

# THE CONTRACTILE BASIS OF AMEBOID MOVEMENT

## II. Structure and Contractility of Motile

### Extracts and Plasmalemma-Ectoplasm Ghosts

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#### ABSTRACT

The role of calcium and magnesium-ATP on the structure and contractility in motile extracts of *Amoeba proteus* and plasmalemma-ectoplasm "ghosts" of *Chaos carolinensis* has been investigated by correlating light and electron microscope observations with turbidity and birefringence measurements. The extract is nonmotile and contains very few F-actin filaments and myosin aggregates when prepared in the presence of both low calcium ion and ATP concentrations at an ionic strength of  $I = 0.05$ , pH 6.8. The addition of 1.0 mM magnesium chloride, 1.0 mM ATP, in the presence of a low calcium ion concentration (relaxation solution) induced the formation of some fibrous bundles of actin without contracting, whereas the addition of a micromolar concentration of calcium in addition to 1.0 mM magnesium-ATP (contraction solution) (Taylor, D. L., J. S. Condeelis, P. L. Moore, and R. D. Allen. 1973. *J. Cell Biol.* **59**:378-394) initiated the formation of large arrays of F-actin filaments followed by contractions. Furthermore, plasmalemma-ectoplasm ghosts prepared in the relaxation solution exhibited very few straight F-actin filaments and myosin aggregates. In contrast, plasmalemma-ectoplasm ghosts treated with the contraction solution contained many straight F-actin filaments and myosin aggregates. The increase in the structure of ameba cytoplasm at the endoplasm-ectoplasm interface can be explained by a combination of the transformation of actin from a less filamentous to a more structured filamentous state possibly involving the cross-linking of actin to form fibrillar arrays (see above-mentioned reference) followed by contractions of the actin and myosin along an undetermined distance of the endoplasm and/or ectoplasm.

Experimental motile model systems have played a significant role in understanding the structural and chemical dynamics of cell movements. Simard-Duquesne and Couillard (31) elicited visible contractions in glycerinated models of *Amoeba proteus* upon the addition of magnesium and ATP. Cytoplasmic contractions and streaming have been

identified both in cytoplasm removed from the plasmalemma (1, 12, 33) and in fractionated cytoplasm (39, 46, 27). In addition, it has been shown that cytoplasm isolated from single specimens of *Chaos carolinensis* contracted upon the addition of at least  $7.0 \times 10^{-7}$  M free calcium ions in the presence of an endogenous magnesium-ATP

energy source. This cytoplasm contained actin and myosin filaments that dissociated upon the addition of a relaxation solution (33, 23). Furthermore, the single-cell cytoplasmic models exhibited streaming patterns identical to those of intact cells when demembrated in a threshold calcium contraction solution (33). However, the actin-containing filaments, as well as the myosin aggregates, were shown to be labile at low divalent cation concentrations (35, 7).

Recently, contractions and relaxations have been induced in local regions of intact amebas by microinjecting test solutions into the endoplasm and ectoplasm (34, 38). Anterior endoplasm and all of the ectoplasm were found to contract immediately upon injecting the threshold calcium concentration, while a delay of a few seconds occurred for observing only weak contractions in the uroid endoplasm. This gradient of contractility was paralleled by a gradient of increasing viscoelasticity in the endoplasm from the uroid to the tips of advancing pseudopods which continued throughout the ectoplasm (34). Furthermore, both low and high calcium ion concentrations caused an ultimate decrease in the cytoplasmic consistency with the high ( $> 10^{-6}$  M) calcium ion concentration first inducing large contractions (34, 35, 38).

Several different investigations have demonstrated that actin plays a structurally dynamic role in cell movements. Thompson and Wolpert (39) originally described the appearance of many thin filaments when cell extracts from *A. proteus* were warmed to room temperature. Pollard and Ito (27) extended this experimental approach and demonstrated the presence of both thick and thin filaments in warmed ameba extracts. Hatano has demonstrated that actin and "actinin" from *Physarum* form variable complexes in the presence and absence of magnesium ions (17). Furthermore, Tilney has shown that the polymerization of actin, as well as the interaction of actin with associated proteins, appears to be involved in the formation of acrosomal processes in various sperm (41, 42).

Recently, actin has been shown to bind to proteins other than known control proteins and myosin. Tilney isolated the acrosomal process from *Limulus* sperm (42), and demonstrated the association of actin with 55,000 mol wt and 95,000 mol wt components. Kane (21) extracted actin from sea urchin eggs with a low ionic strength glycerol solution and observed that the actin formed a gel, apparently in association with 57,000

mol wt and 220,000 mol wt proteins. Furthermore, Tilney and Detmers (43) induced the aggregation of F-actin filaments by the addition of a low ionic strength extract from erythrocyte ghosts which contained spectrin. Finally, Stossel demonstrated that a high molecular weight (ca. 280,000) protein isolated from macrophages would cause F actin to form aggregates (32).

In this study, a motile cytoplasmic extract has been prepared from *A. proteus* in physiological solutions (33), extending earlier experiments performed on single cells (33) and bulk extracts (39, 46, 27). Likewise, the isolation of the plasmalemma and associated ectoplasm was carried out in a manner believed to be least destructive to its function. This investigation attempts to elucidate the control of actin structure and the interaction of actin with myosin, as well as to identify one site of actin filament formation, using motile model systems prepared under physiological conditions. The control, location, and structure of actin in giant free-living amebas are discussed in relation to other motile processes. A preliminary report of this work has appeared (37, 38).

## MATERIALS AND METHODS

### *Ameba Cultures*

*A. proteus* was obtained from Wards Biological Supply and grown in mass culture (13). *C. carolinensis* was cultured as described previously (33).

### *Preparation of Extracts*

The motile extract of ameba cytoplasm was prepared by a modification of the method of Thompson and Wolpert (39) and Pollard and Ito (27) (Fig. 1). Mass cultures were cleaned by repeated aspiration and exchange of the Prescott and James solution. The cultures were starved for 3 days, then pooled to allow the cells to settle. The Prescott and James solution was exchanged twice by aspiration and the pooled cells were kept at 4°C for 12–18 h. All isolation procedures were carried out at 0°–4°C.

The cooled cells were concentrated by centrifugation at 1,000 g in a Sorvall RC-5 centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.). The 2–4 ml of packed cells were centrifuged again for 30 min at 33,000 g in 4-ml tubes in an SS-34 rotor. The amebas fragmented into four layers as described previously (39, 27).

The second layer (1–2 ml) was mixed with an equal volume of stabilization solution which was a modification of that used previously (33) (Table I), and homogenized (30 passes) in a Teflon-glass homogenizer. The pH

## MOTILE EXTRACTS

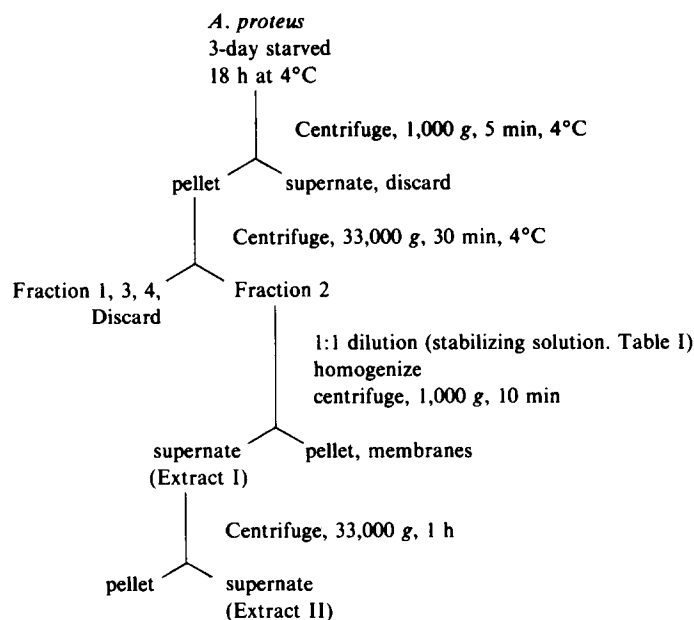


FIGURE 1 Flow diagram describing the methods for preparing extracts I and II.

of the homogenate was adjusted to either pH 6.8 or pH 7.2 by the slow addition of 0.1 N KOH. The homogenate was centrifuged at 1,000 *g* for 10 min and the supernate was designated extract I. Extract I was centrifuged further at 33,000 *g* for 1 h, and the resultant supernate was designated extract II.

### Preparation of the Plasmalemma-Ectoplasm "Ghosts"

Single cells of *C. carolinensis* were rinsed in the relaxation solution (Table I) for 2 min and ruptured as described previously (33). The endoplasm which emptied out of the cell was discarded. The remaining plasmalemma and associated ectoplasm were washed for 3 min more with either the relaxation or the contraction solution (Table I).

The plasmalemma-ectoplasm models were fixed for 3 h in a solution containing 3% glutaraldehyde, 50.0 mM cacodylate buffer, pH 6.8 (33). Some samples were placed in 1.0% osmium tetroxide, 50.0 mM cacodylate buffer, pH 6.8, for 1 h and embedded in Spurr's resin. Light gold and silver sections were stained with saturated uranyl acetate followed by lead citrate. Observations were made with a Philips 301 electron microscope with an accelerating voltage of 80 kV, a condenser aperture of 300  $\mu\text{m}$ , and an objective aperture of 50  $\mu\text{m}$ . Calibration was performed with a no. 1002 crossruled optical grating replica (Ernest F. Fullam, Inc., Schenectady, N. Y.).

### Light Microscope Observations

The experiments were observed with Zeiss Nomarski differential interference optics and Nikon rectified polarized light optics. Birefringence measurements were made with a birefringence detection system manufactured by Custom Instrumentation, Ravena, New York.<sup>1</sup> This system permits the measurement of changes in birefringence with a ca. 0.1 msec time constant.<sup>1</sup> The extracts were placed in observation chambers and allowed to warm to room temperature before the application of test solutions (33).

### Turbidity Measurements

Turbidity measurements were made at 350 nm with a Beckman Acta CIII spectrophotometer (11) (Beckman Instruments, Inc., Fullerton, Calif.). An equal volume of the relaxation solution was added to extract I in a microcell at room temperature. After a base line was established, enough 0.2 M  $\text{CaCl}_2$  was added to make the final free calcium ion concentration ca.  $1.0 \times 10^{-6}$  M, pH 6.8 (33, 35, 7).

