 1.5 mM MgCl₂. They were suspended in 30 mM Tris HCl at pH 8.0 containing 3 mM MgCl₂ and 0.1 mM EDTA, and the residual chromatin was removed by centrifugation. The supernate which contains the bulk of the profil. actin was concentrated and run on an SDS gel. Only three major bands appear on these gels (Fig. 9). As one would expect, one of these bands has an electrophoretic mobility indistinguishable from that of actin (43,000). The other two have mol wt of about 250,000 and 230,000, and usually appear in approximately equal concentrations. In certain preparations this stoichiometry can vary, probably due to limited proteolysis; the lower band appears more concentrated than the upper, and there is an even lower band whose mol wt is about 200,000. These changes are also present when low concentrations of trypsin are added to the preparations, as will be demonstrated below. Some material runs with the dye front. On SDS gels with Tris glycine as the running buffer instead of the phosphate buffer system of Weber and Osborn (27), the dye front runs as a sharp band. The Tris glycine system also tends to make all the bands much sharper. The amount of protein running with the dye front varies from preparation to preparation and appears to result from contaminating materials.

When a preparation of the isolated cups is run, bands similar to the three described above are invariably present. In addition to these three bands, two bands appear which correspond to bands seen when DNase is run by itself. This means that the DNase must be binding to insoluble material or material that is pelletable at 10,000 g for 10 min. Also present in these preparations and moving with the dye front is a great deal of material which, I presume, is made up largely of protamines which would pellet with the contaminating chromatin. The amount of this material varies from preparation to preparation.

Since there are two proteins in this preparation whose mol wt are around 200,000, and since these proteins are bound to actin in the cup, it would be interesting to know whether either one of them has an electrophoretic mobility similar to that of myosin. Therefore, actomyosins isolated from Thyone muscle was coelectrophoresed with an extract of the cups. For this experiment, sperm were treated with 1% Triton X-100 in 30 mM Tris HCl at pH 8.0 containing 3 mM MgCl₂ and 0.1 mM EDTA. Under these conditions, the bulk of the cup is solubilized but the supernate is, of course, contaminated with membrane proteins as well, due to the removal of membranes by the detergent. Running this preparation has an advantage over running a sample of the isolated cups, however, in that there can be no loss of material from the cup before detergent treatment. For example, if I coelectrophoresed actomyosin with the proteins obtained after purification of the cups free from their investing membranes, one might argue that I had inadvertently removed a "myosin-like" protein during membrane extraction or during one of the washing steps. It is clear from Fig. 10 that Thyone muscle myosin has an electrophoretic mobility and, thus, a molecular weight smaller than those of the pair of high molecular weight bands, yet muscle actin coelectrophoreses with sperm actin. In general, nonmuscle myosins have a molecular weight similar to that of muscle myosins. Thus, the fact that the two high molecular

**Figure 9** SDS-gel electrophoresis. Sperm were first demembranated with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4, then washed twice in buffer, and incubated in 30 mM Tris HCl at pH 8.0 containing 3 mM MgCl₂ and 0.1 mM EDTA. This last incubation solubilized most of the profil. actin. The sperm were removed by centrifugation and the supernate was run on this gel. Three major bands are present. One of these is actin (a). The other two have mol wt of 250,000 and 230,000. The arrow indicates the dye front which contains some small polypeptides which are probably contaminating protamines.
FIGURE 10 SDS-gel electrophoresis. On the right hand gel is the supernate derived from sperm which have been incubated in 1% Triton X-100, then treated with 30 mM Tris HCl, 3 mM MgCl₂, and 0.1 mM EDTA at pH 8.0. The left hand gel contains, in addition to the supernate, Thyon muscle actomyosin. Note that the muscle actin (a) comigrates with the sperm actin. The muscle myosin (m), however, has no band of comparable molecular weight in the sperm.

weight substances have a much higher molecular weight than muscle myosin indicates that probably neither one of them is myosin.

