THE ROLE OF ACTIN IN THE TEMPERATURE-DEPENDENT GELATION AND CONTRACTION OF EXTRACTS OF ACANTHAMOEBA

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ABSTRACT

The temperature-dependent assembly and the interaction of *Acanthamoeba* contractile proteins have been studied in a crude extract. A cold extract of soluble proteins from *Acanthamoeba castellanii* is prepared by homogenizing the cells in a sucrose-ATP-ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid buffer and centrifuging at 136,000 g for 1 h. When this supernate of soluble proteins is warmed to room temperature, it forms a solid gel. Upon standing at room temperature, the gel slowly contracts and squeezes out soluble components. The rates of gelation and contraction are both highly temperature dependent, with activation energies of about 20 kcal per mol. Gel formation is dependent upon the presence of ATP and Mg^{++}. Low concentrations of Ca^{++} accelerate the contractile phase of this phenomenon. The major protein component of the gel is actin. It is associated with myosin, cofactor, a high molecular weight protein tentatively identified as actin-binding protein, and several other unidentified proteins. Actin has been purified from these gels and was found to be capable of forming a solid gel when polymerized in the presence of ATP, $MgCl_2$, and KCl. The rate of purified actin polymerization is very temperature dependent and is accelerated by the addition of fragments of muscle actin filaments. These data suggest that *Acanthamoeba* contractile proteins have a dual role in the cell; they may generate the forces for cellular movements and also act as cytoskeletal elements by controlling the consistency of the cytoplasm.

The existence of actin and myosin in nonmuscle cells is now firmly established (see reference 37 for review). Studies of the enzymology, structure, and intracellular localization of these cytoplasmic contractile proteins suggest, but do not rigorously prove that they generate the forces for some cellular movements. A more direct approach is to study *movement* itself in cell-free extracts, which are directly accessible to experimental manipulation. In this paper the latter method is used to investigate some of the cytoskeletal and motile properties of the contractile proteins in a crude extract of *Acanthamoeba*.

There are relatively few published studies wherein the mechanism of cellular movements has been examined in cell-free extracts, but they have yielded much valuable information. Allen et al. (2) discovered that cytoplasm dissociated from *Chaos* retains the ability to stream and contract, proving that the force for cellular movement is generated in the cytoplasm. Thompson and Wolpert (47) subsequently showed that a crude extract of *Amoeba*

proteus cytoplasm streams and contracts at room temperature in the presence of ATP. Movement was inhibited in the cold. High-speed centrifugal fractionation of the extract yielded a pellet of particulate material and a supernate of soluble components. Neither fraction alone was motile, but the recombined fractions moved again (50). In a later study, Pollard and Ito (32) found that there is a large increase in the viscosity of cold *Amoeba proteus* extracts when they are warmed to room temperature. This consistency change was attributed to the temperature-dependent polymerization of thin filaments, later identified as actin (33), from soluble precursors. The cell extracts also contain thick filaments with some morphological features similar to myosin filaments. Since centrifugal fractionation of the extract distributes the soluble actin filament precursors and the thick filaments into the nonmotile supernate and pellet fractions, respectively, Pollard and Ito suggested that both actin filaments and myosin-like filaments might be necessary for movement in *Amoeba proteus*.

A somewhat different approach was taken by Taylor et al. (46), who devised methods for studying the properties of cytoplasm released from single *Chaos* cells. They found that the cytoplasm was viscoelastic and that its physical properties and movement could be manipulated by simple alterations in the Ca^{++} and ATP concentrations of the surrounding buffer. Calcium-dependent movement of the isolated cytoplasm had a threshold of 7 $\times$ 10^{-7} M. Their results suggested that the association and dissociation of a cytoplasmic actomyosin system could influence the consistency of the cytoplasm.

The strength of this work on movement of isolated cytoplasm was that movement itself was examined, rather than some indirect measure of contractile protein function such as ATPase activity. Unfortunately, there are also inherent limitations in the studies. First, all of the work was done on giant carnivorous amoebae which are difficult to grow in very large quantities; consequently, little biochemical information has been available about their contractile proteins. Second, most of the observations were made by microscope examination, so that they were largely qualitative and vulnerable to some subjectivity. To overcome these limitations, it was desirable to develop a cell-free system of motility that would be more quantitative and amenable to biochemical analysis.

Two general approaches were available: (*a*) to combine purified contractile proteins under conditions where they would form threads which would contract or suspensions which would superprecipitate upon the addition of ATP (45); or (*b*) to adapt the crude, motile extract approach to a cell type which could be obtained in large amounts and whose contractile proteins had already been studied. The first approach has been widely used in the study of muscle actomyosin and has been adapted to demonstrate the contractile activity of crude actomyosin from a number of cells, including platelets and *Physarum* (5, 6). The second approach is used in the present study to examine the assembly and activity of the contractile apparatus of the small soil amoeba, *Acanthamoeba castellanii*.

Acanthamoeba was chosen for this work because it can be grown in unlimited quantities and several of its contractile proteins, actin, myosin, and cofactor, have been partially characterized (34, 35, 48, 49). The rationale for working with a crude extract rather than the purified proteins was that extracts might contain components of the motile mechanism which had not been identified in previous biochemical studies. This proved to be true. The design of the present experiments was strongly influenced by Kane's discovery that actin in sea urchin egg extracts forms a solid gel when warmed to 37°C (21). The phenomenon he observed was reminiscent of the behavior of the motile extracts of *Amoeba proteus* and suggested a way of scaling up the motile extract approach for quantitative analysis.

Preliminary studies with *Acanthamoeba* (28, 29) showed that the contractile proteins are extracted from the cells by cold sucrose solutions and that they are components of a solid gel which forms after the extract is warmed to room temperature. Since the gel contracts and squeezes out soluble components, it appeared to be an attractive model system to study the activity of the contractile proteins in some detail. In the present work, a turbimetric assay has revealed that the rates of gelation and contraction are very temperature dependent and that minute changes in the free calcium concentration regulate contraction. Isolation and fractionation of the contracted gel has yielded information on the components of the motile apparatus. This general approach is likely to be useful for studying the contractile proteins of many cells. Stossel and Hartwig (42), for example, have recently applied this approach successfully to analyze the interactions of actin, myo-

sin, cofactor, and actin-binding protein from rabbit macrophages.

MATERIALS AND METHODS

Materials

Reagent grade chemicals were obtained from the following sources: sucrose (Mallinckrodt Chemical Works, St. Louis, Mo.); imidazole (grade III), dithiothreitol, ATP, ethyleneglycol-bis(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA),[1] cacodylic acid (Sigma Chemical Co., St. Louis, Mo.); glutaraldehyde (Taab Laboratories, Emmer Green, Reading, England), Sephadex G-25 fine and G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.); 4% agarose beads (Biogel A 15M, 200-400 mesh; Bio-Rad Laboratories, Richmond, Calif.). Distilled water was deionized with a Barnstead mixed bed deionizer (Barnstead Co., Boston, Mass.).

Methods

PREPARATION OF ACANTHAMOEBA EXTRACTS: *Acanthamoeba castellanii* (Neff strain) was grown in Fernbach flasks or in 15 liter carboys at 29-30°C as described previously (34) and harvested by centrifugation. After two washes in 10-20 vol of ice-cold 0.1 M NaCl, the pelleted cells were stored on ice for 30-60 min. The cells were then homogenized in 2 vol of ice-cold solution of 0.34 M sucrose, 10 mM imidazole chloride pH 7.0, 1 mM ATP, 1 mM EGTA, and 1 mM dithiothreitol (DTT) with six strokes of a Dounce homogenizer with a tight fitting pestle (Kontes glassware from Kontes Glass Co., Vineland, N.J.). Sucrose was used to solubilize the contractile proteins while minimizing the rupture of lysosomes and phagosomes (43). The homogenate was then centrifuged for 1 h at 35,000 rpm in a Spinco 42.1 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) (maximum force of 136,000 g) with the temperature control set at 1°C. The temperature during the run varied between 2°-4°C. At the completion of centrifugation the floating lipid fraction was carefully removed and discarded before pipetting off the clear supernate over the turbid microsomal fraction and the pellet. This supernate will be referred to as "the extract" in this paper. In all of these steps care was taken to keep the preparation as cold as possible to avoid gelation of the extract during the initial centrifugation. To avoid bubble formation when samples of extract were used for turbidity measurements, either the homogenate or the extract were degassed for 10-20 min in a bell jar at 3°-4°C using the vacuum produced by a water pump.

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[1] *Abbreviations used in this paper:* DTT, dithiothreitol; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid, a chelator of calcium; SDS, sodium dodecyl sulfate; and TPCK trypsin, trypsin treated with L-(1-Tosylamido-2-phenyl) ethyl chloromethyl ketone to inhibit contaminant chymotryptic activity.

PREPARATION OF RABBIT SKELETAL MUSCLE PROTEINS: Actin was prepared by a modification of the method of Spudich and Watt (39) with a single cycle of polymerization and sedimentation from 0.8 M KCl. Heavy meromyosin was formed by digestion of myosin with trypsin treated with L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK-trypsin) according to Woodrum et al. (51). Tropomyosin was purified according to Bailey (4) except that only one cycle of ammonium sulfate precipitation was done.

BIOCHEMICAL METHODS: Protein concentration of crude fractions was estimated using the method of Hartree (17) with bovine serum albumin as a standard. The concentration of purified fractions was measured by spectrophotometry with the following extinction coefficients: actin, $E^{1\%}_{280\,nm} = 10.9$ (38); heavy meromyosin, $E^{1\%}_{280\,nm} = 6.0$ (52); and tropomyosin, $E_{280\,nm} = 3.0$ (18). ATPase activity was measured by assaying inorganic phosphate release (34).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was carried out on 5-mm diameter cylindrical gels, using 25 mm Tris-glycine buffer pH 8.3 (40). Samples were boiled for 1 min in 1% SDS, 1% 2-mercaptoethanol, 2.5 mm Tris-glycine. The gels were stained with Coomassie Brilliant Blue R (15) and densitometered at 550 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Viscosity was measured with Cannon-Manning semimicro viscometers containing a sample of 0.6 ml with outflow times for buffer of 28-31 s at 24°C.

Turbidity changes were measured by absorbance at 330 nm, 340 nm, or 360 nm, using a Gilford spectrophotometer fitted with a temperature-controlled sample chamber. The temperature of the samples was monitored using a Yellow Springs Instrument telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) with a Teflon-coated probe placed directly in the sample cuvette or in a blank cuvette in the position adjacent to the sample. DNA was estimated by the method of Burton (9) after perchloric acid extraction.