### Negative Staining of Cytoplasmic Extracts

The negative staining procedure was modified from previously published methods (23, 33, 7). Aliquots of the

<sup>1</sup> Taylor, D. L., and R. Zeh. Manuscript in preparation.

extracts were brought to room temperature and mixed with an equal volume of the appropriate test solution. After standing for 1 min, the mixture was diluted, 1:10 vol/vol for extract I and 1:3 vol/vol for extract II, with the appropriate test solution. A drop of the mixture was applied to Formvar-carbon-coated grids and allowed to settle for 30 s. Excess sample was removed with a drawn-out pasteur pipette; the grid was rinsed three times with 3.0% glutaraldehyde in 50.0 mM cacodylate buffer, pH 6.8, and allowed to fix for 2 min. The fixed sample was rinsed five times with glass-distilled water followed by three rinses with 1.0% uranyl acetate. A thin film of uranyl acetate was allowed to dry on the grid. Preparations treated with heavy meromyosin were rinsed three times with the appropriate test solution lacking ATP before applying the HMM.

### Gel Electrophoresis

The extracts and standards were analyzed by gel electrophoresis on 5.0% sodium dodecyl sulfate (SDS) polyacrylamide gels, by the method of Weber and Osborn (44) or a modification of the method of Fairbanks et al. (10) in which 20% glycerol was used in the gels.

### Preparation of HMM

Heavy meromyosin was prepared from rabbit skeletal muscle (22) and stored at  $-20^{\circ}\text{C}$  as a lyophilized powder. It was dissolved in 5.0 mM PIPES buffer, 30.0 mM KCl, pH 6.8, to give a final concentration of 4.0 mg/ml.

## OBSERVATIONS

### Light Microscope Observations

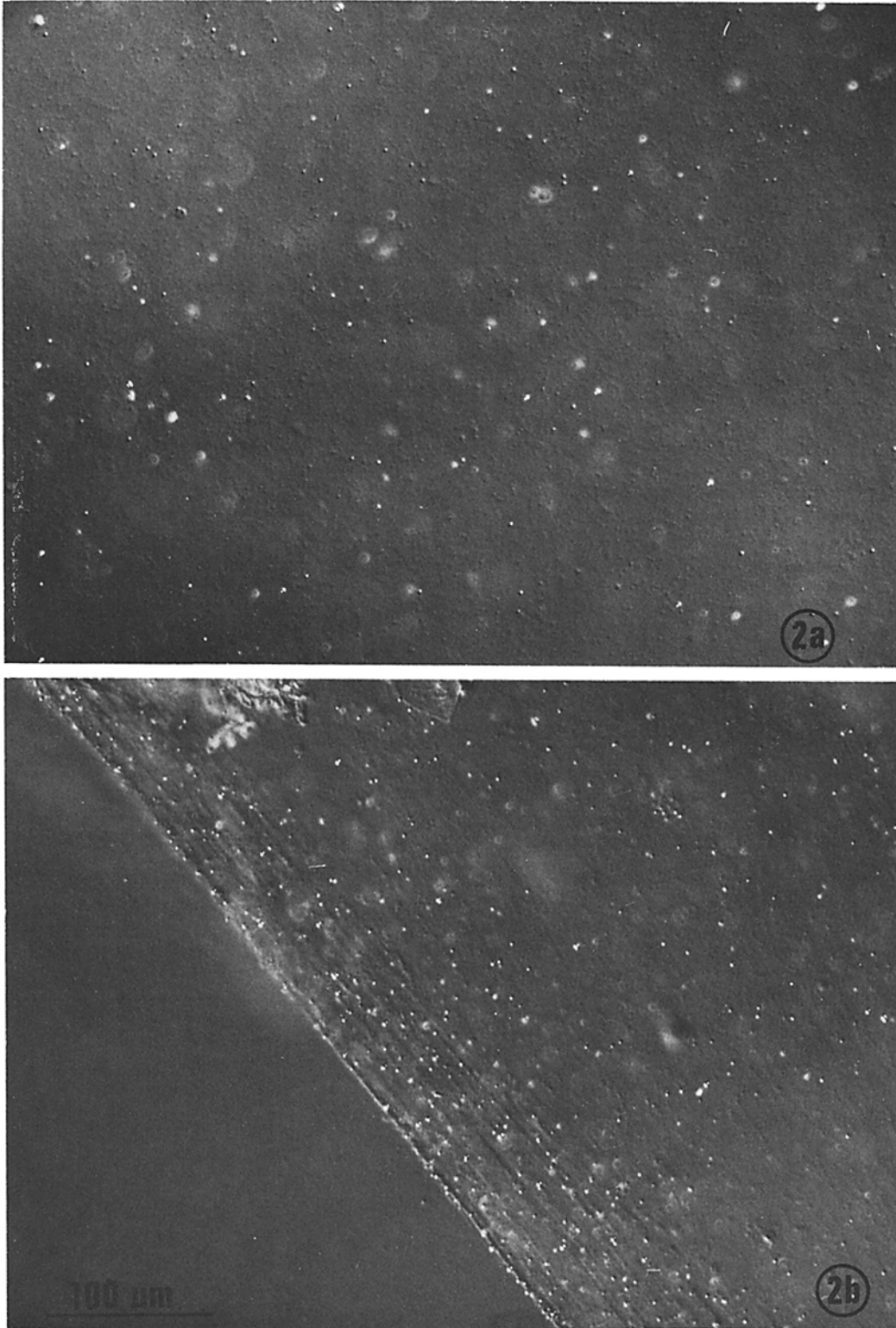
Extract I exhibited no movement during 5 min of warming from  $0^{\circ}\text{C}$  to room temperature. The

addition of the low calcium and ATP solution (relaxation solution) (Table I) to the warmed sample (1:1 vol/vol) caused some fibrils to form but did not elicit contractions or streaming (Fig. 2 a). However, the addition of the contraction solution even at (1:10 vol/vol) (Table I) caused the formation of large arrays of birefringent fibrils during contraction (33) (Figs. 2 b, 3).

The pH and ionic strength of the extract had a dramatic effect on the structure and dynamics of the extract. At pH 7.2 and a low ionic strength (ca.  $I = 0.03$ ), the addition of the relaxation solution (Table I) induced the formation of many fibrils, forming a jelled array that did not contract over a 5-min period. These fibrils were similar in appearance to the fibrils observed in cytoplasm removed from single cells (33). The addition of the contraction solution (Table I) caused the immediate contraction of the jelled extract with some streaming. When extract I was prepared at pH 6.8 at an ionic strength of ca.  $I = 0.05 \pm 0.01$  (reference 33; see Table I), the addition of the relaxation solution did not cause solid gelation, but caused a slight increase in cytoplasmic consistency without contracting. However, the addition of the contraction solution induced contractions and streaming of the extract. The present paper describes experiments performed with the extracts prepared at pH 6.8 at an ionic strength of ca.  $0.05 \pm 0.01$  (33; see Table I). The pH of ameba cytoplasm *in vivo* has been measured over a range from pH 6.4 to pH 7.2 (4). The ionic strength of ameba cytoplasm has been determined to be ca.

TABLE I  
Test Solutions for Extract I

	Stabilizing solution (SS)	Contraction solution (CS)	Relaxation solution (RS)	Threshold contraction (flare solution) (FS)
pH	6.8	6.8	6.8	6.8
mOsM	85-100	85-100	85-100	85-100
Ionic strength ( $\pm 0.01$ )	0.05	0.05	0.05	0.05
Pipes buffer mM	5.0	5.0	5.0	5.0
Dipotassium EGTA mM	5.0	5.0	5.0	5.0
KCl mM	30.0	30.0	30.0	30.0
MgCl <sub>2</sub> mM	1.0	1.0	1.0	1.0
CaCl <sub>2</sub> mM	2.0	4.5	2.0	4.2
Disodium ATP mM	0	1.0	1.0	1.0
DDT mM	1.0	1.0	1.0	1.0
Free Ca <sup>++</sup> M	ca. $10^{-7}$	ca. $1.0 \times 10^{-6}$	ca. $10^{-7}$	ca. $7.0 \times 10^{-7}$



**FIGURE 2** Extract I warmed to room temperature. (a) Extract I remained nonmotile after the addition of the relaxation solution, pH 6.8. (b) Addition of the contraction solution caused the formation of large contracting bundles of cytoplasm, pH 6.8.  $\times 185$ .

0.05 (33). A detailed analysis of the effects of pH and other physiological parameters on the structure and dynamics of the extracts will be discussed in a future publication.

Unlike that in previous experiments on pooled cytoplasmic extracts (39, 46, 27), the calcium sensitivity for contraction demonstrated in single cell extracts (33) was maintained in these motile models. The calcium-sensitive contractions were sometimes lost, however, when extract I was prepared in the relaxation solution at 0°C (Table I) or when cold relaxation solution was added to cold 0–4°C extract I prepared in the usual manner and then warmed to room temperature. Calcium-regulated contractions were maintained in 20 separate experiments when the extracts were prepared in the stabilization solution (Table I) warmed to room temperature, and then tested.

Extract I warmed to room temperature was optically isotropic. However, the addition of the contraction solution (1:10 vol/vol) induced the formation of birefringent, contractile fibrils that shortened (Fig. 3). The birefringence reached a maximum if the cytoplasm did not contract fully (Fig. 4), while the birefringence decayed from the maximum if the cytoplasm continued to contract into a tight mass.

Extract II warmed to room temperature did not contract or stream upon the addition of the relaxation or contraction solutions, although some fibrils formed after the addition of the relaxation solution (Table I) and many fibrils formed after the addition of the contraction solution (Fig. 5 *a, b*). Addition of the pellet from the preparation of Extract II to Extract II (46) permitted contractions and streaming only in the presence of the contraction solution.