The Relation of the High Molecular Weight Substances in Sperm to Erythrocyte Spectrin

The pair of high molecular weight substances in the cup are reminiscent of the pair of bands called spectrin, or so-called bands 1 and 2 in erythrocyte ghosts (4). In fact, if the erythrocyte ghost is extracted with 0.1 mM EDTA, three proteins are removed: actin (band 5) and spectrin (bands 1 and 2) (24). Their relative positions on SDS gels appear similar to those of the proteins making up the profil actin, except that, in the erythrocyte ghost, bands 1 and 2 are present in greater excess than actin. When the proteins in the erythrocyte ghost are coelectrophoresed with the extract of profil actin, sperm actin, and erythrocyte actin coelectrophorese (Fig. 11). Likewise, the upper band (band 1) of erythrocyte ghosts coelectrophoreses with the upper band of the two bands from the cup. The lower of the two high molecular weight bands from the cup migrates somewhat more slowly than band 2 of erythrocyte spectrin, however, indicating that it has a slightly higher molecular weight.

Are the High Molecular Weight Components in the Cup Indeed Proteins?

To determine whether the two high molecular weight substances in the sperm cup are proteins, trypsin was added. Sperm were deflagellated and then treated with 1% Triton X-100 in 10 mM phosphate buffer at 6.4 containing 1.5 mM MgCl₂ to remove the membranes. After the sperm heads were washed twice in phosphate buffer containing Mg²⁺, trypsin (25 μg/ml) was added. Digestion was carried out at room temperature (22°C). Soon after the addition of trypsin, the DNA gels. This is due to the preferential digestion of the protamines (because of their large concentration of arginine and lysine residues) which liberates the DNA. The cups, however, gradually diminish in size and eventually disappear altogether (about 30 min trypsin (50 μg/ml)). In one group of experi-

FIGURE 11 SDS-gel electrophoresis. The center gel is a preparation of purified red cell ghosts. The left hand gel is of the same material illustrated in Fig. 5. On the right hand gel, the proteins of the red cell ghosts and those in Fig. 5 were electrophoresed on the same gel. Note that the actin (a) from red cells and that from sperm comigrates. The upper band comigrates with the upper band of spectrin. The lower spectrin band migrates slightly faster than the corresponding band from sperm.
merits, I halted the reaction by adding soybean trypsin inhibitor after 7 min, at which time the cups have greatly decreased in size and in some cases are no longer discernible. The solution was then centrifuged (10,000 g for 10 min) and the supernate collected, concentrated by precipitation with trichloroacetic acid, and run on an SDS gel (Fig. 12). Under these conditions, actin is still present due to its well-known trypsin insensitivity, but the pair of high molecular components has almost entirely disappeared. The pellet cannot be analysed by SDS-gel electrophoresis because of the amount of DNA. SDS gels, even after DNase treatment, show a smear of stain rather than distinct bands. (In Fig. 12, the gel has been over-loaded; there is a heavy actin band; the high molecular weight bands are very faint; compare with Fig. 9.) Of the remaining high molecular weight components, the lower band is present in striking excess with respect to the upper band. By 25 min, however, the pair of high molecular weight bands cannot be found; another band appears below the actin band (some of this is present after 7 min as well), which presumably is a breakdown product of actin. Running with the gel front is a great deal of material.

Results similar to those described above were obtained when a preparation of cups was digested with trypsin. In these experiments, both the supernate and pellet were combined and run on SDS gels. As before, the high molecular components disappeared. These experiments indicate, therefore, that the two high molecular weight substances are proteins and that these proteins help to maintain the actin in the form of a nonfilamentous aggregate, for, if these are digested or solubilized, the actin is liberated.

Treatment of the Cups with S_{1} of Myosin

When demembranated sperm are incubated in S_{1} and the preparation is examined after a few minutes by negative staining, beautifully decorated filaments can be seen in the preparations (Fig. 13), although some of the cups remain. If
sperm are partially demembranated by glycerination, then incubated in S₁ or heavy meromyosin (HMM) for long periods, decorated filaments are found in place of the cups (25). Thus, the actin can be polymerized from the cups by the addition of S₁ or HMM. It is well known that HMM and S₁ will polymerize actin in vitro to form decorated filaments even when no salt is present (22, 30). Thus, the HMM or S₁ is inducing the polymerization of actin.

In Vitro Transformation of the Actin in the Cups into Filaments

So far, I have not been very successful in inducing the transformation of the actin in the cups into filaments. Some polymerization of the actin in the cups occurs with Mg²⁺ and ATP; some polymerization is achieved with 1 mM ATP and 60 mM KCl. The most favorable conditions involve the addition of 1 mM ATP, 2 mM MgCl₂, and 2 mM CaCl₂. Because the cups are attached to the chromatin, the only assay I have for polymerization is the examination of thin sections of the material.

