THE CONTRACTILE BASIS OF AMOEBOID MOVEMENT

I. The Chemical Control of Motility in Isolated Cytoplasm

D. L. TAYLOR, J. S. CONDEELIS, P. L. MOORE, and R. D. ALLEN

From the Department of Biological Sciences, State University of New York at Albany, Albany, New York 12203

ABSTRACT

Cytoplasm has been isolated from single amoeba (*Chaos carolinensis*) in physiological solutions similar to rigor, contraction, and relaxation solutions designed to control the contractile state of vertebrate striated muscle. Contractions of the isolated cytoplasm are elicited by free calcium ion concentrations above ca. 7.0×10^{-7} M. Amoeba cytoplasmic contractility has been cycled repeatedly through stabilized (rigor), contracted, and relaxed states by manipulating the exogenous free calcium and ATP concentrations. The transition from stabilized state to relaxed state was characterized by a loss of viscoelasticity which was monitored as changes in the capacity of the cytoplasm to exhibit strain birefringence when stretched. When the stabilized cytoplasm was stretched, birefringent fibrils were observed. Thin sections of those fibrils showed thick (150–250 Å) and thin (70 Å) filaments aligned parallel to the long axis of fibrils visible with the light microscope. Negatively stained cytoplasm treated with relaxation solution showed dissociated thick and thin filaments morphologically identical with myosin aggregates and purified actin, respectively, from vertebrate striated muscle. In the presence of threshold buffered free calcium, ATP, and magnesium ions, controlled localized contractions caused membraneless pseudopodia to extend into the solution from the cytoplasmic mass. These experiments shed new light on the contractile basis of cytoplasmic streaming and pseudopod extension, the chemical control of contractility in the amoeba cytoplasm, the site of application of the motive force for amoeboid movement, and the nature of the rheological transformations associated with the circulation of cytoplasm in intact amoeba.

INTRODUCTION

There is now almost general agreement that amoeboid movement is based on contractility and that the cytoplasm undergoes a contraction in one region of the cell. There is still some disagreement regarding the region that contracts, and there are at present only partially substantiated hypotheses regarding the mechanisms of contractility and pseudopod formation and how these processes may be related. The evidence favoring a contraction occurring at pseudopod tips has been reviewed recently by Allen (1). Other authors have preferred to reinterpret new data in the light of older theories (2–4).

As in the study of muscle contraction (5), progress can be expected to be hastened by the availability of model experimental systems less complex than a living intact amoeba. The first successful attempt at the preservation of motility in

a preparation of demembranated cytoplasm was the isolation of "naked cytoplasm" in quartz capillaries (6). Other model systems developed subsequently have helped to elucidate the mechanisms of cytoplasmic streaming and contractility in amoeba. For example, Simard-Duquesne and Couillard showed that glycerinated amoebae could be prepared that would undergo limited contraction on the addition of ATP and magnesium ions (7). Amoeba cytoplasm was shown by them to possess a calcium-activated ATPase activity resembling the ATPase of vertebrate striated muscle myosin (8).

Bulk contractility and unorganized streaming activity have been observed in preparations of pooled cytoplasm from *Amoeba proteus* by Thompson and Wolpert (9). They also were the first to fractionate pooled cytoplasm and to demonstrate the presence of thin filaments in a motile fraction. Pollard and Ito (10) improved the fractionation procedure and demonstrated the presence of both thick and thin filaments in a fraction capable of movement and viscosity changes on the addition of ATP. Motility was depressed by chilling during isolation and observed only in the presence of ATP and on warming to room temperature. However, the manner of movement in this pooled cytoplasm was different from that in intact amoebae. Instead of streaming into pseudopodia, the pooled cytoplasm underwent a massive contraction in which its material shifted in one direction.[1]

The thick and thin filaments found in motile extracts of cytoplasm correspond to those observed in electron micrographs of fixed intact amoebae by Nachmias (11, 12). The 70-Å filaments have been identified as actin by heavy meromyosin binding in *Amoeba proteus* (13) and in *Chaos carolinensis* (14). The thick filaments have not as yet been characterized biochemically. Although ATPase activity has been found in amoebae (8), there is no evidence that it is associated with the thick filaments.

For some years the need for a more physiological cytoplasmic model system has been recognized. The original naked cytoplasm preparations (6) exhibited extended viability but were not ideal for physiological experiments because of the capillary required as a container. Griffin (15) showed that *Chaos* cytoplasm could stream briefly in a limitied quantity of distilled water between a slide and cover glass, presumably because the cytoplasmic contents were diluted only minimally. Gicquaud and Couillard (16) carried this approach a step further by designing a physiological solution in which the motility of *A. proteus* cytoplasm could be extended for 2–15 min. However, their medium lacked the necessary conditions for the complete chemical control of motility.

We have now succeeded in isolating cytoplasm in a lifelike, functional condition from single specimens of *Chaos carolinensis* by rupturing the plasmalemma mechanically in chemically defined, physiological media in which both motility and rheological state can be controlled. The pH, osmolarity, ionic strength, and K^+/Na^+ ratio were chosen to resemble, as much as possible, conditions described in intact amoebae.

MATERIALS AND METHODS

Culture of Amoeba

Specimens of *Chaos carolinensis* were cultured in Marshall's medium (5×10^{-5} M $MgSO_4$, 5×10^{-4} M $CaCl_2$, 1.47×10^{-4} M K_2HPO_4, and 1.1×10^{-4} M KH_2PO_4 in demineralized water) with mixed ciliates for food. Some specimens were subjected to centrifugation and bisection with a glass microneedle to reduce the number of light-scattering inclusions. Only vigorously moving specimens were used in the experiments reported.

Solutions

The solutions used to isolate, stabilize, and control the motility of amoeba cytoplasm are presented in Table I. The were based in large part upon studies on the control of contractility in muscle (17–23). In addition, they reflect published information regarding virtually all of the physiological parameters that might be expected to influence cytoplasmic contractility. The pH, osmolarity, ionic strength, potassium/sodium ratio, and divalent cation concentrations have been taken into account (24–28). Furthermore, each of these factors was varied experimentally over a considerable range to find optimal conditions for the preservation of distinct contractile and rheological states.

Hexokinase solutions were used to study the role of endogenous ATP in contraction. Stabilized cytoplasm (Table I) was treated with test solutions containing various combinations of ATP and divalent cations, both directly and after pretreatment with hexokinase solution (0.5 mM $MgCl_2$, 5.0 mM glu-

[1] T. D. Pollard kindly supplied us with a copy of his film of movements in amoeba extracts (1970).

TABLE I

Solutions Used to Isolate, Stabilize, and Control the Motility of Amoeba Cytoplasm

	Stabilization solution	Contraction solution	Relaxation solution	Flare solution
pH	7.0	7.0	7.0	7.0
mosM	85–100	85–100	85–100	85–100
Ionic strength* (±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
Disodium ATP mM‡	0	0	1.0	0.5
KCl mM	27.0	27.0	27.0	27.0
NaCl mM	3.0	3.0	3.0	3.0
$CaCl_2$ mM	0	4.5	0	4.2
$MgCl_2$§	0	0	0	0.5
Free Ca^{++}	$<10^{-7}$ M	ca. 1.0×10^{-6} M	$<10^{-7}$ M	ca. 7.0×10^{-7} M

* The ionic strength that best preserved the fibrils and consistency in these solutions was 0.05. However, fibrils that were capable of calcium-elicited contractions were visible in an ionic strength range of 0.04–0.12.