### *Turbidity Measurements*

Only a very slow increase in turbidity was observed in the relaxation solution-extract I (1:1 vol/vol) mixture over a 15-min time interval. In contrast, a large and immediate increase in turbidity was induced by titrating the free calcium ion concentration to ca.  $1.0 \times 10^{-6}$  M, while maintaining the pH at 6.8 (Fig. 6). A large, contracted pellet formed in the cuvette after the addition of calcium. The pH of the extract decreased by 0.4 units when the calcium titration was performed in the absence of a high buffer concentration (50.0 mM PIPES buffer, pH 7.0) with the calcium.

### *Negative Staining of Cytoplasmic Extracts*

Extract I warmed to room temperature contained very few F-actin filaments or myosin aggregates, but did contain many barrel-shaped particles which had dimensions similar to those of particles isolated from erythrocyte membrane ghosts (ca. 120 Å in diameter and ca. 165 Å in length) (14, 15) and many amorphous aggregates which appeared to contain the barrel-shaped particles (Fig. 7 *a*). Furthermore, many doughnut-shaped structures were observed that were ca. 120 Å in diameter with ca. 50-Å holes (14, 15), as well as "C"-shaped structures similar to those described as components of the unpolymerized red blood cell ghost extracts (43) (Fig. 7 *a, b*). Addition of the relaxation solution caused the formation of filamentous bundles that appeared to be associated with the amorphous aggregates (Fig. 7 *b*). In contrast, the addition of the contraction solution was characterized by the formation of large arrays of F-actin filaments and the appearance of many myosin aggregates with the same morphology as filaments formed from purified amoeba myosin (6) (Fig. 7 *c*). Furthermore, there was an apparent decrease in the number of large amorphous aggregates after the addition of the contraction solution (Fig. 7 *c*).

Extract II warmed to room temperature contained many small amorphous aggregates that appeared to include the barrel-shaped particles and a few filaments. However, very few free thin filaments and thick filaments were observed. The addition of rabbit muscle heavy meromyosin (HMM) to a warmed extract II resulted in the formation of actin filaments (Fig. 8 *a*). Furthermore, titrating the free calcium ion concentration up to ca.  $1.0 \times 10^{-6}$  M in the absence of exogenous ATP caused only a small number of free actin filaments to appear, but large quantities of a fibrous complex formed, which appeared to be similar to actin-spectrin mixtures (42), as well as actin-actin binding protein complexes observed by Stossel and Hartwig (32) (Fig. 8 *b*).

Addition of the relaxation solution of extract II caused the appearance of some actin filaments in association with the amorphous aggregates (Fig. 8 *c*). In addition, increasing the magnesium ion concentration to 2.0 mM in the relaxation solution (Table I) induced the formation of large bundles of actin filaments in extract II, similar to the fibrils observed in cytoplasm removed from single cells

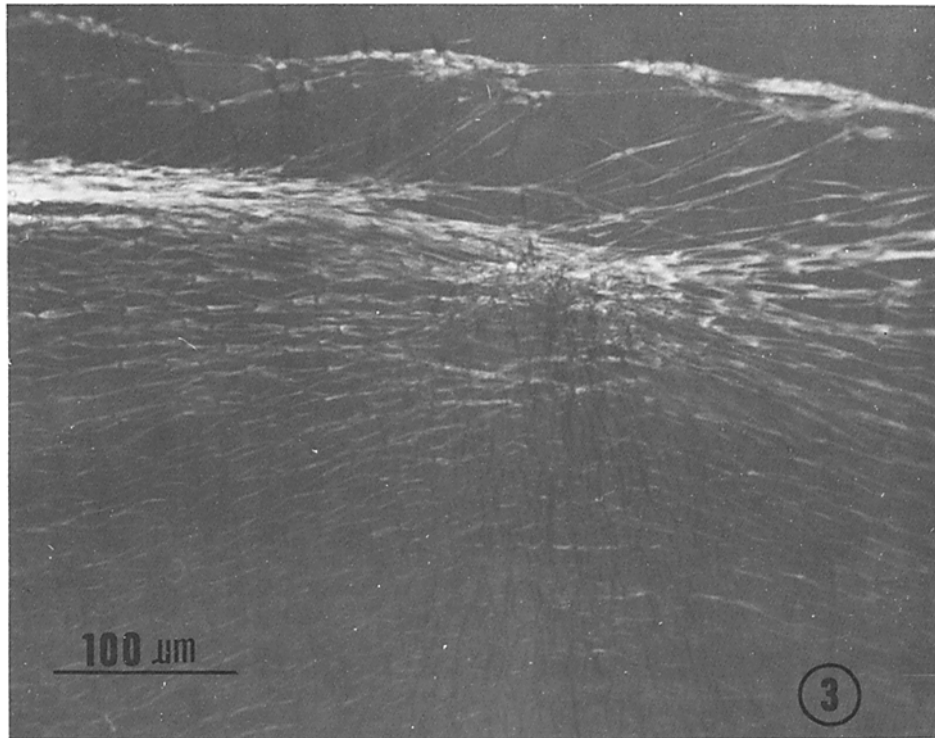


FIGURE 3 Polarized light micrograph of extract I after the addition of the contraction solution. The retardation is ca.  $+185 \text{ \AA}$  with the slow axis parallel to the long axis of the fibrils. Note the opposite contrast at orthogonal directions.  $\times 120$ .

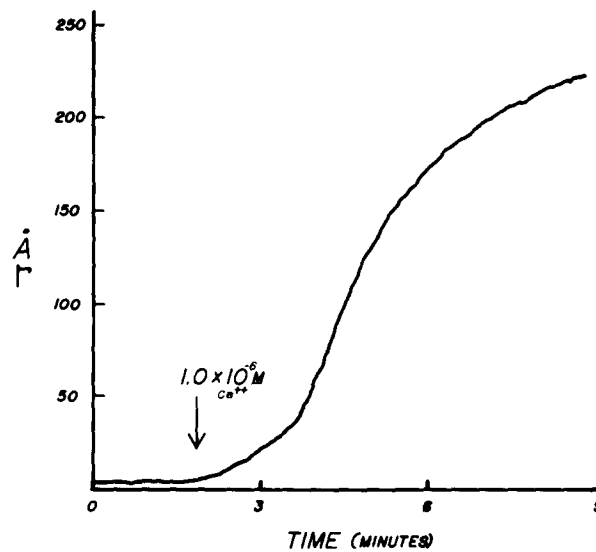


FIGURE 4 Line drawing representing a strip chart recording of the changes in birefringence after the addition of calcium to relaxed extract I. Calcium induced an increase in phase retardation during the contraction.

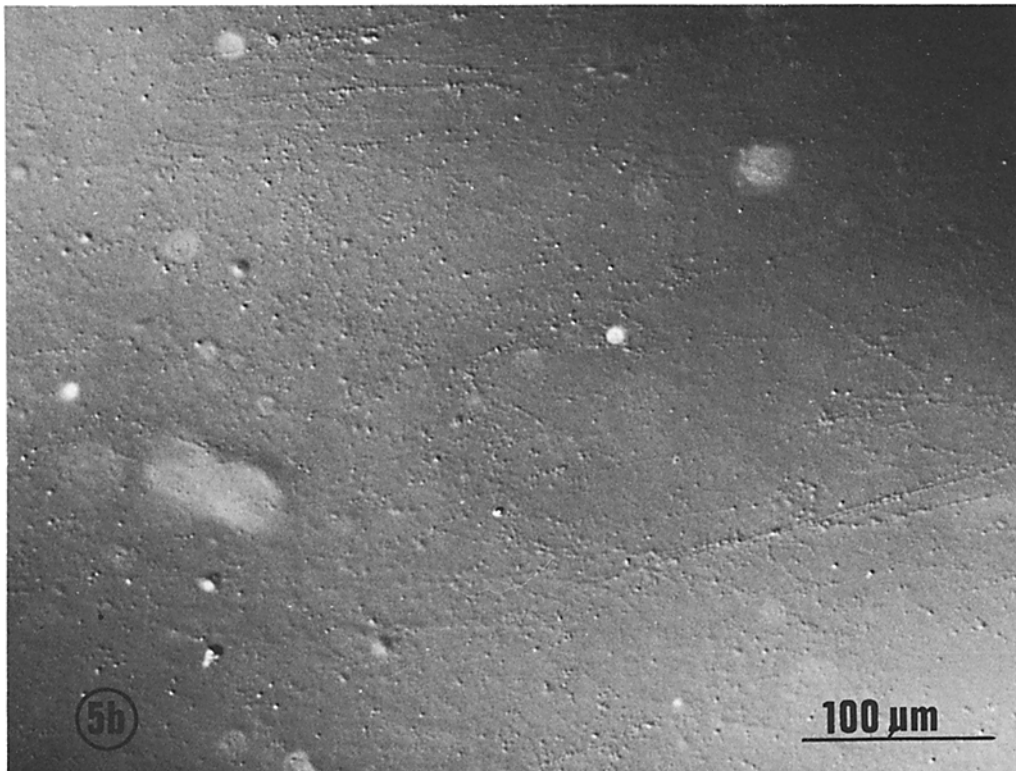
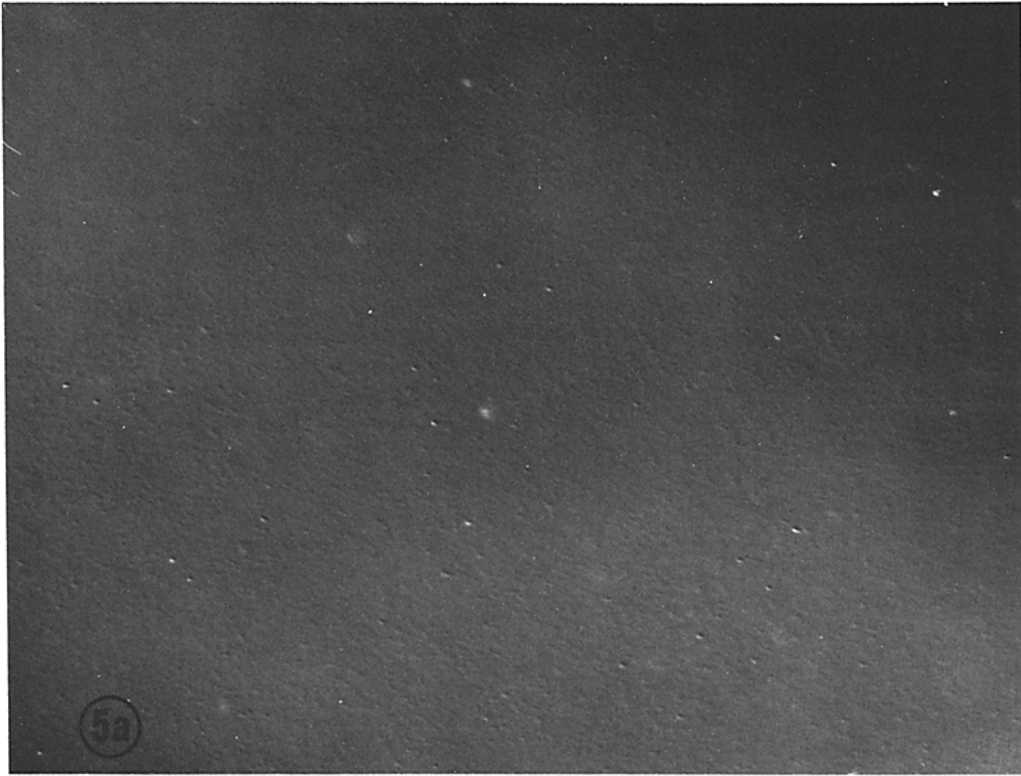


FIGURE 5 Extract II warmed to room temperature. (a) Extract II was nonmotile after the addition of the relaxation solution. (b) Addition of the contraction solution caused the formation of fibrous arrays that did not contract.  $\times 185$ .



