

VISCOMETRIC ANALYSIS OF THE GELATION OF *ACANTHAMOEBA* EXTRACTS AND PURIFICATION OF TWO GELATION FACTORS

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ABSTRACT

We have studied the kinetics of the gelation process that occurs upon warming cold extracts of *Acanthamoeba* using a low-shear falling ball assay. We find that the reaction has at least two steps, requires 0.5 mM ATP and 1.5 mM MgCl₂, and is inhibited by micromolar Ca⁺⁺. The optimum pH is 7.0 and temperature, 25°–30°C. The rate of the reaction is increased by cold preincubation with both MgCl₂ and ATP. Nonhydrolyzable analogues of ATP will not substitute for ATP either in this “potentiation reaction” or in the gelation process. Either of two purified or any one of four partially purified *Acanthamoeba* proteins will cross-link purified actin to form a gel, but none can account for the dependence of the reaction in the crude extract on Mg-ATP or its regulation by Ca⁺⁺. This suggests that the extract contains, in addition to actin-cross-linking proteins, factors dependent on Mg-ATP and Ca⁺⁺ that regulate the gelation process.

The soluble proteins extracted from a variety of cells in the cold form a solid gel when warmed to room temperature under physiological conditions (5, 12, 13, 18, 19, 20, 25, 27, 28, 31). This so-called gelation reaction has attracted considerable attention, because analysis of it promises to reveal how cells regulate the consistency of their cytoplasm. It is already clear that gelation requires polymerization of actin (12, 20) and the cross-linking of the actin filaments by accessory proteins (2, 3, 15, 25, 30). These cross-linking proteins seem to differ from cell to cell.

One goal of research in this area is to explain the gelation phenomenon at the molecular level. This will require the purification of all of the essential components of the gelation system and characterization of their interactions. To prove that all of the essential structural and regulatory components of the gel have been identified, one

must show that the purified components account quantitatively for the gelation properties of the crude extract. To make this comparison, it is necessary to define in some detail the properties of the gelation reaction in the crude extract.

In the present study we use a low-shear falling ball viscometer to describe in detail how environmental conditions influence the rate and extent of the gelation reaction in crude *Acanthamoeba* extracts. We confirm previous qualitative evidence (18, 19) that gelation of *Acanthamoeba* extract requires Mg⁺⁺ and ATP, is reversed by cold, and is inhibited by cytochalasin B. New findings include evidence for an Mg⁺⁺- and ATP-requiring “potentiation reaction” that precedes gelation and for inhibition of gelation by micromolar Ca⁺⁺. Furthermore, we confirm the finding of Maruta and Korn (15) that the extract can be fractionated into a number of low molecular weight compo-

nents each of which will cause purified actin to gel. Neither of two purified gelation factors alone nor any of four partially purified gelation factors can account for the Ca^{++} sensitivity and potentiation reaction characteristic of the unfractionated extract. Our interpretation of these results is that the extract contains, in addition to actin and several cross-linking proteins, some additional factors that regulate the gelation reaction. These characteristics of the *Acanthamoeba* gelation reaction were originally described in brief at the annual meeting of the Biophysical Society in 1978 (14).

MATERIALS AND METHODS

Reagent grade chemicals were purchased from the following sources: sucrose (Mallinckrodt Ltd., St. Louis, Mo.); imidazole chloride (imidazole) (grade III), dithiothreitol (DTT), EGTA, ATP, ADP, AMP, adenosine, GTP, CTP, UTP, cytochalasin B (Sigma Chemical Co., St. Louis, Mo.); ITP (P-L Biochemicals, Inc., Milwaukee, Wis.); adenylyl-imidodiphosphate (AMP-PNP), adenylyl(β - γ -methylene)-diphosphate (AMP-PCP) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); ^{32}P - γ -ATP (New England Nuclear Corp., Boston, Mass.); MgCl_2 , glycerol (J. T. Baker Chemical Co., Phillipsburg, N. J.); anhydrous CaCl_2 (Merck & Co., Rahway, N. J.). Chromatography materials were purchased from the following sources: Sephadex G-25 medium and G-150 (Sigma Chemical Co.); cellulose PEI/F thin-layer chromatography sheets (J. T. Baker Chemical Co.); DEAE-cellulose DE 52, Phosphocellulose P-11 (Whatman, Ltd., Springfield Mill, Maidstone, England); Bio-Gel HTP hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.).

Viscosity and Yield Strength

We used a simple falling ball device, described in detail in the Appendix, to measure the yield strength and apparent viscosity of *Acanthamoeba* extracts. Although this viscometer cannot be used to measure absolute viscosities of non-Newtonian fluids such as the gelling extract, it is very useful for documenting the rapid changes in the apparent viscosity that occur during the gelation process. At 0°C extract was mixed with test solutions, drawn into capillary tubes, and then warmed to 25°C to initiate gelation. The apparent viscosity was measured at several intervals. Rapid changes in apparent viscosity in a single sample were measured by recording the time for the ball to fall past each centimeter level. Data from the occasional sample through which the ball did not fall smoothly were discarded. To avoid disturbing the progress of the gelation reaction, only one ball was used per capillary. We defined a gelled extract as one through which the ball would not fall at an angle of 80° . This represents a yield strength of greater than 140 dyn/cm^2 .

Preparation of *Acanthamoeba* Extracts

An extract of *Acanthamoeba castellanii* (Neff strain) was prepared as described previously (18) with the following modifications: (a) the cells were homogenized with 10 strokes of a Dounce homogenizer (Kontes Co., Vineland N. J.) with a loose-fitting pestle; (b) the homogenate was centrifuged at 4°C for 1 h at 40,000 rpm in a Beckman Instruments, Inc. (Spinco Div., Palo Alto, Calif.) Ti50 rotor (g avg., 106,000); and (c) care was taken to avoid the floating lipid, the incompletely sedimented micro-

somal fraction, and the pellet in pipetting off clear supernatant extract.

Samples of extract (25–30 ml) were desalted by gel permeation chromatography at 4°C on a 2.5×36.5 -cm column of Sephadex G-25 equilibrated with 0.34 M sucrose, 10 mM imidazole (pH 7), 1 mM EGTA, and 0.5 mM DTT. For experiments with 4.5 mM EGTA-Ca buffers, the desalting buffer contained 5 mM EGTA and 30 mM imidazole with DTT and sucrose.

Fractionation of Gelation Factors

Gelation factors were isolated from the extract by a modification of a method described briefly by Maruta and Korn (15). The crude extract was first fractionated by ion exchange chromatography on DEAE-cellulose as described for myosin-II purification (21). To assay for gelation factors, samples were gently vortexed with depolymerized skeletal muscle actin in 30 mM imidazole (pH 7), 5 mM EGTA, 2 mM MgCl_2 , and 1 mM ATP and immediately drawn into capillary tubes. After warming the samples at 25°C for 10 min, we measured the viscosity as described above. Fractions containing gelation factors were identified by their ability to increase the viscosity of pure actin. Active fractions were pooled and purified further by ammonium sulfate precipitation, using 1.5–2.0 M ammonium sulfate with 10 mM EDTA in the saturated stock solution. This was followed by gel permeation chromatography on Sephadex G-150 in 20 mM imidazole (pH 7.5), 1 mM EGTA, and 0.5 mM DTT. The active fractions were applied to Bio-Rad Laboratories HT hydroxylapatite columns in 10 mM imidazole and 1 mM DTT (pH 7.5), and eluted with phosphate gradients.

Biochemical Methods

Rabbit skeletal muscle actin was prepared by a modification of the method of Spudich and Watt (24) with a single cycle of polymerization and sedimentation from 0.8 M KCl. Protein concentrations were estimated using the method of Hartree (10), with bovine serum albumin as a standard.

The amount of pelletable actin in extracts was determined by centrifugation of 1-ml samples for 60 min in a Ti-50 rotor at 40,000 rpm (g max. 150,000) followed by SDS-polyacrylamide gel electrophoresis of pellets and supernates. Actin was determined by densitometry of stained gels (18). It had been shown previously that virtually all of the protein of 43,000 molecular weight was actin (9, 18).