‡ Exogenous ATP is not required for contractions of short duration. Presumably it is bound to the contractile elements and does not diffuse away (see Table II). Exogenous ATP is required only for prolonged flare streaming.

§ Exogenous magnesium ions are not essential either for the production of the rheological states or for contractions of short duration (see Table II). Endogenous magnesium is found in the millimolarity range in amoeba cytoplasm (28). Exogenous magnesium is required only for prolonged flare streaming.

cose, and 15.2 U/ml of hexokinase [Sigma Chemical Co., St. Louis, Mo.]), and both with and without added 0.5 mM KCN. Control preparations were rinsed an equal number of times with stabilization solution (Table I).

Isolation of Cytoplasm

At the low calcium concentrations present in stabilization solution, the plasmalemma of *Chaos* was found to become fragile after a few minutes and could be ruptured with a micropipette 50–100 μm in diameter. The cytoplasm either was allowed to flow out of the ruptured cell or was withdrawn with the micropipette by applying a negative pressure of a few centimeters of water with a manometer. The cytoplasm then slowly spread out over the glass surface of the perfusion chamber, which consisted of a slide and coverglass separated by two nontoxic silicone rubber spacers designed to permit steady flow of solutions through the field of the microscope.

The cytoplasm adhered strongly to the glass slide and permitted the removal of the empty plasmalemma. In experiments that studied contraction and relaxation from the stabilized state, the cytoplasm was oriented with a micropipette controlled by a rack and pinion micromanipulator (Fig. 1).

Solutions in the perfusion chamber were exchanged by introducing test solutions at one end of the chamber and drawing them through with filter paper strips. When individual cytoplasmic fibrils were studied, the solutions were injected into their immediate vicinity with a micropipette. The flow rate of the injected solution was controlled with a manometer.

Some experiments were performed with the cytoplasm in glass tubes to maintain a greater degree of mechanical stability of the cytoplasm during the perfusion of test solutions. Intact amoebae were introduced into the top of a Pasteur pipette (drawn out to a diameter of ca. 100 μm) filled with stabilization solution. The amoeba was forced through the length of the pipette by applying pressure to the solution. The amoeba was demembranated as it approached the end of the tapered pipette. The pipette was broken around the cytoplasmic plug between the isolated cytoplasm and the empty plasmalemma. The pipette section containing the cytoplasm was then placed in the perfusion chamber and the test solutions were introduced into the perfusion chamber as above.

Light Microscope Observations and Measurements

The experiments on isolated cytoplasm were observed with Zeiss strain-free polarized light optics, Nikon rectified polarized light optics, Zeiss Nomarski differential interference, and by Zeiss Jamin-Lebedeff interference microscopy.

Experiments in which isolated cytoplasm was caused to contract and/or stream by treatment with the solutions in Table I were observed directly and recorded on films, selected portions of which were analyzed frame by frame with a Vanguard motion analyzer, Vanguard Instrument Co., Roosevelt, N. Y.). The rates of contraction of fibrils were measured by recording the rate of displacement of particles attached to the fibrils by both frame by frame analysis of selected films and by direct measurement with a calibrated ocular micrometer and stopwatch.

Viscoelasticity where present was also measured quantitatively as stretch-induced strain birefringence monitored by the same automatic, null-seeking photoelectric birefringence detection system (29) employed by Francis and Allen (30) to observe viscoelastic behavior of amoeba endoplasm *in situ*.

Stabilized cytoplasm was stretched at +45° to the axis of transmission of the polarizer. A microbeam of green light (546 nm) was defined by a circular aperture brought to focus in the plane of the cytoplasm. The diameter of the measuring beam at focus was 100 μm, sufficient to illuminate only the cytoplasm. Constant loads were applied to the cytoplasm through a micropipette using a manometer adjusted to a negative pressure of 6 cm of water.

Electron Microscopy

Isolated cytoplasm was fixed by perfusing 3% glutaraldehyde solution buffered to pH 7.0 with 50 mM cacodylate buffer. Specimens were postosmicated 1 h in 1.0% OsO_4, pH 7.0, dehydrated in an ethanol series, and embedded in Spurr's resin. Cured blocks were oriented and trimmed in a LKB pyramitome, and thin sectioned on a Reichert OMU-2 ultramicrotome. Sections were picked up on Formvar-coated grids, stained in 1% uranyl acetate, followed by a lead citrate stain. The method for negative staining was based on that of Huxley (31) but modified for experiments on single cells (32).

Observations were made with an AEI-EM6B electron microscope with an accelerating voltage of 60 kV. A condenser aperture of 250 μm and an objective aperture of 50 μm were used. For all electronmicrographs the electron microscope was calibrated, at the time of use with a no. 1002 cross-ruled optical grating replica (Ernest F. Fullum, Inc., Schenectady, N.Y.).

RESULTS

Control of Contractility

Stabilization solution, relaxation solution, and contraction solution (Table I) successfully control contractility in isolated amoeba cytoplasm. When

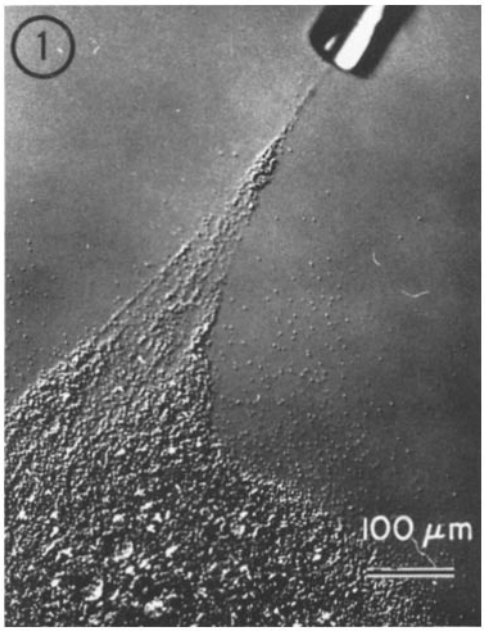

FIGURE 1 Stabilized state cytoplasm stretched with a micropipette demonstrating fibrils. × 120.

placed in stabilizing solution (Table I) containing no exogenous calcium or ATP, amoebae withdraw their pseudopodia, or cytoplasmic streaming is depressed, and the plasmalemma either breaks spontaneously or can be ruptured easily with a micropipette. The cytoplasm flows slowly out of the ruptured membrane and becomes stabilized in a lifelike, viscoelastic condition in which no motility is displayed and numerous fibrils can be observed, especially when the cytoplasm is stretched (Fig. 1). The fibrils are characteristically 0.1–0.4 μm in diameter and are of indefinite length. The cytoplasm remains in this stabilized state for at least 10–20 min or until the chemical composition of the solution is altered.

