

# PREPARATION AND PURIFICATION OF POLYMERIZED ACTIN FROM SEA URCHIN EGG EXTRACTS

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## ABSTRACT

Isotonic extracts of the soluble cytoplasmic proteins of sea urchin eggs, containing sufficient EGTA to reduce the calcium concentration to low levels, form a dense gel on warming to 35–40°C. Although this procedure is similar to that used to polymerize tubulin from mammalian brain, sodium dodecyl sulfate-polyacrylamide gel electrophoresis shows this gel to have actin as a major component and to contain no tubulin. If such extracts are dialyzed against dilute salt solution, they no longer respond to warming, but gelation will occur if they are supplemented with 1 mM ATP and 0.020 M KCl before heating. Gelation is not temperature reversible, but the gelled material can be dissolved in 0.6–1 M KCl and these solutions contain F-actin filaments. These filaments slowly aggregate to microscopic, birefringent fibrils when 1 mM ATP is added to the solution, and this procedure provides a simple method for preparing purified actin. The supernate remaining after actin removal contains the other two components of the gel, proteins of approximately 58,000 and 220,000 mol wt. These two proteins plus actin recombine to form the original gel material when the ionic strength is reduced. This reaction is reversible at 0°C, and no heating is required.

Following the demonstration of an actin-like protein, similar in properties to muscle actin, in myxomycete plasmodium (9, 10), similar methods revealed the presence of a cytoplasmic actin in unfertilized sea urchin eggs. This sea urchin egg actin was first identified and separated through its combination with rabbit muscle myosin (8, 18, 19), and the protein was later prepared directly from egg extracts by salting out with ammonium sulfate (17). These preparative methods gave no information concerning the localization of the actin within the egg, but there is good evidence for its presence in the cleavage furrow during cytokinesis, where it was seen first in the electron microscope as bundles of microfilaments (28, 30, 37). Actin has been localized in the cleavage furrow in a variety of other cell types (1, 21, 27, 29, 34) and this actin has

been specifically labeled with heavy meromyosin (31).

The investigations reported here were begun with the aim of determining whether the methods developed for the polymerization of tubulin to microtubules in mammalian brain (2, 39) could be used to prepare tubulin from the sea urchin egg. Marine eggs are an excellent source of isolated mitotic apparatuses for the investigation of the role of microtubules in chromosome movement, but up to this time only the interaction of mammalian brain tubulin with such isolated mitotic apparatuses has been reported (12, 25). Our attempts to prepare tubulin from extracts of sea urchin eggs with the methods developed for mammalian material were unsuccessful, and a number of modifications of this method were made to adapt it to the

higher tonicity and calcium concentration of the sea urchin egg. These changes did not yield polymerizable tubulin, but instead caused the polymerization of actin and two other cytoplasmic proteins. These preliminary observations were developed into a simple and rapid method for the isolation of actin which has little in common with the more conventional techniques of muscle biochemistry that have been used to prepare actin from the egg, and in several respects it relates more directly to observations (20, 23) on the fibrillar motile system of amoeba. Since the presentation of this method in abstract form (15), it has been adapted to the polymerization of amoeba actin (22).

## MATERIALS AND METHODS

### Preparation of Extracts.

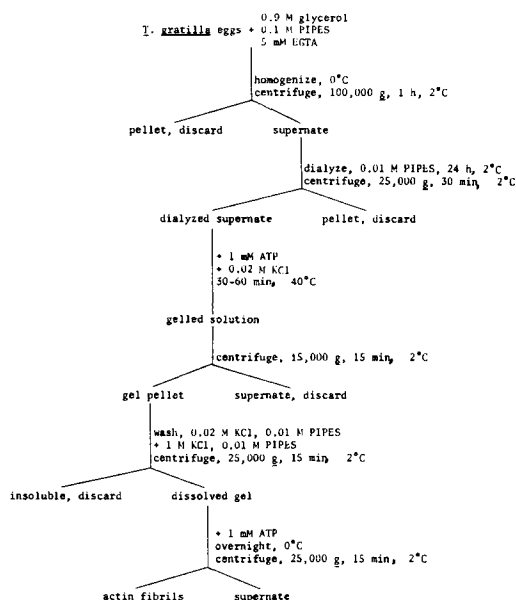
Eggs were obtained from the Hawaiian sea urchin *Tripneustes gratilla* by the injection of isotonic KCl into the body cavity. The jelly coat was removed by titrating the egg suspension rapidly to pH 5 with HCl and washing several times in normal sea water. Eggs were sedimented by hand centrifugation, and the packed egg volumes used in later calculations were estimated after such centrifugation. For extraction, a measured volume of eggs was washed at 25°C in 10 times its volume of a 19 to 1 mixture of isotonic sodium and potassium chlorides, containing 2 mM EDTA to remove seawater divalent ions (14). The eggs were then washed at 0°C in one change of the isolation medium to be used and resuspended in fresh medium at 0°C in the ratio of 3 vol of extraction medium to 2 vol of eggs. The eggs were homogenized by 10–15 passes of a Dounce homogenizer, and the homogenate was centrifuged at 100,000 g for 1 h with a Beckman Spinco Model L ultracentrifuge, using a SW-50 head (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 2–4°C. The procedure for actin isolation is given in Scheme I (details in text).