Free calcium ion concentrations in CaCl_2 -EGTA- MgCl_2 -ATP-imidazole buffers were calculated using the following Ca-EGTA dissociation constants: 1.9×10^{-6} M for pH 6.5, 1.9×10^{-7} M for pH 7.0, 8.0×10^{-8} M for pH 7.2, 3.4×10^{-8} M for pH 7.4, and 2.2×10^{-8} M for pH 7.5 (22).

RESULTS

We measured the viscosity change during gelation of both crude and desalted extracts. The two preparations are similar in many respects, but only the desalted extract is suitable for quantitative analysis, for two reasons. First, the properties of the crude extract change during storage at 0°C , whereas the desalted extract is stable. Second, it is possible to work under defined ionic conditions with the desalted extract, but not with the crude extract. In the following paragraphs we present a

brief description of the properties of the crude extract, followed by a detailed analysis of gelation in the desalted extract.

Crude Extract

PROTEIN COMPOSITION: The polypeptide composition in the crude extract was identical to that described previously (18). The average protein concentration was 10.8 mg/ml. Thus actin, which constituted 14% of the extract protein, was present at 1.5 mg/ml.

PHYSICAL CHANGES DURING GELATION: When the cold extract was warmed to 25°C, the initial apparent viscosity was 1–2 cp, but after a brief lag it increased rapidly to >12,000 cp as it gelled (Fig. 1). At 1 g, this represents a minimum yield strength of 146 dyn/cm². As judged by the centrifugation assay, the yield strength of the gelled extract varied among preparations from 0.6 to 2.9×10^3 dyn/cm². For comparison, a 1.5-mg/ml solution of pure muscle actin filaments in 10 mM imidazole (pH 7) and 2 mM MgCl₂, at 25°C, has an apparent viscosity of ~150 cp in our viscometer and a yield strength of <20 dyn/cm². This is an approximate maximum value for the yield strength of pure actin, because this yield strength is below the accurate range of our apparatus.

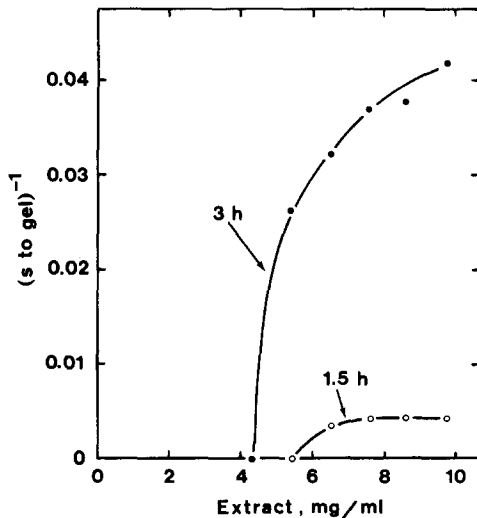


FIGURE 1 Dependence of the time for gelation of *Acanthamoeba* extract upon the concentration of extract and the time after preparation. At 1.5 h (○) and 3 h (●) after preparation of the extract, samples were diluted to various protein concentrations in 0.34 M sucrose, 10 mM imidazole (pH 7), 2 mM MgCl₂, 1.5 mM ATP, 1.5 mM EGTA, and 1 mM DTT, and the time to gel at 25°C was measured.

Using another method, Brotschi (2) found the yield strength of 1.5 mg/ml of muscle actin in 0.1 M KCl is 0.05 dyn/cm². This difference may be in part a result of the buffer composition and the purity of the actin.¹

REQUIREMENTS FOR GELATION: The gelation kinetics of crude extract depend on protein concentration, temperature, and pH. Below a critical concentration of 5–6 mg/ml protein (actin concentration, 0.7–0.8 mg/ml) the extract would not gel (Fig. 1). Gelation occurred at 15°C and the rate was faster at higher temperatures up to at least 30°C. Gelation occurred over a wide pH range, from 6.5 to 9.0, with the optimum rate at pH 7.0.

REVERSIBILITY: Using the low-shear viscometer, we confirmed the earlier qualitative observation (18) that the gelation of crude extract is reversible by low temperature. Fresh extract gelled when warmed to 25°, liquefied when incubated at 0°, and gelled again if rewarmed to 25°C. Both liquefaction and gelation occurred rapidly (<2 min); however, after several cycles of gelation and liquefaction, the crude extract no longer liquefied at 0°C.

AGING OF CRUDE EXTRACT: Although the polypeptide composition of the crude extract was stable for >6 h, even at 25°C (18), its properties changed during the first few hours after preparation while stored at 0°C. Crude extract gelled more rapidly and at lower protein concentrations after storage on ice for 3 h (Fig. 1). Fresh extract did not gel at temperatures below 15°C, whereas 3-h-old extract gelled readily at 12°C. Moreover, gels of aged extract were not liquefied at 0°C. Because this instability of the crude extract complicates detailed analysis, we concentrated our efforts on experiments with the more stable desalted extract.

Desalted Extract

COMPOSITION AND STABILITY: As judged by polyacrylamide gel electrophoresis, the polypeptide composition of desalted extract was quantitatively identical to the crude extract. The average protein concentration of the desalted extract was 9.4 mg/ml, slightly lower than the crude extract, and the actin concentration was about 1.3 mg/ml. The gelation properties of the desalted extract were stable during storage on ice for at least 6 h.

KINETICS OF GELATION: Desalted extract

¹ MacLean-Fletcher, S. and T. Pollard. Manuscript submitted for publication.

gelled when supplied with optimal concentrations of Mg^{++} and ATP and warmed to 25° (18). The gelation kinetics of the desalted extracts (Fig. 2) were similar to those of the crude extract. In both cases, there was an initial lag phase during which the apparent viscosity changed little, followed by an explosive increase in viscosity to >12,000 cp. The duration of the lag depended upon the protein concentration, and below 5 mg/ml desalted extract did not gel (Fig. 2).

REQUIREMENTS FOR GELATION: Gelation of desalted extract was optimal at pH 7.0 and 25–30°C, but it would occur, though less rapidly, over a wide range of pH (6.5–9.0) and temperature (from 12° to at least 35°C). Desalted extract that gelled at 25° liquefied at 0°C through several cycles.

In the presence of 1 mM ATP, gelation of desalted extract required $MgCl_2$ at a concentration ≥ 1.5 mM. At lower concentrations of $MgCl_2$, the viscosity increased slightly after prolonged incubation at 25°C, but a gel did not form. None of several other cations tested substituted for $MgCl_2$ (Table I).

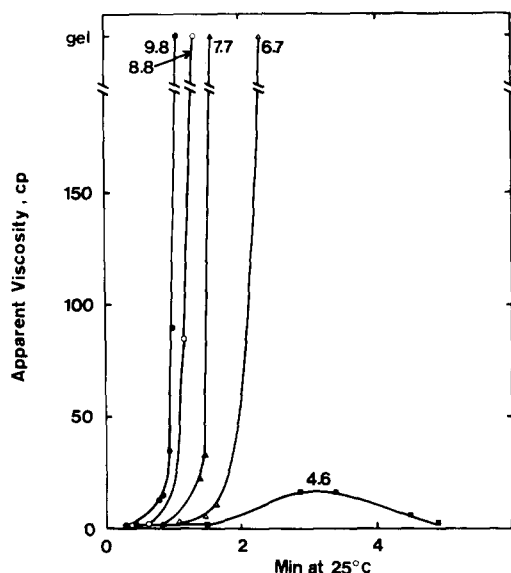


FIGURE 2 Dependence of the time-course of gelation of desalted *Acanthamoeba* extract upon the concentration of extract. We diluted samples to various concentrations in 0.34 M sucrose, 10 mM imidazole (pH 7), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 0.5 mM DTT, and preincubated them for 5 min at 0°C before measuring the time-course of their viscosity changes at 25°C. Protein concentrations in milligrams per milliliter are indicated next to each curve.