When demembranated sperm are incubated in ATP and Mg²⁺ or Ca²⁺ filaments are frequently found extending from the anterior end of the cup (Fig. 14). In general, these newly formed filaments can be seen to be continuous with the bundle of short filaments that is present in the center of the cup (see Fig. 7). Some polymerization of the

![Figure 14](https://example.com/figure14.jpg)

**Figure 14** Electron micrograph of a thin section through a portion of a sperm which has been demembranated with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4. These sperm were then incubated with 1 mM ATP, 2 mM MgCl₂, and 1 mM CaCl₂. Extending out of the anterior end of the cup are some filaments. The cup is surrounded by a "sea" of DNA. × 120,000.
actin occurs on the anterior end of the bundle of short actin filaments when cells are demembranated with Triton in phosphate buffer at pH 6.4, then incubated under conditions in which the cups solubilize (i.e., 30 mM Tris HCl at pH 8.0 containing 3 mM MgCl₂; see Fig. 7). The presence of Mg²⁺ in these preparations is imperative. In both instances mentioned above, the polymerization of actin occurs on the anterior end of the bundle of short filaments, not the basal end or that end which faces the nucleus. In sections through the periacrosomal regions of reacted sperm (see Fig. 4 of reference 25), it is clear that the small compact bundle of filaments that is shown in Fig. 7 is no longer present. I presume that these filaments are now located at the tip of the process. This presumption is consistent with our observations on microvillus growth (23) where elongation most likely occurs by the addition of monomer to the basal end of the filament bundle. Furthermore, the formation of this 90 μm long acrosomal process which occurs in 10–30 s probably is effected by the addition of monomeric actin to the basal end of the forming filament bundle, since it is this end which is sitting in the “pool” of actin in the periacrosomal region. Thus, the actin appears to be polymerizing in vitro from that end which in vivo would be attached to the membrane. Once the membrane is removed, the monomer is able to add on to this “free” end.

In some cases, filaments can be seen extending from both the anterior and posterior ends of the cup (Fig. 15). Of particular interest is that these filaments are attached only to the center of the cup. More specifically, they seem to be attached only to the bundle of short filaments that resides in the center of the cup. Filaments are rarely seen extending from anywhere else in the cup. This indicates that polymerization must be nucleated from the bundle of short filaments.

DISCUSSION
When sperm are demembranated at acid pH and in low-ionic-strength buffer, profil, actin, which is a storage form of actin, remains as a discrete unit even if the solution is kept at room temperature for many hours. I demonstrated that this material is composed of three proteins, actin and two other proteins whose molecular weights are similar to those of erythrocyte spectrin. The actin can be released from this storage form either by raising the pH or the ionic strength and changing the type of buffer used, or it can be released by trypsin.

From these results it is clear that actin can exist in vivo not only in the form of filaments, but also in a bound state in association with two other proteins. When these proteins are removed by solubilization, by digestion, or by overriding their influence by a myosin fragment (S₁), the actin appears to be liberated in the monomeric state from which it then can be induced to polymerize into filaments (in vitro by the addition of myosin, in vivo by the formation of the acrosomal process). Since we and others (3, 21) can see filaments in the center of the cup and since the actin is not pelletable from the solubilized cups under conditions that will pellet actin filaments, it seems clear that the actin in the native cup is not made up of filaments that are
observed by the high molecular weight proteins. Thus, we can diagram the transformation of the actin during the formation of the acrosomal process as follows:

Storage Form of Actin → G-actin → F-actin.