### Microscopy

Photomicrographs were made with a Zeiss universal microscope, using Zeiss phase contrast, interference contrast, and polarization optics. For electron microscopy, protein preparations were negatively stained (11) with 1% uranyl acetate and examined and photographed in a Philips 201 electron microscope operated at 60 kV.

### Protein Measurements

Analytical ultracentrifugation was carried out in a Beckman Spinco Model E analytical centrifuge at 20°C using the rotor temperature indicator and control unit. Distances on the plate were measured with a Nikon



SCHEME I

microcomparator (Nikon, Inc., Div. of EPOI, Garden City, N. Y.).

Sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis was done on 5% gels, using the methods of Weber and Osborn (38).

Protein determinations were made by the method of Lowry et al. (16), using a serum albumin standard.

### Materials

ATP, GTP, and all buffers and protein standards were obtained from Sigma Chemical Co. (St. Louis, Mo.). Rabbit muscle actin, sea cucumber (*Holothuria atra*) myosin, and beef brain tubulin were the gifts of Dr. Richard Himes of the Department of Biochemistry, University of Kansas.

## RESULTS

### Actin Gels from Sea Urchin Extracts

The media that have been used successfully for the extraction and subsequent polymerization of tubulin from mammalian brain have usually contained 0.1 M PIPES or similar buffer, 1 mM EGTA to reduce the calcium concentration to low levels, 1 mM GTP, and 0.5 mM magnesium. Polymerization occurs on raising the temperature of these extracts to 35–37°C (2, 39). This procedure did not yield polymerizable tubulin from urchin egg extracts and was modified by making the solution approximately isotonic through the

addition of 0.9 M glycerol (glycerol was chosen because of its effects on tubulin polymerization (32)) and by increasing the EGTA concentration to 5 mM in view of reports of total calcium of 4 mM in the urchin egg (26). The breakdown of cytoplasmic granules was minimized in this medium, as indicated by the absence in the extract of the 3.5 and 22S yolk proteins (4) and of hyalin from the cortical granules (13). When the supernatant solution resulting from centrifugation of such isotonic homogenates at 100,000 g is warmed, a gel forms in the solution, not at the environmental temperature of the urchin (23–25°C in Hawaii), but at 35–40°C, a temperature much above any to which these cells would normally be exposed. If allowed to proceed without agitation, gelation often converts the entire solution to a solid mass, but the gel later shrinks to a much smaller volume over the course of several hours. Gelation was normally allowed to proceed for 30–60 min, and the gels were then sedimented at 15,000 g, washed, and analyzed. Preliminary electrophoretic studies (similar to Fig. 2 a) showed the presence of no tubulin and a major component in the range of 45,000 mol wt, indicating that actin rather than tubulin was being polymerized by this procedure, and the method was reexamined to determine the conditions necessary for actin polymerization.

The necessity for including glycerol to render the solution isotonic has already been demonstrated in the experiments outlined above, and further studies showed that a buffer concentration of at least 0.1 M was required to control the pH during extraction. A number of buffers developed by Good et al. (5), PIPES, MES, and ADA, were tested, and all were found to give similar results. Although most experiments were carried out at pH 6.8 using PIPES, the procedure was also successful at pH 7.5 using Tris-HCl. Relatively high EGTA concentrations are also required, since gelation was reduced at values of 2.5 mM EGTA and below, indicating that a low calcium concentration is necessary. The elimination of GTP and magnesium had no effect. The role of such physiological components is difficult to determine in concentrated extracts, however, since all of the small mol wt components of the cytoplasm are still present.