TABLE I
Ion Requirements for Gelation

Ion	Viscosity
mM	cp
$MgCl_2$, 1.5	Gel
$CaCl_2$ (without EGTA), 2	1.4
$BaCl_2$, 1.5	1.4
$MnCl_2$, 1.5	1.6
NaCl, 50	1.6
KCl, 50	1.6

Conditions: Desalted extract in 0.34 M sucrose, 20 mM imidazole (pH 7), 2 mM EGTA, 1 mM ATP, and 0.5 mM DTT. Incubation, 15 min at 20°C.

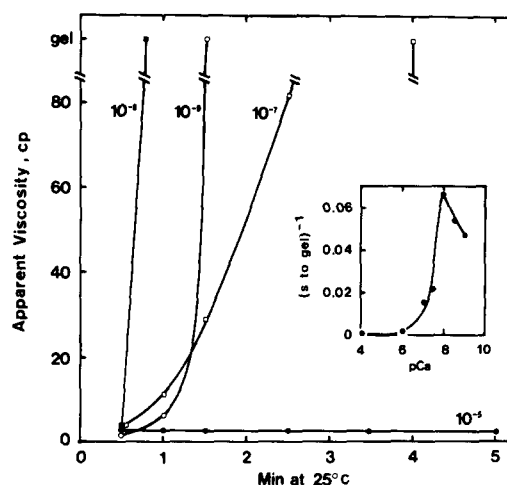


FIGURE 3 Dependence of the time-course of gelation of desalted *Acanthamoeba* extract upon the free calcium ion concentration. We preincubated samples containing 9.2 mg/ml protein in 0.34 M sucrose, 10 mM imidazole (pH 7), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 0.5 mM DTT, and various amounts of $CaCl_2$, for 5 min at 0°C before warming them to 25°C and measuring the time-courses of viscosity changes. Free calcium ion concentrations: 10^{-9} M (○), 10^{-8} M (■), 10^{-7} M (□), 10^{-5} M (●). Inset, a plot of the reciprocal of the time to gel vs. pCa from another experiment.

Gelation of desalted extract with 1 mM ATP and 2 mM $MgCl_2$ was optimal in 0–20 mM KCl. In 40–60 mM KCl, gelation occurred somewhat more slowly, whereas at KCl concentrations >80 mM there was an increase in viscosity, but no gelation. Similar results were obtained with NaCl.

In the presence of Mg-ATP, the optimal free calcium ion concentration for gelation was 10^{-8} M (Fig. 3). Free calcium ion concentrations > 10^{-7} M completely inhibited gelation. The results were identical with either 1 mM or 4.5 mM EDTA-

CaCl₂ buffers, indicating that traces of Ca⁺⁺ contaminating the solutions were not an important factor in determining the free Ca⁺⁺ concentration. The inhibition of gelation by micromolar Ca⁺⁺ occurred over a pH range from at least 6.6 to 7.2.

Inhibition of gelation by Ca⁺⁺ was not reversible at 25°C. An extract in 10⁻⁶ M Ca⁺⁺ did not gel within 4 h of dialysis at 25°C against an EGTA buffer containing 10⁻⁹ M Ca⁺⁺. However, an extract that gelled in ~10⁻⁹ M Ca⁺⁺ could be liquefied at 25°C within ~30 min by dialysis against a buffer containing 10⁻⁶ M Ca⁺⁺.

In the presence of 2 mM MgCl₂, gelation of desalted extract required ATP at a concentration ≥0.5 mM. At lower concentrations of ATP, the viscosity increased slightly after prolonged incubation at 25°C, but a gel did not form. ITP substituted for ATP to give quantitatively identical gelation kinetics. A small increase in viscosity occurred with 1 mM AMP, but there was no viscosity change with 1 mM ADP, AMP-PCP, or AMP-PNP. Thin-layer chromatography on PEI cellulose showed that the ITP and AMP contained <3% ATP. The effects of 1 mM CTP, GTP, or UTP on gelation varied. Typically, the viscosity of the extract increased for a short time before falling back to buffer levels if CTP, GTP, or UTP was substituted for ATP.

POTENTIATION REACTION: When desalted extract was incubated at 0°C with MgCl₂ and ATP, it did not gel, but underwent changes potentiating the gelation that occurred upon subsequent warming to 25°C. This potentiation reaction caused the desalted extract to gel more rapidly, at lower protein concentration, at lower temperatures, and more permanently. These changes in the desalted extract were similar to those observed upon aging of the crude extract, but there is no direct evidence that the two processes involved the same reactions.

The time required for gelation at 25°C decreased by a factor of six (Fig. 4) after 15–20 min preincubation at 0°C with MgCl₂ and ATP (Fig. 5). Both MgCl₂ and ATP were required for this potentiation reaction (Fig. 4). Neither AMP-PCP nor AMP-PNP substituted for ATP in this reaction.

The potentiation effect was maintained even if the MgCl₂ and ATP were removed by a second desalting on Sephadex G-25. If MgCl₂ and ATP were re-added to such a potentiated, MgCl₂- and ATP-free sample, it gelled at 25°C in about one-sixth the time required for gelation of unpoten-

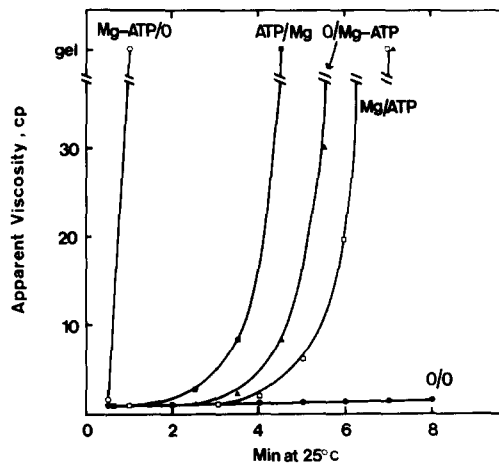


FIGURE 4 Dependence of the time-course of gelation of desalted *Acanthamoeba* extract upon preincubation with MgCl₂ and ATP at 0°C. We preincubated samples containing 7.6 mg/ml protein in 0.34 M sucrose, 20 mM imidazole (pH 7), 2 mM EGTA, and 0.5 mM DTT for 17 min at 0°C under various conditions before warming them to 25°C and measuring the time-course of gelation. Experimental conditions indicated next to each curve: present during 17-min preincubation/added immediately before warming. Conditions: cold preincubation with both 1 mM ATP and 2 mM MgCl₂ (○); cold preincubation with ATP followed by addition of MgCl₂ immediately before warming (■); cold preincubation with MgCl₂ followed by addition of ATP immediately before warming (□); cold preincubation followed by addition of MgCl₂ and ATP immediately before warming (▲); no addition of MgCl₂ or ATP (●).

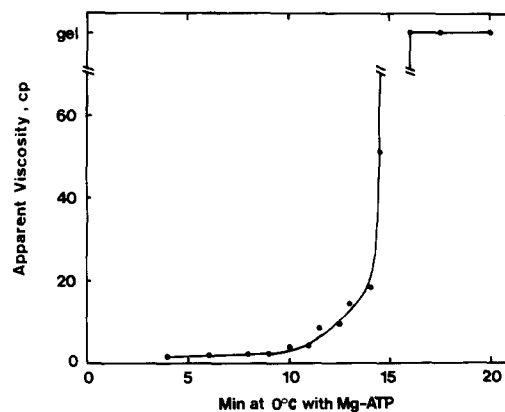


FIGURE 5 Potentiation of gelation of desalted extract at 25°C by preincubation with Mg-ATP at 0°C. We incubated desalted extract containing 7.6 mg/ml protein in 20 mM imidazole (pH 7), 2 mM EGTA, 2 mM MgCl₂, and 1 mM ATP at 0°C for various lengths of time before warming it to 25°C for 1 min, when we measured the viscosity.

tiated desalted extract. Thus, extracts that were potentiated and then desalted retained the effects of the potentiation reaction, even though $MgCl_2$ and ATP were required for gelation. Over a period of 2 h after the second gel filtration, the original potentiation effects were lost and rapid gelation occurred only if extracts were preincubated a second time with $MgCl_2$ and ATP for 15–20 min before warming.

