

# THE CONTRACTILE BASIS OF AMEBOID MOVEMENT

## VI. The Solation-Contraction Coupling Hypothesis

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### ABSTRACT

The contracted pellets derived from a high-speed supernate of *Dictyostelium discoideum* (S3) were investigated to determine the functional activity associated with this specific subset of the cellular motile apparatus. A partially purified model system of gelation and contraction (S6) was prepared from the contracted pellets, and the presence of calcium- and pH-sensitive gelation and contraction in this model demonstrated that a functional cytoskeletal-contractile complex remained at least partially associated with the actin and myosin during contraction. Semi-quantitative assays of gelation and solation in the myosin-free preparation S6 included measurements of turbidity, relative viscosity, and strain birefringence. The extent of gelation was optimal at pH 6.8 and a free calcium ion concentration of  $\sim 3.0 \times 10^{-8}$  M. Solation was favored when the free calcium ion concentration was  $> 7.6 \times 10^{-7}$  M or when the pH was increased or decreased from pH 6.8. Gelation was reversibly inhibited by increasing the free calcium ion concentration to  $\sim 4.6 \times 10^{-6}$  M at pH 6.8. The solation-gelation process of this model has been interpreted to involve the reversible cross-linking of actin filaments. The addition of purified *D. discoideum* myosin to S6 served to reconstitute calcium- and pH-regulated contraction. The results from this study indicate that contraction is coupled functionally to the local breakdown (solation) of the gel. Therefore, solation has been identified as a structural requirement for extensive shortening during contraction. We have called this concept the solation-contraction coupling hypothesis. Fractionation of a preparation derived from the contracted pellets yielded a fraction consisting of actin and a 95,000-dalton polypeptide that exhibited calcium-sensitive gelation at 28°C and a fraction composed of actin and 30,000- and 18,000-dalton polypeptides that demonstrated calcium-sensitive gelation at 0°C.

**KEY WORDS** ameba · gelation ·  
cytoskeleton · calcium · actin

The role of changes in cytoplasmic structure or consistency during cellular movements has been discussed for over 100 yr. Dujardin (13) and Heitzmann (16) were the first to suggest that the struc-

ture of cytoplasm was related directly to the contractility. This concept was recognized by Hyman (18), Pantin (33), Mast (28), and Allen (2) and was incorporated into their theories of ameboid movement (see DeBruyn [12], Allen [2], Pollard [35], and Taylor and Condeelis [43] for reviews).

The absolute structure of cytoplasm has been

debated vigorously during this same period, and the views on cytoplasmic structure have ranged from simple Newtonian fluids to complex thixotropic gels. A combination of structural assays in vivo, cytoplasmic model systems, and ultrastructural investigations have suggested that cytoplasm can exist in distinct cytoskeletal and contractile states varying in absolute structure (sol, gel, and contraction) (see Taylor and Condeelis [43] for a review).

It has been one of the goals of recent research on cytoplasmic structure and motility to reconstitute the cytoskeletal and contractile system from various types of cells with purified proteins. To date, several distinct factors ranging from high to low molecular weights, which duplicate part of the phenomenology observed in crude cell extracts (20, 27, 40, 51, 52) have been identified. However, as a prerequisite to the reconstitution of a physiologically relevant model of the cellular sol-gel-contraction process from purified components, it is necessary to define at least semiquantitatively the physiological requirements for solation, gelation, and contraction that have been identified in crude cellular extracts (9, 11, 25, 30, 36, 48, 45, 53) and to characterize the functional interrelationships between the solated, gelled, and contracted states. These results must then be compared with the results of similar investigations of living cells (42), simple, single-cell models (44), and crude cell extracts (11, 45, 48) to further ensure the physiological relevance of any reconstituted system. A partially purified model system prepared from the contractile complex of *Dictyostelium discoideum* has facilitated separate investigations on the sol-gel and sol-gel-contraction processes while serving as a starting point for purifying and characterizing each component of this functional complex.

The ionic conditions responsible for controlling the nonmotile cytoskeletal state and contraction in ameboid cells have also been characterized in single-cell models (44), crude cell extracts (11, 43, 45), and living cells (42). Potentially the most relevant ionic parameters that modulated both the solated and gelled (cytoskeletal) and the contracted states were the free calcium ion concentration and pH (11, 45, 48). The delicate balance between the gelled state and the contracted state in cell extracts ultimately led us to suggest a possible regulatory role for gelation in ameboid movement (11, 45, 46). In collaboration with John S. Condeelis (46, 49), we have recently described the dynamic interrelationship between the cytoskele-

tal and contractile events, calling it the solation-contraction coupling hypothesis.

The purpose of the present study is threefold: first, to characterize the functional activity in a subset of the contractile-cytoskeletal system; second, to investigate the relationship between cytoplasmic structure and contractility in a simple model; and, third, to begin the identification of the specific component(s) responsible for regulated gelation, solation, and contraction. The results are discussed in relation to our earlier studies in vitro and in vivo.

## MATERIALS AND METHODS

Materials were obtained as follows: EGTA, piperazine-N-N'-bis(2-ethane sulfonic acid) (PIPES), bovine serum albumin (BSA), dithiothreitol (DTT), myoglobin, cytochalasin B, dimethyl sulfoxide (DMSO), and phenyl methyl sulfonyl fluoride (PMSF) from Sigma Chemical Co., St. Louis, Mo.; ATP from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; Trasylol from FBA Pharmaceuticals, Inc., New York; DNase I and phosphorylase A from Worthington Biochemical Corp., Freehold, N. J.

Solutions used in the preparation of models and isolation of proteins were as follows: homogenization buffer (2.5 mM PIPES, 2.5 mM EGTA, 0.5 mM DTT, pH 7.0), KI buffer (2.5 mM PIPES, 2.5 mM EGTA, 0.6 M KI, 5.0 mM MgATP, 0.25 mM CaCl<sub>2</sub>, pH 6.75), depolymerization buffer (2.5 mM PIPES, 0.1 mM CaCl<sub>2</sub>, 0.2 mM ATP, 0.2 mM DTT, pH 7.0), high salt solution (2.5 mM PIPES, 0.6 M KCl, 1.0 mM MgATP, 0.1 mM CaCl<sub>2</sub>, pH 7.0), Tris-KI buffer (10.0 mM Tris-HCl, 1.0 mM EDTA, 0.1 mM DTT, 0.6 M KI, 5.0 mM MgATP, 1.0 mM CaCl<sub>2</sub>, pH 7.5), and buffer A (2.0 mM Tris-HCl, 1.0 mM ATP, 0.5 mM DTT, 0.1 mM CaCl<sub>2</sub>, pH 8.0). Free calcium ion concentrations in calcium-EGTA buffers were calculated using a dissociation constant of  $1.95 \times 10^6 \text{ M}^{-1}$  at pH 6.8. Ca<sup>++</sup>/EGTA equal to 0.05, 0.6, and 0.9 corresponded to free calcium ion concentrations of  $3.0 \times 10^{-8} \text{ M}$ ,  $7.6 \times 10^{-7} \text{ M}$ , and  $4.6 \times 10^{-6} \text{ M}$ , respectively.

### Preparation of Contracted Pellets

Amebas of *D. discoideum* (strain A3) were harvested from axenic culture while in log growth phase at a concentration of  $1 \times 10^7$  cells/ml. The cells were collected by centrifugation at 200 g for 5 min and were washed twice with Sorenson's phosphate buffer (11), pH 6.0. The cells were mixed 1:1 with homogenization buffer and homogenized using a Dounce homogenizer (Kontes Co., Vineland, N. J.) on ice. The high-speed supernate S3 was prepared from the homogenate as described previously (11). Contraction was elicited in the S3 model in the presence of 2.5 mM PIPES, 2.5 mM EGTA, 1.0 mM MgATP, 20 mM KCl, pH 7.0, by increasing the pH to 7.6 or by raising the free calcium ion concentration to  $4.6 \times 10^{-6} \text{ M}$  at pH 6.8 and warming for 10 min at 25°C.

### Preparation of the S6 Model from the Contracted Pellet

The contracted pellet was collected by centrifugation at 25,000 g for 10 min, gently rinsed with homogenization buffer, and

resuspended by homogenization in the KI buffer. After 15 min in the buffer to ensure complete actin depolymerization, the extract was clarified by centrifugation at 100,000 g for 30 min followed by dialysis for 15 h vs. depolymerization buffer. The myosin precipitated with a fraction of actin and other polypeptides during dialysis. The preparation was removed from dialysis and centrifuged at 100,000 g for 1 h, resulting in a myosin-free supernate S5 and a myosin-containing pellet P5 (Fig. 1).