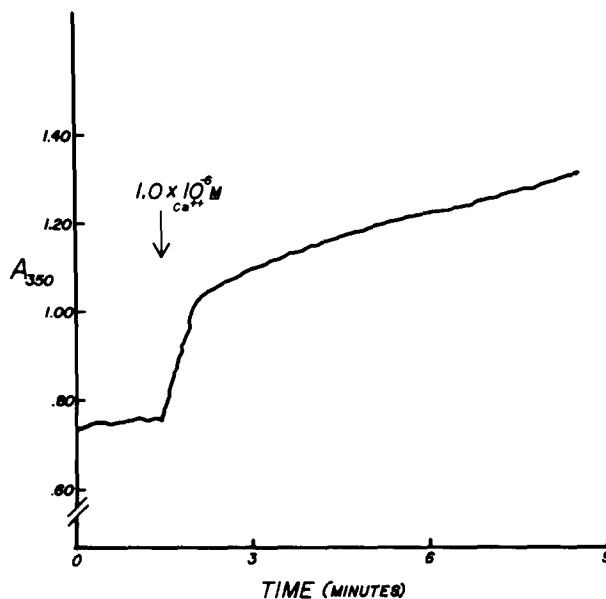


FIGURE 6 A one to one mixture of warmed extract I and relaxation solution exhibited a small increase in turbidity with time. The addition of calcium to a final free calcium ion concentration of ca.  $1.0 \times 10^{-6}$  M, pH 6.8, caused an immediated increase in turbidity. Contracted extract I formed a contracted plug in the bottom of the cuvette.

(Fig. 9 *a*). Some of these fibrils could be labeled with HMM (Fig. 9 *b*). The addition of the contraction solution (Table I) to warmed extract II caused a maximum transformation of actin from the less-organized state to the filamentous state. In addition, under all experimental conditions some  $\sim 100$ -Å filaments of indefinite length were identified that could not be labeled with HMM.

#### *Gel Electrophoresis*

Gel electrophoresis was used to identify the major protein components in the cytoplasmic extracts. Over 30 proteins could be resolved (Fig. 10), of which a protein which comigrated with rabbit skeletal muscle actin was the most abundant in both extract I and extract II. The next most abundant protein in both extracts migrated just above the actin and had a molecular weight of ca. 48,000 daltons. Among the other more prominent bands which were common to both extracts were: one that migrated just above the 48,000-dalton protein, with a molecular weight in the range of 57,000 daltons; proteins that migrated with bands III and IV of human erythrocyte ghost (10); a protein with approximately the same mobility as rabbit skeletal muscle myosin and band 2.1 from erythrocyte ghosts as well as the ca. 225,000 dalton ameba myosin (6). A doublet was present which

comigrated with band I and another doublet which comigrated with band II from erythrocyte ghosts. In addition, a high molecular weight component (ca. 280,000 daltons) was observed in extracts I and II. The relative intensities of the high molecular weight (280,000 daltons) component and the polypeptides comigrating with bands I and II from red blood cell ghosts varied from experiment to experiment. Fig. 10 depicts an experiment in which the 280,000-mol wt component was dominant.

The compositions of extract I and extract II were very similar; only three major proteins were found to decrease in extract II. The largest of these proteins had a mobility between that of bands I and II of erythrocyte ghosts with a molecular weight in the range of 225,000 daltons, which is consistent with the molecular weight determined with purified ameba myosin (6). The second protein, which shows the least decrease of the three major proteins, had a molecular weight of ca. 180,000 daltons. The third protein had a molecular weight of ca. 95,000 daltons (Fig. 10).

#### *Filament Structure in Plasmalemma-Ectoplasm Ghosts*

Cells ruptured in the relaxation solution emptied endoplasm into the observation chamber. The

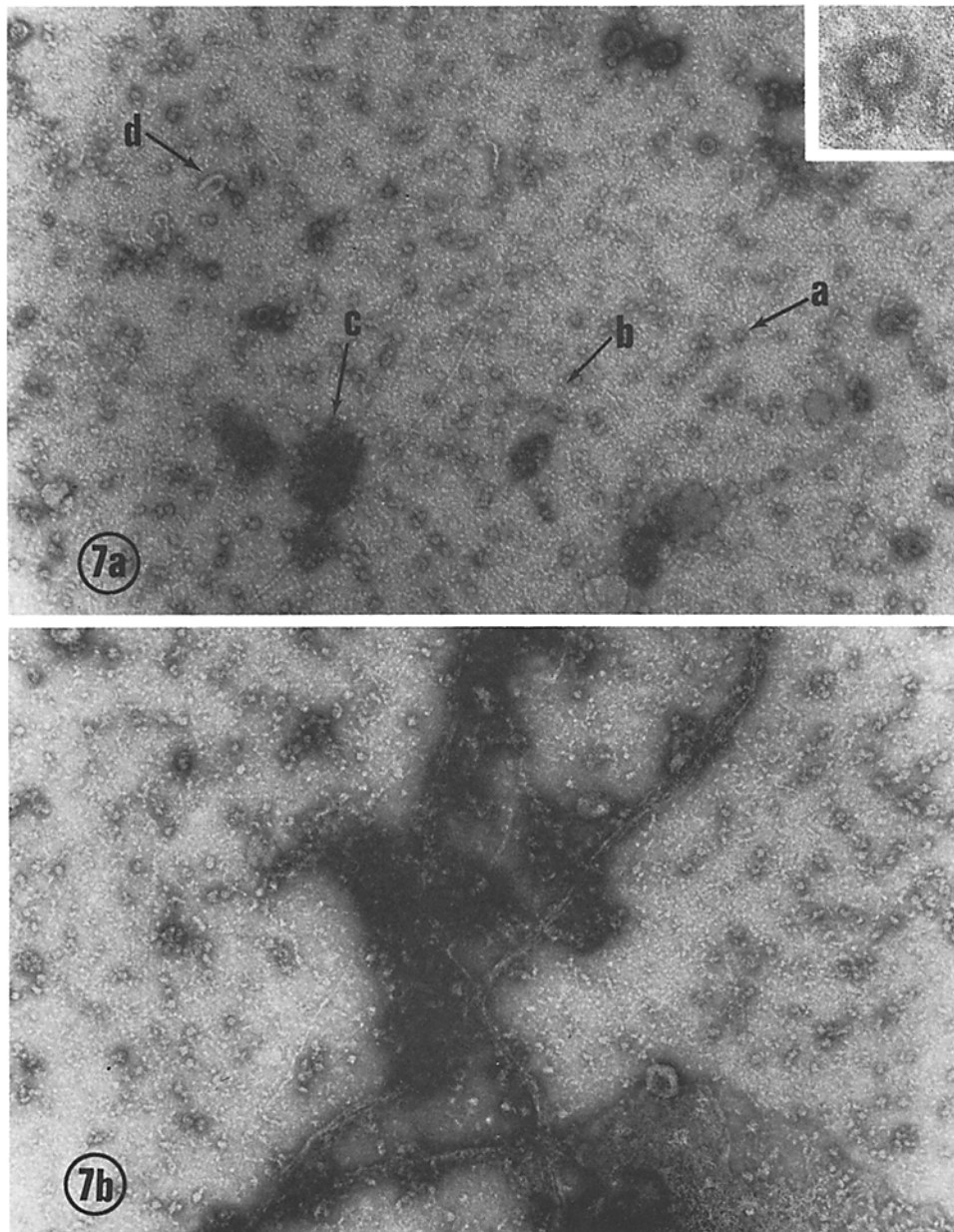
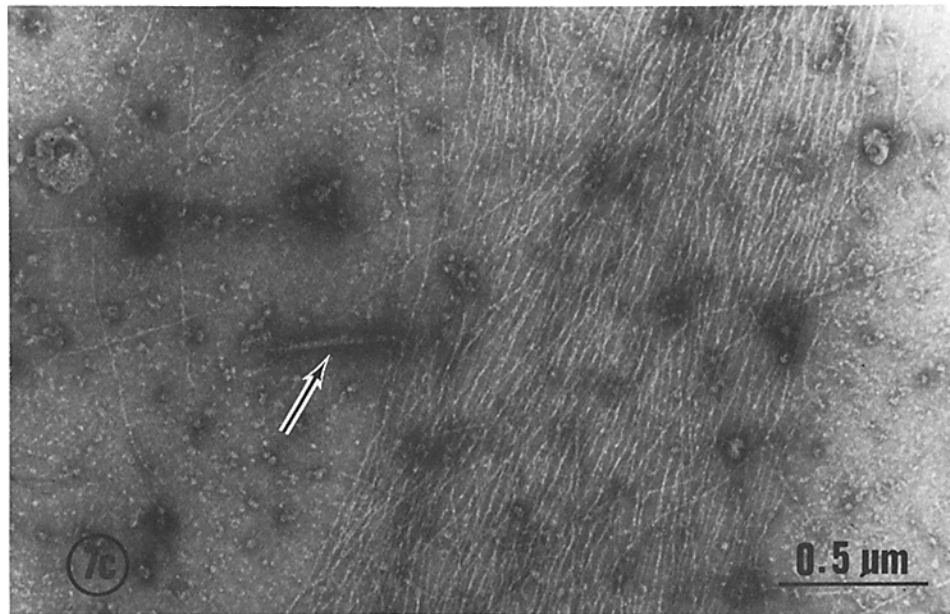