When stabilized cytoplasm was stretched, its birefringence increased (Fig. 2), and upon release the cytoplasm recoiled elastically. The viscoelasticity was characterized by monitoring the changes in retardation due to strain birefringence. The birefringence of stabilized cytoplasm increased when tension was applied up to the fracture point of the constituent fibrils or when the load was released (Fig. 3). The increase in birefringence under constant load is shown in Fig. 3, along with discontinuities caused by breakage of individual fibrils as they reached their respective fracture points. Fig. 3 is a continuous

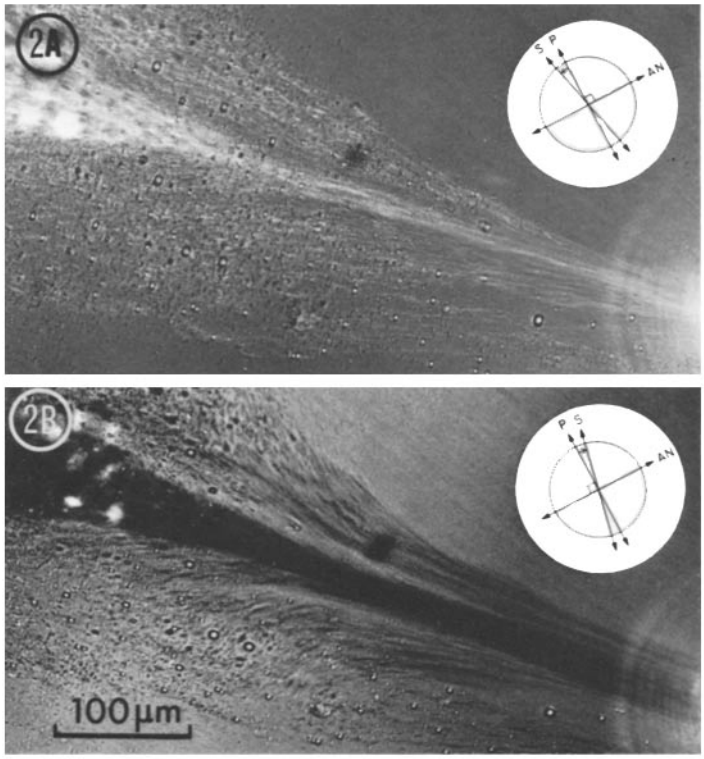

FIGURE 2 Two polarization micrographs of stabilized cytoplasm being stretched by drawing it into a pipette. (A) positive compensation, and (B) negative compensation micrographs photographed a few seconds apart. The retardation is ca. +20 Å with the slow axis parallel to the fibrils. (S) specimen, (P) plane of polarizer, (AN) plane of analyzer. × 185.

pen recording which is representative of many experiments. When the cytoplasm was unloaded the retardation did not return to the original value, but it exhibited a residual positive retardation (positive deformation).

The contraction solution differs from stabilization solution principally in its calcium concentration. When applied to stretched stabilized cytoplasm, it produced directional contractions of the visible fibrils. The diameters of these fibrils inincreased as they shortened to a small fraction of their original length. These contracting fibrils developed tension, since portions of broken glass rods attached to the fibrils were transported during contractions. In an unoriented mass of stabilized cytoplasm, where distinct fibrils were usually not seen, isodiametric contraction occurred as in glycerinated amoeba models (7).

The refractive index of the cytoplasm underwent an obvious increase during contraction. This change was comparable to that observed in intact *Chaos* endoplasm as it becomes everted to form the ectoplasmic tube (33, 34).

The presence of free calcium ions is required for contraction, whereas other free cations at physiological concentrations do not elicit contractions. Contractions were also observed in freshly prepared stabilized cytoplasm on the addition of a simple calcium salt solution in the absence of exogenous magnesium and ATP. When calcium buffer solutions with free calcium ion concentrations between 10^{-8} and 10^{-5} M were applied to stabilized cytoplasm, the threshold for contraction was found to be ca. 7.0×10^{-7} M. The rate of contraction increased with calcium concentration as shown in Fig. 4. The time-course of contraction of a single fibril contracting toward its point of attachment to the glass slide in the presence of 1.0×10^{-3} M free calcium ion is shown in Fig. 5. The rate of contraction decreased noticeably as the fibril shortened beyond one-half of its rest length.

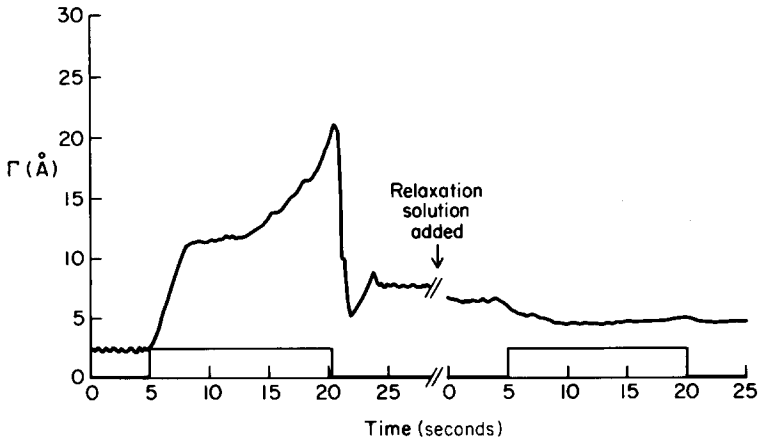

FIGURE 3 Photoelectrically measured changes in retardation because of birefringence on stretching stabilized cytoplasm by drawing it into a micropipette under constant negative pressure (6 cm of H_2O). The increased birefringence fell off sharply when the deforming force was removed, but some birefringence remained. When the same experiment was performed after washing with relaxing solution, no increase in birefringence was observed. The decrease observed is because of a diminishing amount of oriented material as it is pulled apart. (Γ) gamma retardation.

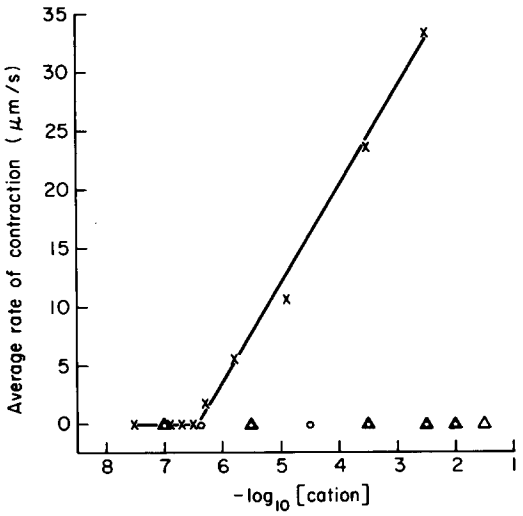

FIGURE 4 Average velocity of contraction in a population of fibrils. The data were obtained using frame by frame ciné analysis of the velocity of displacement of individual particles attached to the fibrils. (X-X) calcium, (O-O) magnesium, (△-△) potassium and sodium.