MICROSCOPY: Electron micrographs were made with Siemens Elmiskop 1A or 101 microscopes. Samples were prepared by negative staining with 1% uranyl acetate (51) or by fixation at room temperature overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, followed by washing with 0.1 M cacodylate buffer, treatment with 1% OsO_4 in 0.1 M cacodylate at room temperature for 1 h, 1% uranyl acetate in water for 1 h, dehydration in a series of ethanol solutions, and embedding in Epon. Thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Gelation of Extracts

GENERAL DESCRIPTION: When the *Acanthamoeba* extract is warmed to room temperature *without stirring*, it forms a solid gel (Fig. 1).
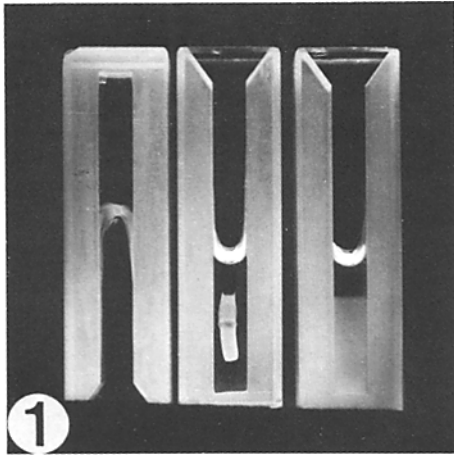
FIGURE 1 Examples of gelled and contracted *Acanthamoeba* extract. The sample on the left has gelled after 25 min at 25°C. The contracted sample in the middle was warmed to 25°C for 6 h. After solidifying during the first 20 min, the gel contracted slowly in all directions to form a small, opaque mass suspended in expressed fluid. The sample on the right contained 40 μM added $CaCl_2$. As shown in Fig. 9, $CaCl_2$ causes a rapid opacification of the gelled extract and in this case the opaque gel fragments settled to the bottom of the cuvette.
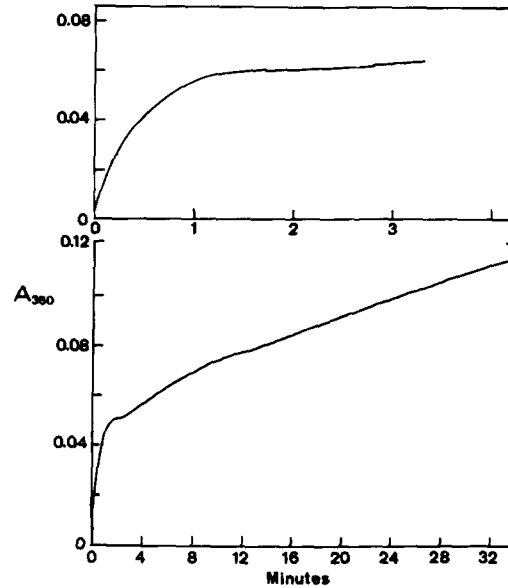

FIGURE 2 Turbidity changes of extract warmed to 30.2°C. Absorbance of the extract is monitored after the addition of 0.7 ml of ice-cold extract to a cuvette prewarmed to 30.2°C. The upper curve has an expanded time scale to show the first phase of the reaction. An extract gels in about 1 min at this temperature.

Even beakers containing over 150 ml of gelled extract can be inverted without spilling. Frozen extract, when it is thawed and warmed to room temperature, forms a gel in approximately the same time as fresh extract. During the first hour after gelation, gelled extract liquifies in 5–15 min after cooling to 0°C. Three cycles of gelation and liquification have been carried out successfully on a single sample. The gelled extract has properties of a weak solid, as it fractures into chunks when stirred or strongly agitated. If the extract is stirred during warming to room temperature, a white precipitate forms, rather than a gel. Consequently, viscometry and flow birefringence are not useful for following the course of gel formation.

TURBIMETRIC ASSAY FOR GELATION AND A QUANTITATIVE ANALYSIS OF TEMPERATURE DEPENDENCE: The gelled extract has a slightly greater turbidity than the liquid extract, so that the formation of the gel can be followed by monitoring absorption at 330–360 nm (Fig. 2). The longer wavelength is preferable, because absorption changes due to oxidation or reduction of nicotinamide nucleotides are minimized. When the cold extract is placed in a warm cuvette, there is an increase in absorption, the rate

of change of which decays exponentially (Figs. 2 and 3). There is a parallel increase in right-angle light scattering. The source of the turbidity change is not known, although it is temporally related to the gel formation. If one assumes that the extent of gel formation is proportional to the absorbance change, the formation of the gel at 25°C approximates a first order reaction during the initial 80–90% of the turbidity change; thereafter the rate increases slightly, probably due to warming (Fig. 3).[2]

The rate of the turbidity change and the gelation time are temperature dependent; thus the solidification of the extract takes less than 1 min at 35°C and requires 4–6 h at 0°C. Since the gelation of the

[2] The temperature of the ice-cold sample does not immediately reach that of the cuvette and the sample compartment. For example, with the cuvette at 33°C the temperature of an ice-cold sample jumps to 23°C within 5 s and then increases at an exponentially decaying rate to 33°C over a period of about 4 min. Consequently, the temperature of the sample would increase slightly during the gelling phase, which takes about 30–40 s at this temperature, and the actual temperature of the extract is lower than the temperature of the cuvette.

extract at 25°C can be reversed initially by lower-
ing its temperature to 0°C, the gel which forms
after many hours at 0°C must differ in some
unknown way from the gel which forms at higher
temperatures.

The data for turbidity changes at cuvette tem-
peratures between 20° and 35°C indicate apparent
first-order rate constants dependent upon tempera-
ture (Fig. 3). A linear Arrhenius plot yields a
calculated activation energy of about 22 kcal per
mol. This is probably a minimum value, because
the sample temperature is somewhat lower than
the cuvette temperature, especially at higher tem-
peratures. Nonetheless, this estimated high activa-
tion energy indicates a very strong temperature
dependence for the gelation reaction. This will be
examined in more detail in future studies. At
temperatures below 20°C the initial turbidity
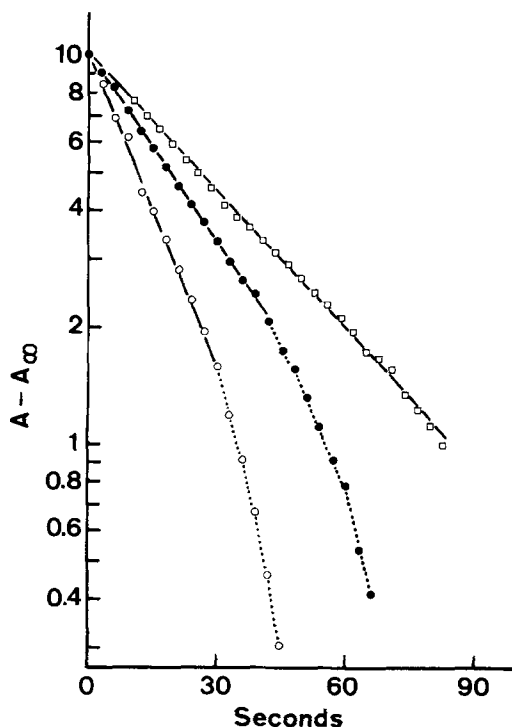change does not plateau before the secondary rise

FIGURE 3   Temperature dependence of extract turbidity
changes. At time zero 0.7 ml samples of ice-cold extract
were added to cuvettes prewarmed to 24°C (□), 30.2°C
(●), or 33°C (O), and absorbance monitored. The
absorbance at each time point, less the absorbance
plateau at the end of the initial rapid change in absorb-
ance, is plotted in arbitrary units on a log scale. The
linear part of each curve is drawn with a solid line.

in turbidity attributed to contraction (see below).
Since this complicates the analysis of gelation
kinetics, they have not been studied quantitatively
at these temperatures.

ION REQUIREMENTS: All of the ions and
small organic molecules required for gelation of
the cold crude extract are contained in the ex-
tract. The effects of adding KCl or $CaCl_2$ have
been examined. Although the addition of KCl
increases the extent of the temperature-dependent
turbidity change in proportion to the final KCl
concentration, the addition of more than 50–100
mmole/liter prevents gel formation. The addition
of $CaCl_2$ to the cold crude extract is considered
below.

To determine which ions are important for gel
formation, samples of extract were desalted on
Sephadex G-25 fine columns equilibrated with 0.34
M sucrose, 10 mM imidazole pH 7, 1 mM DDT
with or without 0.5 mM EGTA. The influence of
KCl, $MgCl_2$, $CaCl_2$, and ATP upon gelation are
described semiquantitatively in Table I. The com-
bination of $MgCl_2$ and ATP strongly stimulate gel
formation; in their absence, gelation was slow or
did not occur. This provides a convenient method
for preventing gelation during storage of the
desalted extract.

MICROSCOPY: By light microscopy (Fig.
4), fragments of the gel are amorphous or wrin-
kled phase-dense masses with smooth or rough
edges. If lipid droplets or microsomal vesicles con-
taminate the extract, they become trapped in the
gel. The texture and contrast of the gel resembles
the hyaline ectoplasm of living cells. Occasionally
there are a few fibrils in the preparations as well.
Microscope observation through crossed polars
reveals that the gel and the fibrils are birefringent
(Figure 4 b).

Electron microscopy of thin sections of gelled
extract reveals dispersed fragments of fibrous
material (Fig. 5 a). Filaments are somewhat more
evident in pellets of gelled extract (Fig. 6 a), but
here too they are mostly short fragments with
bends and branches.

Since there was biochemical evidence that the
gel is composed of actin (see below), and since the
disordered appearance of the fixed gel did not
account for the strong birefringence of unfixed
specimens, we studied the effects of fixation upon
actin filaments. We have fixed and embedded
pellets of pure rabbit muscle actin filaments by the
method used for the Acanthamoeba gel and moni-
tored the birefringence of these actin filament

TABLE I

Ion Requirements for Gel Formation

Additions	Time at 25°C		
	15 min	1 h	2 h
None	0	0	0
30 mM KCl	0	0	0
30 mM KCl, 1 mM MgCl₂	0	0	0
30 mM KCl, 1 mM ATP	+	+	+
30 mM KCl, 1 mM MgCl₂, 1 mM ATP	+++	+++	+++
1 mM ATP	0	++	++
1 mM ATP, 1 mM MgCl₂	+++	+++	+++
1 mM MgCl₂	+	±	±
1 mM ATP, 0.1 mM CaCl₂	0	+	++
1 mM MgCl₂, 1 mM ATP, 0.1 mM CaCl₂	+++	+++	+++
30 mM KCl, 1 mM MgCl₂, 1 mM ATP, 0.1 mM CaCl₂	++	++	++

A 13-ml sample of extract was desalted at 4°C on a 2.5 × 12.5-cm column of Sephadex G-25 fine equilibrated with 0.34 M sucrose, 10 mM imidazole chloride pH 7, 1 mM DTT, and 0.5 mM EGTA. Various additions were made to 0.4-ml samples and gel formation at 25°C assessed by observation. *Scale:* 0, liquid; +, semisolid gel which would pour out of an inverted tube; ++, solid gel which would pour out of an inverted tube intact; and +++, solid gel which would not pour out of an inverted tube.

pellets during fixation. In electron micrographs the appearance of these bona fide actin filaments is similar to that of the gelled extract (Fig. 6 *b*). In addition, we observed a marked decrease in birefringence during osmium treatment (31, 44). The loss of birefringence probably corresponds to a transition of the actin filaments from their straight, native configuration to the bent and fragmented configuration of the fixed and embedded actin filaments. In addition, both pure muscle actin (31, 44) and the actin filaments in the *Acanthamoeba* gel are stable during fixation if they have tropomyosin (Fig. 6 *c*) or muscle heavy meromyosin (Fig. 6 *d*) bound to them. These experiments, in addition to indicating the destruction of actin filaments during conventional fixation, identify the filaments composing the *Acanthamoeba* gel as actin filaments and show that they are a major morphological component of

the gel. No thick filaments are present, but there are a few microtubules in the gel (Fig. 6 *a*).