FIGURE 7 Negatively stained preparations of extract I warmed to toom temperature. (a) Extract I contained very few F-actin filaments and myosin aggregates. Many barrel-shaped particles (arrow *a* and *inset*) and doughnut-shaped structures (arrow *b*) (14, 15) were observed in addition to amorphous aggregates (arrow *c*). In addition, many structures resembling Cs (43) were also observed that were 200–500 Å in length (arrow *d*). *Inset* magnification, 100,000. (b) Addition of the relaxation solution caused an increase in the number of filaments that were usually associated with the larger amorphous aggregates. (c) Addition of the contraction solution caused a dramatic increase in the number of F-actin filaments and the appearance of many myosin aggregates (arrow).  $\times 45,000$ .



plasmalemmas which appeared to “constrict” during the emptying process became less wrinkled after a few minutes in the relaxation solution. Thin sections of the cytoplasmic surface of the plasmalemma exhibited relatively few straight F-actin filaments but some less filamentous material (Fig. 11 *a*) which has been shown to contain F-actin after the addition of HMM (5). Furthermore, the surface coat, or glycocalyx, consisted of many relatively straight filaments.

In contrast, the plasmalemmas treated with the contraction solution remained wrinkled. Thin sections of the cytoplasmic surface of the plasmalemmas exhibited massive arrays of straight F-actin filaments (Fig. 11 *b*). Many regions exhibited both actin and thicker filaments which appeared similar to myosin aggregates (33, 23, 7) (Fig. 12 *a*), as well as areas suggestive of a transformation from a less filamentous to a more filamentous state of actin (Fig. 12 *b*). The glycocalyx was much more condensed upon the addition of the contraction solution.

## DISCUSSION

### *Filament Structure and Dynamics in Cytoplasmic Extracts*

Various observations have indicated that actin and possibly myosin might undergo reversible

transformation<sup>2</sup> from a less-structured state into a more-structured filamentous state during the normal process of some cell movements, including amoeboid movement. Thompson and Wolpert (39) and Pollard and Ito (27) described the formation of thin filaments when motile extracts were warmed to room temperature. The thin filaments, which were later identified as actin (28), were found to be cold labile and required heat as well as ATP for formation. In addition, Hinssen (18) demonstrated that nonfilamentous actin, present in *Physarum* endoplasm, could be transformed into actin paracrystals by the addition of high magnesium concentrations.

Myosin aggregates have also been suggested to form from monomers in the cytoplasm. Holberton and Preston (19), and Hinssen and D’Haese (8) demonstrated that myosin-like aggregates formed in glycerinated models of amoebas and *Physarum* actomyosin preparations, respectively, upon the

<sup>2</sup> Transformation is used instead of polymerization, since polymerization suggests the formation of filaments from a homogeneous pool of G-actin subunits. It has not been established whether the filament bundles observed in single cells or cell extracts consist of actin filaments formed directly from G-actin monomers, from the copolymerization of different proteins, or from the lateral association of less structured (less filamentous) actin polymers.

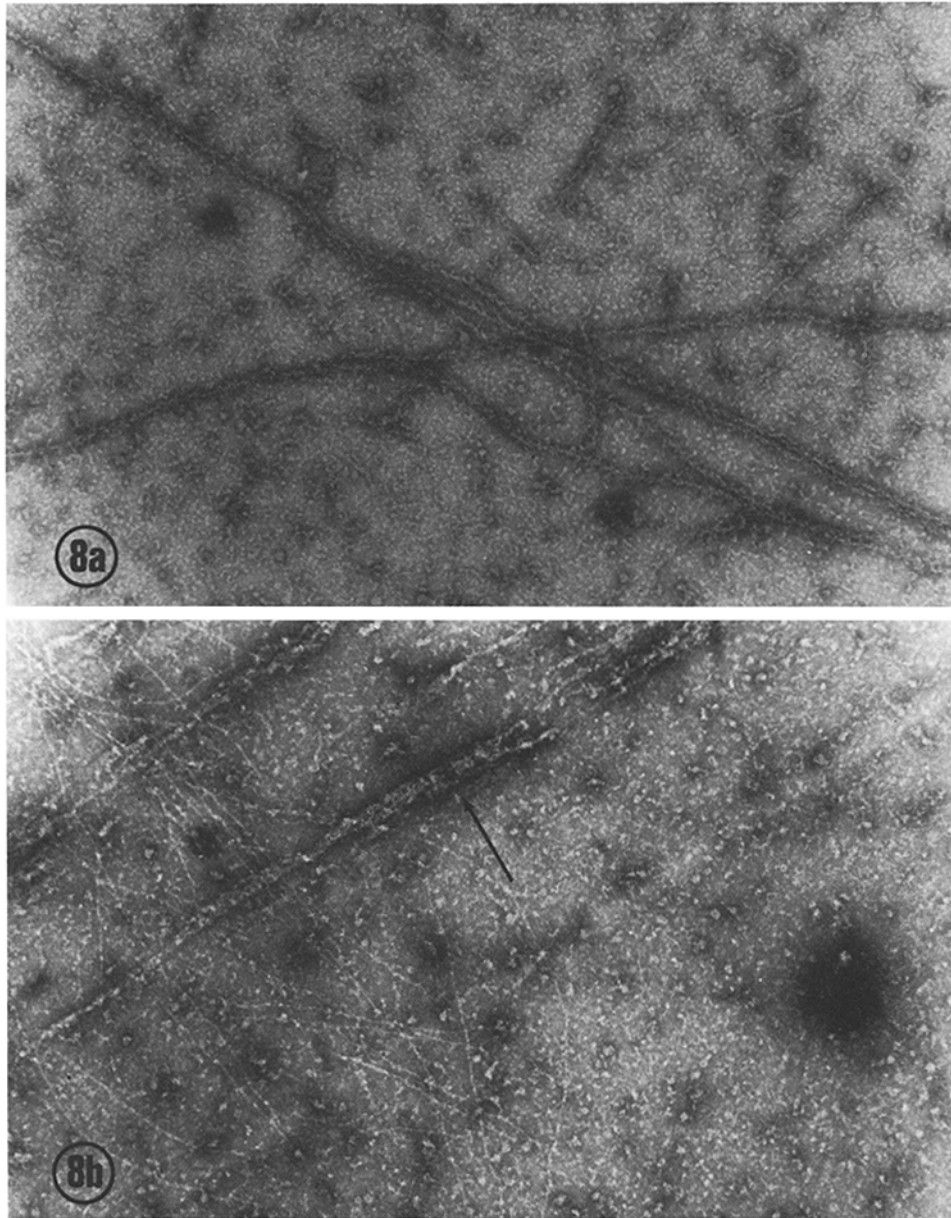
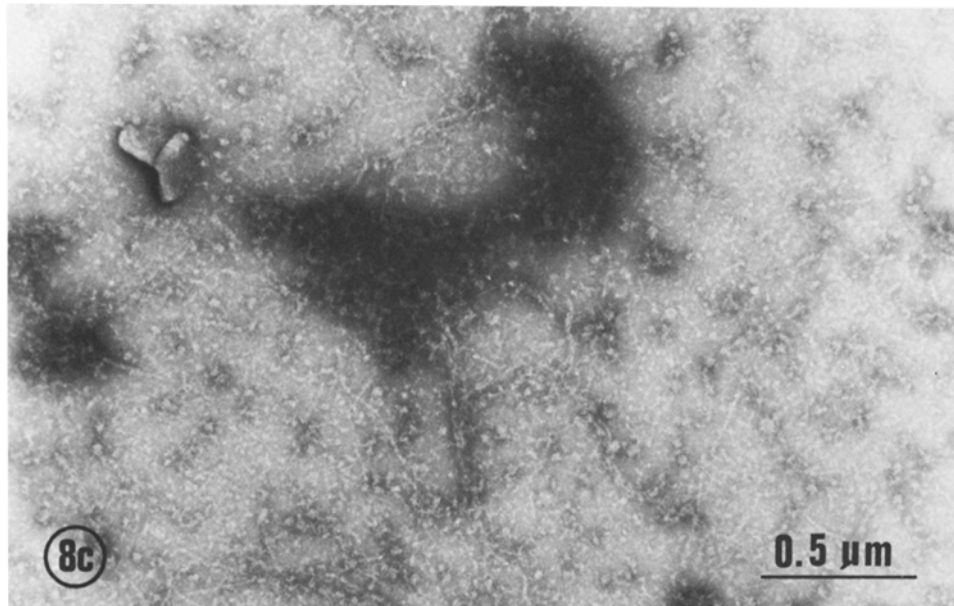


FIGURE 8 Negatively stained preparations of extract II warmed to room temperature. (a) Addition of ca. 4.0 mg/ml rabbit HMM resulted in the appearance of F-actin. (b) The addition of calcium in the absence of exogenous Mg-ATP caused a small increase in the number of fibrous bundles that did not label with HMM (arrow). (c) Addition of the relaxation solution containing 2.0 mM magnesium chloride caused the appearance of actin filaments that appeared to be associated with amorphous aggregates.  $\times 45,000$ .

addition of magnesium and ATP. More recently, it has been shown that primarily actin filaments and also myosin aggregates depolymerized under conditions of low divalent cation concentrations (35,

7). Therefore, reversible transformations from less-filamentous to more-filamentous states might play a role in normal cell movements.