S5 was made 0.6 M KCl, 1 mM MgATP, 0.1 mM CaCl<sub>2</sub>, pH 7.0, incubated at 25°C for 45 min and centrifuged at 100,000 g for 1.5 h to reduce the actin concentration, resulting in S6, a myosin-free supernate with an actin concentration ranging from 0.5 to 1.3 mg/ml and a pellet, P6, containing actin.

S6 was dialyzed vs. depolymerization buffer for 15 h and then tested for gelation under various conditions. The ability of S6 to regulate contraction was studied by the addition of purified *Dictyostelium* myosin to a final concentration ranging from 0.04 to 0.15 mg/ml.

The actin pellet P6 was resuspended in buffer A and dialyzed vs. buffer A for 24 h. The actin was further purified by a cycle of polymerization and depolymerization.

### Isolation of Myosin and Calcium-sensitive Gelation Factors

P5 was chromatographed on a 1.5 × 60-cm column packed with agarose (Bio-Rad A15, 100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) according to the method of Clarke and Spudich (7). The column buffer was 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT, 0.6 M KCl, 2 mM ATP. P5 was resuspended in a small volume of Tris-KI buffer, gently homogenized, and centrifuged for 30 min at 100,000 g. The clear supernate, typically 2 ml at 10 mg/ml, was applied to the column. The flow rate was 4 ml/h and 2 ml-fractions were collected.

The elution profile was as shown in Fig. 2. The fractions containing *Dictyostelium* myosin were pooled, and the myosin was collected and assayed as described by Clarke and Spudich (7). The specific Ca<sup>++</sup> ATPase activity of the myosin averaged 0.1 μmol Pi/min per mg myosin. Fractions containing a 95,000-dalton polypeptide with actin (fraction I) and fractions containing primarily actin with 30,000- and 18,000-dalton polypeptides (fraction II), as determined by SDS polyacrylamide slab gel

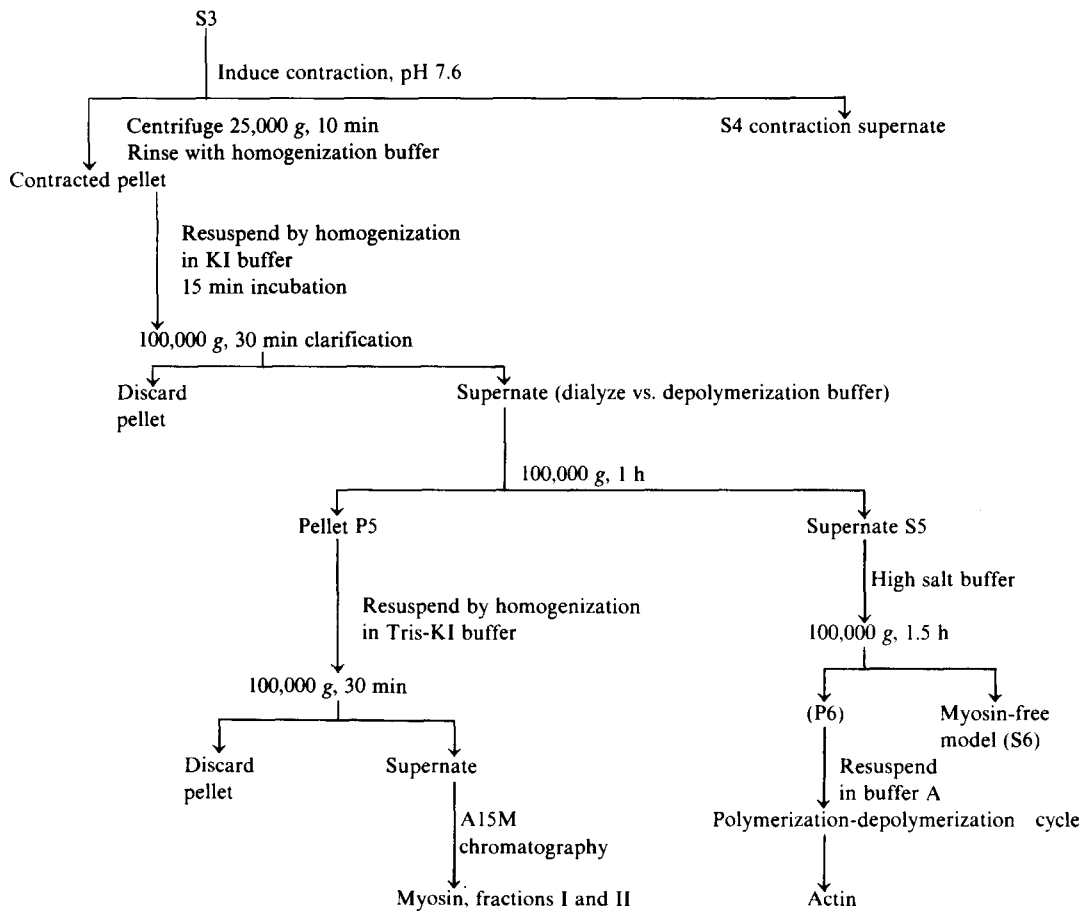


FIGURE 1 Flow diagram of preparative methods.

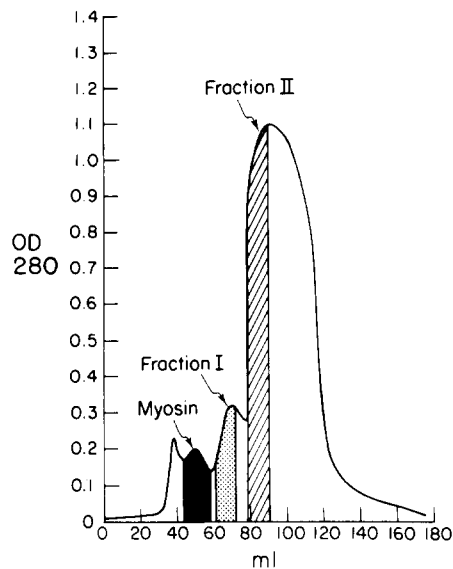


FIGURE 2 Elution profile of P5 on agarose A15M, 100–200 mesh. Peak fractions 42–59 contained *Dictyostelium* myosin, based on  $\text{Ca}^{++}$  ATPase assays and SDS polyacrylamide gel electrophoresis. Peak fractions 62–72 contained a 95,000-dalton polypeptide and actin (fraction I). Fractions 78–92 contained actin and 30,000- and 18,000-dalton polypeptides (fraction II). Fraction 92–160 contained primarily *Dictyostelium* actin. OD, optical density.

electrophoresis, were pooled separately and dialyzed vs. depolymerization buffer to remove the KCl. The fractions were concentrated two- to threefold by dialysis vs. solid sucrose, dialyzed vs. depolymerization buffer, and centrifuged at 100,000 g for 30 min before being assayed for gelation activity. No other fractions from the column exhibited gelation activity.

To obtain a sample of the contracted pellets without myosin (CPI-myosin), the contracted pellets were fractionated as described for P5. Similar elution profiles were obtained. All fractions not containing myosin were pooled and treated as described for fractions I and II. Myosin was removed to prevent contraction from interfering with gelation.

### Assays for the S6 Model

**MEASUREMENT OF TURBIDITY:** Turbidity changes in S6 were measured at 350 nm using a Beckman Acta III spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The carrier was fitted with an aperture in the bottom fifth of the cuvette as described previously (11). An increase in turbidity upon warming S6 to 25°C could be correlated with the formation of a solid mass of gelled material detected by inverting the test tubes (11, 45), a method used to assay gelation under the various conditions described below. The absence of myosin from S6 permitted the use of changes in turbidity as a nonperturbing assay of gelation because the possibility of precipitation resulting from contraction was removed (11, 45). Initiation of contraction in samples reconstituted with myosin was measured as an initial rise in turbidity resulting from the precipitation of contracting material. This was followed by a sharp drop in turbidity as the contracting material formed a tight knot that shrank up out of the light path.

**VISCOSITY MEASUREMENTS:** The relative viscosity of S6 and purified actin was assayed in an Ostwald semimicro viscometer at 25°C. The outflow time for buffer was 31 s. S6 at 4°C was drawn up into the viscometer and allowed to warm to 25°C for various periods of time, and then a single measurement of viscosity was made for each sample. Multiple samples were measured for each condition. This assay was valuable only as a semiquantitative assay of the structure of solated extracts because it is a high shear technique that can break down gels.