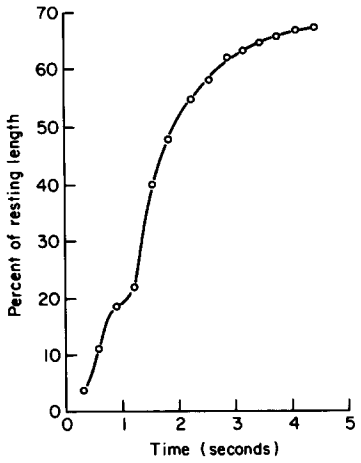

FIGURE 5 Time-course of shortening as percent of the resting length of a single fibril contracting in the presence of 1.0×10^{-3} M free calcium ion. The average rate of contraction of this fibril is 30.5 μm/s as described in Fig. 4.

Stabilized cytoplasm treated with relaxation solution gradually lost its viscoelasticity and birefringence (Fig. 3). The visible fibrils became very plastic and neither cohered nor fractured when drawn out. The relaxed state could be reversed by the addition of stabilizing solution. The addition of magnesium salt to the relaxation solution in Table I was unnecessary for the relaxation of amoeba cytoplasm. Thus, for simplicity, the relaxation solution without magnesium salt (Table I) was adopted as the standard relaxation solution for all the experiments reported in this paper.

The ionic strength that best preserved the fibrils and consistency in these solutions was 0.05. However, functional cytoplasm was observed in an ionic strength range of 0.04–0.12.

The visible fibrils could be caused to contract,

relax, and contract again by carefully injecting the required solutions with a micropipette into the vicinity of the cytoplasm. Successful cycling required the use of minimal calcium concentrations (ca. 7×10^{-7} M); otherwise, the fibrils contracted rapidly and irreversibly.

The isolated cytoplasm could also be converted from one state to another inside a glass micropipette. If the micropipette was placed in the perfusion chamber and the solutions were gradually exchanged, the mechanical and chemical integrity of the preparation were preserved to a greater extent than in naked cytoplasm in the perfusion chamber. In the pipette, cyclic changes of state could be performed for up to 4 h at room temperature.

When stabilized cytoplasm was incubated in the hexokinase solution for 5 min, the rates of contraction were shown to be dependent not only on calcium concentration (Fig. 4), but on the presence of magnesium and ATP. Calcium, magnesium, and ATP caused contractions eight times more rapid than calcium and ATP alone (Table II). The values in Table II are the average of 10 experiments.

TABLE II

Test solution	Average velocity of contraction μm/s	
	Untreated stabilized cytoplasm	Stabilized cytoplasm after hexokinase treatment
0.5 mM Ca^{++}	23.0	5.5
0.5 mM ATP	0	0
0.5 mM Ca^{++} + 0.5 mM ATP	20.0	3.0
0.5 mM Mg^{++}	0	0
0.5 mM Mg^{++} + 0.5 mM ATP	0	0
0.5 mM Ca^{++} + 0.5 mM Mg^{++} + 0.5 mM ATP	22.0	25.0

Stabilized cytoplasm was treated with test solutions containing various combinations of ATP and divalent cations, both directly and after pretreatment with standard hexokinase solution (0.5 mM $MgCl_2$, 5.0 mM glucose, and 15.2 U/ml of hexokinase) and both with and without added 0.5 mM KCN which did not affect the results. Velocity of contraction was measured as in Fig. 4.

Electron Microscopy

The ultrastructural basis for the viscoelastic properties of stabilized cytoplasm has been explored both by negative staining procedures (32) and by thin sectioning of oriented fibrils in cytoplasm isolated from single amoebae (Fig. 6). The fibrils visible in the light microscope consist of bundles of numerous 70-Å thin filaments with 150–250-Å thick filaments and a few smaller, uncharacterized ca. 45-Å filaments (Fig. 6 *a* and *b*).

At the ultrastructural level, relaxation solution caused separation of the filaments and permitted a more detailed view of the thick filaments (Fig. 6 *c*). In higher resolution, negatively stained electron microscope images, the morphology of the thick filaments from *Chaos* was found to be identical with that of myosin aggregates from vertebrate striated muscle and from *Physarum* (35, 36). Upon relaxation, the cytoplasmic fibrils dissociated into individual myosin aggregates and actin filaments (Fig. 6 *c*).

Naked Pseudopodium Formation and Locomotion

The "flare solution" containing ATP, magnesium ions, and threshold calcium caused naked pseudopodia to extend from isolated cytoplasm (Fig. 7). In flare solution, freshly isolated cytoplasm retained its ability to stream and form pseudopodium-like structures. There was a direct correlation between the motility of isolated cytoplasm in flare solution and the motility of the intact cell from which the cytoplasm was isolated. The most active preparations were always obtained from the most actively moving amoebae, regardless of their nutritional state. Neither the exogenous magnesium nor the exogenous ATP in the flare solution was required for short-term streaming. However, the combination of magnesium and ATP was found to increase the duration of motility by a factor of 10. In flare solution, the peripheral layer of cytoplasm came into contact with the solution and a contraction was elicited in such a way that loop-shaped pseudopodia up to several hundred micrometers in length erupted from the surface of the cytoplasm (Fig: 7). The local application of flare solution from a micropipette at a restricted area of stabilized cytoplasm also caused localized flare formation. Similarly, cytopalsm could be transferred to flare solution from