To further clarify the requirements for this gelation reaction, the extracts were dialyzed to remove the small mol wt components. Dialysis was carried out for 1–2 days at 2–4°C against a large

volume of 0.01 M PIPES, pH 6.8. A small amount of insoluble material sometimes forms on dialysis, but does not involve any of the proteins of interest here and is removed by centrifugation at 25,000 g for 30 min. These dialyzed and centrifuged extracts, which contain approximately 30 mg of the total of 140 mg of protein per ml of cells, do not gel on warming, but the original gel response can be restored by the addition of ATP and KCl. The minimum ATP concentration required is of the order of 0.5 mM, and 1 mM was used routinely. GTP or ADP can be substituted for ATP in this reaction, but not AMP. Some dialyzed extracts will gel on the addition of ATP alone, but most require the addition of 0.01–0.05 M KCl. Aliquots of each extract are checked for KCl requirement; 0.02 M was used in most experiments. No added magnesium appears to be required and magnesium concentrations equal to or greater than the ATP concentration block the reaction. Higher magnesium concentrations can be counteracted with increased ATP, the reaction going successfully as long as the ATP concentration exceeds that of magnesium by approximately 0.5 mM. As might be expected from the necessity for EGTA in the extraction, gelation in dialyzed extracts is blocked by calcium concentrations in the range of 0.1 mM and increased ATP will not reverse this effect.

The gelled material which results when a dialyzed extract supplemented with 1 mM ATP and 0.02 M KCl is raised to 40°C for 30–60 min is illustrated in Fig. 1 a, b, and c. When centrifuged from the solution and washed, these gels contain approximately 1 mg of protein per ml of cells, or approximately 3% of the soluble protein present in the extract. Gelation is not temperature reversible, as once formed at 40°C the gel remains insoluble in low salt solutions at 0°C.

The gelled material could be studied in the ultracentrifuge through the use of a method reported by Morgan (20) in an investigation of the composition of microfilamentous bundles from amoeba. This author found that the solubilization of these bundles in 0.5% Sarkosyl reduced the tendency of the protein to aggregate in solution and allowed ultracentrifugal analysis. The washed gel pellet resulting from treatment of an urchin egg extract at 40°C dissolved rapidly in 0.5% Sarkosyl in 0.01 M PIPES, and the resulting solution showed one major peak in the analytical ultracentrifuge, with a sedimentation coefficient of 2.8S at 20°C (Fig. 3). This sedimentation value is the same

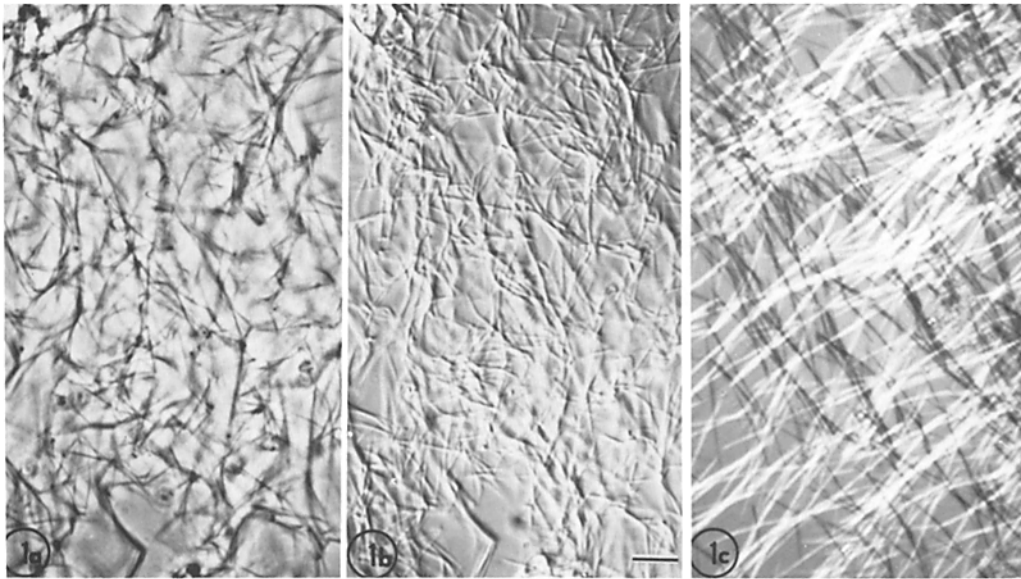


FIGURE 1 Photomicrographs of gels formed in 45 min at 40°C in dialyzed extracts containing 1 mM ATP and 0.025 M KCl. (a) Phase contrast, (b) interference contrast, (c) polarization optics. All  $\times 600$ . Reference mark = 10  $\mu\text{m}$ .