During the time required for the potentiation reaction, gamma phosphate from ATP was incorporated into many of the proteins of the desalted extract. The rate of phosphorylation at $0^\circ C$ was about $0.07 \text{ nmol } ^{32}P/\text{mg protein per min}$ for ~ 7 min and about $0.02 \text{ nmol } ^{32}P/\text{mg protein per min}$ thereafter. This protein phosphorylation required $MgCl_2$, but was not affected by the presence or absence of micromolar concentrations of Ca^{++} . Autoradiographs of extract polypeptides separated by gel electrophoresis in SDS showed that many polypeptides (excluding actin) were phosphorylated during the potentiation reaction (Fig. 6). The first and most prominent phosphorylated polypeptide had the same electrophoretic mobility as the myosin-II heavy chain. In the low molecular weight region of the gel, the most highly phosphorylated polypeptides had molecular weights of 37,000, 33,000, 27,000, and 23,000.

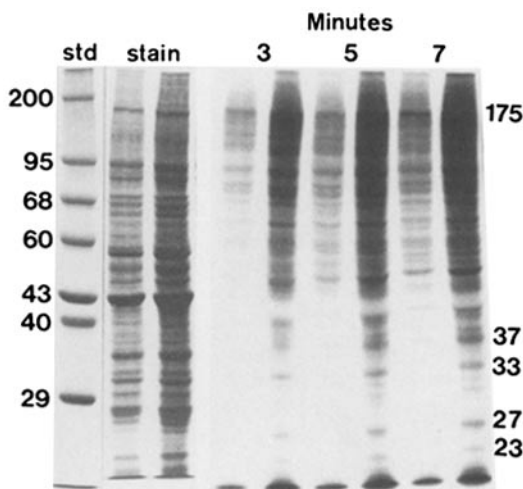


FIGURE 6 Phosphorylation of *Acanthamoeba* proteins during incubation of desalted extract with $MgCl_2$ and ^{32}P -ATP at $0^\circ C$. Polyacrylamide gel electrophoresis was used to separate the proteins. From the left: standards with molecular weights given in thousands; two stained gels with light and heavy loadings; and autoradiograms of pairs of gels with samples incubated with ^{32}P -ATP for 3 min, 5 min, and 7 min.

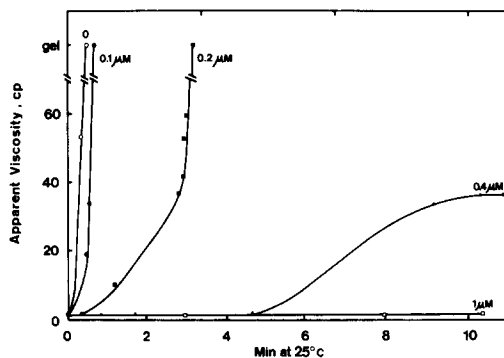


FIGURE 7 Inhibition of gelation by cytochalasin B. We preincubated desalted *Acanthamoeba* extract containing 9.2 mg/ml protein in 0.34 M sucrose, 10 mM imidazole ($\text{pH } 7$), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 0.5 mM DTT, and various concentrations of cytochalasin B (with $\leq 0.1\%$ DMSO) for 3 min at $0^\circ C$ before warming it to $25^\circ C$ and measuring the time-course of viscosity changes. Concentrations of cytochalasin B: $0 \pm 0.1\%$ DMSO (\circ), $0.1 \mu\text{M}$ (\bullet), $0.2 \mu\text{M}$ (\blacksquare), $0.4 \mu\text{M}$ (\blacktriangle), $1 \mu\text{M}$ (\square).

CYTOCHALASIN B INHIBITION: Micromolar concentrations of cytochalasin B (<1 cytochalasin B/30 actins) completely inhibited gelation, whereas submicromolar cytochalasin B reduced both the rate and extent of the reaction in a concentration-dependent fashion (Fig. 7). At low shear in the falling ball viscometer, the viscosity of $10 \mu\text{M}$ polymerized muscle actin was also decreased $\sim 90\%$ by $1 \mu\text{M}$ cytochalasin B. A comparable decrease in viscosity of muscle actin by cytochalasin B was not detectable by Ostwald viscometry. On the basis of these observations, we believe that cytochalasin B influences actin filament-filament interactions but not the extent of actin polymerization.¹

ACTIN POLYMERIZATION: We used a pelleting assay to estimate the amount of actin polymerized during various treatments of the extract (Table II). Both above and below the critical extract concentration for gelation (Fig. 2), $\sim 30\%$ (range in four experiments, 25–45%) of the extract actin pelleted (Table II). At the critical concentration for gelation, there was about 0.2 mg/ml polymerized and 0.5 mg/ml unpolymerized actin. When depolymerized muscle actin was added to cold extract, the amount of actin that pelleted after warming to $25^\circ C$ equaled the sum of the exogenous muscle actin and the expected endogenous actin polymer. Thus, all added pure actin seemed capable of polymerization in the extract. When gelation was reversed by cooling, the amount of

TABLE II
Actin Polymerization in the Extract

	Total actin	Actin pelleted	Total actin pelleted	Gel
	mg	mg	%	
A. Extract, mg/ml				
1. 8.5	1.2	0.37	31	+
6.6	0.9	0.25	28	+
4.7	0.7	0.22	32	-
2.8	0.4	0.13	33	-
2. 8.5 + 0.2 mg muscle actin	1.4	0.53	38	+
2.8 + 0.2 mg muscle actin	0.6	0.35	58	-
B. Gel reversed by cold				
	1.2	0.38	32	-
C. MgCl ₂ , mM				
0	1.2	0.24	20	-
0.2	1.2	0.34	28	-
2.0	1.2	0.37	31	+
D. ATP, mM				
0	1.2	0.38	32	-
0.1	1.2	0.34	28	-
1.0	1.2	0.37	31	+
E. Ca ⁺⁺ , mM				
0	1.2	0.37	31	+
10 ⁻⁹	1.2	0.34	28	+
10 ⁻⁸	1.2	0.35	29	+
10 ⁻⁷	1.2	0.28	23	+
10 ⁻⁶	1.2	0.18	15	-

Pelleting assay described under Materials and Methods. Conditions: (A1) 1-ml Samples with various concentrations of extract were warmed at 25°C for 15 min in 0.34 M sucrose, 30 mM imidazole (pH 7.0), 2 mM MgCl₂, 1 mM ATP, 1 mM EGTA, and 0.5 mM DTT, and then centrifuged at 25°C. (A2) 0.2 mg/ml depolymerized chicken skeletal muscle actin was added to two different concentrations of extract in 1 ml total volume and treated as in A1. (B) Gel reversed by cold. A sample of 8.5 mg/ml was warmed to 25°C to gel as in A1, liquefied by incubation at 0°C for 30 min, and then centrifuged at 3°C. (C) Mg concentration. 8.5 mg/ml samples of extract were treated as in A1, except that the MgCl₂ concentration was varied from 0-2 mM. (D) ATP concentration. 8.5 mg/ml samples of extract were treated as in A1, except that the ATP concentration was varied from 0-1 mM. (E) Free calcium ion concentration. 8.5 mg/ml samples of extract were treated as in A1, except that CaCl₂ was added to yield a free Ca⁺⁺ concentration ranging from 10⁻⁹ to 10⁻⁶ M.

pelletable actin did not change (Table II). Actin polymerization does not seem to be the limiting factor in the Mg⁺⁺ and ATP requirement for gelation, because actin pelleting was normal even

at concentrations of Mg⁺⁺ (0.2 mM) or ATP (0.1 mM) that failed to support gelation (Table II). Compared with 10⁻⁹ M Ca⁺⁺, only half as much actin pelleted at 10⁻⁶ M Ca⁺⁺ (Table II).