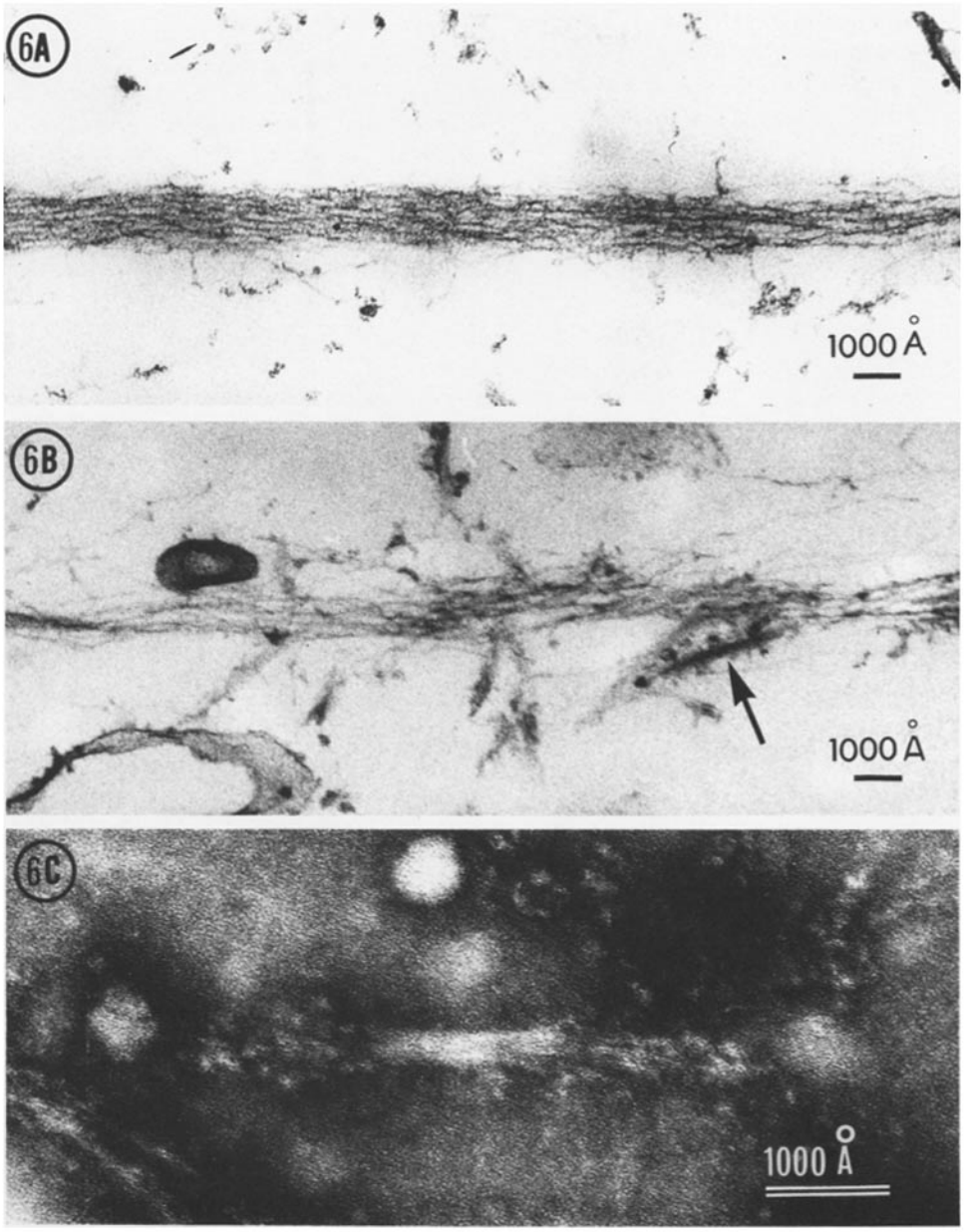

FIGURE 6 Longitudinal sections of stabilized state fibrils demonstrating: (A) The approximately parallel arrangement of the ca. 70 Å thin filaments within the fibril. × 65,110. (B) The presence of thick filaments (arrow) within the fibril. × 65,100. (C) This electronmicrograph shows a negatively stained myosin-like aggregate seen after relaxing stabilized state cytoplasm. The aggregate is ca. 0.5 μm long, has a bare central region 0.1–0.2 μm long, and irregular projections at either end. × 165,000.

stabilizing solution and exhibit flare pseudopod formation.

The pattern of streaming in intact pseudopodia varies from a symmetrical fountain pattern through differing degrees of asymmetry to its extreme, a loop pattern (6). Previous studies on isolated cytoplasm have demonstrated the breakdown of the fountain pattern into loops (6). Isolated cytoplasm

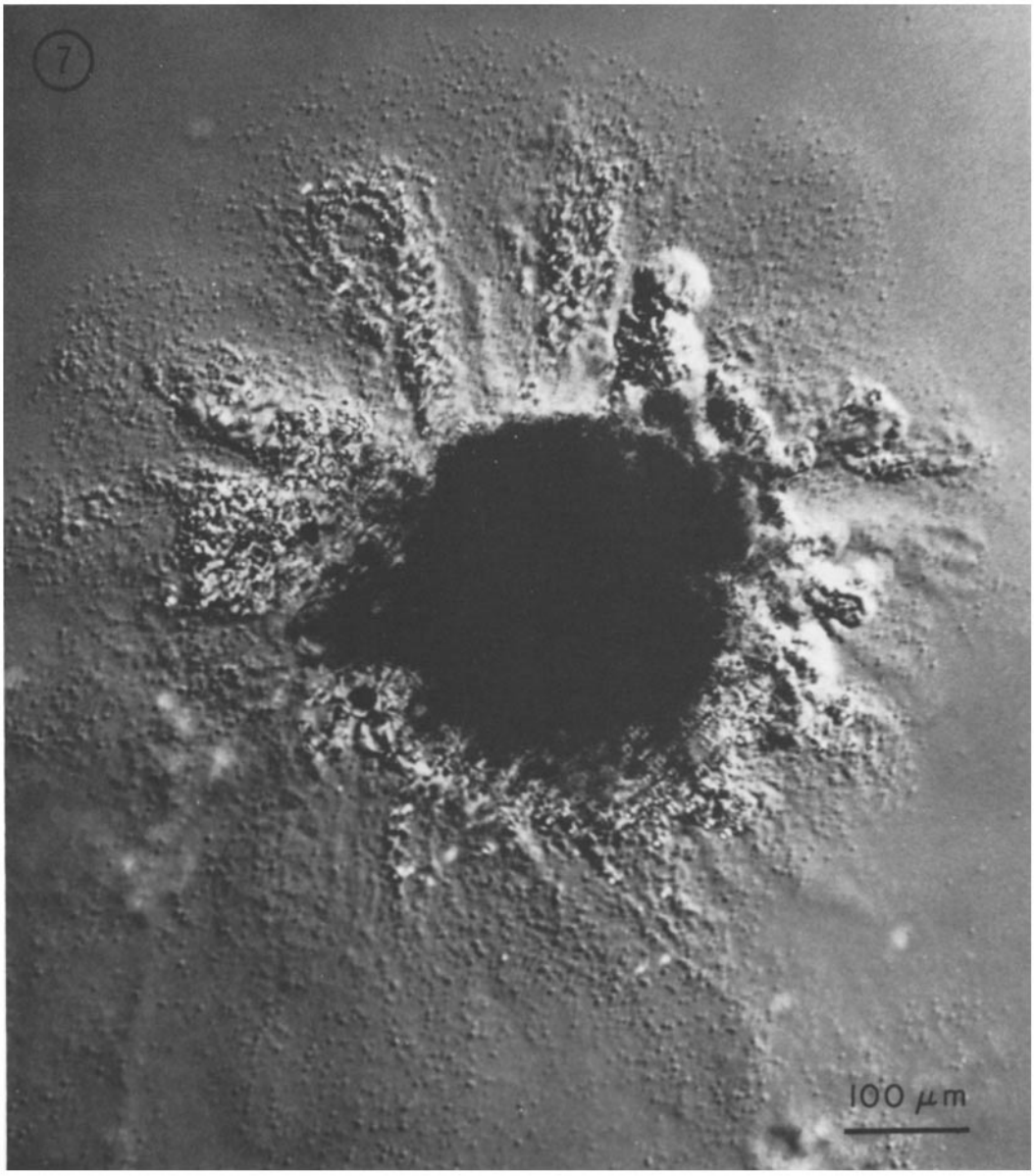

FIGURE 7 A naked cytoplasmic droplet from the giant amoeba, *Chaos carolinensis*, in flare solution from which pseudopodia extend into the solution. × 170.

in flare solution more often streams in the loop pattern, presumably because the cytoplasm is not confined (Fig. 8).