MACROMOLECULAR COMPOSITION OF THE GEL: The extract is composed of about 60% of the protein and 8% of the DNA in the homogenate (Table II). The subunit polypeptides of actin and myosin can be identified after polyacrylamide gel electrophoresis of the extract (Fig. 7 A). Their concentrations have been estimated by densitometry (Fig. 8) and are given in Table III.

It was hoped that freshly formed gel could be separated from soluble components of the extract by ultracentrifugation, so that the composition of the gel could be determined directly, but the gel does not form a compact pellet after centrifugation at 100,000 *g* for 1 h. Instead, it compresses somewhat so that the bottom two thirds of the tube consists of a slightly opaque gel, while the upper third is clear fluid. Consequently, a large fraction

of the soluble proteins remains trapped in the pellet of compressed gel, which makes a direct determination of its structural components impossible. An alternative mode of analysis is thus necessary. By comparing the composition of the original extract and the composition of the clear supernate over the centrifugally compressed gel, it is possible to estimate the composition of the gel indirectly (Fig. 8 and Table II). About 25% of the protein sediments with the gel, a large part of which is probably trapped and carried down nonselectively with the gel. This is suggested by the general lowering of all the peaks in scan 8 b, relative to scan 8 a. Only a few of the proteins are sedimented selectively, and it is thought these few proteins are likely to be structural components of the gel. These include part of the actin and myosin and all of a high molecular weight polypeptide (about 280,000 daltons). The concentrations of these presumed structural components are given in Table III. It is remarkable that a solid gel forms from such low a concentration (about 0.1%) of these proteins.

If the gelled extract is incubated for a much longer time, for example for 4 h at 25°C and then ultracentrifuged, three layers form: an opaque pellet; a clear semi-solid layer filling the bottom two thirds of the tube; and a clear liquid layer at the top. On the basis of light microscope appearance, it is thought that the middle layer is compressed gel which does not completely sediment. The opaque pellet also consists of gel fragments, but it is heavily contaminated with vesicles and membrane fragments which impart its opaque appearance. The opaque pellet comprises about 14% of the extract protein, but little DNA (Table II). The main polypeptide in the pellet has the electrophoretic mobility of actin (Fig. 7 B), but most of this component remains in the upper layers (Fig. 7 C). In addition to actin, there are a large number of other polypeptides in the pellet, including a small amount of myosin.

Contraction of Gelled Extracts

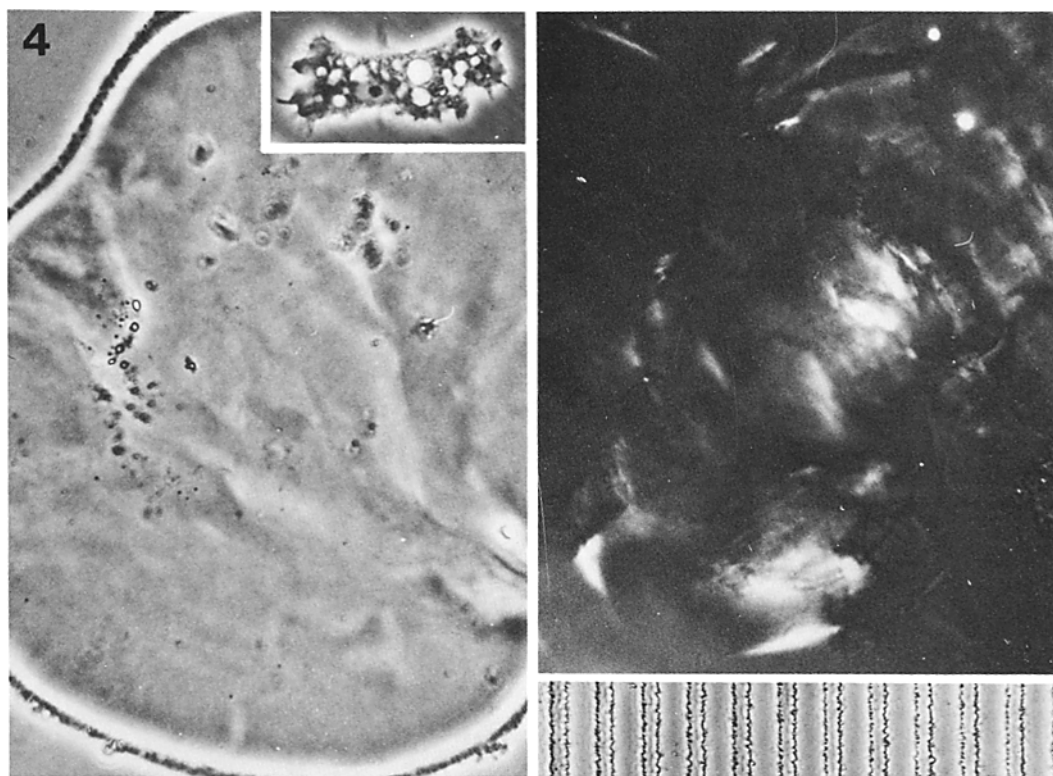GENERAL DESCRIPTION: If the gelled extract stands at room temperature in a small con-

FIGURE 4   Light micrographs of fragments of gelled extract, phase contrast on the left, and polarization optics on the right. The inset shows Acanthamoeba at the same magnification. Calibration bars = 10 μm.
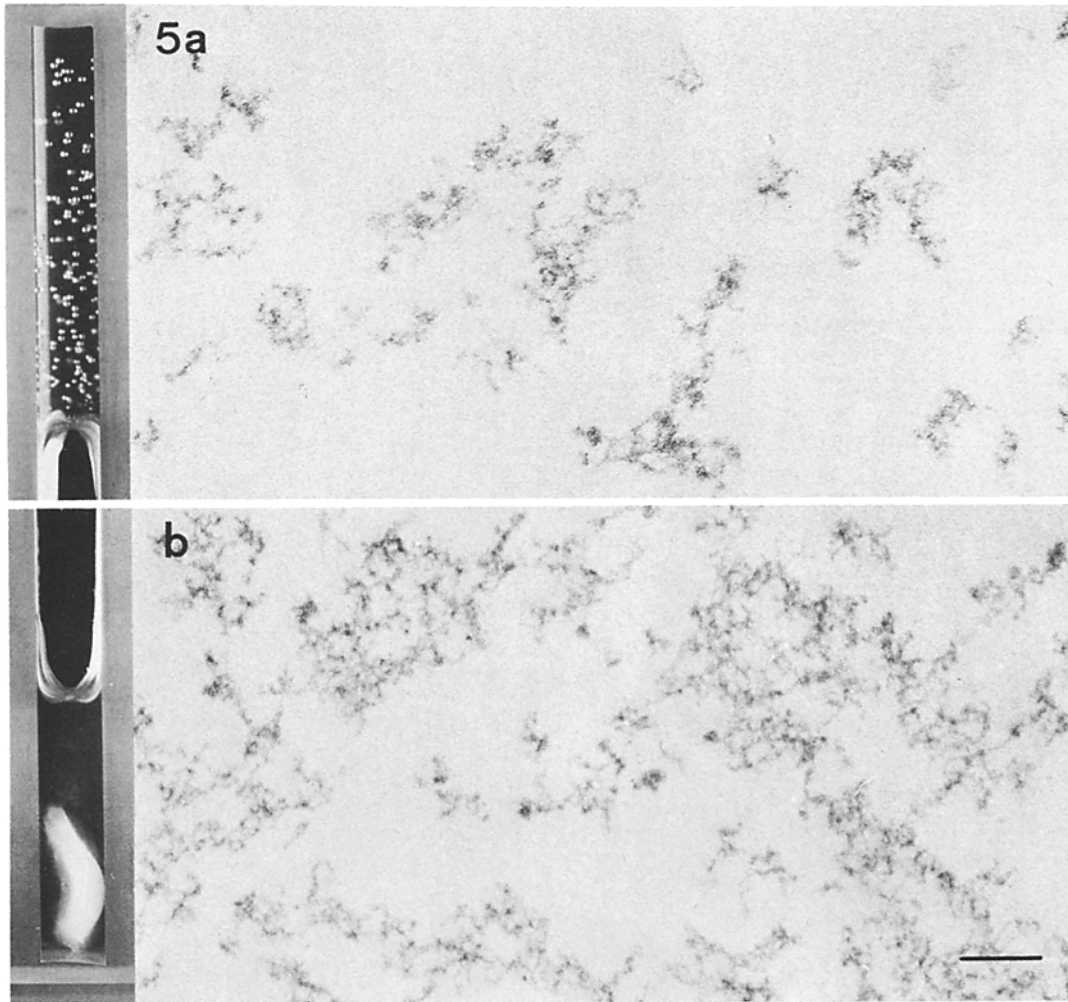
FIGURE 5   Electron micrographs of thin sections of gelled extract before (*a*) and after (*b*) contraction. The *insets* show the gross appearance of similar samples. Magnification, 76,420. Calibration bar = 0.1 µm.

tainer, the gel slowly shrinks away from the walls of its container to form a condensed white mass (Figs. 1 and 5 *b*). Because the volume of the gel may decrease over 80% during this transofrma-tion, I will refer to the phenomenon as a "contrac-tion." The soluble components which are ex-pressed from the gel during contraction can undergo further gelation, because the contracted gel is sometimes surrounded by a clear semi-solid layer (Fig. 1). In other cases, the contracted gel is attached to the meniscus (29) or sinks to the bottom of the cuvette (Fig. 5 *b*). Grossly, this re-action resembles blood clot retraction, because in both cases soluble components are squeezed from a solid gel. In contrast, the superprecipitation of actomyosin involves the condensation of fine pre-cipitate.

TURBIMETRIC   ASSAY   FOR   CONTRAC-TION   AND   A   QUANTITATIVE   ANALYSIS OF   TEMPERATURE   DEPENDENCE: The contraction of the gel is accompanied by a turbid-ity increase which can be monitored by changes in absorbance (Figs. 2 and 9). After the rapid, small amplitude increase in turbidity during gel forma-tion, there is a slower, larger increase in turbidity. In the 20°–35°C temperature range, there is typi-cally a plateau in the absorbance curve after the gel forms, followed by a more or less linear in-crease in turbidity. After 1 or more hours, there may be a further increase in the rate of turbidity

change until the contracting gel pulls out of the spectrophotometer beam and absorbance drops. The rate of turbidity change is very temperature dependent, and the process has an activation energy of about 19 kcal per mol in the temperature range of 15°–33°C.