**OPTICAL METHODS:** Strain birefringence was performed with the Polar Eye birefringence detection system (50). The strain birefringence measurements were performed on the partially purified model S6 placed in optically isotropic chambers as described previously (11, 45). This assay was useful as a semiquantitative indication of extensive actin cross-linking (gelation).

**ELECTRON MICROSCOPY:** S6 was negatively stained by methods described previously (11, 45). The negatively stained preparations were observed with a Phillips 301 electron microscope at 80 kV. The negatively stained images of S6 provided a qualitative view of the possible molecular structures responsible for gelation and solation (22, 37, 38).

**METHODS FOR REVERSIBLY INHIBITING GELATION:** S6 was incubated at 25°C for 15 min in the presence of 1 mM MgATP, 2.5 mM PIPES, 50 mM KCl, and a free calcium ion concentration of  $\sim 4.6 \times 10^{-6}$  M (Ca/EGTA = 0.9) at pH 6.8 to inhibit gelation. To reverse inhibition, S6 was then returned to 0°C, and the free calcium ion concentration was adjusted to  $\sim 3.0 \times 10^{-8}$  M (Ca/EGTA = 0.05) by the addition of 100 mM EGTA while maintaining pH 6.8. Changes in turbidity upon warming S6 at both stages were monitored at 350 nm and were correlated with the formation of a gel.

### Assay for Calcium-Sensitive Gelation Activity of Fractions I and II

**FALLING BALL VISCOMETRY:** Falling ball viscometry<sup>1</sup> (14) was valuable as a semiquantitative assay for detecting increases in viscosity using minimal volumes of dilute samples. The reproducibility of measurements was maximized when the concentration of the fractions was such that the final viscosity was no greater than 300 centipoise. Increases in viscosity were correlated with the formation of a solid gel when the fractions were concentrated two- to threefold, as judged qualitatively (11, 45).

Fractions I, II, and CPI-myosin were tested under the optimal conditions for gelation discussed for S6 (see below). Calcium inhibition of gelation was tested by adjusting the free calcium ion concentration to  $4.6 \times 10^{-6}$  M at pH 6.8. Rabbit actin was added to all samples to a final concentration of 0.8 mg/ml. Triplicate samples were taken up into 100- $\mu$ l capillaries (Accu-fill 90 Micropet, Becton, Dickinson & Co., Rutherford, N. J.), sealed with plasticine at one end, and then incubated at 0 or 28°C for 30 min. After incubation the time required for a stainless steel ball (Micro Ball Co., Peterborough, N. H.) to fall through the sample was measured. The time taken by the ball to fall a certain distance with the capillary tipped at various angles was converted to centipoise using calibration curves derived from

<sup>1</sup> T. D. Pollard and his collaborators kindly provided us with samples and advice on the falling ball viscometer used in their laboratory.

measurements made with glycerol.<sup>2</sup> This technique could not be used to determine the absolute viscosity because the samples were not Newtonian fluids.

Muscle actin was purified from acetone powder of rabbit skeletal muscle according to the method of Spudich and Watt (39).

**POLYACRYLAMIDE GEL ELECTROPHORESIS:** Electrophoresis of the various preparations was performed on SDS polyacrylamide gels according to the method of Laemmli (23) modified to slab gel configuration. Samples were solubilized before electrophoresis by heating at 100°C for 2 min in 60 mM Tris HCl, pH 6.8 (2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.005% bromophenol blue). The percent of acrylamide used in each case is noted in the figure legends. SDS gels were fixed, stained, and scanned as described previously (11). Molecular weights were determined using standard curves derived from muscle myosin heavy chains (200,000 daltons), phosphorylase A (100,000 daltons), DNase I (31,000 daltons), and myoglobin (16,000 daltons).

**PROTEIN DETERMINATIONS:** Determinations of total protein were made by the method of Lowry (24) and the modification of Hartree (15) using BSA as a standard. Quantitative scanning densitometry of Coomassie Blue-stained gels was used to determine the relative amount of polypeptides present, with the assumption that all polypeptides bound the same amount of dye per unit weight.

## RESULTS

We have found that a partially purified, myosin-free fraction (S6) derived from the contracted pellet of *D. discoideum* extracts retains the most important properties of gelation described previously for crude extracts of *D. discoideum* (11, 45). In what follows we first describe the preparation and composition of the contracted pellet and the models derived from the contracted pellet and then detail the calcium- and pH-sensitive properties of gelation. We then demonstrate that calcium- and pH-sensitive contractility can be restored to the S6 model by the addition of purified myosin. Finally, we show that calcium-sensitive gelation at 28°C occurred with a fraction containing actin and a 95,000 dalton-polypeptide (fraction I) derived from the contracted pellet. In addition, calcium-sensitive gelation at 0°C was demonstrated with a fraction containing actin and 30,000- and 18,000-dalton polypeptides (fraction II).

### Preparation of the Contracted Pellet

The preparation of a partially purified model system containing fewer proteins than our previous extract, S3 (11), was based on the working hypothesis that at least some of the required contractile-cytoskeletal proteins would remain associated with actin and/or myosin during contrac-

TABLE I  
*Distribution of Actin during Preparation of the Contracted Pellet*

Source	Volume	Protein	Total actin
		concentration	
	<i>ml</i>	<i>mg/ml</i>	<i>mg</i>
Cell homogenate	58.0	39.5	193.0
S3	26.0	21.1	63.7
Contracted pellets P4	8.0	9.6	21.7
Contraction supernate S4*	25.0	18.8	42.0

These values represent an average of three experiments. \* The contraction supernate was centrifuged at 100,000 g for 2 h to pellet any F-actin that was not concentrated in the contracted pellet. During this step, 3.25 mg of actin was pelleted.

tion of S3. The distribution of actin during the preparation of S3 and the contracted pellet is presented in Table I. One-third of the total cellular actin was present in the S3 model, and 34% of the actin present in S3 was incorporated into the contracted pellet. Centrifugation of the contraction supernate at 100,000 g for 2 h pelleted an additional 5% of the actin. Thus, 61% of the actin in S3 was nonsedimentable under conditions that favored the polymerization of pure actin. This nonsedimentable actin was not denatured because it polymerized following purification by diethylaminoethyl cellulose chromatography (data not shown). Thus, the actin in the contracted pellet represented the readily polymerizable actin originating from the total soluble actin in the cell extracts.

### Composition of the Contracted Pellet and the S6 Model

The polypeptide composition of the contracted pellet is given in Table II where it is compared with that of the earlier model S3. Actin and myosin were significantly enriched (Fig. 3), whereas the 250,000-dalton polypeptide remained in the contraction supernate. The 95,000-dalton polypeptide was a major component of the contracted pellet present in a 1:18 molar ratio with actin. However, it was also present in the contraction supernate that was capable of gelling. 10% of the 95,000-dalton polypeptide remained with actin during contraction (Tables I and II). The actin, 35,000-, 30,000-, 25,000-, and 18,000-dalton polypeptides were present in the contracted pellet in an approximate molar ratio of 7:1:1:1:2.5, respectively. Al-

<sup>2</sup> Calibration curves were prepared by V. Fowler.

TABLE II  
Composition of S3 and the Contracted Pellet

	S3*	Contracted pellet‡
	%	%
250,000	0.85	—
Myosin	2.8	6.5
95,000	3.9	3.4
75,000	5.0	2.3
55,000	7.3	3.0
50,000	2.8	1.8
Actin	8.6	29.4
35,000	NM§	2.6
30,000	NM	3.0
28,000	0.9	0.9
25,000	NM	2.9
18,000	NM	4.8

Average protein concentration (mg/ml): S3, 16.2; contracted pellet, 9.6.

\* Data from Condeelis and Taylor (11).

‡ Percent determined by quantitative densitometry of SDS polyacrylamide gels.

§ NM, not measured.

though the 75,000-, 55,000-, and 50,000-dalton polypeptides were also major components of the contracted pellet, they were found at higher concentrations in the contraction supernate. Preparations made in the presence of sucrose, Trasylol, PMSF, iodoacetic acid (pH 8.0), 5.0 mM sodium pyrophosphate, and 15 mg/ml BSA in various combinations with contraction induced at 4 or 25°C had identical polypeptide compositions.