This process must, however, be more complex than that outlined above. This appears to be due to the inability of the actin in the cup to nucleate spontaneously. This conclusion is based on the following observations. When demembranated sperm are incubated in ATP and divalent salts, the filaments do not grow at random out of material in the periacrosomal region. Instead, polymerization seems to take place exclusively from the bundle of short filaments that preexists in the cup. Actin monomers appear to be able to add on to either the basal or apical end of this bundle of filaments. This also is true for the situations in which the sperm are demembranated at low pH and then the cup is solubilized by increasing the pH. Under conditions in which Mg++ is present, the preexisting filaments increase in length, but filaments other than the elongated preexisting filaments are not found associated with the cup. The observation that existing filaments appear to be the only place where polymerization of actin occurs demonstrates, at least under the conditions applied in vitro, that the actin does not appear to nucleate spontaneously or that at least self-nucleation is unfavored. Thus, I conclude that the polymerization of actin may require oriented nuclei which are present as a bundle of short filaments. This may be very important for a normal acrosomal reaction in vivo as well. For example, if the actin were to polymerize randomly all over the cup, the anterior end of the sperm would mushroom out to form a large, fat pseudopod instead of a thin cell extension. Similarly, if nonmuscle actin does not nucleate readily in vivo, but requires centers of existing filaments, this could explain how the polarity of the filaments relative to the cell surface could be controlled, a feature which must play an important role in the functioning of the system in relation to myosin in the cell. So far, the polarity of actin filaments (as judged by the direction of the arrowheads after HMM or S1 decoration) in relation to the limiting membrane has been established in only two systems, microvilli (15, 16) and *Mystus* sperm (22). In both these systems, the “arrowheads” point away from the membrane. Preliminary observations in echi nodermsperm (25) also suggest that the arrowheads point away from the tip of the acrosomal process.

Five years ago, we (23) established that, in the formation of microvilli in the brush border of intestinal epithelial cells, nucleation of actin filaments seems to occur from small densities attached to the plasma membrane. This material is now known to consist at least in part of the protein α-actinin, (19) the main component of the “Z” line of skeletal muscle. Thus, if spontaneous nucleation of the actin in nonmuscle cells does not occur or occurs inefficiently, the actin polymerizing either on the tips of existing filaments or from nucleating centers such as the dense tips of microvilli, the polarity of the actin filaments could be controlled. Yet, since muscle actin as well as purified nonmuscle actin will spontaneously nucleate in vitro (18), obviously something must have been either added to or subtracted from monomeric actin during purification to allow the actin to spontaneously nucleate.

These observations on the inefficiency of the nucleation of actin in vivo and in vitro help to explain one other puzzling fact. I have wondered for some time why there are not at least a few randomly oriented filaments in the periacrosomal region before induction, since the actin concentration here must be at least 100 mg/ml. For example, if one looks at a pellet of G-actin at these concentrations (see the preceding paper), some filaments are present even in the absence of salt, presumably due to the fact that in very high concentrations of actin the equilibrium between the monomer and the polymer allows some of the actin to polymerize. Furthermore, salt (Mg++) must be present in vivo for Mg++ is required for the function of dynein in flagellar movement, and clearly Mg++ must be present in the nucleus in order to keep the DNA condensed. Since Mg++ pushes the equilibrium maximally in favor of the polymer (F-actin), in concentrations above 0.5–1 mg/ml some of the monomeric actin, although inhibited, should polymerize. It may be unreasonable to expect that the two high molecular weight proteins by themselves could maintain all the actin in the unpolymerized state; there must be an additional controlling factor (either the presence or absence of some substance) that keeps the actin from polymerizing. For example, when the periacrosomal material is solubilized by changes in pH and buffer (3 mM Mg++ is present in these
solutions as well), or when the isolated cups are digested with trypsin, the liberated actin does not form filaments as seen by negative staining. Since the high molecular weight proteins cannot be found on gels after trypsin digestion, whereas the molecular weight of the actin is unchanged, the actin should be competent to polymerize. Thus, the failure of actin to polymerize is probably not due entirely to the high molecular weight proteins but may also be due to the lack of spontaneous nucleation.

The Homology between Erythrocyte Spectrin and the Pair of High Molecular Weight Proteins in Sperm

Let me begin by listing the similarities between erythrocyte spectrin and the two high molecular weight proteins in sperm. Both appear to bind to actin (see reference 24 for evidence on erythrocyte spectrin), and the complex produced by the association of spectrin and actin is morphologically similar to profil. Both spectrin and the high molecular weight substances in sperm appear to inhibit the polymerization of actin. Furthermore, the addition of S1 to either sperm or erythrocyte ghosts leads to the polymerization of actin, and in both systems not only are the spectrin or spectrin-like molecules very trypsin sensitive but also, upon the initiation of digestion, the lower molecular weight component becomes much stronger (this report: 4). The molecular weights of band 1 of spectrin and of the larger protein band in sperm are identical; band 2 of spectrin and the lower molecular weight band in sperm are similar but not identical (this may be due to species differences, i.e., sea cucumbers are being compared to humans, or may indicate that a family of spectrin molecules exists). And finally, we know that the solubilities of these proteins from the two sources are similar. For example, in both systems Mg++ seems to be important in keeping the actin and the spectrin or the actin and the high molecular weight components intact. If the Mg++ is removed, the cup of profil. actin in sperm swells and the spectrin and actin in the erythrocyte ghost go into solution. Further work is necessary to strengthen the homology between erythrocyte spectrin and the high molecular weight proteins in sperm, but already the structural and chemical similarities are very striking. One wonders, then, if there is a functional homology between the spectrin and actin complex in the erythrocyte ghost and the spectrin-like proteins and the actin in sperm. The answer to this is a qualified "yes." Basically, this question revolves around an understanding of what controls the polymerization of actin in cells and what the cell cortex is. Let me begin to explore this question with a brief discussion of the cell cortex.