MICROSCOPY: By light microscopy the appearance of the contracted gel is very similar to that of the gelled extract. By electron microscopy (Fig. 5 b), the contracted gel is shown to consist of the same fibrous material as the initial gel (Fig. 5 a), but to be arranged more compactly as ex-
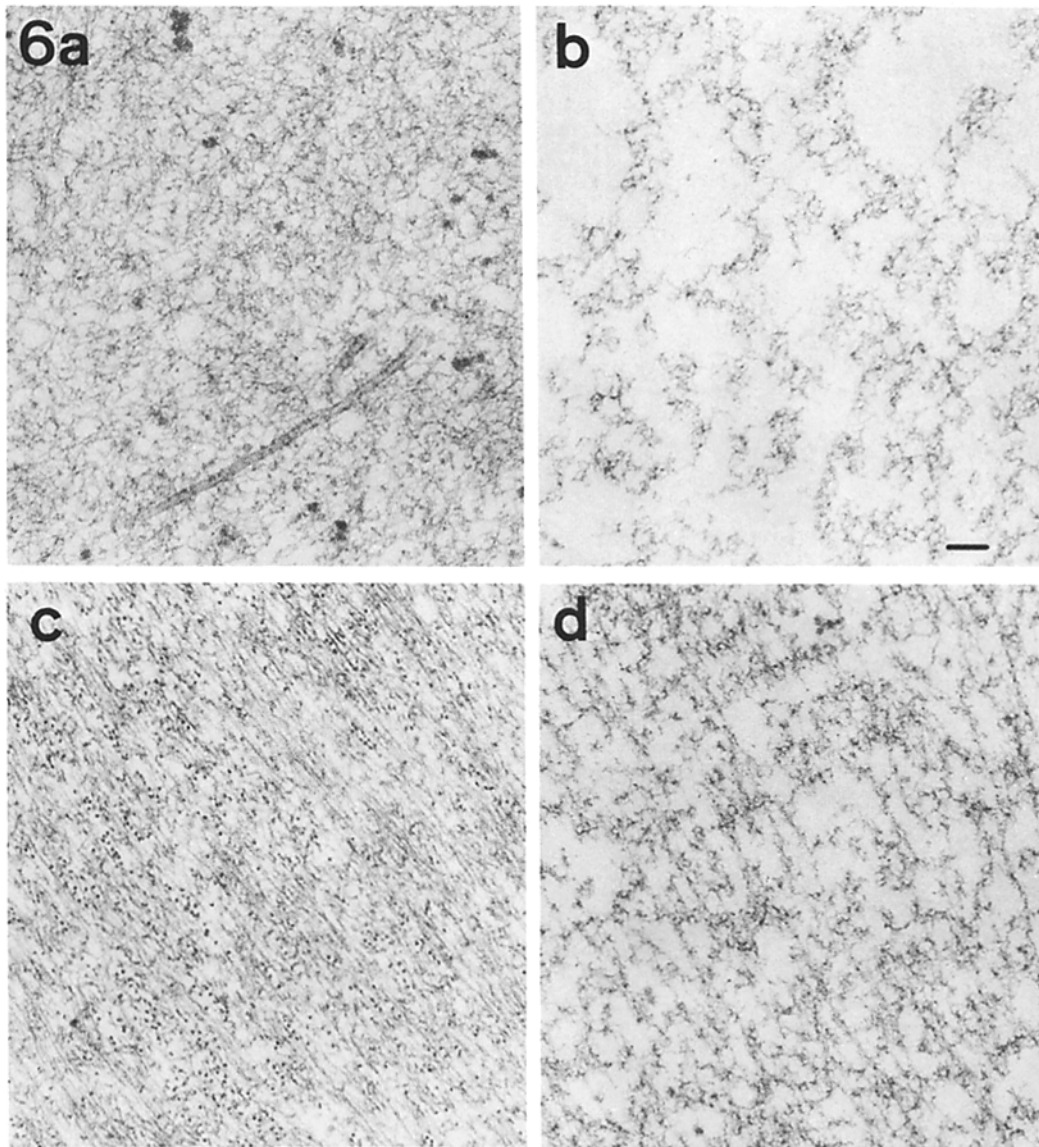

FIGURE 6 Electron micrographs of thin sections of (a) a pellet of gelled extract, (b), a pellet of pure muscle actin filaments, (c) a pellet of extract which gelled with added muscle tropomyosin (1.5 mg/ml) and MgCl₂ (2.6 mM), and (d) a sample of gelled extract to which muscle heavy meromyosin (2.4 mg/ml) was added before pelleting. Note the nonlinear appearance of the filaments in (a) and (b) and the linear appearance of the filaments in (c) and (d). Repeating arrowhead-shaped complexes are seen along some of the filaments in (d). The filaments in (c) have a larger diameter than the bare actin filaments in (a) and (b). Magnification, 50,000. Calibration bar = 0.1 μm.

Fractionation of Acanthamoeba Extract

Fraction*	Vol-ume	Protein		DNA		K+EDTA ATPase‡		Ca++ ATPase‡		Gel electro-phoresis§
		mg/ml	Total mg	µg/ml	Total mg	Total	Sp act	Total	Sp act	
	ml	*mg/ml*	*Total mg*	*µg/ml*	*Total mg*					
Homogenate	274	25.6	7014	73	20.0	27.9	4.0	42.2	6.0	
Extract	182	14.6	2657	6	1.1	14.7	5.5	17.1	6.6	7 A
4 h at 25°C pellet	21.5	17.8	383	1	0.1	0.7	1.8	8.2	21.3	7 B
4 h supernate	148	11.8	1746	4	0.6	10.7	6.1	10.7	6.1	7 C
4 h supernate after 16 h at 25°C	148	11.4	1687	—	—	10.7	6.3	5.7	3.4	7 D
16 h supernate	135	8.5	1148	2	0.3	5.5	4.8	2.4	2.1	7 E
16 h pellet	32	11.6	371	11	0.4	3.8	10.3	3.9	10.5	7 F
Low-ionic-strength ex-tract of 16 h pellet	31	7.4	229	—	—	2.5	10.6	0.3	1.5	7 G
Ghosts	12	12.2	146	—	—	1.7	11.6	6.2	42.3	7 H
KI-ATP extract of ghosts	10.5	7.6	80	—	—	0.9	11.6	4.2	53.0	12

* 90 g of *Acanthamoeba* were extracted and fractionated as described in the text.
‡ ATPase is expressed in total µmoles P_i per minute and nmoles P_i per minute-milligram protein.
§ Figure reference.

pected after a contraction. There are no thick filaments visible in these preparations.

COMPOSITION OF THE CONTRACTED GEL: Electrophoretic analysis (Fig. 7) reveals that the polypeptides in the extract are very stable during incubation at 25°C for periods of 20 h. The composition of the supernate obtained after 4 h incubation (Figure 7 C) is unchanged after incubation for an additional 16 h (Fig. 7 D). Except for the absence of the peptides removed by centrifugation at 4 h (Fig. 7 B), the 4-h supernate (Fig. 7 C) is unchanged from the original cold extract (Fig. 7 A), which was frozen immediately after it was made. Hence, there is little or no proteolytic activity in the extract as detectable by electrophoretic analysis. The ATPase activity is also rela-

tively stable during the long incubation of the extract at 25°C (Table II). During incubation for 20 h at 25°C, the inorganic phosphate concentration in the extract increases from 2 to 6 mM and ATP, detected semiquantitatively by thin-layer chromatography, disappears.

After 20 hours at 25°C, the gel is contracted and can be isolated as a hard, white pellet by centrifugation (35,000 or 136,000 g for 1 h), so that its composition can be determined directly. This pellet of contracted gel contains about 20% of the protein in the starting material, but little DNA (Table II). The polypeptide compositions of this pellet and its supernate are compared in Fig. 7 F and E. The pellet contains virtually all of the 42,000-dalton actin band, the 160,000-dalton myo-
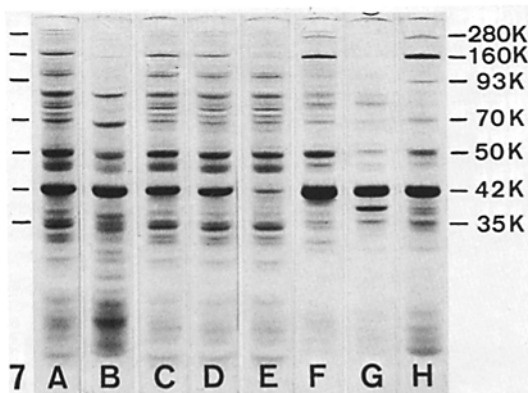
FIGURE 7 Electrophoresis on 7.5% polyacrylamide gels in SDS: (A) extract; (B) opaque pellet resulting from 136,000 g centrifugation of extract warmed to 25°C for 4 h; (C) supernate from extract centrifuged after incubation for 4 h at 25°C (this includes the partially compressed gel layer over the pellet); (D) supernate from extract incubated at 25°C for 4 h, after incubation for an additional 12 h at 25°C; (E) 136,000 g supernate from extract incubated for a total of 16 h at 25°C; (F) 136,000 g pellet from extract incubated a total of 16 h at 25°C; (G) low-ionic-strength extract of the 16-h pellet; and (H) pellet from the low-ionic-strength extract of the 16-h pellet ("ghosts"). Identification of bands: 280 K = actin-binding protein; 160 K = myosin heavy chain; 93 K = cofactor; 70 K and 50 K are unidentified; 42 K = actin; 35 K is unidentified. 15–20 μg of protein were applied to each gel.

sin band, and the 280,000-dalton band. Numerous unidentified polypeptides are also found in the pellet, the most prominent of which has a mol wt of about 50,000 daltons. Most of these latter polypeptides are also found in the supernate, so they could have been trapped nonspecifically in the pellet. If selective concentration in the pellet is used as the criterion for identifying the structural components of the contracted gel, then it is composed of actin, myosin, and the high molecular weight polypeptide. This is, of course, the same conclusion reached above regarding the composition of the freshly formed gel. Evidence for the presence of several other proteins in the gel will be considered in the section on fractionation of the contracted gel, which follows.

Influence of Calcium Ion on Gelation and Contraction

Although calcium ions control the contraction of muscle (13) and isolated cytoplasm of Chaos-

chaos (46), previous studies with isolated contractile proteins from Acanthamoeba failed to produce any evidence that Ca^{++} regulated their activity (30, 35). In fact, experiments showed that, although muscle troponin-tropomyosin could interact with Acanthamoeba actin (14), it could not serve as a calcium-modulated regulator of Acanthamoeba myosin and cofactor with actin (30). Therefore, it was interesting to test the effect of Ca^{++} on the gelation and contraction of these Acanthamoeba extracts in the hope of finding a previously missed regulatory system. Calcium has a dramatic effect on the contraction of the gelled extract!