S6 derived from the contracted pellets contained all of the major polypeptides present in the contracted pellet with the exception of myosin, as shown in Fig. 3. The final protein concentration of S6 ranged from 2.0 to 3.0 mg/ml. One fraction of the 95,000-, 75,000-, 55,000-, 30,000-, and 18,000-dalton polypeptides and actin sedimented with the myosin (P5) (Fig. 9) during its removal from the solubilized contracted pellet and was used for the isolation of myosin and fractions I and II.

#### Regulation of Gelation in S6

As observed in our previous study with the crude extract S3 in which the ionic conditions for gelation were investigated in detail (11), gelation of S6 occurred upon warming to 25°C in the presence of 2.5 mM PIPES, 2.0 mM EGTA, 1 mM MgATP, 20 mM KCl, and Ca/EGTA = 0.05 ( $1.0 \times 10^{-8}$  M free calcium), pH 7.0. However, a slightly higher ionic strength of 50 mM KCl and

a lower pH of 6.8 gave an optimal extent of gelation of S6. A minimum actin concentration of 0.5 mg/ml was required for gelation, and the gel was optically isotropic, as described for the crude cell extract (11, 45).

The effects that the free calcium ion concentration and pH had upon the extent of structure of S6 were determined by measurements of turbidity, relative viscosity, and strain birefringence, (Figs. 4 and 5). These semiquantitative measurements were also correlated with the formation of a solid gel, as judged qualitatively (11, 45) under the optimal gelation conditions described above. A free calcium ion concentration  $>7.6 \times 10^{-7}$  M or pH  $>$  or  $<6.8$  inhibited gelation (favored the sol state) but had no detectable effect upon the polymerization of purified *D. discoideum* actin at the same concentration as found in S6 (Figs. 4 and 5).

The relative viscosity of S6 obtained at free calcium ion concentrations which favored solation was similar to the viscosity of purified *D. discoideum* F-actin at the same concentration (Fig. 4 b)

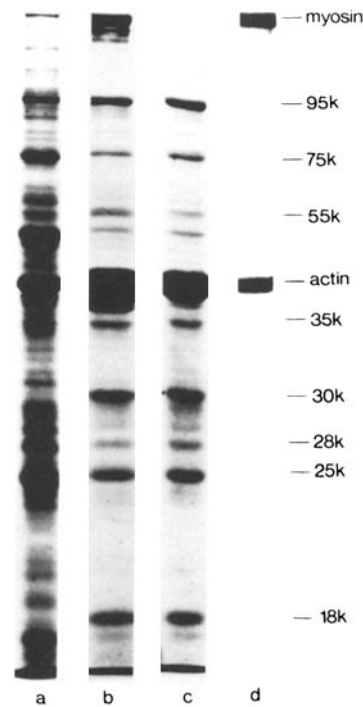


FIGURE 3 10% SDS polyacrylamide slab gel electrophoresis of (a) high-speed supernate S3, (b) proteins brought down from S3 during contraction, (c) S6, the myosin-free supernate, and (d) purified *Dictyostelium* myosin and actin. Numbers correspond to the molecular weights.

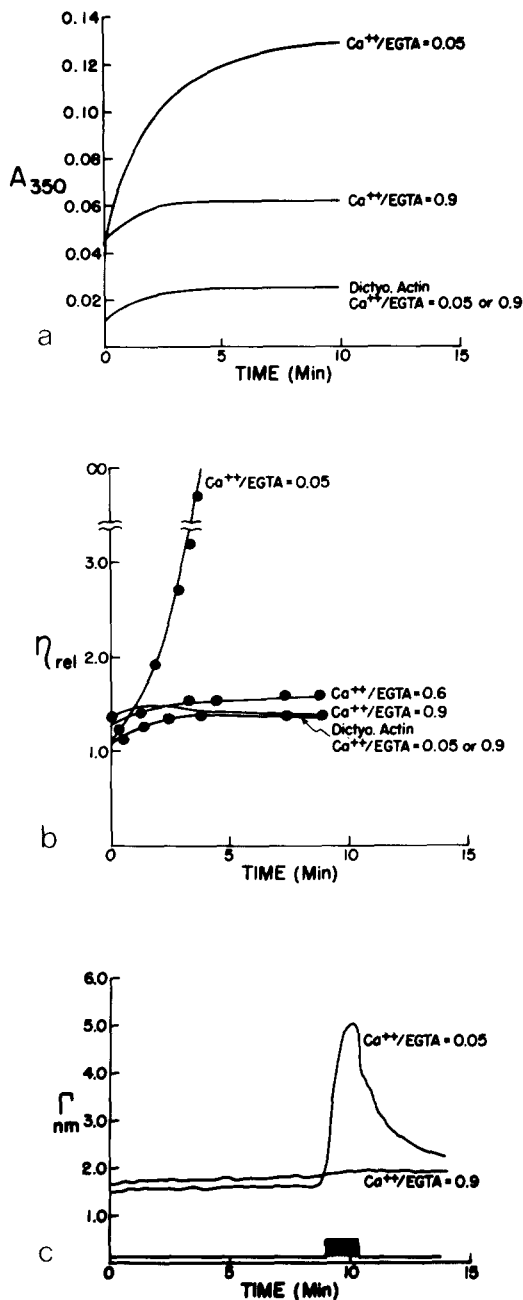


FIGURE 4 Effect of  $\text{Ca}^{++}$  on gelation of S6.  $\text{Ca}^{++}/\text{EGTA} = 0.05, 0.6,$  and  $0.9$  corresponded to free calcium ion concentrations of  $\sim 3.0 \times 10^{-8}, 7.6 \times 10^{-7},$  and  $4.6 \times 10^{-6}$  M, respectively. (a) Turbidity changes at 350 nm in response to  $\text{Ca}^{++}/\text{EGTA}$  ratios of 0.05, which optimized gelation, and 0.9, which inhibited gelation. Only slight changes in turbidity occurred upon polymerization of purified *Dictyostelium* actin (0.5 mg/ml) at  $\text{Ca}^{++}/\text{EGTA} = 0.05$  or 0.9. (b) Relative viscosity measurements

and was correlated with the presence of F-actin filaments observed in negatively stained preparations (Fig. 6a). In contrast, gelled material at free calcium ion concentration of  $\sim 3.0 \times 10^{-8}$  M exhibited an abundance of random meshworks containing actin (Fig. 6b).

#### Reversible Inhibition of Gelation

Gelation of S6 was inhibited by adjusting the free calcium ion concentration to  $\sim 4.6 \times 10^{-6}$  M before warming. After warming the solated S6 contained F-actin filaments (Fig. 6a) that gelled again when the free calcium ion concentration was returned to  $\sim 3.0 \times 10^{-8}$  M at pH 6.8 (Fig. 7). Thus, inhibition of gelation with  $4.6 \times 10^{-6}$  M calcium can be reversed. The solution of a gelled extract by adding micromolar calcium has been shown previously (45).

Thus, in the absence of myosin, gelation of S6 was optimal at low calcium ion concentrations, and solation occurred as the free calcium ion concentration was increased above  $\sim 7.6 \times 10^{-7}$  M or as the pH was increased or decreased from pH 6.8. The observed structural changes suggested the reversible cross-linking of actin filaments.

#### Reconstitution of Contraction in S6

Calcium- and pH-sensitive contraction were reconstituted by the addition of purified *D. discoideum* myosin to S6. The effect of calcium and pH on the reconstituted system was studied by measuring the turbidity changes at 350 nm over time (Fig. 8a and b). At pH 6.8 and a free calcium ion concentration of  $\sim 3.0 \times 10^{-8}$  M, contraction was inhibited, and an increase in turbidity indicated that gelation had occurred. However, the turbidity at the plateau was greater than the sum of the individual turbidities of gelled S6 and myosin. Therefore, even though contraction, as defined by the condensing of the gel, did not occur under these conditions, some actin-myosin interactions and some actin-gelation factor interactions were suggested. When the free calcium ion concentra-

tion of S6 at  $\text{Ca}^{++}/\text{EGTA} = 0.05$  showed a dramatic increase in structure as gelation occurred. Comparatively small changes in relative viscosity of S6 occurred at  $\text{Ca}^{++}/\text{EGTA} = 0.6$  or 0.09, which inhibited gelation, and upon polymerization of pure *Dictyostelium* actin at  $\text{Ca}^{++}/\text{EGTA} = 0.05$  or 0.9, which inhibited gelation. Only slight changes in turbidity occurred upon polymerization of purified *Dictyostelium* actin (0.5 mg/ml) at  $\text{Ca}^{++}/\text{EGTA} = 0.05$  or 0.9. (c) Strain birefringence was induced at  $\text{Ca}^{++}/\text{EGTA} = 0.05$ , demonstrating the viscoelastic properties of gelled S6. At  $\text{Ca}^{++}/\text{EGTA} = 0.9$  strain birefringence could not be induced (see text).