The present results indicate that the addition of



the relaxation solution (Table I) induces the formation of some fibrils that include actin filaments which do not contract; while the contraction solution, specifically a threshold calcium concentration (33) and ATP, induces a rapid formation of F-actin filaments followed by contractions of extract I. The increase in turbidity (Fig. 6) and birefringence (Fig. 4) was correlated with an increase in the number of filaments observed by negative staining (Fig. 7). It is interesting in this regard that the contracted pellet of extract I contained actin and myosin, but showed a dramatic decrease in the amount of ca. 48,000 mol wt, ca. 57,000 mol wt, and ca. 95,000 mol wt proteins and the high molecular weight proteins (ca. 280,000, 240,000, and 220,000 daltons). A 55,000 mol wt and a 95,000 mol wt protein have been identified recently in *Limulus* sperm and apparently bind actin in a coiled state (42). Furthermore, Kane (21) has shown that the removal of actin from a "jelled" extract of marine eggs leaves 58,000-mol wt and 220,000-mol wt components in the supernate which reforms the gel when recombined with actin. Work is now in progress to characterize the major polypeptides in ameba extracts. In addition, some of the similarities between the electrophoretic patterns of red blood cell ghosts and ameba extracts are being investigated, particularly the presence of 48,000 mol wt

and 57,000 mol wt polypeptides in erythrocyte ghosts.

It is known that spectrin (43)<sup>5, 4</sup> and actin-binding protein (32) will bind actin. Therefore, the addition of the contraction solution to extract I under conditions that should support complete actin polymerization might alter the association of actin with one or more of the associated proteins, permitting a transformation from a less-filamentous to a more-filamentous state possibly involving the aggregation of actin filaments. Therefore, at least part of the calcium sensitivity observed in ameba cytoplasm (33, 34) might involve the control of the transformation of actin. This transformation might be similar to the transformation from the Mg polymer to F actin in *Physarum* (17), or the transformation of actin in the acrosomal processes of some sperm (41). It is interesting that greater than ca.  $10^{-4}$  M calcium ion concentrations appear to inhibit cytoplasmic streaming (39). Therefore, calcium could be inhibitory at both low ( $<ca. 10^{-6}$  M) and high ( $>ca. 10^{-4}$  M) free calcium ion concentrations. It has been demonstrated that the calcium ion concentration fluctuates during cytoplasmic streaming (36, 25).

<sup>5</sup>D. Shotten and D. L. Taylor. Unpublished observations.

<sup>4</sup>D. Shotten. Unpublished observations.

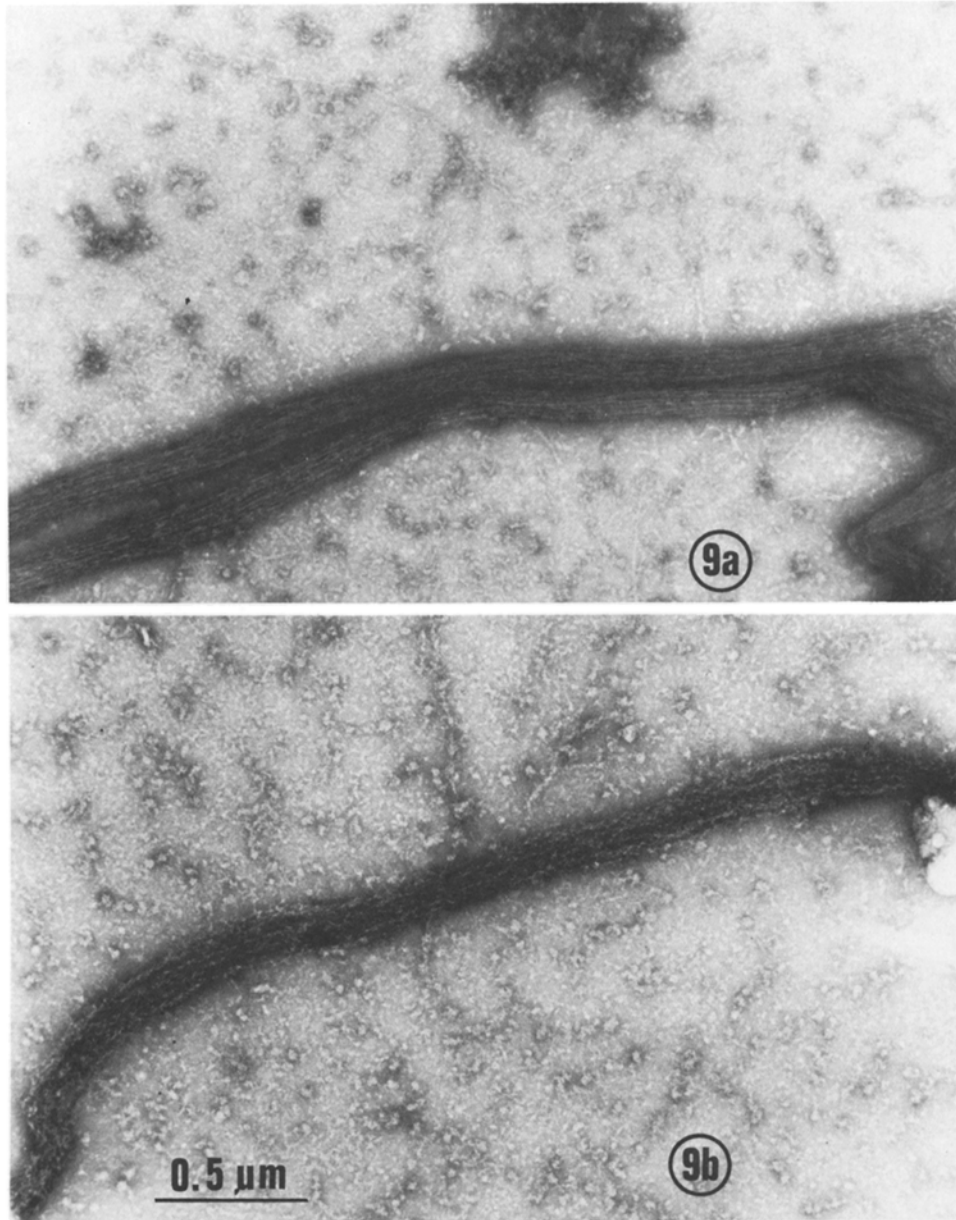


FIGURE 9 Addition of the relaxation solution containing a total of 2.0 mM  $MgCl_2$  also induced the formation of large fibrous bundles containing actin. (a) The fibrils were similar in appearance to the viscoelastic fibrils from single cell extracts (33). (b) Some of the fibrous bundles of thin filaments labeled with HMM.  $\times 45,000$ .

The ameba myosin has a molecular weight of ca. 225,000 daltons (6) and migrates in a position between bands I and II from erythrocyte ghosts (Fig. 10). It is apparent that the ca. 225,000 dalton ameba myosin was pelleted in extract II along with

the ca. 95,000 dalton component (Fig. 10). The loss of ameba myosin from extract II probably accounts for the observed loss of motility in extract II (39, 46, 27).

Preliminary results indicate that the contracted

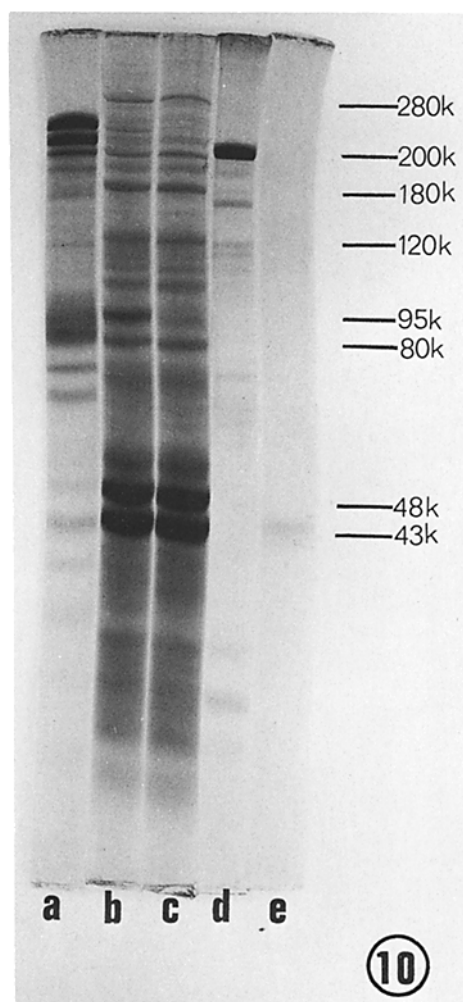


FIGURE 10 SDS gel electrophoresis of (a) erythrocyte ghosts; (b) extract I; (c) extract II; (d) rabbit psoas muscle myosin; and (e) rabbit actin.

pellet that formed after the addition of the contraction solution to extract I contained the ameba myosin (7), actin, the ca. 80,000-dalton protein, and a protein whose mobility was greater than that of rabbit actin (ca. 32,000 daltons). Apparently, the major contractile proteins exclude most of the other proteins during contraction.