GELATION OF PURIFIED MUSCLE ACTIN BY EXTRACT: Small amounts of crude extract from *Acanthamoeba* caused pure actin to form a gel (15). We confirmed this observation, using the viscometric assay: a mixture of actin monomers (≥ 0.4 mg/ml) and extract (≥ 0.1 mg/ml) in 0.34 M sucrose, 10 mM imidazole (pH 7), 2 mM MgCl₂, 1 mM ATP, 1 mM EGTA, and 0.5 mM DTT formed a gel upon warming. Under these conditions, the actin polymerized in the presence of the extract. When actin filaments were used in place of actin monomers, gelation occurred, but only if mixing were gentle. For example, upon warming, a gel formed at the interface of a solution of actin filaments in 1 mM ATP and 2 mM MgCl₂ layered over low concentrations of desalted extract.

Most of the conditions that influenced the time-course of gelation in the crude and desalted extracts also influenced gelation in the mixtures of muscle actin and small amounts of desalted extract. Gelation was completely inhibited in the absence of MgCl₂ or in the presence of micromolar concentrations of free calcium ion or cytochalasin B. Gelation occurred slowly or not at all without added ATP.

Two properties of the extract were not reconstituted when small amounts of desalted extract were mixed with muscle actin. First, the rate of gelation of the reconstituted system was not potentiated by preincubation with 1 mM ATP and 2 mM MgCl₂. Second, the gel formed in the reconstituted system at 25° was not liquefied at 0°C.

FRACTIONATION OF GELATION ACTIVITY: As demonstrated by Maruta and Korn (15), the gelation activity of *Acanthamoeba* extracts can be separated into a number of fractions, each of which causes solutions of pure skeletal muscle actin to form a gel. They purified four different proteins that accounted for the bulk of the gelation activity in their extracts. Using a procedure similar to that described briefly by Maruta and Korn (15), we separated the gelation activity of the extract into a number of distinct fractions, including two of high purity.

The cold extract was first fractionated on a large DEAE-cellulose (DEAE) column (Fig. 8), which separated three peaks of gelation factor activity (designated A, B, and C) from the two classes of

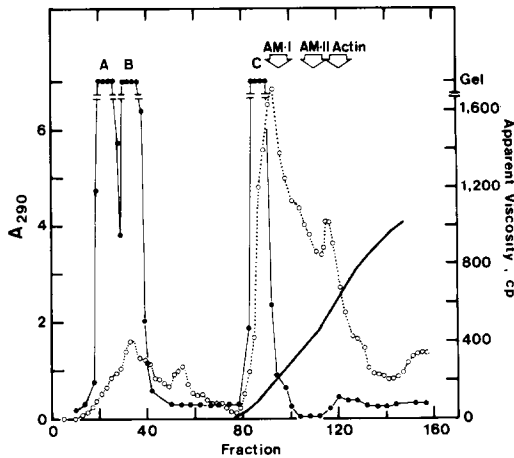


FIGURE 8 Fractionation of *Acanthamoeba* extract on DEAE as previously described (21). 500 ml of extract was applied to a 4×24 -cm column and eluted with a 1,500 ml 0–0.5 M KCl gradient (solid line). Fraction volume, 17 ml. The gelation factor activity was measured as the low-shear viscosity of 0.5 mg/ml muscle actin mixed with 0.01 part of column fraction. Arrows indicate the elution positions of myosin-I (AM-I), myosin-II (AM-II), and actin. \circ , A_{290} ; \bullet , gelation activity in centipoise measured with 0.5 mg/ml actin and 0.01 part of column fraction.

myosin and from actin.² Although each of these three fractions can gel actin, none of these partially purified fractions had the millimolar Mg-ATP requirement or the Ca^{++} and cold sensitivity of the crude extract. Each of the three peaks of gelation activity was fractionated by ammonium sulfate precipitation and gel permeation chromatography, revealing that there were at least six distinct fractions of gelation activity.

We purified two of these gelation factors from DEAE peak A by use of gel permeation chromatography (Fig. 9) and hydroxylapatite chromatography (Fig. 10). During the purification, we assessed the specific activity by determining the dependence of the viscosity of actin upon the concentration of gelation factor (Fig. 11). The viscosity was not directly proportional to gelation factor concentration; rather, the curves were hyperbolic. Low concentrations of gelation factor had little effect on the viscosity, but over a narrow concentration range there was an abrupt transition

² Assays of the DEAE column for gelation activity also revealed that fractions 105–115 decrease the low-shear viscosity of actin filaments. Similar activity is found in muscle actin preparations. See footnote 1.

in the viscosity, as noted previously for other actin-cross-linking proteins (2). Consequently, the specific activity of a fraction must be estimated by the concentration required to yield a viscosity, e.g., 1,000 cp, just above the transition point. By this criterion, the specific activity of DEAE peak A was about four times higher than that of the crude extract, but this activity declined by ~50% during the concentration of the gelation factor activity by

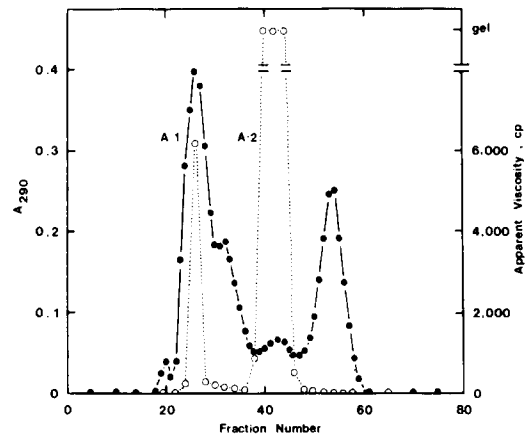


FIGURE 9 Fractionation of ammonium sulfate-concentrated DEAE peak A on a 2.5×52 -cm column of Sephadex G-150. Fraction volume, 4.0 ml. \bullet , A_{290} . Gelation factor activity (\circ) measured with 0.5 mg/ml actin and 0.1 part of column fraction.

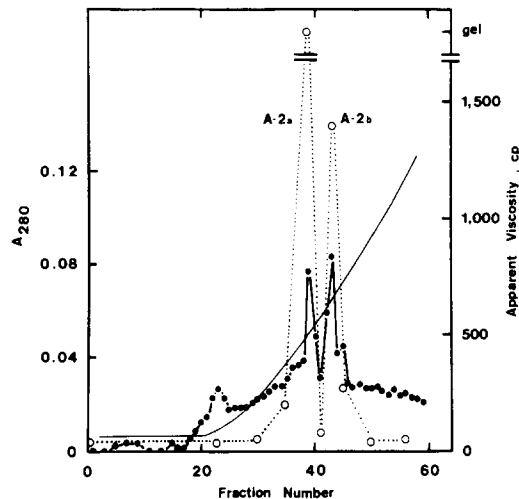


FIGURE 10 Fraction of Sephadex G-150 peak A-2 on a 1.5×9 -cm hydroxylapatite column as described in Materials and Methods. Fraction volume, 4.7 ml. \bullet , A_{280} . Gelation factor activity (\circ) measured with 0.5 mg/ml actin and 0.1 part of column fraction.