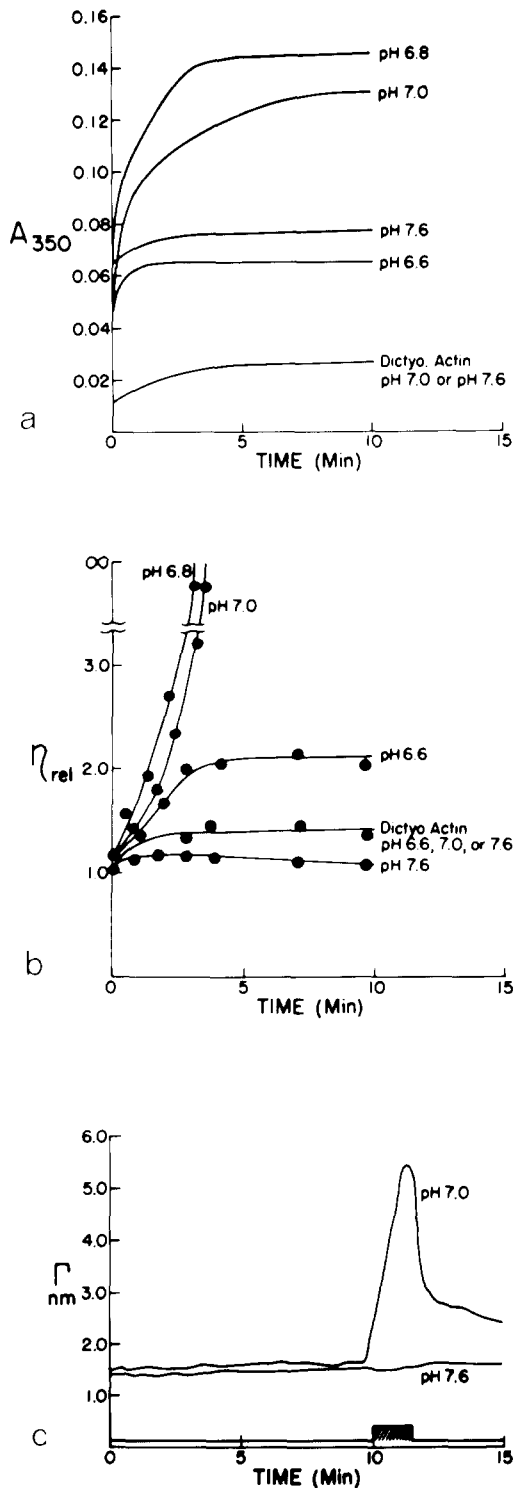


FIGURE 5 Effect of pH on gelation of S6. (a) Turbidity changes at 350 nm in response to pH 6.8 and 7.0, which

tion was increased above  $\sim 7.6 \times 10^{-7}$  M at pH 6.8, or when the pH was increased above 6.8, contraction occurred. A pH below 6.8 inhibited both gelation and the formation of a contracted knot (Fig. 8a).

The results in Figs. 4, 5, and 8 demonstrate the effect of the absence or presence of myosin on the behavior of S6. In the absence of myosin, S6 was a sol containing free F-actin filaments at a free calcium ion concentration  $> 7.6 \times 10^{-7}$  M or at a pH  $> 6.8$ . Contraction occurred when myosin was added to S6 under these conditions. In contrast, contraction was inhibited when myosin was added to S6 under conditions that optimized gelation.

#### Calcium-sensitive Gelation Factors

P5 was a useful by-product obtained during the preparation of S6 (Fig. 1) because it could be used to isolate myosin and several components of S6. To begin the identification of the specific component(s) responsible for calcium-regulated gelation, P5 was fractionated by gel filtration. Two fractions were obtained that exhibited calcium-sensitive gelation. Fraction I consisted of a 95,000-dalton polypeptide with actin, and fraction II contained primarily actin and 30,000- and 18,000-dalton polypeptides (Fig. 9). The gelation (actin cross-linking) activity recovered in these fractions was compared to the activity of the contracted pellet without myosin, which, taken together, represented the total activity contributed by the polypeptides associated with actin and myosin during contraction.

Increases in viscosity measured by falling ball viscometry were used to obtain a semiquantitative comparison of the actin cross-linking activity in dilute samples of fractions I, II, and in the contracted pellet without myosin (Fig. 10). None of the fractions exhibited increases in viscosity without the addition of rabbit actin because the endogenous actin concentrations were  $< 0.5$  mg/ml (see legend, Fig. 10). Therefore, 0.8 mg/ml rabbit actin

optimized gelation, and pH 6.6 and 7.6, which inhibited gelation. Only slight changes in turbidity occurred upon polymerization of pure *D. discoideum* actin (0.5 mg/ml) at pH 7.0 or 7.6. (b) Relative viscosity measurements of S6 showed that gelation was inhibited at pH 6.6 and 7.6, although the relative viscosity of S6 at pH 6.6 was greater than at 7.6 (see text). Only slight changes in relative viscosity occurred upon polymerization of purified *D. discoideum* actin at pH 6.6, 7.0, or 7.6. (c) Strain birefringence was induced in gelled S6 at pH 7.0 but not at pH 7.6, where gelation was inhibited.



was added to each fraction. The contracted pellet without myosin exhibited calcium-sensitive increases in viscosity at both 0 and 28°C, after incubation for 30 min. At 28°C, fraction I, which contained the 95,000-dalton polypeptide at a concentration comparable to that found in the contracted pellet without myosin, also exhibited calcium-sensitive increases in viscosity. At 0°C, frac-

tion I showed no increase in viscosity above that of rabbit actin alone.

In contrast, fraction II, which contained 30,000- and 18,000-dalton polypeptides, exhibited calcium-sensitive increases in viscosity at 0°C. At 28°C, fraction II also showed an increase in viscosity that was less calcium-sensitive than that at 0°C.