In all of the work described to this point, the extract contained 0.67 mM EGTA, so that the Ca^{++} concentration was very low during gelation and contraction. The concentration of Ca^{++} in the
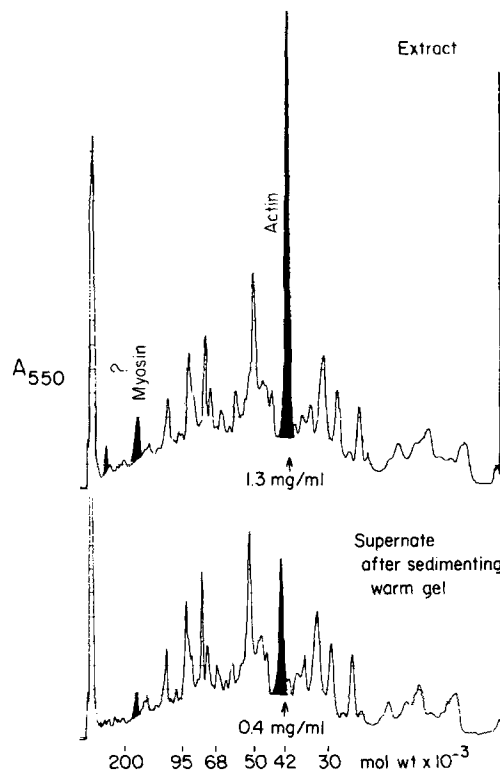

FIGURE 8 Densitometer scans of electrophoretic gels stained with Coomassie Blue. The upper scan is a sample of extract. The lower scan is a sample of the supernatant fluid obtained by warming extract to 25°C for 15 min and then centrifuging at 185,000 g for 15 min. The 280,000-dalton polypeptide is labeled with a question mark. The shaded areas were used to calculate the concentration of each component (Table III).

TABLE III

TABLE III

Protein Components of the Gel

Protein	Extract		Supernatant		Gel	
	mg/ml	(%)	mg/ml	(%)	mg/ml	(%)
Total protein	12.7		9.2		3.5	
280,000-dalton protein	0.04	(0.3)	0	(0)	0.04	(1.1)
Mysoin	0.13	(1.0)	0.05	(0.6)	0.08	(2.3)
Actin	1.34	(10.7)	0.38	(4.1)	0.96	(27.4)
Other proteins	11.2	(88)	8.8	(95)	2.4	(69)

A sample of extract was warmed to 25°C for 15 min to allow gel formation and then centrifuged at 180,000 g for 15 min at 25°C to compress the gel. Samples of the original extract and the clear supernate over the compressed gel were analyzed by gel electrophoresis (Fig. 8) and the quantities of high mol wt 280,000-dalton protein (labeled with a question mark), myosin, and actin estimated by densitometry of the stained gels. The quantities of the proteins in the gel fraction are calculated by subtracting their concentrations in the supernate from their concentrations in the extract.

extract is not known precisely, because neither the amount of calcium contributed by the amoeba nor the amount of calcium bound to amoeba components is known. Preliminary estimates from atomic adsorption spectrometry indicate that the total calcium concentration in the extract is about 0.1 mM.[3] If all of this calcium were free to bind to the EGTA, the calcium ion concentration would be on the order of 10^{-8} M in the routine extracts.

When low concentrations of $CaCl_2$ are added to the extract, there is a modest increase in the rate and extent of the initial temperature-dependent turbidity change and a pronounced acceleration of the secondary turbidity change (Fig. 9). As in the samples without added calcium, the initial turbidity change is associated with gelation, but in contrast to the samples without added calcium, there is no overall contraction of the gel during the second phase. Instead, the gel rapidly becomes opaque, and fragments into small bits that may settle to the bottom of the container (Fig. 1). One is given the impression that this violent reaction is due to rapid contractions which tear the gel apart. Characteristically, there is a short lag after gelation before the sharp increase in turbidity occurs; the meaning of this lag is not known. The maximum acceleration of the second phase of the turbidity increase occurs with added calcium-to-
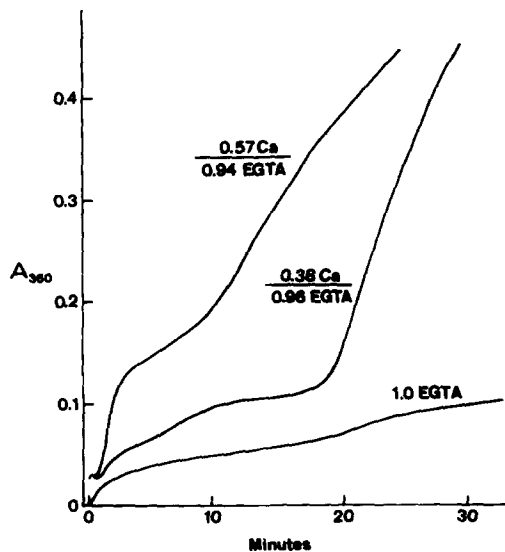
FIGURE 9 Influence of calcium on the turbidity changes of extract. At time zero 0.7-ml samples of ice-cold extract were added to cuvettes prewarmed to 25°C. The extract contained 1.0 mM EGTA and $CaCl_2$ was added to two of the samples as indicated by the Ca:EGTA ratios next to each curve.

EGTA ratios of 0.4 or 0.6 to 1, corresponding to Ca^{++} concentrations of about 2-4 × 10^{-7} M. These experiments provide the first evidence that Ca^{++} may regulate motility in *Acanthamoeba* and suggest that its threshold for activating the con-

tractile apparatus is in the submicromolar range. If $CaCl_2$ is added in excess of the EGTA concentration, the extract becomes turbid as soon as it is warmed to room temperature, but it usually fails to gel.

Fractionation of the Isolated Gel and Identification of Its Minor Protein Constituents

By fractionating the gel isolated from *Acanthamoeba* extracts, it is possible to obtain purified actin in high yield and to identify cofactor and several other polypeptides as likely gel components. Most of the work on fractionation has been done with contracted gel, because the incompletely contracted gel does not form a compact pellet during ultracentrifugation. The pellets of con-

tracted gel are solubilized in two steps: first, a low-ionic-strength extraction of actin monomers and then a high-ionic-strength extraction of residual actin and the associated proteins. The extracted proteins can then be separated from each other by gel filtration.

LOW-IONIC-STRENGTH ACTIN EXTRACTION AND PURIFICATION: Low-ionic-strength extraction of the contracted gel, with a buffer which depolymerizes actin, selectively extracts actin monomers from the gel. In 36 h, over 60% of the protein, consisting largely of actin, is solubilized by this treatment (Fig. 7 G and Table II). Additional actin can be extracted by prolonging the extraction at low ionic strength. Chromatography of the low-ionic-strength extract on Sephadex G-150 (Fig. 10), removes minor con-
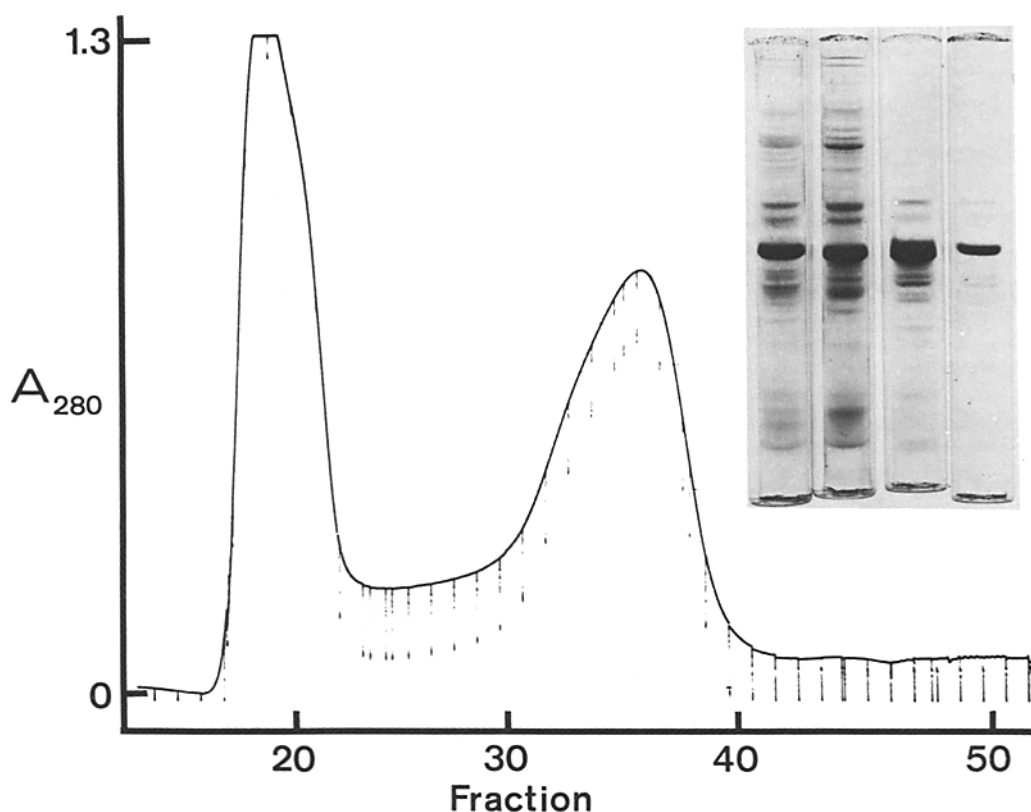

FIGURE 10   Purification of *Acanthamoeba* actin by gel filtration at low ionic strength. The 2.6 × 90-cm column of Sephadex G-150 was equilibrated and eluted with a low-ionic-strength buffer (2 mM Tris-chloride pH 8, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, 3 mM sodium azide, 40 μM phenyl methyl sulfonyl fluoride). The sample was 12.5 ml of a low-ionic-strength extract of contracted gel pellet. The curve is a monitor record of Absorbance at 280 nm, with full scale equal to 1.28. *Inset:* polyacrylamide gel electrophoresis patterns. Left = column sample. Second = voided fraction (first peak). Third = 25 μg of the second peak, consisting mainly of actin. Right = 5 μg of the second peak.

taminants and yields a preparation of monomeric actin which is at least 95% homogeneous (Fig. 11A). The only consistent contaminants are a faint band with a mol wt of about 50,000 daltons, consisting of less than 1% of the stained protein, and a band of variable density with a mol wt of about 37,000 daltons. The latter polypeptide arises *de*
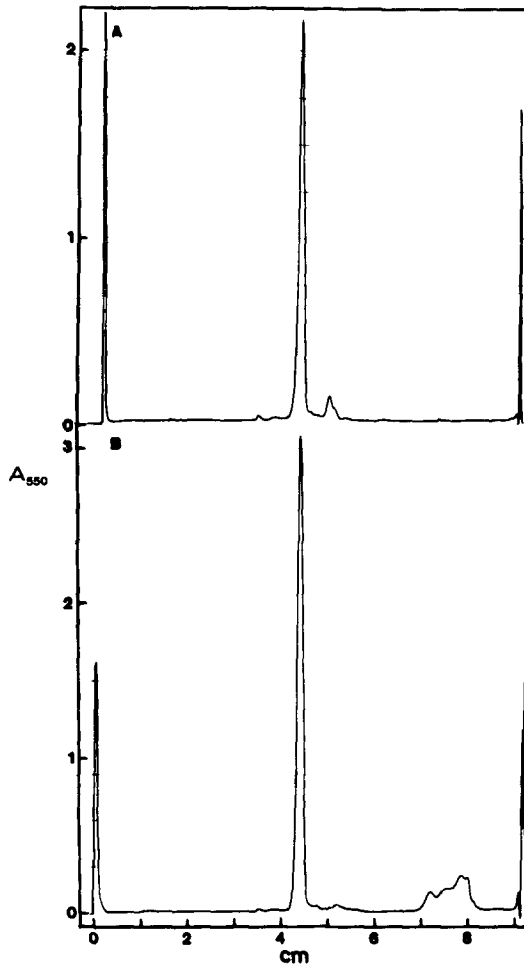