From investigations on erythrocyte ghosts from which some of the peripheral proteins such as spectrin have been removed (4) and on lipid membranes formed in vitro, it is clear that proteins, more specifically peripheral proteins, have a marked effect on the stability of the lipid bilayers (6). It has been well established that beneath the cell membrane in many cell types is a gelated surface layer commonly called the "cortex." Most of the work in this area has been carried out on sea urchin eggs or on amphibian eggs largely because of their size. Various experimental procedures have been used; measurements of the extent and "stiffness" of this layer have been made with fine pipettes (elastimeters) (13, 14) or a compression technique (2, 7), by watching the amount of pressure needed when applied with centrifugation to displace cortical pigment granules (1, 12, 31) and by the use of magnetic particles (8). Measurements of the size and stiffens of the cortex vary according to the method employed, but it has been established that the cortex in sea urchin eggs is a layer 1.6–6 μm in thickness (29). The stiffness of this layer increases after fertilization and, again, at cleavage (8, 12, 29, 31). Although much has been written about the mechanical properties of this cortical layer, little information is available as to its chemical composition. Furthermore, changes in the stiffness of the cortex have not been correlated with morphological changes except during cleavage when filaments can be seen in the furrow. In fact, thin sections through the cortex so far have not been informative. The isolated sea urchin cortex contains large amounts of actin and myosin (11), as is also true of the cortex of certain other cell types, i.e., the isolated apical surface of intestinal epithelial cells (26), the erythrocyte ghost (24), HeLa cells, and fibroblasts (5), Dictyostelium (20) and the Acanthamoeba (17). In certain cases this actin is filamentous, but in other cases the nature of the actin in vivo remains unestablished. The point I would like to make before continuing is that the cortex of the erythrocyte ghost is analogous to that of a sea urchin egg. The difference between the two may be merely the
exceptions, the actin functions in relationship to
describable cortex in both and actin is present in
the plasma membrane. For example, motile proc-
tesses associated with actin include the acrosomal
reaction, cytokinesis, phagocytosis, microvillar
movement, ruffling of the cell surface, platelet
contraction, pseudopod contraction, etc. Thus, the
localization of actin in the cell cortex may be of
considerable importance to cells. It may also be
that this actin contributes to the gel strength and
the stiffness of the cortex. Such a hypothesis is not
unreasonable. The ideal way to increase the gel
strength of the cortex is to create an anastomosing
network, such as must lie beneath the erythrocyte
cortex (24), formed by the association of
spectrin and actin. A similar "gel" seems to exist
in sperm. The actin can be mobilized from this gel
and polymerized to produce the force for the
formation of the acrosomal process. It is interesting
that in the erythrocyte ghosts, in the isolated
sea urchin cortex, and in Thyone sperm, Mg** is
important in maintaining the density and integrity
of the gel. In sperm, if the Mg** is removed the
cup of profil. actin tends to swell; if Mg** is
added, the cup becomes very compacted. Likewise,
if EDTA is added to erythrocyte ghosts, spectrin
and actin go into solution. Thus, the gel seems to
be stabilized by Mg**. Further work is needed to
verify the idea that in most cells the cortices are
chemically homologous and are composed of actin
and spectrin-like proteins. Obviously, at this stage
we can only suggest that the cortices of cells may
be similar. This idea does give us direction,
however, as we can test to determine whether there
are high molecular weight proteins coupled to the
actin, and whether these proteins are similar. We
can also ask about the state of the actin in the
cortex: is it filamentous, nonfilamentous, or both?

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