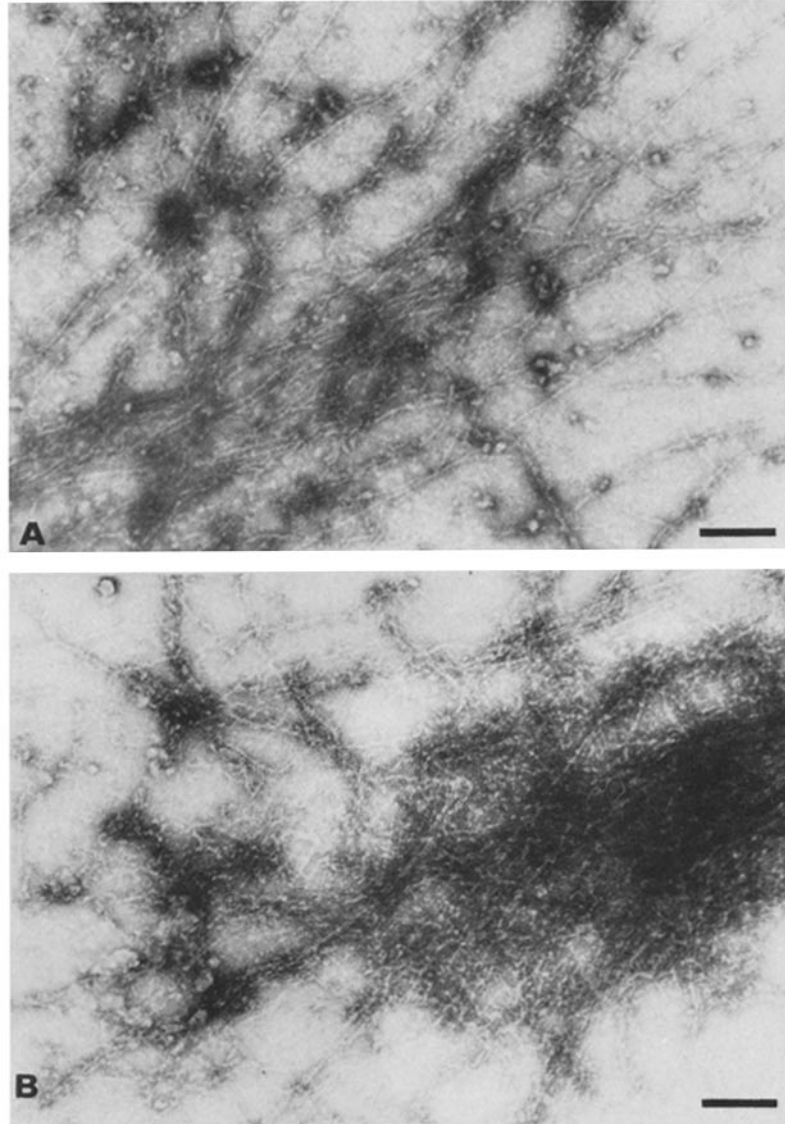


FIGURE 6 Electron micrographs of negatively stained S6.  $\text{Ca}^{++}/\text{EGTA} = 0.05$  and  $0.9$  corresponded to free calcium ion concentrations of  $\sim 3.0 \times 10^{-8}$  and  $4.6 \times 10^{-6}$  M, respectively. (A) After warming in relax solution at  $\text{Ca}^{++}/\text{EGTA} = 0.9$ , pH 6.8, showing free F-actin filaments when gelation was inhibited. (B) After warming in relax solution at  $\text{Ca}^{++}/\text{EGTA} = 0.05$ , pH 6.8, showing undefined meshworks containing actin in the gelled material. Bar,  $0.5 \mu\text{m}$ .

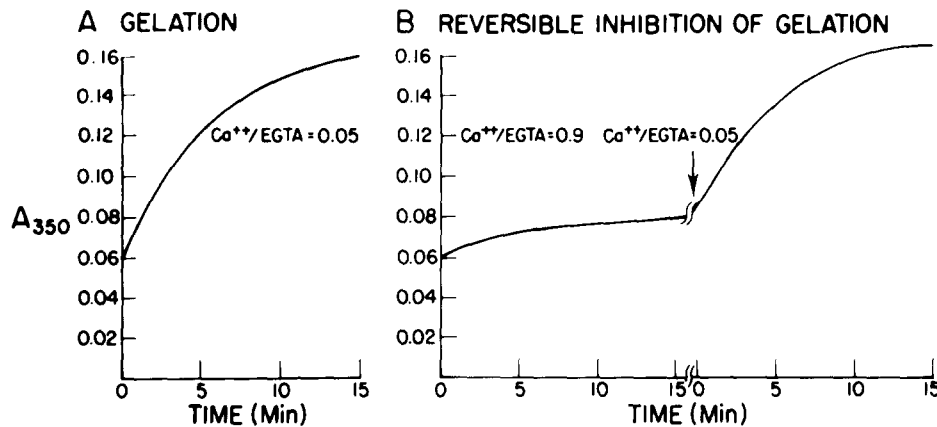


FIGURE 7 Reversible inhibition of gelation.  $\text{Ca}^{++}/\text{EGTA} = 0.05$  and  $0.9$  corresponded to free calcium ion concentrations of  $\sim 3.0 \times 10^{-8}$  M and  $4.6 \times 10^{-6}$  M, respectively. (A) Turbidity measurements at 350 nm show optimal gelation of S6 at  $\text{Ca}^{++}/\text{EGTA} = 0.05$ , pH 6.8, upon warming. (B) At  $\text{Ca}^{++}/\text{EGTA} = 0.9$ , pH 6.8, gelation is inhibited upon warming. However, if after inhibition the  $\text{Ca}^{++}/\text{EGTA}$  is titrated back to  $0.05$ , pH 6.8, gelation proceeds as in A.

All samples that exhibited increases in viscosity also formed solid gels, as judged qualitatively (11, 45), when concentrated two- to threefold.

#### DISCUSSION

This study has focused on the polypeptides that remained associated with actomyosin during contraction of the S3 model. The calcium- and pH-regulated gelation and contraction observed in S3 were reproduced in the S6 model, which was derived from the contracted pellet. It is important to note that S3 contained all of the soluble actin from the cell, whereas the S6 model consisted only of the readily polymerizable actin concentrated during contraction (4, 6). Therefore, the structural properties of S6 do not characterize the complete actin cytoskeleton, but rather the filamentous subset. Also, the fact that the contraction supernate exhibited calcium-sensitive gelation (11) suggests that not all of the gelation and regulatory factors remain completely associated with actin and myosin during contraction, and/or that other actin-binding proteins are present. For example, the 250,000- and 120,000-dalton polypeptides present in S3 and in the contraction supernate have recently been shown to form a gel in the presence of actin.<sup>3</sup>

The discovery of high molecular weight actin-binding proteins in other cell extracts (20, 25, 40, 51, 52) and the presence of a 250,000-dalton poly-

peptide in S3 and in the contraction supernate of our earlier gelation models (11, 48, 45) alerted us to the possibility that the high molecular weight protein might be proteolyzed during contraction, yielding active fragments. However, the absence of detectable changes in the SDS gel pattern of the contracted pellets after the possibility of proteolysis had been minimized suggests that the active gelation factors in fractions derived from the contracted pellets might be distinct from the high molecular weight ( $\sim 250,000$  daltons) polypeptide in the contraction supernate (11, 45) (see Taylor and Condeelis [43] for a review). However, uncontrolled proteolysis, yielding functional fragments, cannot be ruled out entirely. The possible presence of other "actin-binding proteins" on the plasmalemmas pelleted during the first stage of preparation or the presence of any other actin-binding proteins not associated with the contracted actin and myosin will be of interest in completing the cytoskeletal models (43).

#### Calcium-sensitive Gelation and Contraction Activity Derived from the Contracted Pellet

The slightly higher ionic strength and lower pH required for optimal gelation in S6 as compared with S3 might be related to the large differences between them in both the total number of major polypeptides (10 vs. 28) and the total protein concentration (2.5 vs. 16.0 mg/ml). A definite relationship between the states of gelation and contraction has been demonstrated by comparing

<sup>3</sup> J. Condeelis. Personal communication.

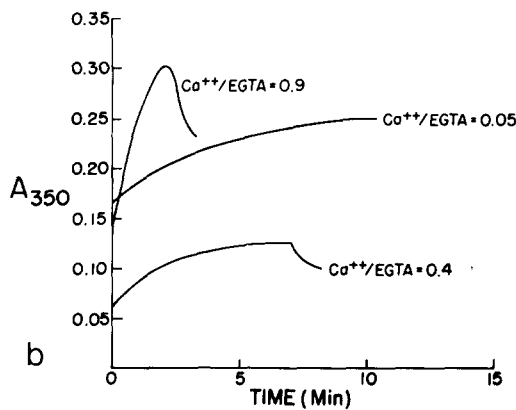
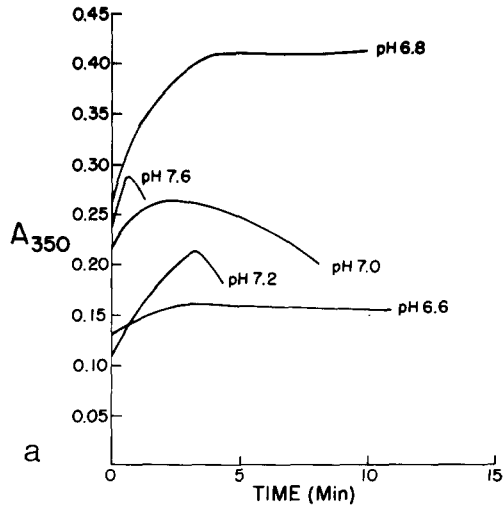


FIGURE 8 Effects of pH and  $\text{Ca}^{++}$  on the myosin reconstituted system.  $\text{Ca}^{++}/\text{EGTA} = 0.05$  and  $0.9$  corresponded to free calcium ion concentrations of  $\sim 3.0 \times 10^{-8}$  M and  $4.6 \times 10^{-6}$  M, respectively. (a) Turbidity measurements at 350 nm showed that at pH 6.6 gelation and contraction were inhibited. Gelation occurred at pH 6.8, and contraction was inhibited. As the pH was raised above 6.8, contraction, seen as a sharp drop in turbidity, occurred. The rate of contraction increased at higher pH. (b) The characteristic increase in turbidity was observed during gelation at  $\text{Ca}^{++}/\text{EGTA} = 0.05$ , and contraction was inhibited. However, raising the  $\text{Ca}^{++}/\text{EGTA}$  to  $0.9$  resulted in contraction, as is demonstrated by the sharp drop in turbidity (see text).

the changes in turbidity, viscosity, and strain birefringence between the myosin-free S6 model (Figs. 4 and 5) and the S6 model reconstituted with myosin (Fig. 8). Solation of the gel occurred

before or during contraction. The simplest interpretation of the semiquantitative assays and the negatively stained preparations is that the solation involves a decrease in the cross-linking of actin filaments that leaves them free to slide during contraction. In addition, the reversibility of the calcium inhibition of gelation in the S6 model demonstrates that solation induced by calcium is not a destructive process.