FIGURE 11 Densitometer scans of Coomassie Blue-stained electrophoretic gels of actin purified by the two methods described in the text. (A) Purification by low-ionic-strength extraction and chromatography (Fig. 10). (B) Purification by KI-ATP extraction and chromatography at high ionic strength (Fig. 12). These samples show the maximum amount of contamination of these purified actin preparations. For example, the KI-ATP actin in Fig. 12 does not have the low molecular weight polypeptides shown in part (B) of this figure. In addition, the density of the actin bands on these gels is out of the range of proportionality to the amount of actin in the band, while the contaminants are all in the proportional range.

novo during the low-ionic-strength extraction (Fig. 7 F and 7 G) and chromatography of the actin. It is thought to be a fragment of the actin arising from bacterial proteolysis of the monomeric actin. The polymerized actin remaining in the pellet is not fragmented by proteolysis (Fig. 7 H). The presence of bacteria in the actin and the breakdown of the actin are minimized by including azide and phenylmethylsulfonyl fluoride in the low-ionic-strength buffer. Without these inhibitors, more than half of the actin can be converted to the 37,000 dalton component during extraction and chromatography. A similar contaminant was noted in *Acanthamoeba* actin prepared by a previous method (48, 49) which also involved gel filtration in a low-ionic-strength ATP buffer. Muscle actin is also subject to a similar bacterial proteolysis under these conditions.[4]

COMPOSITION AND SOLUBILTY OF GEL "GHOSTS": The material which is insoluble after low-ionic-strength extraction of the contracted gel looks similar to the fragments of the original gel by phase contrast microscopy, except that it is much less dense. Because of this gossamer appearance, I refer to this insoluble material as gel "ghosts." Actin is the major component of this fraction (Fig. 7 H). It is associated with myosin and a relatively small number of other polypeptides. Their identifications are considered below.

Gel ghosts dissolve in concentrated buffers containing 0.6 M KCl or KI: the actin and all of the polypeptides marked in Fig. 7 H are solubilized (Fig. 12 *inset*). The material insoluble in 0.6 M salt is heterogeneous by electrophoretic analysis and includes a number of poorly resolved, low molecular weight bands. It is interesting that high-ionic-strength buffers are required to solubilize the myosin from the contracted gel, because isolated myosin is soluble in the low-ionic-strength buffer. Even after solubilization with 0.6 M KCl, the mixture of proteins extracted from the gel ghosts reprecipitate when dialyzed against dilute buffers, suggesting that the association of myosin with the other components makes it insoluble at low ionic strength.

FRACTIONATION OF GEL GHOSTS: Actin can be separated from the other components of the gel ghosts by solubilization with KI-ATP

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[4] T. D. Pollard, R. S. Adelstein, and E. Eisenberg. Unpublished observation.

buffer and chromatography on 4% agarose (Fig. 12), a procedure used previously to fractionate platelet actomyosin (36). The resulting actin is highly purified (Figs. 11 B and 12 *inset*). All of the ATPase activity from the gel ghosts elutes from the column in one peak, along with the 160,000-dalton myosin in heavy chain and associated light chains (Fig. 12 *inset*). The fractions containing the 93,000-dalton band overlap the myosin peak. The fractions containing the 280,000-dalton polypeptide have not been identified.

IDENTIFICATION OF THE MINOR POLY-PEPTIDES ASSOCIATED WITH ACTIN: Six polypeptides are associated with actin in the gel ghosts. On the basis of this association, it is tentatively concluded that they are bound together in some meaningful way, although they might simply have similar solubility properties. The molecular weights of these polypeptides have been determined by gel electrophoresis with internal standards (Fig. 13) and are indicated in
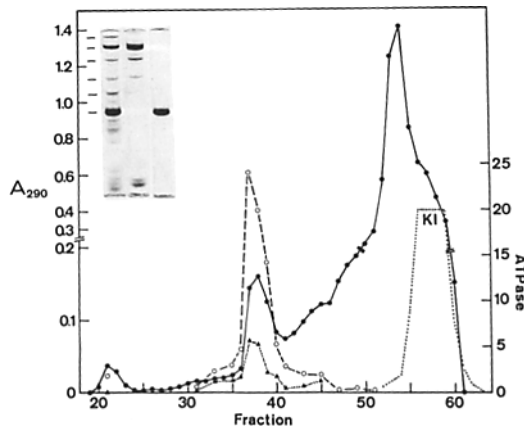

FIGURE 12 Fractionation of gel proteins by gel filtration at high ionic strength. The 2.6 × 90 cm column of Bio-gel A-15M (200–400 mesh) was equilibrated with 0.6 M KCl, 20 mM Tris-chloride pH 8, 1 mM DTT, 0.1 mM MgCl₂, 0.1 mM ATP, and 3 mM sodium azide. The sample was 9.5 ml of a KI-ATP buffer extract of gel ghosts clarified by ultracentrifugation. This sample was preceded by 10 ml of KI-ATP buffer. A_{290} (●——●), Ca⁺⁺ ATPase (O——O), K⁺EDTA ATPase (▲···▲), KI-ATP buffer zone measured by A_{260} (.......). Units of ATPase activity are nmoles/minutes·milliliter. *Inset* shows gel electrophoresis of (left) column sample, (middle) fraction 38, and (right) fraction 54. The marked bands have mol wt of 280, 000, 160, 000, 93, 000, 68, 000, 50, 000, and 42,000 daltons as shown in Fig. 13. The pair of bands at the bottom of the middle gel are the light chains of the myosin.
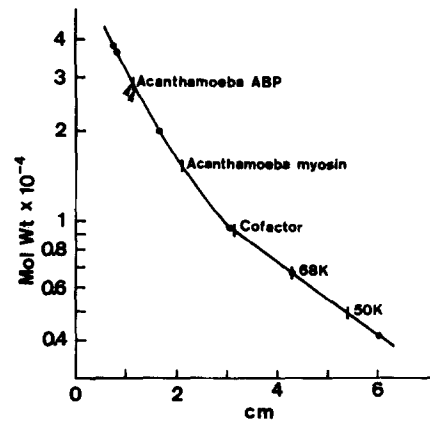

FIGURE 13 Molecular weight determination of *Acanthamoeba* polypeptides associated with the actin gel. Electrophoretic mobility in SDS on a 5% polyacrylamide is plotted vs. molecular weight. The standards (●) are sea urchin α-dynein (380,000 daltons, references 8 and 16), sea urchin β-dynein (360,000 daltons, references 8 and 16), rabbit muscle myosin heavy chain (200,000 daltons), phosphorylase-a (95,000 daltons), bovine serum albumin (68,000 daltons) and rabbit muscle actin (42,000 daltons). The *Acanthamoeba* polypeptides are marked with vertical bars. The mobility of human neutrophil actin-binding protein (see footnote 5), which split into a tightly spaced doublet, is marked with two arrowheads on either side of the polypeptide tentatively identified as *Acanthamoeba* actin-binding protein (*ABP*). Human spectrin has a slightly greater electrophoretic mobility than these actin-binding proteins.

Fig. 7. In paragraphs which follow, a few remarks are made on their identity.

The 280,000-dalton polypeptide has tentatively been identified as *Acanthamoeba* actin-binding protein since it has several properties in common with the actin-binding protein recently discovered by Hartwig and Stossel (19, 41) in rabbit macrophages. The *Acanthamoeba* protein has the same electrophoretic mobility as actin-binding protein from human neutrophils,[5] and it is found associated with actin in the gelled extracts under the same conditions in which the macrophage protein binds to actin.

The 160,000-dalton polypeptide is the heavy chain of *Acanthamoeba* myosin because it co-chromatographs with the ATPase activity, is associated with the characteristic light chains, and has the appropriate partition coefficient. The differ-

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ence between this polypeptide and a subunit with a mol wt of 140,000 daltons previously reported (34) may be attributable to differences in electrophoretic systems. The ATPase activity of *Acanthamoeba* myosin isolated from the gel is lower than that purified by other methods.

The 93,000-dalton polypeptide is probably the cofactor protein. Cofactor is required for actin to activate the *Acanthamoeba* myosin ATPase, and it has been tentatively identified as a polypeptide with a mol wt of about 95,000 daltons (35). Like the cofactor activity studied earlier, the 93,000-dalton polypeptide elutes from gel filtration columns near the myosin (Fig. 12).

Smaller polypeptides with mol wt of 68,000, 50,000, and 35,000, are associated with the gel ghosts, but they have not been identified. Their selective association with the known contractile proteins suggests that they may also be part of the *Acanthamoeba* contractile machinery; perhaps they are responsible for the influence of Ca^{++} on the contraction.

Reconstitution Experiments

Efforts to reconstitute the *Acanthamoeba* motile apparatus in vitro from purified components began with a surprise: purified *Acanthamoeba* actin forms a solid gel when polymerized at room temperature in the presence of $MgCl_2$, ATP, and KCl. Because this finding is unprecedented, the polymerization of *Acanthamoeba* actin has been studied. Less effort has been put into reconstitution of the contraction phase and only limited success can be reported.

GEL FORMATION BY PURIFIED ACANTHAMOEBA ACTIN: All of the experiments reported here were carried out in the low-ionic-strength buffer containing ATP and $CaCl_2$ which is used for chromatography (Fig. 10). Actin purified by gel filtration at low ionic strength was used in every experiment, though similar results were obtained with actin purified by the KI method.

In KCl alone, *Acanthamoeba* actin polymerizes but does not form a solid gel. Figure 14 compares the polymerization of muscle and *Acanthamoeba* actin in KCl. As is well documented in the literature (27), muscle actin polymerizes rapidly, attaining a viscosity of about 11 dl/g. Polymerization of muscle actin begins immediately after salt is added, because actin prepared by polymerization and depolymerization contains small fragments which nucleate the assembly (27). In contrast,

Acanthamoeba actin polymerizes more slowly under these conditions, and the final viscosity is lower; the value is about 4 dl/g, as reported earlier for *Acanthamoeba* actin purified by a different method (48). The lower viscosity of the *Acanthamoeba* actin is unexplained, but the lag in the polymerization is likely to be due to the absence of nucleating fragments in the actin prepared by gel filtration. Adding a very low concentration of muscle actin fragments eliminates the lag period in *Acanthamoeba* actin polymerization (Fig. 14). If the muscle actin nucleates the polymerization of the amoeba actin, the two types of actin must be capable of copolymerization. Experiments of a different type have suggested that *Physarum* and muscle actin can also copolymerize (20).