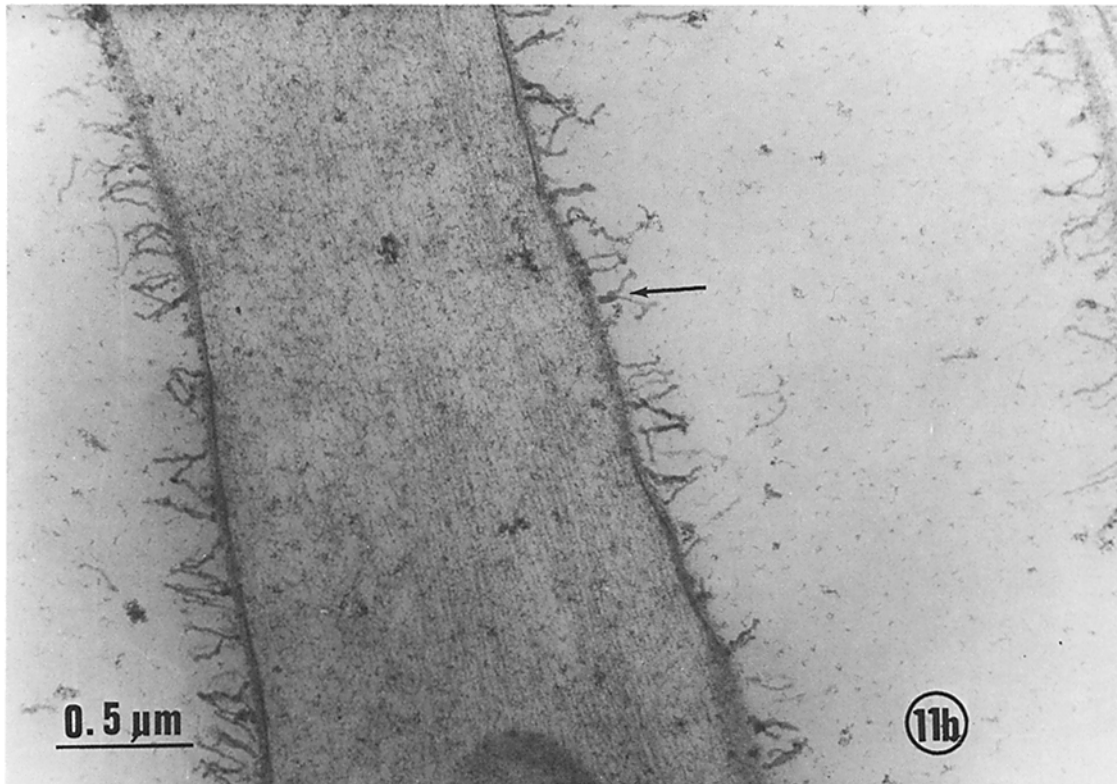
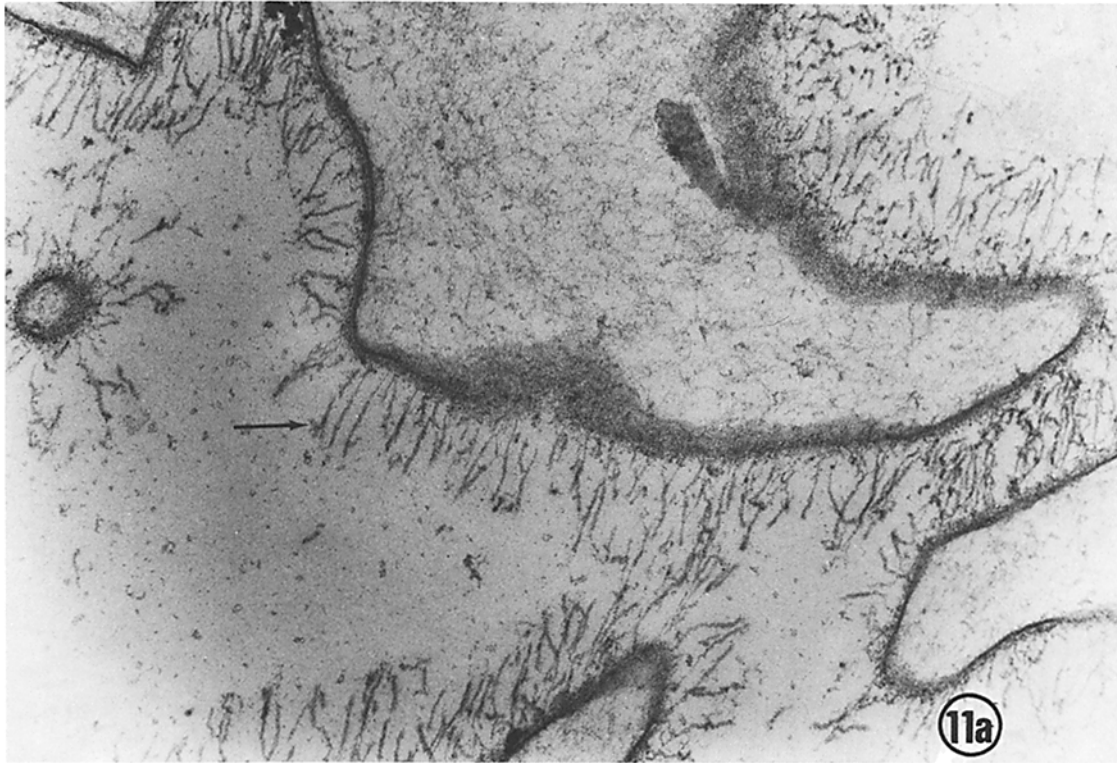
The formation of fibrils in the presence of low magnesium ion, ATP, and just-subthreshold calcium ion concentrations (2.0 mM  $MgCl_2$  relaxation solution, Table I) is probably significant physiologically (Fig. 9). The fibrillar arrays that were observed in the relaxation solution (Table I)

could be similar to some regions of the intact cell. However, a micromolar calcium ion concentration was required to induce contractions. Therefore, the dynamic events in the cell could be separated into two different processes: (a) the formation of a structured cytoskeletal network containing actin filaments involving the transformation of actin to a more filamentous state in the presence of Mg-ATP and a subthreshold (33) calcium ion concentration; and then (b) the contraction of actin and myosin initiated by a threshold calcium ion concentration (33).

Alternatively, in the presence of a threshold calcium ion concentration (33) and Mg-ATP the dynamic events would include the transformation of actin to the more filamentous state followed directly by contractions between actin and myosin. This mechanism is optimal at a slightly lower pH (ca. pH 6.8) and a higher ionic strength (ca.  $0.05 \pm 0.01$ ). Both of these processes probably can occur in the intact cell. Portions of the ectoplasmic tube that are stationary (not shortening) perform a cytoskeletal function for the cell (26). In this state, the actin and myosin network is formed, but the calcium ion concentration would be subthreshold for contraction. However, contractions can be induced by the increase in the free calcium ion concentration to threshold (34, 38). The direct transition from the less-structured, nonmotile state to an actively contracting state can be induced experimentally in the intact cell by microinjecting a threshold calcium ion concentration into the endoplasm (34, 38). The endoplasm contracts in response to the calcium without first forming a temporally separated, nonmotile network of actin, myosin, and any associated proteins. In addition, the ectoplasm, which is a highly structured region of the cell, contracts immediately upon the microinjection of the threshold contraction solution.

The variable effect of pH on the ability of extract I to form fibrous arrays of actin ("gelation") might have physiological relevance. The local pH at the membrane-cytoplasm interface could influence the consistency of cytoplasm *in vivo*, while maintaining a constant ionic strength. A subthreshold calcium ion concentration for contraction and ATP could induce "gelation" of the cytoplasmic extracts at pH 7.2, while much less gelation would occur at pH 6.8. Therefore, transient changes in cellular pH could effect the structural interactions between actin and possibly associated proteins.





**FIGURE 11** Thin sections of ameba plasmalemma-ectoplasm ghosts after removing the endoplasm and incubating in either the relaxation or contraction solutions (Table I). (a) Appearance of the plasmalemma-ectoplasm ghost after a 3-min incubation in the relaxation solution. There are only a few straight F-actin filaments but a large amount of less filamentous actin. Note the extended glycocalyx (arrow). (b) Appearance of the plasmalemma-ectoplasm ghost after a 3-min incubation in the contraction solution. Massive arrays of straight F-actin filaments fill the lumen of the plasmalemma. Note the retracted appearance of the glycocalyx (arrow).  $\times 63,000$ .



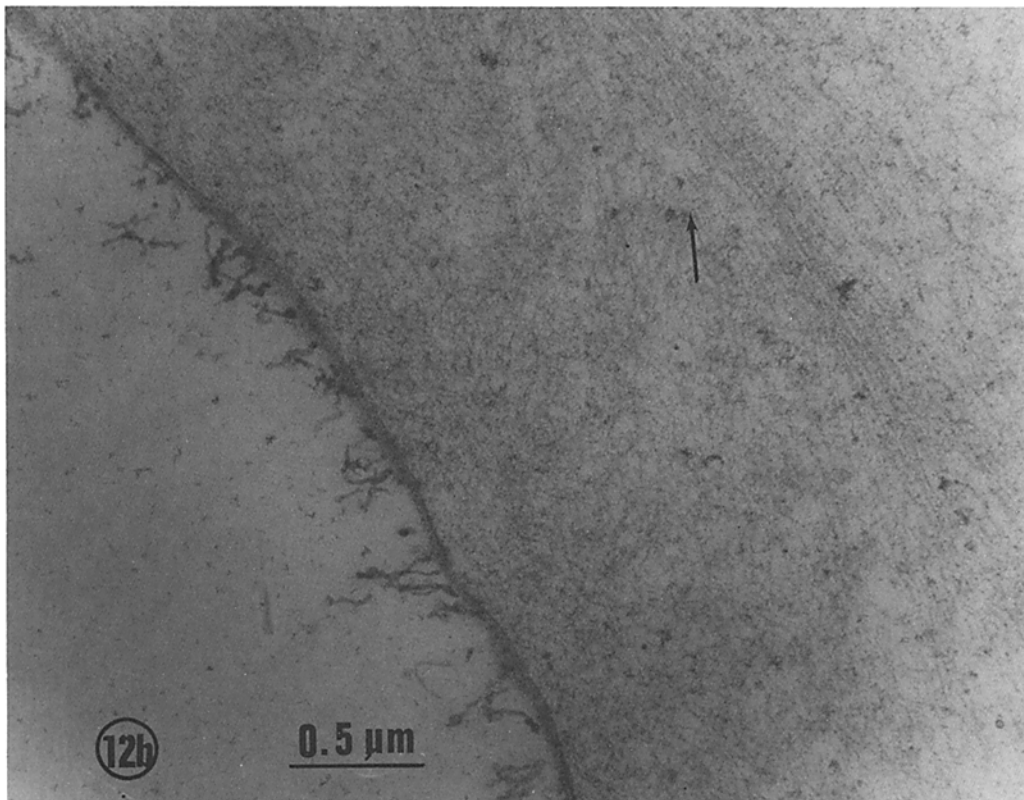
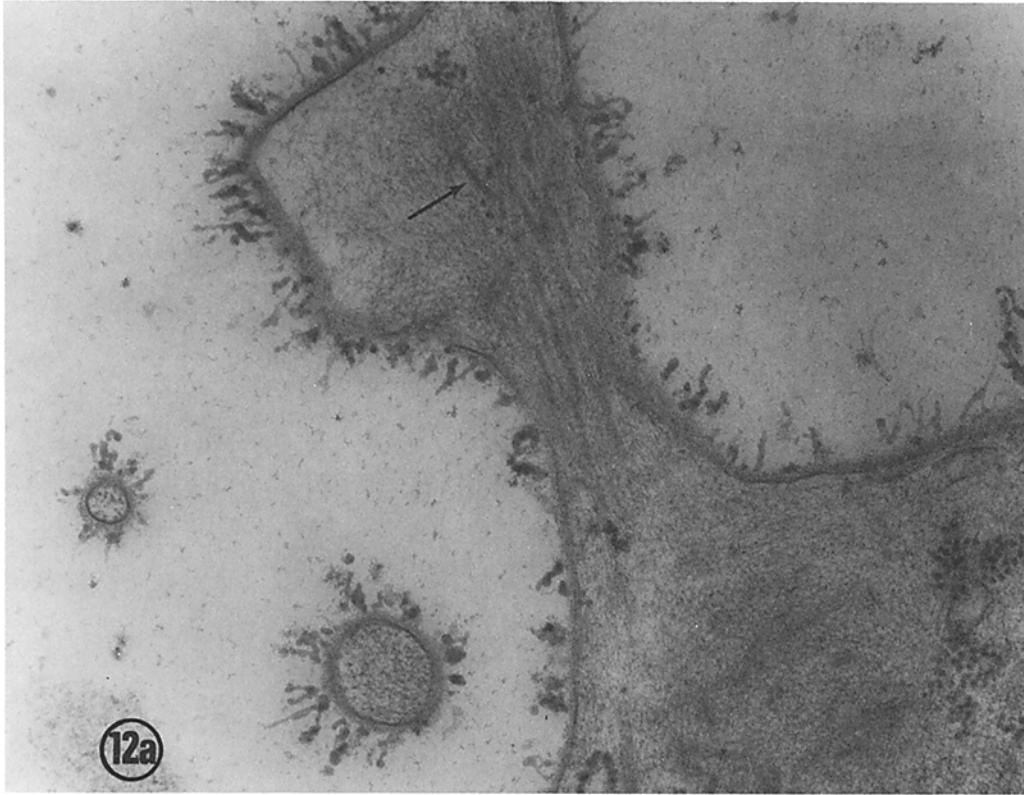