#### Calcium-sensitive Gelation Activity of Fractions I and II

The results presented in Fig. 10 demonstrate that both fractions I and II exhibited calcium-sensitive increases in viscosity that were correlated with the formation of a solid gel. Fractions I and II were more active than the contracted pellet without myosin, even when the concentration of the 95,000-, 30,000-, or 18,000-dalton polypeptides were comparable to those found in the contracted pellet without myosin. This observation suggests that processes might be competing in the whole system. The presence of at least two calcium-sensitive gelation fractions with distinct temperature

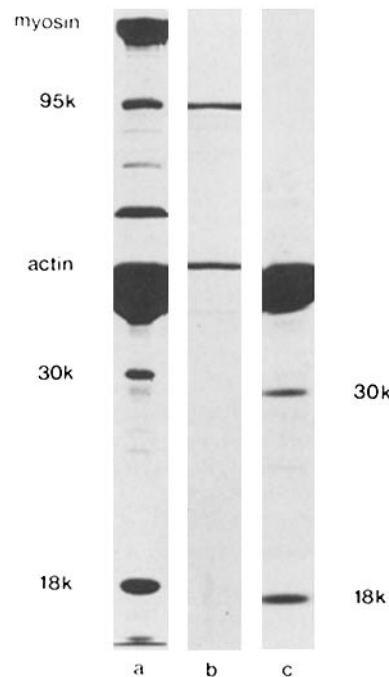


FIGURE 9 10% SDS polyacrylamide slab gel electrophoresis of (a) P5, (b) fraction I from gel filtration of P5, and (c) fraction II from gel filtration of P5. Numbers correspond to molecular weight.

dependences support the possibility of competing and/or synergistic interactions. Therefore, in this study we did not attempt to sum all of the gelation activity from individual fractions for a comparison with the contracted pellet without myosin. Quantitation of total gelation activity is important but cannot be presented at this time (5). A complete

characterization of these factors will be presented elsewhere.

Determination of the physiological relevance of various gelation factors will require more extensive investigations. The increased viscosity measured using the falling ball viscometer could represent the functional gelation factor(s) present in vivo, proteolytic fragments of higher molecular weight gelation factor(s), or simply the cross-linking of actin by other actin-associated proteins. For example, Podlubnaya et al. (34) have demonstrated that muscle  $\alpha$ -actinin would cross-link F-actin at 0°C and Ishiwata (19) has demonstrated a gel-filament transformation when purified striated muscle actin, tropomyosin, and troponin are mixed. This gel-filament transformation at 0°C is calcium sensitive. These facts are especially interesting in view of the low-temperature, calcium-sensitive gelation activity detected in fraction II. Also, the stoichiometry of the low molecular weight proteins in the contracted pellet is similar to the stoichiometry of actin and the tropomyosin-troponin complex in vertebrate striated muscle. If an actin-linked regulatory system analogous to troponin and tropomyosin exists in *D. discoideum*, it might be expected to remain associated with actin and myosin during contraction. Actin-linked regulation of contraction has been demonstrated in *Physarum* (21, 31, 41) and in platelets (10) and has been indicated in *D. discoideum* (29), which suggests the possibility of multiple regulatory mechanisms (43). The effect of fractions I and II on actin-activated *D. discoideum* myosin ATPase must be tested in the future to complete the characterization of these fractions.

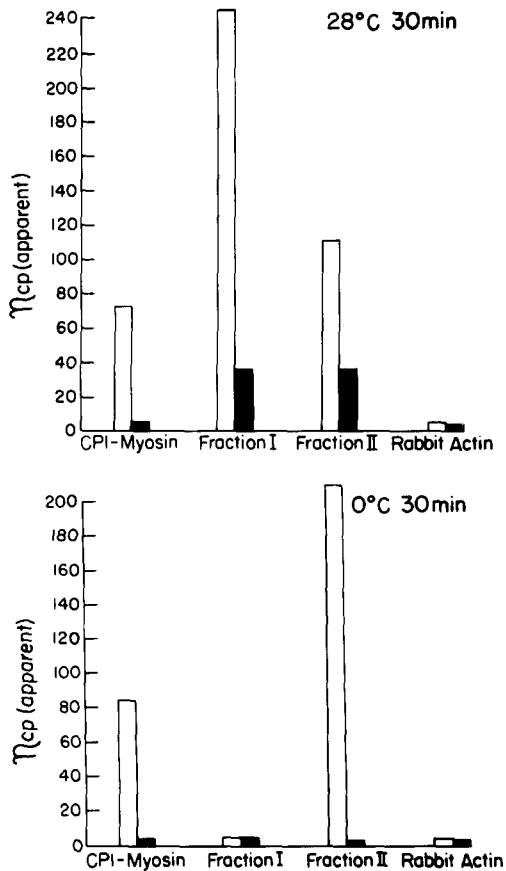
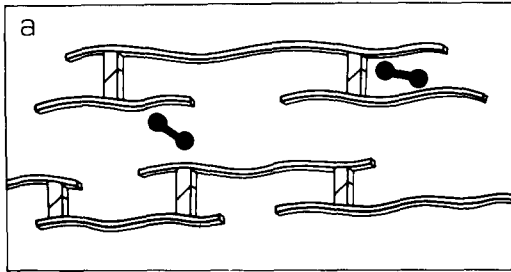


FIGURE 10 Bar graph of viscosity as measured using the falling ball viscometer after incubation at 28 or 0°C for 30 min. Unshaded areas represent experiments performed at  $Ca^{++}/EGTA = 0.05$ , pH 6.8, and shaded areas represent those performed at  $Ca^{++}/EGTA = 0.9$ , pH 6.8. In all cases, increases in viscosity were obtained only upon the addition of 0.8 mg/ml rabbit actin. The data represent an average of three experiments. The concentration of various components in the fractions tested was as follows: CPI-myosin: 95,000 = 0.025 mg/ml, *Dictyostelium* actin = 0.246 mg/ml, 30,000 = 0.027 mg/ml, and 18,000 = 0.043 mg/ml; fraction I: 95,000 = 0.036 mg/ml, actin = 0.050 mg/ml, 30,000 = not detected (ND), and 18,000 = ND; fraction II: 95,000 = ND, actin = 0.174 mg/ml, 30,000 = 0.044 mg/ml, and 18,000 = 0.05 mg/ml; rabbit actin = 0.8 mg/ml.

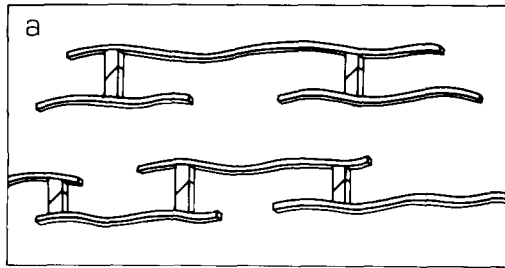
#### Solution-Contraction Coupling Hypothesis of Cell Movement

The hypothesis that local solution of the gel is required for maximal contraction has evolved over the last few years of research on cell extracts (11, 42, 44, 45, 46, 48, 49). We first formulated this concept with John S. Condeelis (11, 45) and have called it the solution-contraction coupling hypothesis. The physiological role of the cross-linked actin gel is that of a cytoskeleton that determines cell shape and transmits tension from contacting regions to the cell substrate attachment site(s). According to this model (Fig. 11), the cross-links involved in the gelled state must be dissociated locally to permit the sliding of actin filaments during contraction. Work is performed when these