When *Acanthamoeba* actin polymerizes in the presence of 1 mM $MgCl_2$, it forms a solid gel which will not pour from a small container like a cuvette, even if shaken sharply. An actin concentration of 0.7 mg/ml is sufficient to form a firm gel. In contrast, muscle actin at this concentration
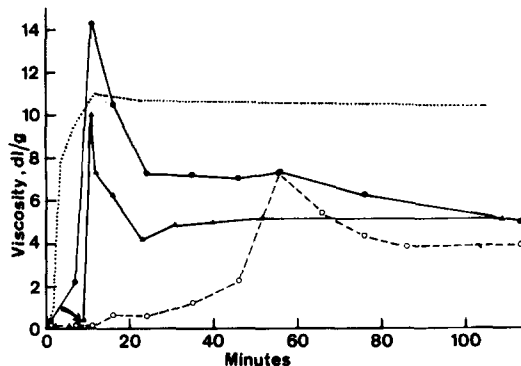

FIGURE 14 Effect of KCl, $MgCl_2$, and muscle actin-nucleating fragments on the polymerization of *Acanthamoeba* actin. Temperature is 24°C. *Acanthamoeba* actin was purified by the low-ionic-strength method and used at a concentration of 0.74 mg/ml. At time zero, KCl was added to a concentration of 0.1 M (O- -O), or KCl and $MgCl_2$ were added to concentrations of 0.1 M and 1 mM, respectively (●—●). For comparison, a sample of rabbit muscle actin at the same concentration was polymerized by the addition of KCl to 0.1 M (.......). In another experiment (▲—▲), KCl to 0.1 M was added to *Acanthamoeba* actin at time zero; after 10 min, sonicated muscle actin filaments in 0.1 M KCl were added to a concentration of 0.076 mg/ml as indicated by the arrow. This concentration of muscle actin would contribute a viscosity of 0.8 dl/g if fully polymerized. Therefore, at least 90% of the peak viscosity is attributable to the polymerization of *Acanthamoeba* actin.

polymerizes to form a thixotropic viscous liquid. This difference in the two types of actin is reflected in their changes in viscosity (Fig. 14) and turbidity (Fig. 15) during polymerization. The "viscosity measurements" do not accurately reflect the extent of polymerization, because the actin solution changes from a fluid to a gel, after which the flow time from the viscometer depends in part upon how much the gel is fragmented by shearing. Nevertheless, viscometry has revealed some interesting information about the time-course and temperature dependence of the polymerization and gel formation.

The polymerization of *Acanthamoeba* actin is more rapid in the presence of $MgCl_2$ than in its absence (Fig. 14), so $MgCl_2$ may promote nucleus formation. $MgCl_2$ also increases the extent of the viscosity change. The viscosity attained after 10–15 min may even stop the flow from the viscometer. At this point unsheared samples have solidified. As the sample flows through the viscometer during subsequent measurements, a fine precipitate forms, and the viscosity drops to a lower plateau. The precipitate is thought to be fragments of the gel arising from shearing, since no precipitate forms in unsheared samples. The fragmentation of the gel probably accounts for the drop in the viscosity.

The time-course of the polymerization and gel formation can also be followed by turbidity measurements (Fig. 15). During the polymerization of muscle actin there is a small rapid increase in turbidity. When KCl is added to *Acanthamoeba* actin there is a slow increase in its absorbance. Polymerization of *Acanthamoeba* actin in $MgCl_2$ and KCl results in a large, rapid change in turbidity during the time that it gels. These turbidity changes associated with the gelation of the purified *Acanthamoeba* actin are similar in amplitude but slower than the turbidity changes accompanying the gelation of the extract (Fig. 2).

The purified actin gel consists of sheets and bundles of actin filaments. By light microscopy there are variable proportions of birefringent tactoids and birefringent hyaline sheets (Fig. 16). The hyaline sheets are morphologically similar to the gel fragments observed in the extract. By electron microscopy, the tactoids are bundles of actin filaments. There are also free actin filaments. In one experiment, the proportions of individual filaments and large aggregates was found to be about 35% and 65%, respectively. The aggregates were pelleted by centrifugation at 6,000 *g* for 15
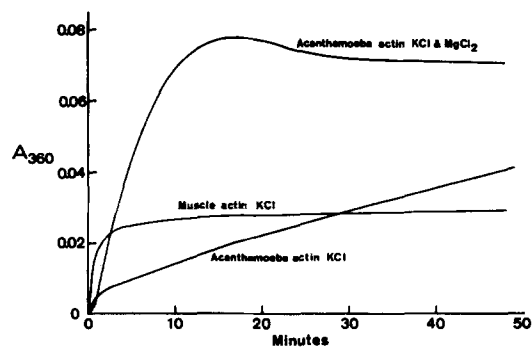

FIGURE 15   Turbidity changes of *Acanthamoeba* and muscle actin during polymerization. *Acanthamoeba* actin was purified by the low-ionic-strength method and was used at a concentration of 0.74 mg/ml. Muscle actin was used at the same concentration. Samples of monomeric actin were preequilibrated in cuvettes at 25°C and polymerization initiated by the addition of KCl to 0.1 M with or without $MgCl_2$ to 1 mM, as indicated. The *Acanthamoeba* actin with KCl and $MgCl_2$ formed a solid gel within 15 min.

min, leaving free actin filaments in the supernate. Both fractions had the same polypeptide composition, so that none of the minor contaminants discussed above were specifically associated with the aggregates.

The strong effect of temperature on the polymerization and gelation of *Acanthamoeba* actin is illustrated in Fig. 17. One sample of actin was polymerized at 23°C with $MgCl_2$. It rapidly increased in viscosity and gelled. An identical sample was treated in the same way at 4°C. It polymerized slowly after a long lag of about 2 h. A third sample was incubated at 4°C for 40 min; when it was shifted to 23°C, it polymerized immediately and formed a gel. Thus, the *rate* of polymerization of *Acanthamoeba* actin is very temperature dependent like muscle actin (22). Because the gelation of *Acanthamoeba* actin interfered with the viscosity measurements, it has been impossible to assess the influence of temperature on the *extent* of polymerization.

RECONSTITUTION   OF   CONTRACTION:
The gel formed by polymerizing *Acanthamoeba* actin in the presence of $MgCl_2$ does not contract or undergo the secondary phase of turbidity change associated with the contraction of the gelled extract, so muscle myosin or *Acanthamoeba* myosin have been added in an effort to reconstitute contraction. Addition of muscle myosin to purified *Acanthamoeba* actin before gelation at room temperature in 0.1 M KCl, 1 mM $MgCl_2$, 0.18 mM
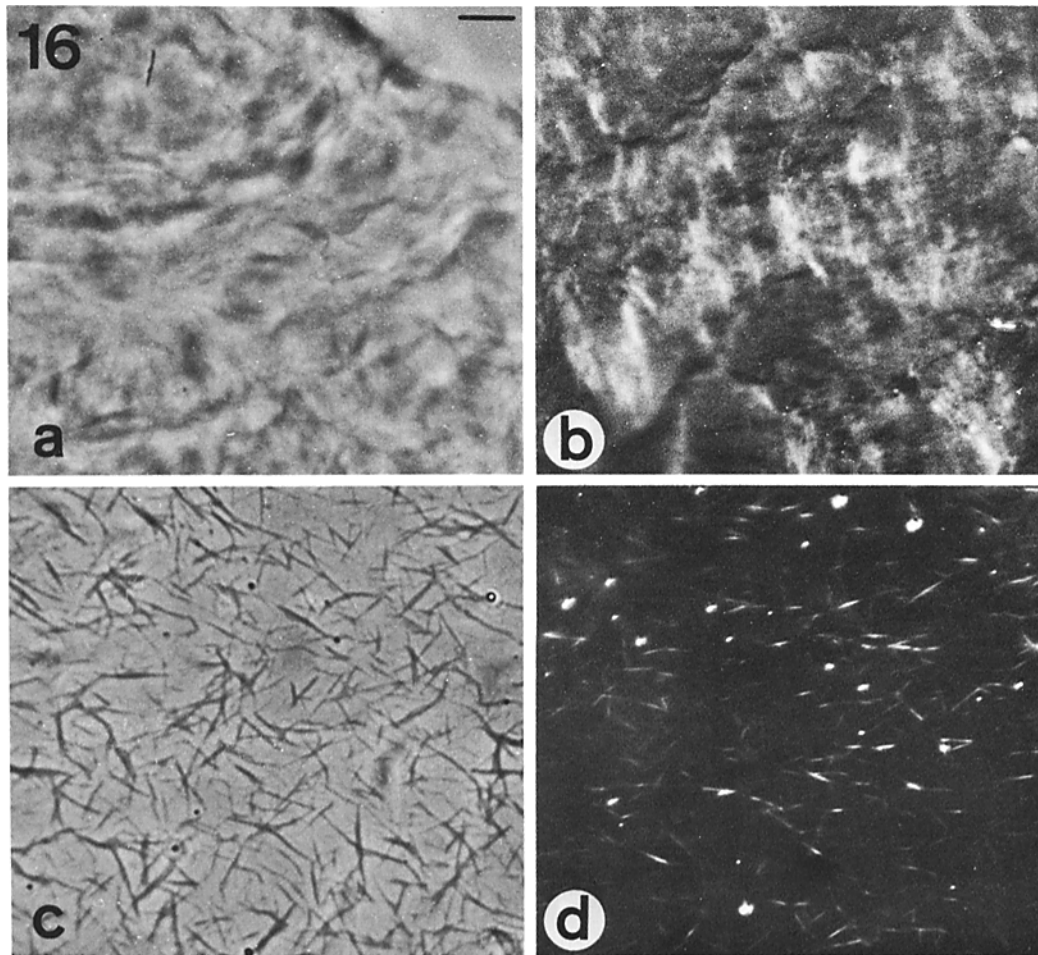
FIGURE 16 Light micrographs of *Acanthamoeba* actin polymerized with KCl and MgCl₂. Several different forms have been observed, usually sheets (a and b) or tactoids (c and d). The tactoids in (c) have been dried on the glass slide, while the other samples are suspended in the KCl-MgCl₂ buffer. Phase contrast (a) and (c), polarization (b) and darkfield (d) micrographs. Bar is 10 μm.

ATP, and 0.18 mM CaCl₂ resulted in a firmer actin gel. After the initial turbidity change associated with gelation, there was rapid secondary rise in turbidity. There was no overall contraction or syneresis of fluid under these conditions, but the secondary increase in turbidity may reflect a small internal contraction. The addition of muscle heavy meromyosin to *Acanthamoeba* actin does not give this secondary rise in turbidity after gelation. Addition of *Acanthamoeba* myosin from the KI gel filtration column (Fig. 12) to actin before forming a gel with MgCl₂ results in a firmer and somewhat more turbid actin gel, but there is not a secondary turbidity increase or contraction. The

low ATPase activity of myosin prepared by this method may have been a problem.