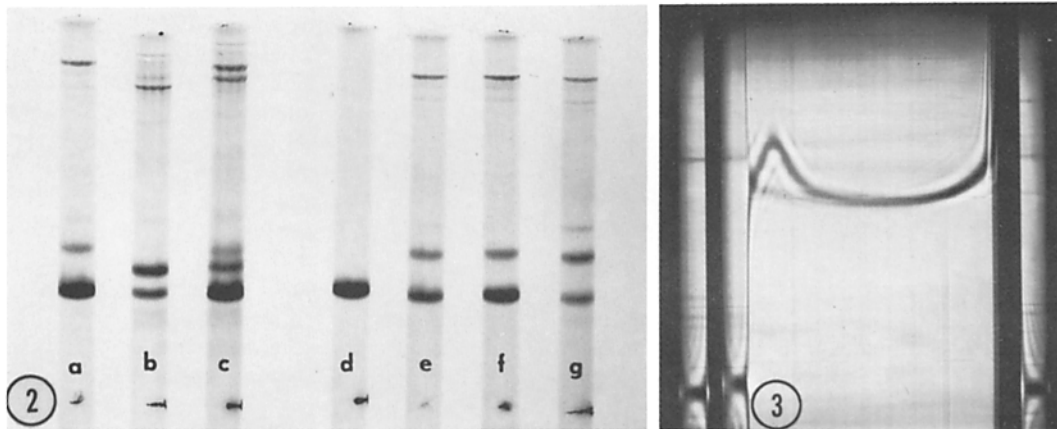


FIGURE 2 SDS-polyacrylamide gel electrophoresis. (a) Gelled material formed at 40°C in extract containing 1 mM ATP and 0.020 M KCl, removed and dissolved in 1 M KCl; (b) standard containing rabbit muscle actin, beef brain tubulin, and sea cucumber myosin; (c) combination of (a) and (b); (d) fibrils induced in (a) by 1 mM ATP, removed, and dissolved in 0.01 M PIPES; (e) 1 M KCl supernate remaining after removal of fibrils; (f) gelled material formed on dialysis of (e); and (g) supernate after dialysis. All at similar concentrations except (g), which was loaded at four times the others.

FIGURE 3 Ultracentrifugation of 40°C gel dissolved in 0.5% Sarkosyl, 0.01 PIPES. 36 min after reaching speed of 56,000 rpm, bar angle 30°.

as that measured by Miki-Noumura (17) for actin prepared from sea urchin eggs by a different method.

The gelled material can be dissolved at higher salt concentrations, and we have used 0.6-1 M KCl

for this purpose. After washing, the gel is taken up in a small amount (usually 1/10th the volume of extract from which the gel was prepared) of 0.6 or 1 M KCl and the material gently homogenized at 0°C for 30-60 min. A small amount of insoluble