At the concentration of free calcium given in Table I for the flare solution, the velocity of outward cytoplasmic streaming averaged ca. 30 μm/s (range 19–43 μm/s). When the cytoplasm reached the tip of the flare, it contracted on turning, underwent an increase in refractive index, and then either remained stationary or migrated back into the droplet much less rapidly (0–12 μm/s). Such "flare streaming" persisted for up to 10 min.

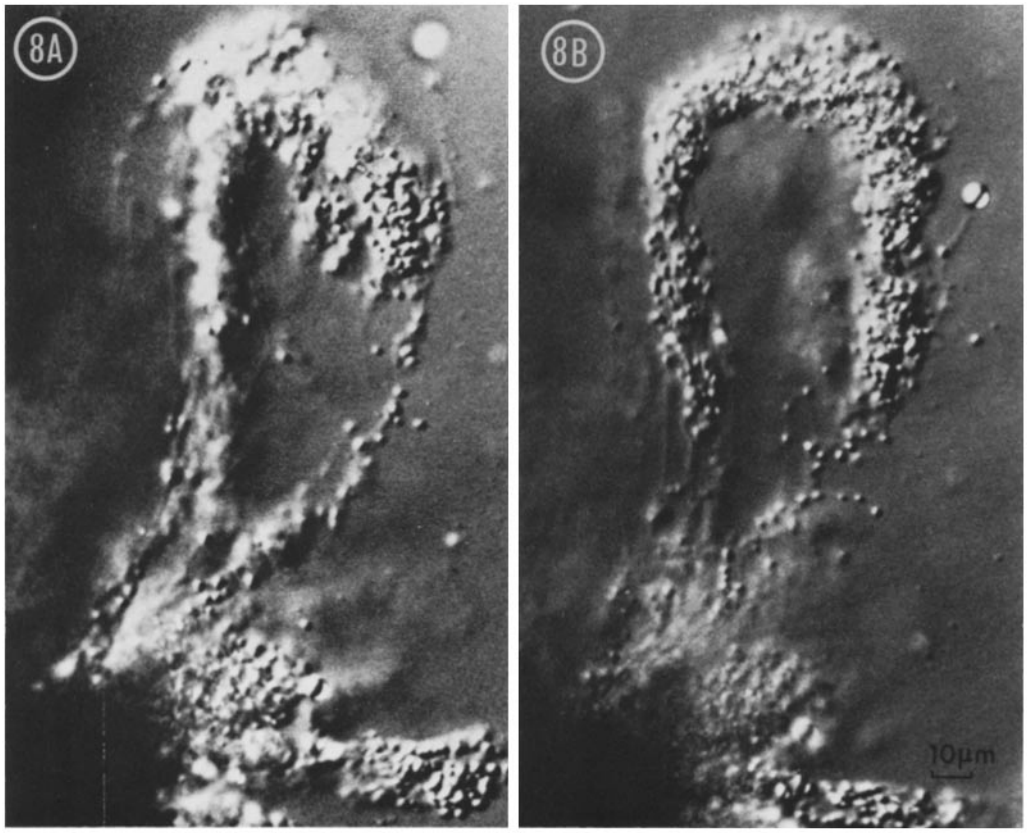

FIGURE 8 A loop streaming in a plane parallel to the glass substratum in flare solution. These two micrographs (A and B) are of the same loop taken a few seconds apart. The cytoplasm which is capable of supporting tension is initially pulled out by a contraction at the bend where the cytoplasm thickens and increases in refractive index. × 540.

Although ATP is required for long-term streaming, changing the ATP concentration over a range of 0.1 mM-1.0 mM had no effect on the velocity of streaming.

Locomotion of isolated cytoplasm often occurred when the returning cytoplasm in a flare-streaming loop attached temporarily to the substratum. The extent of locomotion was often several millimeters over a few minutes.

If the free calcium concentration was increased within a factor of two, the rate of streaming increased dramatically but lasted for a shorter time. Above 1.0×10^{-6} M free calcium, the naked pseudopodia contracted into the cytoplasmic mass.

The volume of cytoplasm in these experiments was always less than 1/1000th that of the solution. The concentrations of calcium and magnesium in the cytoplasm are not precisely known. However, the results of experiments showed a dependence of the duration of motility on the concentrations of calcium, magnesium, and ATP.

The absence of an external membrane surrounding the cytoplasm in the stabilized, relaxed, and flare states has been established with confidence on the basis of the following observations. Neither a steep gradient in optical path nor a birefringent surface could be detected under optical conditions adequate for the detection of unit membrane. In addition, not only could cytoplasmic "threads" be drawn away from the fabric of the isolated cytoplasm with a micropipette (Fig. 1), but the dye eosin Y penetrated the cytoplasmic mass

within seconds while minutes were required for penetration into intact amoebae.

DISCUSSION

Advantages of Isolated Cytoplasm as a Model System

Isolated cytoplasm prepared by the present method has several advantages over other model systems: (*a*) No extreme mechanical or chemical pretreatment (such as centrifugation, glycerination, or chemical skinning by detergents or enzymes) is required; (*b*) the chemical environment can be controlled in order to investigate the conditions necessary for contractility and streaming; (*c*) the physical integrity and, to some extent, the rheological consistency of cytoplasm are maintained; (*d*) contractility and the ability to form organized pseudopodial extensions are preserved. A qualitative indication of the success of this approach is that high quality preservation of cytoplasmic organelles is achieved in both light and electron micrographs.

The stabilization solution (Table I) constitutes a "rigor solution" for amoeba cytoplasm because it lacks ATP and free calcium ions. Furthermore, it is buffered to maintain the free calcium concentration below the threshold concentration for contraction.

The contraction solution differs from stabilization solution principally in the presence of free calcium ions (ca. 10^{-6} M). The increase in ionic strength because of added calcium is insignificant, as shown by control experiments with added monovalent cations. Short-term contraction does not require exogenous ATP. The availability of endogenous ATP in isolated cytoplasm is suggested by the results of experiments with hexokinase. The relaxation solution differs from stabilization solution principally by the presence of 1 mM disodium ATP.

Cytoplasmic Contractility

It is now apparent that isolated amoeba cytoplasm can be induced to exist in rigor (stabilized), relaxed, and contracted states by solutions similar to those used to control contractility in vertebrate striated muscle (17, 18, 37).

The rigor (stabilized) and relaxed states can be distinguished in amoeba cytoplasm by criteria similar to those employed in the study of vertebrate striated muscle. On passing from rigor to the relaxed state, muscle becomes highly extensible because of the loss of cross-linking between actin and myosin. That a similar transition occurs in amoeba cytoplasm is demonstrated in Fig. 3. There is an increase in retardation in the stabilized cytoplasm under a constant load and an instantaneous decrease in retardation when the load is removed. These viscoelastic properties of stabilized (rigor) cytoplasm can be accounted for by crossbridges between the actin and myosin filaments. This argument is supported by the loss viscoelasticity of the cytoplasm in the presence of relaxation solution which should break cross bridges between actin and myosin (Fig. 3).