DISCUSSION

The gelation and the contraction of *Acanthamoeba* extract provide new information about the molecular basis of two important features of living cells: cytoplasmic structure and cellular motility. The present experiments suggest that contractile proteins may have a *dual role* in cellular function: (a) they may generate the force for cellular movement, and (b) they may also serve as cytoskeletal elements by forming a solid gel in some parts of

the living cell. These dual functions are discussed in turn below.

Motility

The shrinkage of the gel at room temperature has been interpreted as a contraction; the volume of the solid gel decreases and fluid is squeezed from the gel interstices. Analysis of the gel contraction has provided new information concerning three important aspects of the *Acanthamoeba* contractile apparatus: (*a*) the involvement of myosin, actin, and cofactor in force generation; (*b*) the mode of association of myosin with the other proteins necessary for contraction; and (*c*) the regulation of the contraction rate by Ca^{++}.

The selective association of *Acanthamoeba* actin, myosin, and cofactor in the contracted gel supports previous data on the properties of the proteins (34, 35, 48, 49) which have indicated that they are the motility proteins of the cell. An interesting aspect of the gel contraction is that it occurs in the apparent absence of a conventional, high-molecular-weight myosin. Thus the "mini-myosin," which is the only ATPase bound to the gel, may be capable of generating the force for the contraction of the gel. Although this small myosin molecule can cross-link actin filaments in vitro (35), it does not form bipolar filaments and must
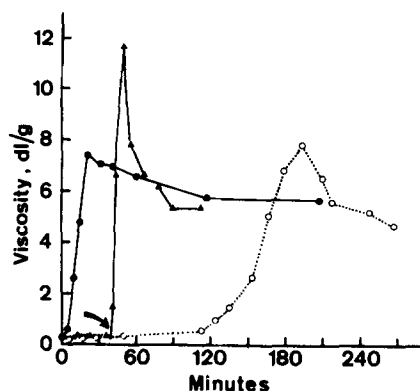

FIGURE 17   Influence of temperature on the polymerization of *Acanthamoeba* actin. The actin was purified by the low-ionic-strength method and used at a concentration of 0.74 mg/ml. At time zero, polymerization was initiated by the addition of KCl to 0.1 M and MgCl$_2$ to 1 mM. One sample (●—●) was at 23°C throughout. A second sample (O·····O) was a 4°C throughout. The third sample (▲—▲) was at 4°C for 38 min and then was rapidly warmed to 23°C (arrow).

therefore use an unconventional mechanism to generate contractile force. In this regard, the discovery that the solubility properties of the *Acanthamoeba* myosin are altered in the presence of the components comprising the gel ghost is particularly important. In vivo a number of myosin molecules might be associated with other proteins to form a multisubunit force generating unit. If this is true, it would explain the failure to reconstitute gel contraction from purified actin, *Acanthamoeba* myosin, and cofactor.

Another new feature of the *Acanthamoeba* contractile apparatus revealed in these experiments is that calcium may regulate the rate of its contraction. Slight variations of calcium ion concentration in the submicromolar range have a profound influence on the contraction of the gel. At about 10^{-8} M Ca^{++} there is a slow overall contraction of the gel. At about 2×10^{-7} M Ca^{++} the gel fragments rapidly due to what appears to be violent contractions. It was previously known from the work of Taylor et al. (46) that Ca^{++} concentration determines the rate of contraction of isolated *Chaos-chaos* cytoplasm; the extension of this concept to *Acanthamoeba* is important because of the unusual properties of the *Acanthamoeba* myosin and because earlier experiments suggested that its interaction with actin might not be controlled by Ca^{++} (30). The identity of the *Acanthamoeba* calcium receptor is unknown, but it may be one of the proteins bound to the contracted gel. Whatever its nature, it must have a very high affinity for Ca^{++}, since Ca^{++} has its effect on contraction at such low concentrations.

Cytoplasmic Structure

The present results support the concept that cellular contractile proteins have an important cytoskeletal function by virtue of their ability to form a solid gel. Although Pollard and Ito (32) were the first to suggest that the reversible polymerization of actin might influence the consistency of cytoplasm, the idea that cytoplasmic structure and motility might have a common basis is 141-yr old. As discussed by Allen (1) in his review of amoeboid movement, the early cytologist Dujardin believed (in 1835) that all cells were composed of a material called "sarcode," which possessed both structural and contractile properties. Dujardin was right!

Before the application of electron microscopy to biological research, the "structure of living protoplasm" was a popular area of investigation which reached its height during the 1930's, when it was established that cytoplasm is a dynamic thixotropic gel (see reference 23 for review). Early scientists had no information about the molecular basis of cytoplasmic structure, but they devised clever experiments on whole living cells (usually giant amoebae or marine eggs) which showed that cytoplasm has a gel-like consistency. It was appreciated that this gel is unevenly distributed in some cells; it is found primarily in the ectoplasm of amoebae (1, 26), for example. Efforts were made to measure the viscosity of the cytoplasmic gel by a number of methods, and the results, while they varied, indicated that the consistency of some regions of cytoplasm is very high (see reference 1 for review).

Although the cytoplasmic gel is very strong, it is also labile. The cytoplasm of giant amoebae is cyclically converted from a high consistency form to a low consistency form and back again as it moves (1, 26). The gel appears to be thixotropic, since cells round up and their cytoplasmic consistency decreases when they are agitated (3), although the mechanical stimulation might not act directly on the gel. Since cells round up and their cytoplasmic gel is liquified by high pressure or low temperature, gel formation is an endothermic process which is accompanied by a positive volume change (24). These properties of cytoplasm are not unique to amoebae and marine eggs; Crick and Hughes (12) showed that cultured chick cell cytoplasm is a thixotropic gel with slight elastic properties.

Movement of cells is apparently dependent upon the presence of gelled cytoplasm, since treatments which destroy cytoplasmic structure, such as low temperature, high pressure, and mechanical stimulation (see for example a fascinating experiment of Griffin and Allen described in reference 1), also stop movement. One popular early theory of amoeboid movement (26) even supposed that the transformation of cytoplasm from a sol to a gel provided the motile force.

Interest in cytoplasmic structure and its relation to motility seems to have waned in the early days of biological electron microscopy, perhaps because OsO₄ fixation destroyed the structure of the cytoplasmic matrix and nothing was found there to account for the previously established properties of the cytoplasm. When improved fixation methods revealed microtubules in many cells, they were thought to be cytoskeletal elements, although some cells like giant amoeba have few if any microtubules to account for the structured property of their cytoplasm. In these cells, other structures must be responsible for the gel-like consistency of the cytoplasm, while in cells with microtubules there may be two complimentary cytoskeletal systems.

The first information on the molecular basis of cytoplasmic gels came from work with motile cytoplasmic extracts of giant amoebae, which showed that contractile proteins may influence the consistency of cytoplasm. Two general mechanisms of action were proposed. Pollard and Ito (32) thought that the assembly and disassembly of actin filaments might control cytoplasmic consistency. Their correlation of actin filament assembly with viscosity increases in extracts of *Amoeba proteus* and their discovery that these actin filaments were cold labile accounted for some of the previously established properties of cytoplasm. Taylor et al. (46) suggested that the association and dissociation of actin and myosin filaments might control cytoplasmic consistency, and they showed that variation of the concentrations of Ca⁺⁺ and ATP could regulate these interactions. The present experiments with *Acanthamoeba* and the recent reports on sea urchin eggs (21) and macrophages (42) show that the gelation phenomenon is widespread and suggest that both the assembly and the association of actin filaments may influence cytoplasmic consistency, although the molecules involved may differ from cell to cell.

It is thought that the formation of the gel in the *Acanthamoeba* extract corresponds to the assembly of the amoeba's motile apparatus from dispersed, soluble components and that the reaction is due to the polymerization and association of actin filaments. Actin is the major component of the gel which forms in the extract, but the actin filaments must be bound to each other in some way to form a solid gel. The mechanism of this interaction is incompletely understood. Since purified *Acanthamoeba* actin alone forms a solid gel when polymerized in the presence of MgCl₂ and ATP, these actin filaments may bind directly to each other, although a minor contaminant protein might be involved. Whatever the mechanism, it is not inhibited by added tropomyosin.

Gel formation by purified actin has not been found in another system. Muscle actin filaments have a slight tendency to form a weak network in

solution, but this complex is destroyed by very low shear forces (25). Other investigators have shown that proteins bound to actin are necessary for gel formation. Heavy meromyosin (10) or alpha-actinin (25) enhance the gelation of muscle actin filaments. Kane (21) finds both a high molecular weight and a 58,000-dalton protein in association with actin in the gelled extract of sea urchin eggs, and Stossel and Hartwig (42) have evidence that a high-molecular-weight, actin-binding protein promotes the gelation of purified macrophage or muscle actin.

Thus, although it is generally found that actin is the major structural component of gels in cytoplasmic extracts, various cells seem to have different ways of cross-linking the actin filaments. Each cell may actually use several mechanisms to stabilize its cytoplasmic gel. In *Acanthamoeba*, a special property of the actin is important, but in addition, any or all of the proteins selectively associated with the actin filaments in the gel may contribute to stabilizing its structure, especially the myosin and the protein tentatively identified as actin-binding protein.

At the present time only temperature, ATP, and $MgCl_2$ have been identified as potential regulators of gel formation, but it is clear that other regulatory mechanisms must exist to control the dynamic state of the gel inside the cell. The *rate* of gel formation is very temperature dependent, but the influence of temperature on the *equilibrium* between the gel and its precursors is complex. Two observations indicate that there is a highly temperature-dependent equilibrium between precursors and gel: (*a*) the gel components are soluble in the cold during extraction; and (*b*) the gel which forms at 25°C can initially be liquified by cooling. On the other hand, the gel becomes refractory to cold liquification upon aging, and, given sufficient time, the extract will gel even at 0°C. The explanation of these observations is not known, but they indicate that the extract must be modified in some way during aging; this allows it to gel in the cold. Thus, there must be factors in the cell which regulate gelation and which remain to be identified.

Recent morphological evidence supports the concept of a structural actin filament network in the cytoplasm. Comly (11) found numerous actin filaments in the ectoplasm of glycerinated *Chaos* treated with heavy meromyosin. Electron microscope studies of whole cultured cells by Buckley (7) show a filamentous network throughout the cytoplasm. Although these filaments have not been positively identified as actin, their interconnected structure is suggestive of a supportive gel. This actin filament cytoskeleton may not have been fully appreciated in conventional thin-sectioned material because actin filaments may be distorted during fixation (44). This impression is confirmed by the elaborate network of straight actin-tropomyosin filaments found in the cortex and permeating the cytoplasm of *Acanthamoeba* after glycerination in the presence of tropomyosin (31). Hopefully, improved fixation procedures will reveal the true extent of these cytoskeletal elements in intact cells.

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