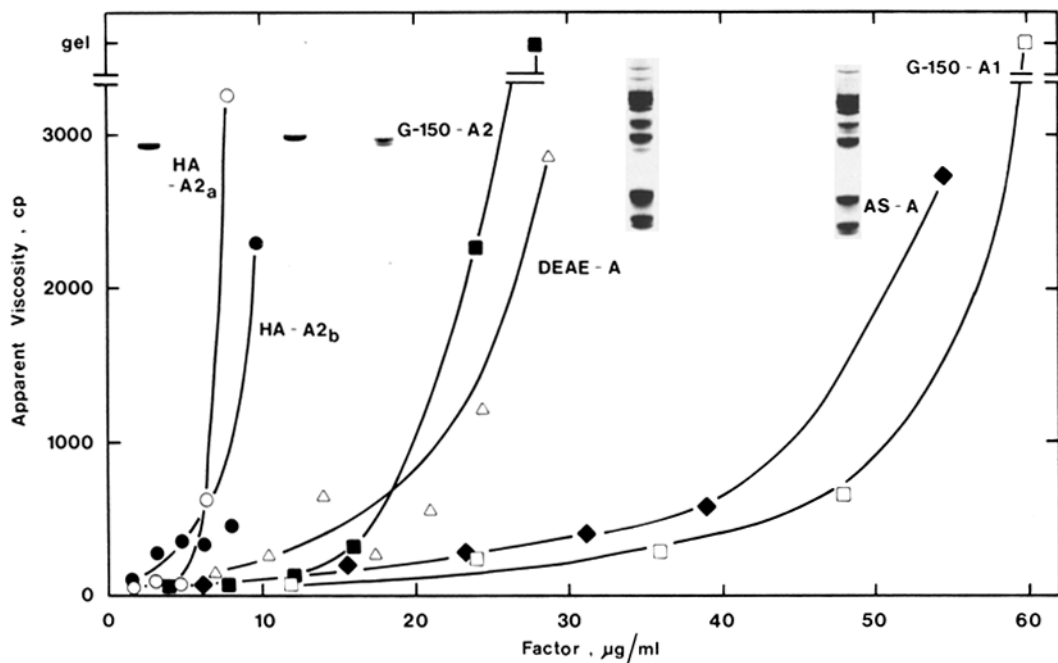


FIGURE 11 Dependence of actin low-shear viscosity on the concentration of gelation factor fractions. Actin concentration: 0.5 mg/ml in 2 mM MgCl₂, 1 mM ATP, 1 mM EGTA, and 10 mM imidazole (pH 7). Hydroxylapatite fraction A-2_a (○), hydroxylapatite fraction A-2_b (●), Sephadex G-150 fraction A-2 (■), DEAE fraction A (△), ammonium sulfate fraction A (◆), Sephadex G-150 fraction A-1 (□).

ammonium sulfate precipitation. Chromatography on Sephadex G-150 (Fig. 9) separated the activity into two peaks (*A-1* and *A-2*). Peak *A-2* had a much higher specific activity than the column sample or peak *A-1* and was composed of only two major polypeptides (Fig. 11). Both of these polypeptides had gelation activity after separation on hydroxylapatite (Fig. 10) into purified fractions *A-2_a* and *A-2_b*. The two purified gelation factors had molecular weights of 33,000 and 37,000, respectively, so they may be the gelation factors III and IV isolated by Maruta and Korn (15). However, their gelation factor IV bound to DEAE, whereas *A-2_b* did not. It is important to note that although our procedure resulted in substantial purification of these two polypeptides, the increase in specific activity was only ~12-fold compared with the crude extract and 3-fold compared with the DEAE fraction *A*. Moreover, <10% of the total activity in the DEAE-*A* fraction was recovered in the *A-2_a* and *A-2_b* fractions.

Neither of these purified gelation factors can account for the behavior of the extract. Although each can cross-link actin to form a gel, in neither case was gelation dependent on ATP or inhibited

by micromolar Ca⁺⁺, as was the crude extract or the mixture of pure actin with a small amount of crude extract.

CONTRACTION: Under our standard conditions, extract remained gelled for ~45 min, and thereafter the gel pulled away from the sides of the container to form a contractile thread. The ability of the thread to do work was demonstrated by its movement of the steel ball a distance of several centimeters overnight.

DISCUSSION

We divide the following discussion into two parts. Because we have introduced a new method for measuring gelation, we first evaluate the falling ball method and compare it with other assays. Second, we comment on the new features of the gelation reaction revealed by our experiments.

Gelation Assays

The actin-containing gels from *Acanthamoeba* and other cells are complex materials, possessing the properties of both liquids (above their yield points) and solids (below their yield points). Moreover, microscopic observations (5, 26) suggest that

they are elastic as well. It is also likely that their physical properties change with time under stress. Eventually it will be desirable to make a thorough analysis of the physical properties of these gels, including a complete stress/strain curve vs. time for the viscous component and a restoring force vs. displacement curve for the elastic component. No simple assay can yield all of this information, but of the approaches one might take in a kinetic study, the falling ball method has distinct advantages.

The falling ball assay is simple, inexpensive, and highly reproducible. The sample size is small. The sample is stationary before interaction with the ball. The viscosity range is wide. Kinetic data are obtained with ease. It is possible to keep the maximum shear rate for Newtonian fluids below 10 s^{-1} for samples with viscosities $>6 \text{ cp}$ by choosing an appropriate angle of inclination. For comparison, Ostwald viscometers typically have shear rates $>100 \text{ s}^{-1}$.

Although the falling ball method has many advantages, it has three main limitations in studies of gelation. First, only two structural parameters (apparent viscosity and yield strength) can be measured. Second, for a given sample the shear rate can only be varied by a factor of ~ 12 when using gravitational acceleration and angles of inclination between 10° and 80° . Finally, even at the relatively low shear rates in the falling ball device, the stress applied to these delicate samples by the falling ball is destructive.

The ball centrifugation assay is a simple approach to measuring yield strength. Its major limitations at the present time are some uncertainty about the value of the geometrical constant in Eq. 3 (see Appendix) and the crude, stepwise application of force by centrifugation. A better method would be to observe the ball continuously as the centrifugal force is gradually increased.

A number of other assays have been used to study gelation of cytoplasmic extracts and systems reconstituted from purified actin and various cross-linking proteins. In most cases, the assay has been the inversion of test tubes to demonstrate sample consistency (3, 5, 12, 15, 18, 19, 25, 30). This assay is both subjective and impossible to quantitate. Other inadequate assays include capillary viscometry, strain birefringence, pelleting, and turbidity. Capillary viscometry has been used to show that the viscosity increases during gelation, but the apparent viscosities obtained are unreliable because the gel is fragmented during flow through

the capillary (20). Moreover, stationary samples allowed to gel in such viscometers will not flow because their viscosity exceeds the range of the viscometer, an observation no more quantitative than test-tube inversion. Strain birefringence measurements (5) could be made quantitative if the stress were applied quantitatively, but this has not been done. Because cross-linked actin filaments will sediment at low centrifugal forces, it has been possible to use a pelleting assay for gelation (2, 13). This assay may be misleading under some conditions because aggregated proteins also pellet. Turbidity changes have been used to follow the progress of the gelation reaction (5, 18, 19, 25). Although the method is ideal for kinetic analysis, turbidity changes resulting from other reactions, including precipitation, complicate this approach.

Perhaps the only satisfactory assay for gelation previously described is a measurement of yield point (2). This assay is, however, cumbersome for kinetic analysis.

Physical Properties of the Gelled Extract

The most important physical properties of the gelled extract are its rigidity, reflected in its high yield strength of $\sim 10^3 \text{ dyn/cm}^2$, and its sensitivity to mechanical shearing. Both rigidity and thixotropy are also properties of cytoplasm. These features of the gelled extract can be accounted for by a continuously cross-linked network of actin filaments. Such a model has been inferred previously from the gooey nature of the gels, from electron micrographs of fixed gels (5, 18, 19, 20, 27), and from the requirement for both actin and actin-binding gelation factors to form a gel (2, 3, 12, 15, 20, 25, 30). The high yield strength of the gel, as compared to that of the same concentration of actin filaments, proves that the filaments are physically cross-linked to one another.

The measured rigidity and high viscosity of these samples are properties of the bulk material. For particles as small as organelles, the viscosity must also be very high, judging from the absence of Brownian movement (18). On the other hand, it seems highly likely that the microscopic viscosity, on the level of solute molecules, is much lower, because small molecules should be free to diffuse throughout the interstices of the actin filament network.

As in other cross-linked fibrous gels and in cytoplasm, mechanical shearing disrupts the structure of the gelled extract. At the high shear rates

found in Ostwald viscometry or mechanical mixing, the gel is fragmented into a precipitate that never recovers the original viscosity. The level of shearing in our falling ball viscometer also disrupts the gel, but given time, the latter recovers, demonstrating its thixotropic nature.

Brotschi et al. (2) used a "gelmeter" to measure the yield strength of gels composed of actin and macrophage actin-binding protein and found values of <10 dyn/cm². We cannot account for why our measurements of the yield strength of the *Acanthamoeba* extract gel are $100 \times$ higher, but we believe the difference must be related to methods used to measure rigidity rather than to major differences in the properties of the gels.