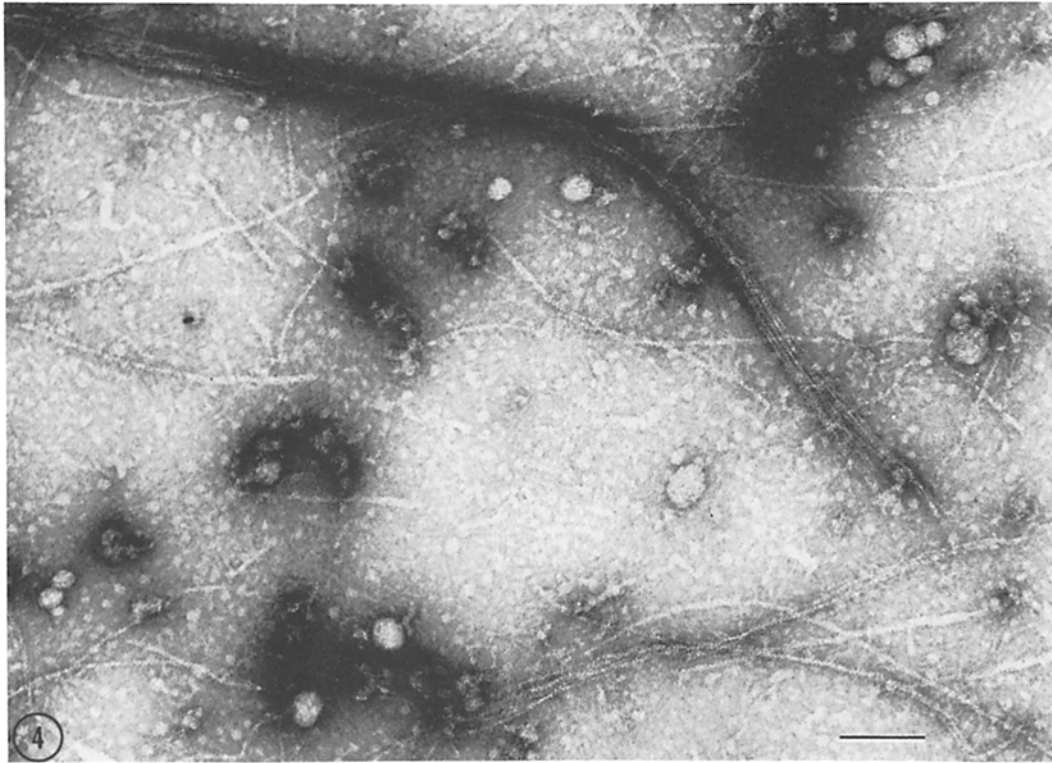


FIGURE 4 40°C gel material dissolved in 0.6 M KCl and negatively stained with 1% uranyl acetate. 70-80-Å thin filaments are present singly and in small aggregates.  $\times 112,500$ . Reference mark = 0.1  $\mu\text{m}$ .

material is removed by centrifugation at 25,000  $g$  for 15 min, the pellet is reextracted with a small volume of fresh KCl, and recentrifuged, and the resulting clear supernatant solutions are combined.

When run on SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn (38), the major component in the 1 M KCl solution has a mobility in the range of 45,000 mol wt and this component comigrates with rabbit muscle actin (Fig. 2 *a, b, c*). A second component of approximately 58,000 mol wt is always present in the gels. This protein is heavier than the tubulin standard and migrates separately from it (Fig. 2 *a, b, c*). A third, high mol wt component is present in smaller and more variable amounts in these gels. This component is larger than the heavy chain of rabbit myosin (200,000); to insure that no phylogenetic differences were involved, it was also compared to an echinoderm (sea cucumber) myosin. This myosin also travelled at 200,000 while the mol wt of the heavy component from the gels was estimated at 220,000, and it did not comigrate with myosin (Fig. 2 *a, b, c*).

#### *Preparation of Purified Actin*

When negatively stained and examined in the electron microscope the 0.6-1 M KCl-soluble material of the gel contained 70-80-Å filaments, similar in appearance to muscle F-actin filaments (7), both free and in small aggregates (Fig. 4), plus other amorphous material. The filaments present in these KCl solutions can be aggregated and separated from the other components by a simple method. If 1 mM ATP is added to the KCl solution and the preparation held at 0°C overnight, microscopic, birefringent fibrils form in the solution (Fig. 5 *a, b, c*). The fibrils differ in appearance from the gel material and when removed from the KCl solution by centrifugation at 25,000  $g$  for 15 min and run on SDS-polyacrylamide gel electrophoresis, only actin can be detected (Fig. 2 *d*). When negatively stained and observed in the electron microscope (Figs. 6,7), these microscopic fibrils are seen to be bundles of aggregated actin filaments, whose substructure is not in register.