Marked changes in rheological properties such as are observed in the cytoplasm of amoebae could conceivably be caused by the polymerization and depolymerization of either or both types of filaments as was inferred from the viscometric experiments of Pollard and Ito (10) on pooled amoeba cytoplasm. However, a more likely explanation for cyclic rheological changes in the intact cell would be control exercised by endogenous free calcium ions and ATP on the interaction of actin and myosin in the intact cell.

The evidence that contraction is calcium dependent and that the rate of contraction is proportional to the calcium concentration makes calcium the most probable control ion. Vesicles believed to constitute part of a calcium sequestering system have recently been described (38).

The observed increase in the rate of contraction of fibrils with increase in calcium concentration is thought to be because of the increase in the number of calcium-activated filaments interacting. Thus, a saturation for calcium activation is not observed, since calcium ions are not delivered to every contractile unit simultaneously as in vertebrate striated muscle.

The rapid decrease in the shortening rate of the fibril during the last one-half of the contraction described in Fig. 5 is believed to be because of the compression and disorientation of the filaments. This disorientation may be analogous to the "supercontraction" of muscle. The inability to relax fibrils contracted into a mass supports this suggestion.

The contraction of stabilized cytoplasm with the addition of buffered calcium salt solutions indicates that an energy source and any necessary cofactors for contraction remained available to the

contractile elements. The hexokinase experiments shown in Table II suggest the requirement of both magnesium and ATP for maximum rates of contraction. These results indicate that both ATP and magnesium ions are contained in freshly isolated stabilized cytoplasm. Since the volume of the isolated cytoplasm is about 1/1000th that of the solution, it is reasonable to assume that these substances may be bound to some elements in the cytoplasm.

Ultrastructure

The identity of the thin filaments as actin or actin-like has already been indicated by the heavy meromyosin binding studies of Pollard and Korn (13) for *Amoeba proteus* and of Comly (14) for *Chaos*. The thick filaments have remained unidentified from electron micrographs of thin sections although several authors have speculated that they might be myosin. The negative staining results indicate the presence of myosin aggregates. While positive identification of these structures as myosin aggregates requires biochemical confirmation, it is interesting to note that *Chaos* is the first cellular primitive motile system in which such presumptive myosin aggregates have been documented.

Site of Application of the Motive Force

The details of streaming in flares permit us to draw conclusions about how the motive force for this movement is applied. The flare solution was designed to test the prediction that localized contraction in a viscoelastic cytoplasm would result in stable patterns of cytoplasmic streaming.

Because the cytoplasm does not flow out through any kind of tube or other confining structure, the flow could not conceivably be caused by a pressure gradient. Because the "arms" of the cytoplasmic loops are often not contiguous, the force cannot be generated by any lateral interaction (or "active shear") between the outward and inward flowing masses. Therefore, the type of active shearing postulated to operate in *Nitella* (39) or *Allogromia* (40) could not account for pseudopod extension and streaming in *Chaos*. These results appear to invalidate the recent suggestion of an active shearing model for amoeboid movement (41).

The ratio of streaming velocities in the outward and inward streaming arms of the loops and the increase in refractive index at the tips of the loops demonstrate that the site of contraction occurs at the bend (Fig. 8). The site of change of refractive index and the ratio of streaming velocities in the flare streaming model are identical to the measurements made on intact cells (33, 34). Therefore, the flare loops are believed to represent a component of the "fountain" pattern described by Allen (42).

Flare streaming can be prolonged only by the addition of both ATP and magnesium ions. Increasing the ATP concentration over a range of 0.1 mM–1.0 mM had no effect on the velocity or mode of streaming. However, since the ATP and mangesium ion concentrations cannot be controlled rigorously in these whole cytoplasm preparations, the complete understanding of the role of ATP and magnesium ions awaits further biochemical analysis.

When the free calcium ion concentration is increased above 10^{-6} M, the naked flare pseudopodia contract rapidly into the cytoplasmic mass. This result is expected if enough free calcium ions are suddenly made available to activate a large portion of the contractile elements in the cytoplasm.

The fact that different types of motility can be induced in isolated amoeba cytoplasm merely by manipulating the free calcium ion concentration suggests the possibility that different modes of locomotion in related primitive motile systems might be attributable to differences in the control system which varies the free calcium ion concentration. For example, rapid pseudopod retraction, which occurs infrequently on strong mechanical or electrical stimulation in *Chaos*, could be because of massive release of calcium from storage in vesicular elements (38). Similarly, forcible contraction of pseudopodia in *Difflugia* might be because of a calcium control system capable of releasing amounts of calcium sufficient to cause a forcible contraction of a highly organized bundle of contractile elements (43). Eckert[2] has demonstrated the presence of numerous vesicles along the entire length of the microfilament bundles believed to be responsible for rapid contraction of *Difflugia* pseudopodia.

The velocity of streaming in intact cells is variable over a considerable range and very sensitive to changes in environmental conditions. It is

[2] B. Eckert. 1973. Pattern organization of thick and thin microfilaments in contracting pseudopodia of *Difflugia*. In preparation.

now possible to infer that at least two factors play a role in determining the velocity of streaming. One is the rate of shortening of the cytoplasm and the other is the degree of shortening. From our results it appears that free calcium ions affect both of these variables. A likely third factor is viscous or viscoelastic resistance in which the concentration of ATP very likely also plays a role.

Role of the Plasmalemma

The plasmalemma with its attached glycocalyx has long been recognized to represent both the permeability barrier of the amoeba and the structure by means of which the cell normally attaches to its substratum. The plasmalemma has also been postulated to play a role in supposed ectoplasmic contraction (44), in generating a gradient in bioelectric potential that might cause streaming (45), and in controlling movement through local surface charges (46).

It is now evident that pseudopodia can form in the absence of a plasmalemma or other limiting membrane. Furthermore, cytoplasmic contraction and relaxation can be induced by controlling the calcium and ATP concentration without the presence of a limiting membrane. Thus, whatever ATPase may be involved in motility does not reside in the plasmalemma. It is more likely that the thick filaments are myosin and that ATPase activity resides there. However, this remains to be demonstrated biochemically.

It has long been known that a "surface precipitation reaction" occurs whenever naked cytoplasm comes into contact with a medium containing free calcium ions (47). We have observed such a reaction in experiments in which calcium salts are added to stabilized cytoplasm. However, the membrane formation observed was always incomplete and neither interfered with nor was necessary for contraction.

While much remains to be learned about the role of the plasmalemma in amoeboid movement, it appears not to be required for cytoplasmic streaming into naked flare pseudopodia which are frequently deployed in a manner remarkably similar to the deployment of intact pseudopodia. Thus, some of the suggested roles for the plasmalemma can probably be discarded. One important role must be emphasized: amoeboid locomotion can occur only if the ectoplasm is anchored to the substratum. It is local attachment of the plasmalemma to the ectoplasm on the one hand and to the substratum on the other that provides this anchor. Isolated cytoplasm can show amoeboid locomotion only when the returning cytoplasm (ectoplasm) of a loop adheres directly to the slide.