The Gelation Process

KINETICS: The gelation reaction occurs in two steps: a lag period of duration approximately proportional to concentration⁻², followed by a rapid rise in viscosity. Maximum rates of change exceed 100 cp/s. The rate of this rapid phase of the gelation reaction seems to be sufficiently fast to account for the rapid changes in consistency that occur in the cytoplasm of living cells (1, 27).

SOLUTION CONDITIONS: The optimal conditions for gelation are likely to be close to those found inside the *Acanthamoeba*: millimolar Mg-ATP, low ionic strength, neutral pH, and submicromolar free Ca⁺⁺. Similar conditions favor gelation in extracts of other cells such as *Dictyostelium* (5), alveolar macrophages (25, 32) and Ehrlich ascites tumor cells (13, 17).

Micromolar concentrations of calcium ion completely inhibit the gelation reaction and partially inhibit the polymerization of actin in the extract. Micromolar Ca⁺⁺ also inhibits the gelation of mixtures of muscle actin and small amounts of extract. In contrast, micromolar calcium does not inhibit the gelation of exogenous actin mixed with any of the four gelation factors purified by Maruta and Korn (15) or any of the gelation factors we have identified. Our interpretation of these observations is that the extract contains one or more calcium-regulatory factors that are separated from the gelation factors by DEAE chromatography. The identification and characterization of the factors mediating Ca⁺⁺ control will be most interesting, because they may regulate the consistency of the cytoplasm within the cell. Such factors could regulate gelation either by controlling actin polymerization (as suggested by the experiment in Table

II) or by affecting the cross-linking of actin filaments by gelation factors. There is evidence for such a calcium-sensitive regulatory factor in macrophages (32).

Several observations indicate that Mg-ATP participates in the gelation reaction, above and beyond its role in actin polymerization. The Mg-ATP concentration required for gelation is considerably higher than that needed for actin polymerization. Both Mg⁺⁺ and ATP are required for the cold potentiation of gelation. The inability of nonhydrolyzable analogues of ATP to substitute for ATP suggests that ATP hydrolysis is necessary for gelation. One possibility is phosphorylation of one or more proteins involved in gelation, because many *Acanthamoeba* proteins, including four with approximately the same molecular weights as the Maruta and Korn gelation factors, are phosphorylated during the potentiation reaction.

Whatever the mechanism of this potentiation reaction, it is likely to occur both during the gelation reaction at 25° and before gelation at 0°C. If potentiation is necessary to activate one or more of the components of the gelation system, these gelation factors may be missed during fractionation of the extract, if they become separated from their activators.

Actin Polymerization in the Extract

Actin filaments are required for gelation, as we expected from the cross-linked filament model for the gel. Thus, actin filaments must form from depolymerized actin in the extract before gelation. This step may account for the lag in the kinetic curves (Fig. 2). The actin filament concentration is probably the factor limiting gelation when the extract is diluted, because the extract contains a vast excess of gelation activity (15). In both reconstitution experiments with pure actin plus 0.2 mg/ml of extract protein and in extract-dilution experiments, 0.2–0.3 mg/ml of actin filaments is necessary for gelation. Consequently cellular regulation of actin polymerization may be an essential factor in determining the cytoplasmic consistency.

The observation that the fraction of extract actin sedimentable at 25°C is constant and independent of the total protein concentration suggests that some factor in the extract limits the polymerization of a fixed fraction of the actin. The experiment suggests that actin is present in $\sim 1.5 \times$ the concentration of this presumed inhibitor, because within the limits of our assay, one-third of the endogenous

actin, and any additional exogenous actin, pellet. This inhibitor is separated from the actin by chromatography on DEAE, because thereafter virtually all of the actin can polymerize (9). A low molecular weight *Acanthamoeba* protein (23) similar to mammalian profilin (4) may be responsible for the inhibition of actin polymerization that was observed in the extract.

Reconstitution

Maruta and Korn (15) isolated four low molecular weight proteins from *Acanthamoeba*, each of which caused pure actin to form a gel. Together they seemed to account for most of the gelation factor activity of the extract. In our efforts to fractionate the gelation factor activity, we have separated at least six different low molecular weight components, none of which can account for the activity of the extract.

Further efforts to reconstitute the gelation reaction from purified components will require the final purification of all the actin-cross-linking proteins as well as any associated regulatory proteins. This study suggests that both a calcium-binding regulator of cross-linking and a Mg^{++} - and ATP-requiring activating enzyme should be sought.

APPENDIX

In this appendix we describe the theory, design, and testing of a small falling ball device that can be used to measure both yield strength and viscosity. From the force balance in the system, we derive an expression relating the yield strength of any sample to the maximum force per unit mass that can be applied to the system without the ball moving through the sample. The same force balance and the Stokes' equation yield the well-known (29) expression relating the velocity of a ball falling through a Newtonian sample to the viscosity of the sample. We use the velocity of the ball in standard Newtonian fluids to determine the empirical calibration constants for the viscometer, which we then use to measure the apparent viscosities of non-Newtonian fluids. Finally, we discuss the caution necessary in using this viscometer to estimate the apparent viscosity of non-Newtonian fluids such as the cross-linked actin gels studied in this paper.

Theory

The force balance for a ball immersed in a medium is:

$$F_b = F_y + F_m + F_f, \quad (1)$$

where the force acting on the medium (the ball force, F_b) is balanced by the forces acting on the ball (the static yield force, F_y , the buoyant force, F_m , and the dynamic frictional force, F_f). Eq. 1 is valid when there is no acceleration in the system, i.e., when the ball is either stationary or moving at a constant velocity.

$$F_b = 4/3 \cdot \Pi \cdot R^3 \cdot \rho_b \cdot \alpha, \quad (2)$$

where R is the radius of the ball, ρ_b is the density of the ball, and α is the force per unit mass applied to the ball.

$$F_y = T_o \cdot \Pi \cdot R^2 \cdot C, \quad (3)$$

where the static yield strength, T_o , is the maximum force per unit area that the medium will support without displacement and C is a geometrical constant to correct for the nonhomogeneous distribution of shear stress around the ball. For a sphere, C has been determined empirically to be about 1.75 (11).

$$F_m = 4/3 \cdot \Pi \cdot R^3 \cdot \rho_m \cdot \alpha, \quad (4)$$

where ρ_m is the density of the medium and α is the force per unit mass applied to the medium.

$$F_f = f \cdot u, \quad (5)$$

where f is the dynamic frictional coefficient and u is the velocity of the ball.

YIELD STRENGTH: In the case where the medium is strong enough to support the ball, $F_f = 0$ and $F_b = F_y + F_m$. Under these conditions, it is possible to estimate the static yield strength, T_o , of the medium by determining α_o , the maximum force per unit mass that can be applied to the system without movement of the ball. As described in a latter section, this is accomplished by centrifuging a capillary containing a ball immersed in the test medium. This applies to the system a force per unit mass of $\omega^2 r$, where ω is angular velocity in radians per second and r is the radius. Solving for T_o ,

$$T_o = \frac{4/3 \cdot \Pi \cdot R^3 \cdot \alpha_o (\rho_b - \rho_m)}{1.75 \cdot \Pi \cdot R^2} = 0.76 \cdot R \cdot \alpha_o (\rho_b - \rho_m). \quad (6)$$

VISCOSITY: If the medium is not strong enough to support the ball, the ball will accelerate

until it reaches a constant "terminal" velocity. At this point the forces are balanced and the absolute viscosity of a Newtonian fluid can be determined from the terminal velocity using Stokes' equation,

$$\eta = \frac{f}{6 \cdot \Pi \cdot R} \quad (7)$$

If F_y is negligible compared with the other forces in the system (i.e., $F_b - F_m - F_f$ is small), then $F_f = F_b - F_m$, and

$$f = \frac{m \cdot 4/3 \cdot \Pi \cdot R^3 \cdot \alpha(\rho_b - \rho_m)}{u} \quad (8)$$