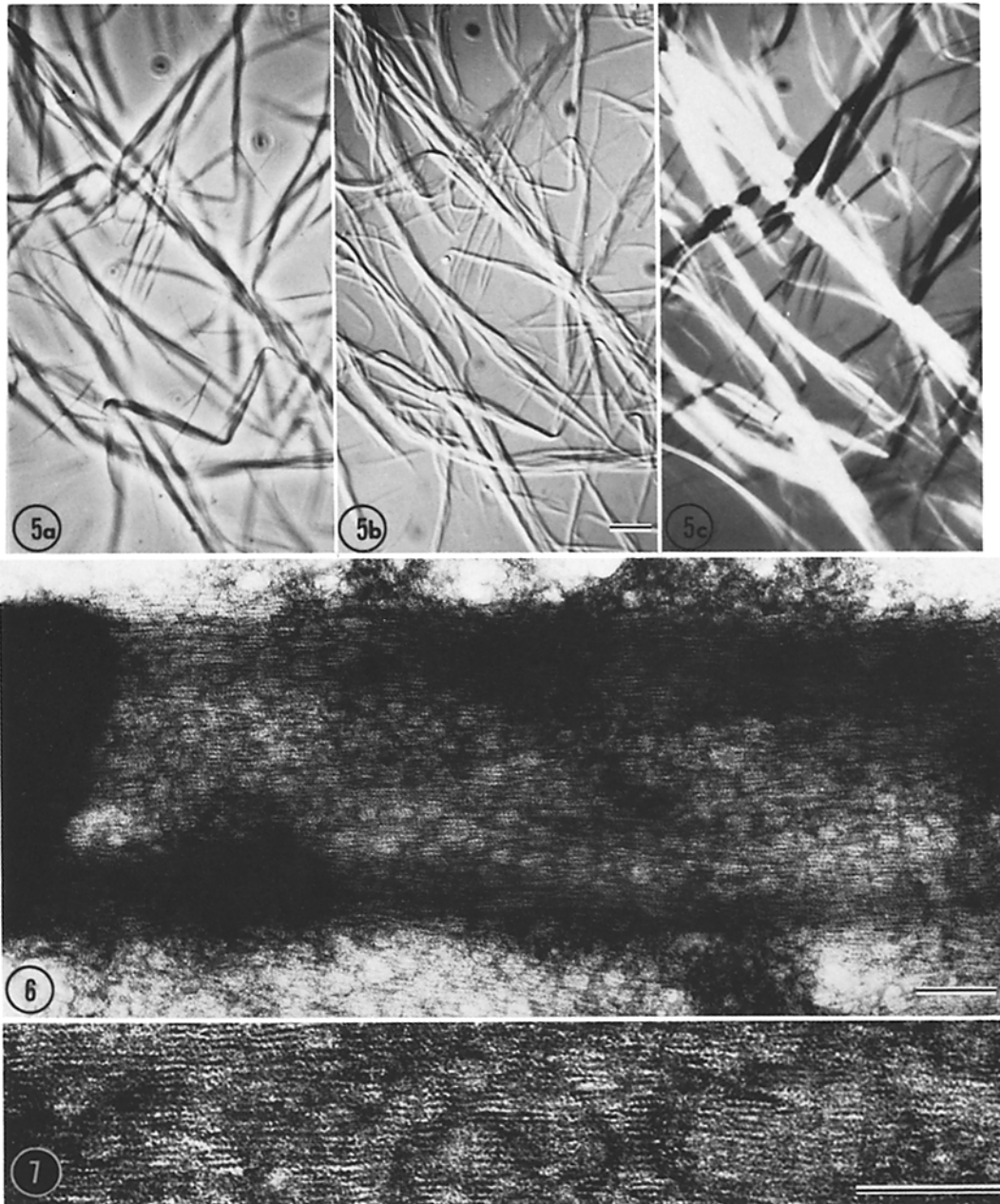


FIGURE 5 Photomicrographs of fibrils formed overnight at 0°C after the addition of ATP to 40°C gel material dissolved in 1 M KCl. (a) Phase contrast; (b) interference contrast; and (c) polarization optics. All  $\times 600$ . Reference mark = 10  $\mu\text{m}$ .

FIGURE 6 Negatively stained preparation of same fibrils as Fig. 5.  $\times 112,500$ . Reference mark = 0.1  $\mu\text{m}$ .

FIGURE 7 Same preparation as Fig. 6.  $\times 210,000$ . Reference mark = 0.1  $\mu\text{m}$ .



FIGURE 8 Negatively stained preparation of actin paracrystals formed on the addition of 0.05 M  $MgCl_2$  to fibrils dissolved in 0.01 PIPES.  $\times 37,500$ . Reference mark = 0.1  $\mu m$ .

FIGURE 9 Same preparation as Fig. 8.  $\times 210,000$ . Reference mark = 0.1  $\mu m$ .

If these microscopic actin fibrils are not removed but allowed to remain in 1 M KCl solution at 0°C, they gradually disappear over 3–4 days but will reform if fresh ATP is added. They will also reform without ATP addition if the solution is warmed to 25°C and will disappear again on cooling; this cycle can be repeated. The fibrils are stable indefinitely at 0°C if 1 mM EDTA is added with the ATP.