Question of the Reality of Amoeba Fibrils

The idea that amoeboid movement is caused by the action of cytoplasmic fibrils can be traced back to observations on fixed cells during the 19th century (cf. de Bruyn [48] for review). The question of reality versus artifact has always been present wherever fixation or experimental manipulation is involved in determinations of cytoplasmic structure. In the present experiments on stabilized cytoplasm the fibrils are sometimes visible as soon as the cytoplasm pours out of the broken cell membrane. They are made more easily visible in cytoplasm oriented by stretching. This does not necessarily mean that they are not artifacts, for either the stabilizing solution and/or mechanical deformation might cause their formation by lateral aggregation, under stress, of previously oriented submicroscopic linear elements (actin and myosin). It is tempting to suggest that factors internal to the amoeba may control the degree of lateral interaction of linear elements from a state observable as diffuse birefringence to a condition in which discrete fibrils may be seen. Such factors as local divalent cation concentration and mechanical stress might control such interactions. Nakajima and Allen (49) observed such differing degrees of lateral aggregation in the plasmodial cytoplasm of the acellular slime mold, *Physarum polycephalum*. Wohlfarth-Botterman (50) found an increase in the number of cytoplasmic fibrils when plasmodial strands were hung so that streaming had to overcome gravitational acceleration. Kamiya, Allen, and Zeh (51) have more recently observed that moderate mechanical stretching of plasmodial strands increases the force they can develop on contraction. These data are consistent with the hypothesis that mechanical stress may increase both the efficiency of contraction and the degree of lateral aggregation under in vivo conditions.

Theory of Amoeboid Movement

It has long been known that the cytoplasm of an amoeba circulates in a fountain pattern through the inner and outer regions of the cell (48). Mast

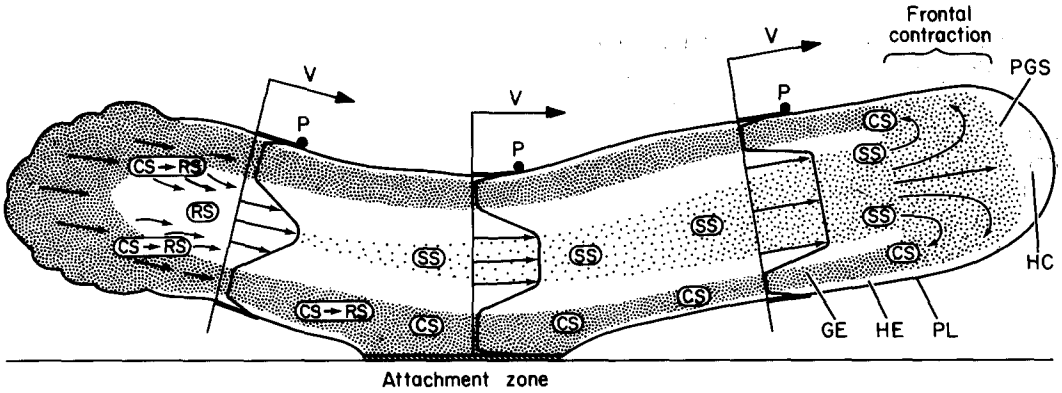

FIGURE 9 A schematic diagram of processes in amoeboid locomotion. Velocity (V) profiles show the patterns of plasmalemma (*PL*) displacement and cytoplasmic streaming in the front, middle, and rear regions. The region of ectoplasm-plasmalemma-substrate attachment is indicated. The relationship among the region of frontal contraction, the hyaline cap (*HC*), and plasmagel sheet (*PGS*) are shown at the pseudopod tip. Regions of the cytoplasm corresponding qualitatively with the rheological states of isolated cytoplasm are labelled *SS* (stabilized state), *CS* (contracted state), *CS→RS* (transition from contracted state to relaxed state), and RS (relaxed state). (HE) hyalin ectoplasm, (GE) granular ectoplasm.

(52), in his classical ectoplasmic contraction "sol-gel" theory of amoeboid movement, incorporated the well-known fact that the consistency of the cytoplasm underwent changes in different regions of the cell. Although these changes have long been described in oversimplified terms as a "sol-gel transformation," there is no doubt that the inner region of the cell, the endoplasm, has a "softer," more compliant consistency. However, even the endoplasm has been shown to possess viscoelasticity (30), and therefore it is not correct to refer to it as a "sol."

The results of the present experiments offer useful insights into the possible significance of the rheological changes in intact amoebae that Mast (52) referred to as sol-gel transformations. The cytoplasm streaming into pseudopodia is known to be viscoelastic; therefore, it is qualitatively similar to stabilized, isolated cytoplasm. The presence of viscoelasticity is significant because it allows tension from frontal contraction to be transmitted along the endoplasm backward toward the tail.

Release of free calcium in the pseudopodial tip region presumably causes contraction in the region where the endoplasm becomes everted to form the ectoplasmic tube. As the cytoplasm contracts, it squeezes out some of its interstitial water into the hyaline cap and increases in refractive index (33, 34). Presumably, contraction also results in an increase in rigidity as in muscle and isolated cytoplasm. Such a change probably represents the "gelation" postulated to occur as the endoplasm becomes everted to form the ectoplasmic tube.

The cytoplasm of the amoeba circulates around the cell in a fountain pattern as the latter advances in relation to its substratum. The ectoplasm in the posterior region of the cell "relaxes", analogous to the loss of viscoelasticity in relaxed stabilized cytoplasm (Fig. 3), then forms the material from which the endoplasmic stream is recruited. The relaxation is probably accounted for by assuming that the cell has a mechanism for sequestering calcium and controlling the local concentration of endogenous ATP. The tail endoplasm has a weaker birefringence than that of the anterior cytoplasm (53), suggesting that its degree of actin-myosin interaction is less. If this were not so, then the tension in the endoplasm would increase until its fracture point was reached, and steady-state pseudopodial extension could not occur.

Although free calcium ions have been shown to control contractility in amoeba cytoplasm, it is not known whether exogenous free calcium may enter pseudopod tips from the environment or whether endogenous calcium is sequestered and released by organelles.

The fact that isolated cytoplasm can be made to cycle repeatedly through stabilized, contracted, and relaxed states suggests that the rheological cycle in the intact amoeba during locomotion may

be similar. Fig. 9 represents an attempt to depict the major processes in amoeboid locomotion, including the patterns of cytoplasmic streaming, the site of the generation of the motive force, the locations of steps in the rheological cycle, and the role of the plasmalemma in locomotion and cell attachment.

Many unsolved problems remain. How pseudopodium formation begins is still unknown as are the factors that control the number and form of pseudopodia. Much remains to be learned about the respectives roles of the cytoplasm and the plasmalemma in excitability and behavior. It can be seen that the extended frontal contraction theory offers some fruitful insights into mechanisms of behavior (54).

At the molecular level, the thick filaments need to be characterized in order to establish their identity by biochemical criteria. Similarly, the macromolecular basis of calcium control remains obscure. It is not known whether amoeba cytoplasm contains a system of calcium-sensitive proteins similar to or analogous with the troponin-tropomyosin system of vertebrate striated muscle (55–57) or whether the light chains of myosin might be responsible for calcium control as in more primitive muscles (58). Experiments are now in progress to find answers to these questions.

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