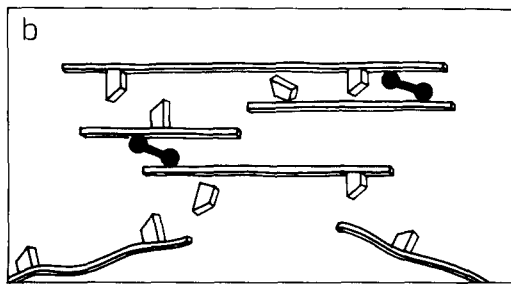
### THE GEL - WITH MYOSIN



### THE GEL - NO MYOSIN

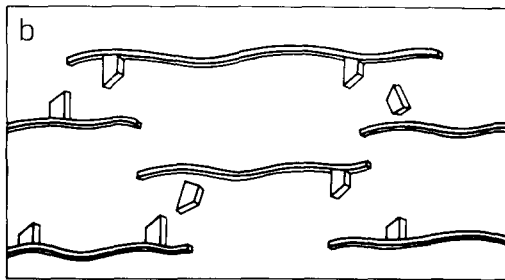


### GEL BREAKDOWN AND CONTRACTION



11

### GEL BREAKDOWN - NO MYOSIN



12

FIGURE 11 Diagrammatic representation of the solation-contraction coupling hypothesis. The actin is represented by the bars, myosin by the dumbbells, and gelation factors by the blocks. Only the basic events are presented. The exact factors and molecular interactions remain to be defined in detail. (a) In the presence of myosin, at pH 6.8 and  $\text{Ca}^{++}/\text{EGTA} = 0.05$ , gelation occurs, and contraction is inhibited. Resistance to contraction is extensive because the gel consists of cross-linked actin filaments. The actin filaments cannot readily slide when cross-linked in the gelled state. (b) If the pH is raised to  $>6.8-7.0$  or if the  $\text{Ca}^{++}/\text{EGTA}$  of 0.05 is raised to 0.9 in the presence of myosin, the cross-links are decreased, releasing actin filaments that are then free to interact with myosin, resulting in shortening as a result of contraction. In microdomains devoid of myosin, solation occurs under the same ionic signal.

FIGURE 12 Diagrammatic representation of the gel-sol transition in the absence of myosin. (a) At pH 6.8 and  $\text{Ca}^{++}/\text{EGTA} = 0.05$ , gelation occurs, represented here as the cross-linking of actin filaments. (b) If the pH is raised to  $>6.8-7.0$ , or if the free calcium ion concentration is raised to  $>7.6 \times 10^{-7}$  M, solation occurs, represented here as the dissociation of cross-links (gelation factors) from each other, from actin, or from both, leaving free actin filaments.

actin filaments pull adjacent gelled regions closer together. This model is supported by the results obtained from myosin-free models, in which only solation occurs under conditions that support contraction (Fig. 12). Also, the presence of calcium-sensitive gelation activity in the contraction supernate and in the fractions derived from the contracted pellets suggests that at least this gelation factor did not remain completely associated or dissociated with actin filaments. The exact distribution of gelation factors under different conditions has not yet been determined, but the present working hypothesis takes this complex association

into account (Figs. 11 and 12). This working hypothesis emphasizes the fundamental relationship between solation and contraction and does not attempt to define all the specific gelation factors or their exact mode of action.

This hypothesis also predicts that microdomains in the models or in the living cells not containing myosin would solate instead of contracting under the same ionic signal. The latter process would be valuable in regions of various cells that decrease in volume and change in structure in the region of contraction. For example, some amoeboid cells contract in the tail ectoplasm, whereas ectoplasm is

also recruited into the endoplasm (solation) at the same site. The solation-contraction coupling hypothesis depicted in Fig. 11 explains this apparent paradox. Both solation and contraction occur at the same site. Microdomains of the cytoplasm not containing myosin (the myosin/actin ratio is low in nonmuscle cells) would only solate, whereas adjacent regions containing myosin would solate-contraction. The force of these contractions would then drive the solated cytoplasm forward (28, 33, 42). It is also reasonable to think that transition from the gelled to solating-contracting states can occur anywhere in the cytoplasm (1, 3, 42), depending on the local pH or pCa. This basic hypothesis can also be used to explain some of the dynamics of cytokinesis and movements of fibroblasts (43).

The structural dynamics of our previous models (11, 42, 44, 45, 48) could be interpreted in relation to the solation-contraction coupling hypothesis. The "flare streaming" model from single cells was originally explained as contractions of peripheral cytoplasm pulling the viscoelastic cytoplasm toward the site of contraction. Alternatively, "flare streaming" could result from a coupling of solation and contraction within the cytoplasmic droplet. The threshold calcium concentration in the medium could induce solation and contraction of the gelled cytoplasm, and the force of the contractions could initially squeeze the solated cytoplasm out into the medium (flare loops). The solated cytoplasm, however, would tend to return to the gelled state because the pH and pCa of the medium are just at the threshold for contraction. The threshold contraction conditions would induce contraction when the cytoplasm regained minimal viscoelasticity. Therefore, a delicate balance between the gelled, solated, and contracted states would be maintained to permit cyclic streaming. Future studies in vivo and in vitro will be required to select among these and any other possible mechanisms.

Although the regulation of the cytoplasmic structure is believed to play a fundamental role in controlling force generation and movement, it is probably not the only regulatory mechanism of cell movement. The possible regulatory mechanisms for nonmuscle cell motility have been reviewed recently (8, 17, 43). These mechanisms include phosphorylation of myosin heavy and light chains, calcium regulation of myosin light chains, calcium regulatory components of F-actin filaments, regulation of filament formation, and

the control of gelation. Multiple regulatory mechanisms might be operable in the same system. For example, Kuzmarski and Spudich<sup>4</sup> have identified an effect of light chain phosphorylation of the actin-activated myosin ATPase, and Mockrin and Spudich (29) have demonstrated an uncharacterized calcium-dependent regulation that required *D. discoideum* myosin. In addition, calmodulin has been identified in *D. discoideum*.<sup>5</sup> Finally, the gelation factors might play a role in regulating the interaction between actin and myosin (11, 45, 46, 49).

#### *Relationship between Regulation In Vitro and In Vivo*

The relationship between the regulation of cell-free extracts, partially purified models, and reconstituted models must ultimately be related to the modulation of potential regulatory parameters in living cells. It is interesting in this regard that Gerisch and co-workers (26, 54) have identified changes in the extracellular calcium ion concentration and the extracellular pH induced by the addition of nanomolar concentrations of cAMP, which induces cellular aggregation through amoeboid movement. These results at the cellular level were consistent with our working hypothesis in vitro, namely that either fluctuations in the free calcium ion concentration and/or pH can modulate both the cytoskeletal and contractile states (11, 42, 45, 46, 48).

Recent evidence<sup>6</sup> (47) has indicated that the free calcium ion concentration is maximal in the tails of *C. carolinensis* during locomotion, and that movement occurs at free calcium ion concentrations somewhere between  $1.0 \times 10^{-7}$  and  $6.0 \times 10^{-7}$  M. Also, spontaneous fluctuations in the intracellular free calcium ion concentration were also detected during movement (47). These direct measurements of the free calcium ion concentration in *C. carolinensis* were consistent with the spontaneous influx of calcium detected over the entire cell and with the calcium currents entering the tails of *C. carolinensis* measured with a vibrating probe (32).

It cannot presently be determined whether pCa

<sup>4</sup> E. Kuzmarski and J. Spudich. Personal communication.

<sup>5</sup> T. Means and his collaborators kindly assayed a *D. discoideum* extract for cross reactivity with an antibody to calmodulin. Positive results were obtained.

<sup>6</sup> Taylor, Blinks and Reynolds. Unpublished results.

or pH regulates cytoplasmic structure and contractility in vivo. The free calcium ion concentration could change in response to changes in intracellular pH and vice versa, or both could be modulated independently. Calcium is the most widely understood second messenger, but transient changes in pH, especially localized at a membrane, are plausible.

We would like to thank R. E. Stephens for discussing with us the dangers of proteolysis. We thank P. H. Cobbold for communicating his calculations on the effect of pH on the dissociation constant of Ca/EGTA buffers. We have adopted the dissociation constants used in his laboratory. We would also like to thank T. D. Pollard, V. Fowler, J. Heiple, S. Virgin, and Yu-Li Wang for their critical comments. The formulation of the solution-contraction coupling hypothesis has evolved over the last 6 yr of investigations with several valued collaborators. Theoretical and experimental investigations with John S. Condeelis were particularly valuable.

This work was supported by the Institute of Arthritis, Metabolism and Digestive Diseases research grant AM 18111 and by a Research Career Development award to D. L. Taylor.

Received for publication 24 July 1978, and in revised form 20 August 1979.

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