Unlike the gel material from which they originated, these fibrils are soluble in 0.01 M PIPES buffer and the addition of 0.05 M magnesium to this solution results in the formation of characteristic paracrystals (Figs. 8,9) similar to those described with muscle (6) and platelet (33) actin. This observation provides additional confirmation of their actin composition. These actin fibrils account for approximately one-quarter of the protein in the original gel or the order of 1% of the total soluble protein of the extract, but fibril for-

mation does not result in the quantitative removal of actin from the solution (Fig. 2 e); as a result these values do not represent the total actin present.

#### *Role of Other Proteins in Gel Formation*

Although actin is a major component of the gel material prepared from extracts at 40°C, the two other proteins present also appear to be involved in gel formation. This is indicated by the following experimental sequence. Gel material prepared at 40°C is dissolved in 1 M KCl, and some of the actin is removed through the induction of actin fibrils with ATP and their sedimentation. The KCl supernate after the removal of these fibrils contains the other two proteins of the original gel as well as a reduced concentration of actin (Fig. 2 e). If the salt concentration of this solution is reduced by dialysis against 0.01 M PIPES at 2–4°C, gel material is formed within 1 h, smaller

in amount but similar in microscopic appearance to the original 40°C gel. When collected by centrifugation, this material is found to have the same composition (Fig. 2 *f*) as the original gel (cf. Fig. 2 *a*). Actin is the major component, followed by the 58,000 mol wt protein and the heavier component of 220,000 mol wt. The supernate remaining after the removal of this gel is much reduced in protein concentration, but when loaded at four times the previous concentration, it is seen to be relatively enriched in the 58,000 mol wt protein (Fig. 2 *g*).

These results suggest that the original 40°C gel is a polymer of actin plus the 58,000 and 220,000 mol wt proteins, which is insoluble in low salt solution. When transferred to high salt concentration the complex appears to dissociate, releasing the actin in the form of F-actin filaments, some of which can then be aggregated to fibrils by the addition of ATP and removed by centrifugation. The resulting supernatant solution is poorer in actin and thus forms a smaller amount of insoluble gel complex when dialyzed back to low salt conditions. Some of the 58,000 and 220,000 mol wt proteins, which were originally complexed with the actin removed as fibrils remain in the soluble supernatant solution. Thus both actin and the other proteins appear to be separately soluble in low salt, but combine to form an insoluble gel complex when present together in solutions of low ionic strength. This behavior is most simply demonstrated by dissolving the gel in high salt concentration and then dialyzing against 0.01 M PIPES; a gel of the original structure and composition reforms as the ionic strength decreases.

## DISCUSSION

The procedure described here provides a simple method for the preparation of relatively pure actin from the sea urchin egg, based on two processes: (*a*) the polymerization of actin and other proteins from low calcium extracts at temperatures of 35–40°C in the presence of ATP and low concentrations of KCl, and (*b*) the subsequent separation of actin from this material by dissolving it in 0.6–1 M KCl and inducing the F-actin present to aggregate into microscopic fibrils by the addition of ATP at 0°C. The protein of these fibrils comigrates with rabbit muscle actin and forms characteristic actin paracrystals on the addition of magnesium. Yield values for actin have been measured, although they will obviously be low in view of the

unrecovered actin still present after fibril removal from the KCl solution. The actin recovered is approximately 1% of the total soluble protein present in the extract and one-quarter of this (0.25%) on the basis of total egg protein. The first value is probably of more significance in comparison to other cell types as it relates actin to the soluble cytoplasmic proteins of an isotonic extract, while the latter includes the much larger amount of stored material characteristic of eggs but not present in other cells.

The observations made and methods developed here are more closely related to investigations on actin-like proteins from amoeba than to previous sea urchin investigations. The preparation by Thompson and Wolpert (85) of motile cytoplasm from amoeba has led to a series of investigations on the nature of the cytoplasmic units involved and to methods for their isolation and investigation. Motility in such isolated cytoplasm is normally induced by the addition of ATP to a preparation at low temperature, followed by warming to room temperature. This motility is accompanied by the appearance of various filamentous or fibrillar units in the cytoplasm. It has been established by complexing with heavy meromyosin that the so-called thin filaments (approximately 70 Å) present in amoeba cytoplasm are actin (24). The aggregation of these thin filaments into microscopic fibrils can be induced by ATP or EDTA, and these fibrils have been studied by a variety of techniques *in situ* by Pollard and Ito (23) and after isolation by Morgan (20).