The term m is a calibration constant required when the ball and medium are confined to a narrow tube and inclined at angle θ . It corrects for drag on the ball resulting from wall effects (29) and friction at the point of contact between the ball and the wall (8). The value of m is determined empirically below and is a function of the radius of the ball, the radius of the tube, and the angle of inclination. The force per unit mass (α) along the axis of a tube inclined at angle θ from the horizontal is $g \cdot \sin \theta$. Solving Eq. 7 and Eq. 8 for η ,

$$\eta = \frac{0.22 \cdot m \cdot R^2 \cdot g \cdot \sin \theta (\rho_b - \rho_m)}{u} \quad (9)$$

Design of the Falling Ball Apparatus

A capillary tube, 1.3 mm i.d., 12.6-cm long (100- μ l micropipet from VWR Scientific Inc., Univar Corp., San Francisco, Calif.), is filled with about 170 μ l of sample using a Clay Adams Pipet Filler and sealed at one end with Seal Ease (Clay Adams, Div. Becton, Dickinson & Co., Parsippany, N. J.). A Plexiglas stand is used to hold the micropipet at 10°, 50°, or 80° of inclination from the horizontal plane in a temperature-controlled water bath. A stainless-steel ball (0.64-mm diam., density 7.2 g/cm³, grade 10, gauge deviation \pm 0.000064 mm, material 440C from the Microball Company, Peterborough, N. H.) is placed on the meniscus of the sample by hand and pushed through the meniscus with a thin metal wire to initiate its fall. The velocity of the ball is measured by recording with a printing timer (Chronomix All Sport Timer, Chronomix Corp., Sunnyvale, Calif.) the time required for the ball to pass 2-cm intervals beginning about 1 cm below the meniscus.

If the sample has a yield strength >146 dyn/cm², the ball will fail to move at an angle of 80°

under the influence of gravity alone. A measurement of yield strength can be made by increasing the ball force. This is accomplished by placing the ball about 0.5 cm below the meniscus and centrifuging in a stepwise fashion for 30 s at progressively higher speeds. For samples with $T_o < 146$ dyn/cm², a rough estimate of yield strength can be made by placing the ball in a horizontal tube and slowly increasing the angle of inclination until the ball moves. In this case, $\alpha_o = g \cdot \sin \theta$.

Testing the Falling Ball Apparatus

The viscometer was tested with glycerol/water mixtures, which are Newtonian solutions of known viscosity. The ball has a constant velocity throughout the measuring section of the capillary for any given sample over the entire viscosity range tested (1–12,000 cp). At any angle between 10° and 90°, u^{-1} is proportional to viscosity up to the point where the ball will not move (Fig. 12). The measurements are highly reproducible with a standard deviation from the mean velocity of <2% over the entire range. The variation is larger at high viscosity than at low viscosity. Tilting the capillary at various angles θ allows one to vary the useful range of the apparatus. Most of our measurements are made at 50° or 80° because the samples have high viscosities. An angle of 10° is more convenient for low-viscosity samples.

We determined the calibration constant m for the 0.32-mm radius stainless-steel ball in tubes of

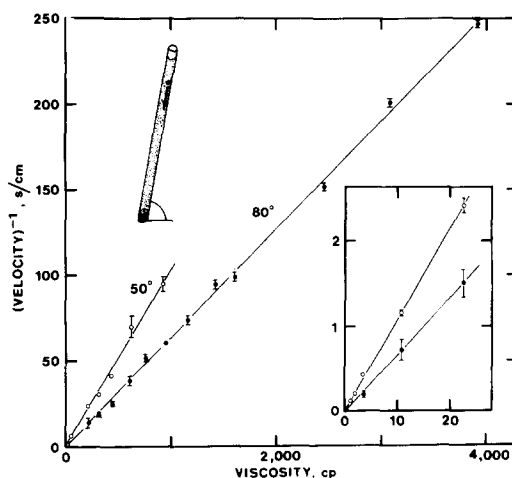


FIGURE 12 Calibration of the inclined falling ball viscometer with glycerol solutions of known viscosity. Angle of inclination 50° (○), 80° (●). Vertical bars represent \pm one standard deviation.

various radii held at several angles. In tubes with radii $R_c > 16$ mm, $m = 1$. For R_c between 2.7 and 16 mm, m can be calculated from the empirical equation of Faxén (6) quoted in reference 29. For $R_c < 2.7$ mm, m must be determined empirically and is a function of both R_c and θ . For the capillaries we use with R_c of 0.65 mm, m is 0.13 at 80° , 0.09 at 50° , and 0.06 at 10° . These values do not vary with the viscosity of the sample (Fig. 12). The linearity of this relationship justifies, for these Newtonian fluids, our omission of the F_y term from the viscosity equation. (This result is expected from the magnitude of the forces in the system. At 80° , $F_b - F_m = 0.82$ dyn. For $F_y = 1\%$ of this force, $T_o = 1.46$ dyn/cm². The standards used for calibration have yield forces even less than this.) These tests establish that the viscometer can be used to measure the absolute viscosity of Newtonian solutions in the range of 1–12,000 cp with an error of <2%.

The use of the apparatus for yield strength measurement was tested with solidified gelatin. The precision of this measurement is limited by the crude method of applying centrifuged force in graded steps, but we obtained values close to those in the literature: 2×10^4 dyn/cm² for 4% gelatin and 1×10^4 dyn/cm² for 3% gelatin (7). This confirms that the empirical geometrical factor (1.75 in Eq. 3) is approximately correct for our apparatus. Using gelatin, we measured the same value for the yield strength in tubes with radii from 0.65 to 14 mm, demonstrating that there was no detectable wall effect on yield strength measurement.

Application of the Falling Ball Viscometer to Non-Newtonian Fluids

In a falling ball viscometer with acceleration provided by gravity, the velocity of the ball, and hence the maximum shear rate of a Newtonian fluid, $d = 1.5 R/u$ (29), varies inversely with viscosity. This is of no consequence with a Newtonian fluid in which viscosity is independent of shear rate.

In contrast, the absolute viscosity of a non-Newtonian fluid varies with the shear rate, so that the observed ball velocity is used to calculate an "apparent viscosity" from Eq. 5. These apparent viscosities are expressed in centipoise for comparison with the absolute viscosities of Newtonian fluids through which the ball falls at the same velocity, with the full understanding that these apparent

viscosities are not absolute, but depend on the shear rate around the ball. Absolute viscosities could be calculated directly from these apparent viscosities if the shear rate around the ball could be determined exactly and the dependence of the absolute viscosity upon the shear rate were established. However, to our knowledge, there is no theoretical treatment of shear rates around balls falling through non-Newtonian fluids, nor have we or others established the dependence of the absolute viscosity of our samples on the shear rate.

A second note of caution is that the shear dependence of the viscosity of non-Newtonian samples such as actin filaments (16) will amplify differences in the apparent viscosity. Take, for example, the case of actin filaments and other materials in which viscosity is inversely proportional to shear rate. If sample A has a higher viscosity than sample B when the two are measured at the same shear rate, the velocity of the ball falling under the influence of gravity will be less in A than in B. Thus the shear rate in A will be less than in B. As a result of the lower shear rate in A, the observed difference in the viscosity of A and B is greater than the difference measured at constant shear rates. In contrast, with rheopexic materials the apparent difference will be less than the actual difference.

A final point is that the static yield strength of a sample will contribute to the apparent viscosity, if the magnitude of the yield force approaches that of the other forces in the system. In our device at 80° inclination, F_y would have to be >0.02 dyn to have a detectable ($>2\%$) effect on the observed viscosity. F_y of 0.02 dyn corresponds to T_o of 3 dyn/cm², which is greater than the T_o of low concentrations of actin filaments (2), so we expect that F_y has little or no effect on the apparent viscosity of actin filament solutions. Even if the yield strength of a sample were 70 dyn/cm² ($F_y = 0.41$ dyn), it would increase the viscosity by only a factor of 2. On the other hand, when $F_y = F_b - F_m$, $u = 0$ and the observed viscosity is, of course, infinite. These are maximum effects of yield strength on observed viscosity, because it is possible that the dynamic yield strength is much less than the static yield strength, in which case the F_y term is less consequential than stated here.

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