FIGURE 12 The plasmalemma-ectoplasm ghosts contain (a) thick filaments as well as F-actin filaments after the addition of the contraction solution (arrow); (b) some of the "contracted" plasmalemma ectoplasm ghosts exhibit regions where filamentous arrays appear to extend from more amorphous regions (arrow).  $\times 63,000$ .

A relationship exists between the possible pH transients and the calcium ion concentration. Reznikoff and Pollack (29) demonstrated that the microinjection of calcium ions into amebas pretreated with pH indicators caused a decrease in the cytoplasm pH. A similar observation was made on cytoplasmic extracts. When extract I was titrated with calcium chloride to a final free calcium ion concentration of ca.  $10^{-6}$  M, there was a decrease in cytoplasmic pH. Therefore, cytoplasm appears to contain calcium-binding compounds (other than EGTA) that release hydrogen ions upon binding calcium. This effect of calcium on the cytoplasmic pH could be an indirect calcium control of cytoplasmic consistency. It has been shown that a strong calcium current enters *C. carolinensis* in the uroid and that a smaller transient calcium current enters anterior regions during normal cell movements (25). In addition, elevated free calcium ion concentrations have been observed in the uroid and tips of advancing cells when *C. carolinensis* was oriented in an electric field (36). Work is in progress to quantify the cellular pH and to identify any possible pH gradients across the plasmalemma-ectoplasm interface in relation to known calcium gradients.

One of the most abundant structures in the negatively stained preparations of both extracts I and II were the hollow cylinders (barrel-shaped particles) and the doughnut-shaped structures (Fig. 7) which are similar in dimensions to the "particles" and "torus proteins" isolated from erythrocyte ghosts (14, 15).<sup>4</sup> The hollow cylinders observed in extracts from erythrocyte ghosts have been shown to contain a tetramer of the high molecular weight "torus" protein (doughnut-shaped particles). It has been determined that those structures probably exist on the inner surface of the erythrocyte membrane (16). If the hollow cylinder and doughnut-shaped structures in the ameba extracts are similar to the particles from erythrocyte membranes, then they might also be localized on the cytoplasmic surface of the plasmalemma and might be involved in the association of actin with the plasmalemma. No exact function has yet been determined for the ca. 100 Å filaments.

#### *Filament Formation at the Ectoplasm-Plasmalemma Interface*

The ectoplasm-plasmalemma models fixed in the relaxed state (33) exhibited a less filamentous

organization on the cytoplasmic side of the plasmalemma than the contracted models. Apparently, the actin is in a less-filamentous or highly labile state at low calcium ion concentrations (Fig. 11 *a*). However, a threshold calcium concentration (33) in the presence of Mg-ATP maintained or induced the highly filamentous state (Fig. 11 *b*). A transformation from a less-filamentous state to a stable filamentous state of the contractile proteins and possibly associated proteins exists which is regulated by the free calcium ion and ATP concentrations (38). Therefore, part of the ectoplasm which is closely "associated" with the plasmalemma could be in a less-ordered state when not activated by calcium. Upon activation with calcium, the actin could become maximally filamentous, permitting interactions with myosin aggregates. Furthermore, myosin could be in competition with other proteins for actin binding (32), depending on the calcium ion and ATP concentrations, as well as the local pH of the cytoplasm.

#### *Relation of Actin Transformation to the Structure of Cytoplasm*

The plasmalemma-ectoplasm interface appears to be the region that changes dramatically during movement. It is at the cytoplasmic side of the plasmalemma where the endoplasm everts to form the ectoplasm which is readily contractile (34, 38). In addition, the uroid ectoplasm decreases in structure to become part of the endoplasm. Therefore, the plasmalemma with associated proteins is the logical site for the transduction of information from the environment to the cytoplasmic contractile machinery.

The eversion of endoplasm to ectoplasm at the tips of advancing cells could involve a transformation of actin from a less-filamentous state to a more-structured filamentous state possibly involving cross-linking with other proteins, followed by contractions along some unknown distance of the ectoplasm. Monopodial cells would result when the ectoplasm remained highly structured (filamentous) and contractile along the length of the cell. The ectoplasm to endoplasm conversion, primarily in the uroid, would involve dissociation of the contracted actin and myosin ("relaxation") at the inner margin of the ectoplasmic tube, followed by a transformation of actin from the filamentous contractile state to a less-filamentous state (Fig. 13). Polypodial cells would occur when the ectoplasm "relaxed" and contracted in multi-

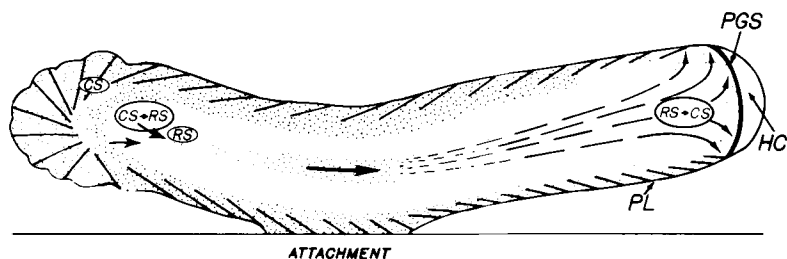


FIGURE 13 Schematic diagram summarizing the structure and contractility in various regions of the amoeba. The region of one ectoplasm-plasmalemma-substrate attachment is indicated. The plasmagel sheet (PGS), hyalin cap (HC), and plasmalemma (PL) are shown. The endoplasm gains in structure (34, 38) and contractility (34, 38) as it approaches the tips of advancing pseudopods. The plasmagel sheet forms by peeling off the cytoplasmic side of the plasmalemma and passes back over the extending pseudopod (34, 38). This region involves a transformation from the relaxed (RS) to the contractile (CS) state (34, 38). The ectoplasm remains in the contractile or contracting state until it is recruited primarily in the uroid. The ectoplasm serves a cytoskeletal as well as a motile function. The ectoplasm decreases in structure probably involving a "relaxation" (33) and a transformation of actin to a less-structured state (CS  $\rightarrow$  RS).

ple regions. The membrane is an important structure for both the attachment of actin and the control of cell movement.

#### *Relationship of Amoeba Motile Models to Other Motile Phenomena*

Many cellular motile processes occur or are initiated at the cytoplasmic side of a membrane. For instance, *Physarum* (20) forms "contractile" bands in the ectoplasm. Cytokinesis (30) involves the transient formation of filaments under the plasmalemma, while the brush border of intestine contains filaments in close proximity to the plasmalemma (24, 40). Furthermore, *Dictyostelium discoideum* (45, 9), a testate amoeba, extends a pseudopod by cytoplasmic streaming, but then rapidly forms a birefringent filamentous array in the ectoplasm just under the plasmalemma that contracts. In addition, platelets (2, 3), acrosomal processes (41), fibroblasts, and many other motile systems involve a rapid formation of filaments. The central process in all of these systems appears to be the transformation of actin from a less-filamentous state to a more-filamentous state. It is possible that the basic mechanisms are the same or very similar. Calcium in the presence of Mg-ATP could initiate the transformation of actin and then contraction where it is involved. Decreasing the calcium concentration would induce relaxation (33) and the reverse transformation. The major difference between cytoplasmic streaming in *Physarum*, *A. proteus*, *Dictyostelium*, and fibroblast movements, as well as other nonmuscle motile processes, could be the localization of the actin transformation and

contraction (if involved) mediated by the plasmalemma. *Physarum*, *A. proteus*, *Dictyostelium*, and fibroblasts, and cells undergoing cytokinesis, etc., represent motile systems in which all of the possible processes occur in a cyclic fashion. Other motile systems such as the platelets (2) utilize both the transformation from less filamentous to more filamentous state and contraction processes, while the formation of some acrosomal processes appears to involve only the rapid transformation of actin to the ordered filamentous state (41). A motile extract from *Dictyostelium discoideum* produced almost identical results as the *A. proteus* extracts (37).<sup>5</sup>

Work is now in progress to identify the complete, physiological control of filament formation and dynamics in intact cells and motile extracts and to identify the proteins involved in the cytoskeletal and contractile processes. The goal of these investigations is the reconstruction of a calcium-sensitive motile model prepared from purified proteins.

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