The methods used to induce the formation of fibrils in amoeba cytoplasm preparations resemble the methods developed here for the induction of actin gels in urchin egg extracts, in that both processes depend on the addition of ATP to a preparation at 0°C, followed by warming. But the products obtained in the two cases are different; instead of the fibrils and tactoids of aggregated actin filaments induced in amoeba (20, 23), warming of the low calcium extract of sea urchin egg cytoplasm causes the formation of dense gel composed of actin and two other proteins which is not temperature reversible. The fine structure, the chemical composition, and the behavior of the fibrils obtained with ATP or EDTA in amoeba can be compared more directly to those of the fibrils induced in the dissolved urchin gel material by the addition of ATP. In both cases, microscopic, birefringent fibrils are formed by the aggregation of F-actin filaments into bundles of varying sizes,



with no evidence of periodicity in their substructure. In addition, after their initial formation and gradual disappearance at 0°C the urchin fibrils then reappear at room temperature and display cold lability, i.e., behavior similar to that reported for amoeba fibrils (23). The slow disappearance of the fibrils at 0°C in urchin material and their reappearance on ATP addition might suggest a direct dependence of the fibers on the presence of ATP, but their reappearance at 25°C without added ATP appears to contradict this. The mechanism of this lability and its prevention by EDTA is unexplained.

The gelation process induced by temperatures of 35–40°C in egg extracts of low calcium concentration differs from fibril formation in that it involves the polymerization of actin with two additional cytoplasmic proteins. One of these proteins of approximately 58,000 mol wt is in the size range of tubulin but is distinguishable from it electrophoretically. A protein of similar mol wt (55,000) which can also be distinguished from tubulin has recently been reported by Tilney (36) in the acrosomal process of the horseshoe crab, *Limulus polyphemus*, where it is also associated with actin. This protein was present in preparations of *Limulus* leg muscle and had previously been found bound to actin in insect flight muscle (3), where it could be distinguished from tropomyosin and troponin. On the basis of his evidence, Tilney suggests that this protein is attached to the surface of the actin subunits in the acrosomal process, thus accounting for the change in the center-to-center spacing from the 50 Å of pure actin to the 85 Å measured in the acrosomal process and also for the absence of heavy meromyosin binding to the actin in the presence of the 55,000 mol wt protein.

There is no direct evidence available to allow identification of the protein in urchin egg extracts with this protein from *Limulus*, but it is of similar mol wt and may bind to actin in the same fashion. Electron microscope observations on the dissolved gel material from sea urchin extracts show it to have F-actin filaments as a major component, indicating that these filaments may be released from their previous association with the other proteins by the breakdown of the gel in high salt. Electrophoretic evidence indicates that this protein occurs in the gel in an approximately constant ratio to actin, and the removal of some actin from the dissolved material reduces the amount of gel obtained on lowering the ionic strength and leaves some of this protein in the supernate. Further

studies are needed to clarify the interaction of these two proteins.

A second protein of 220,000 mol wt is associated with actin in variable amounts in the gel complex and is distinguishable from the heavy chain of myosin. The role of this protein in the complex is also unknown, but it can be compared to the actin-binding protein of comparable size found in mammalian macrophages by Hartwig and Stossel.<sup>1</sup> This protein is present in extracts of macrophages made in low ionic strength solutions containing ATP and EDTA and approximately isotonic in sucrose. It complexes with actin to form a precipitate when 0.075–0.10 M KCl is added and the extract is held at 25°C for 1.5 h. This procedure is comparable in some respects to the gelation of isotonic urchin egg extracts at 35–40°C in the presence of ATP and KCl, and the subsequent separation of the actin from the complex by dissolving in 0.6 M KCl is also similar. However, the actin-binding protein was precipitated from this solution at 0.05 M KCl, while the protein of this mol wt in sea urchin extracts is soluble under these conditions in the absence of actin.

The necessity for heating of the urchin egg extracts to induce gelation remains unexplained (perhaps the heat denaturation of some other component is required), but once the gel has formed and has been separated from the solution, its polymerization can be controlled by ionic strength changes. The gel is soluble in high ionic strength solutions, and the original gel containing all three components reforms when the ionic strength is reduced by dialysis. The presence of F-actin filaments in the dissolved gel indicates that the other components may be bound to these filaments to form the gel, but the localization of the other components is presently unknown and under investigation.

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<sup>1</sup> Hartwig, J. H., and T. P. Stossel. 1975. Isolation and properties of actin, myosin and a new actin-binding protein in rabbit alveolar macrophages. *J. Biol. Chem.* In press.

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