MOTILITY IN *ECHINOSPHAERIUM NUCLEOFILUM*

II. Cytoplasmic Contractility and Its Molecular Basis

KENNETH T. EDDS

From the Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222. Dr. Edds' present address is the Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

ABSTRACT

Echinosphaerium nucleofilum exhibits at least three kinds of movement: locomotion by the bending and shortening of its many axopodia, feeding by means of food-cup pseudopodia formed from its cortical cytoplasm, and saltatory motion of cytoplasmic particles, especially in the cortex and axopodia. Since previously presented evidence indicated that the microtubular axoneme is not essential for particle motion, the cytoplasm was investigated for the possible existence of contractile behavior and for the possible presence of linear elements other than microtubules.

Cytoplasm can be isolated in physiological media in which rigor, relaxation, and contraction can be induced, as in muscle, by manipulating the concentrations of calcium ions and magnesium-adenosine triphosphate. Contraction is initiated by calcium ions at concentrations above 2.4×10^{-7} M. The rigor-to-relaxation transition occurs at subthreshold calcium concentrations on the addition of 10^{-3} M ATP.

Negatively stained preparations of isolated cytoplasm show two types of filaments: thin filaments identified as cytoplasmic actin by virtue of their binding heavy meromyosin from striated muscle in characteristic arrowhead arrays, and thicker filaments which do not strictly resemble myosin aggregates from muscle or amoeba but could conceivably be myosin aggregated in an unfamiliar form.

Nearly all cases of eucaryotic cellular motility, including intracellular motion, appear to be related to the presence of either microtubules, cytoplasmic actins and myosins, or both. Motility associated with organelles derived from centrioles or basal bodies appears to be associated with microtubules at the ultrastructural level (13, 16) and with tubulin at the molecular level (9). At least in cilia and flagella, a microtubule-associated ATPase (dynein) is undoubtedly involved in producing motions (14). On the other hand, amoeboid movement (15), shuttle streaming in acellular slime molds (7), and rotational streaming in characeans (e.g., *Nitella*) (11) are only a few of the motile systems associated with the presence of one or more types of microfilaments at the ultrastructural level, and with the presence of cytoplasmic actins, and sometimes myosin, as molecular counterparts (12). In neurons, three types of linear elements are present: microtubules, neurofilaments, and microfilaments. However, their respective roles are not yet well defined (1, 8).

In *Echinosphaerium* the prevalence of cytoplasmic microtubules has suggested a possible role for

microtubules in intracellular motility. Recently, however,[1] it has been demonstrated that the microtubular axoneme is not required for normal particle motions and that single microtubules are unlikely candidates for motive force production in *Echinosphaerium*.

The present work was undertaken to investigate the possibility that cytoplasmic actin and myosin might be present in *Echinosphaerium*, and thus account for certain types of movements.

MATERIALS AND METHODS

Cultures of *Echinosphaerium nucleofilum* were obtained from Carolina Biological Supply Co. (Burlington, N. C.) and cultured in Marshall's medium containing mixed ciliates as a food source.

Light microscope observations were carried out with Zeiss differential interference and Nikon rectified polarizing optics. Electron microscope observations were made with an AEI EM6B electron microscope operated at an accelerating voltage of 60 kV.

Solutions used to stabilize, relax, and contract the cytoplasm were identical to those described by Taylor et al. (15) for *Chaos carolinensis*. The *stabilization* solution is believed to induce a rigor-like condition in the cytoplasm, as in muscle, by the absence of ATP and low Ca^{++} concentration. Under these conditions the constituents of an actomyosin system are believed to be bound together by the interaction of actin and the "head" portion of the myosin molecule. The *relaxation* solution contains 1 mM ATP and a Ca^{++} concentration below that required for contraction. The ATP is thought to break the actomyosin complex, thereby relaxing the system and allowing it to be more easily stretched because the filaments are now free to slide past one another. The *contraction* solution contains no ATP, but does contain a Ca^{++} concentration sufficient to initiate a contraction. The *flare* solution contains, in addition to the threshold Ca^{++} concentration and ATP, 0.5 mM Mg ions. This solution was so named because it induced the formation of flaring loops of cytoplasm in the amoeba, *Chaos carolinensis*.

Intact organisms were placed in the stabilization solution, and then the relaxation, contraction, or flare solutions were introduced into the vicinity of the stabilized cytoplasm with a micropipette. Since the cells were partially lysed by these solutions, it was not necessary to rupture the cell mechanically for the contractility experiments. The response of the cytoplasm to the solutions was filmed for later analysis.

The negative staining procedure of Moore et al. (6) for single cells was used to observe the cytoplasm with the electron microscope. The cytoplasm was first placed in the stabilization solution, and then drawn into a micropipette containing the relaxation solution to rupture the cell. The cytoplasm was then expelled from the micropipette onto a Formvar-coated grid, rinsed several times with fresh relaxation solution, fixed with 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7), rinsed with buffer, stained with 1% uranyl acetate and then air dried.

Heavy meromyosin (HMM), at a concentration of 0.2 mg/ml in stabilization solution, was applied to a grid containing initially stabilized cytoplasm before fixation and staining. The HMM solution was rinsed off the grid with fresh stabilization solution and then prepared as described above. Control preparations for the HMM experiments differed in that the 1 mM ATP-containing relaxation solution was used to rinse the grids after the HMM was applied; otherwise they were prepared in an identical manner.

RESULTS

Cytoplasmic Contractility

All motions in the cytoplasm of *E. nucleofilum* stopped immediately when the organism was placed in either stabilization or relaxation solutions. The axopodia then began to shorten and by 10 min had been reduced to wisps of cytoplasm containing no birefringent axoneme (Fig. 1). No particle motions occurred during retraction of the axopodia.

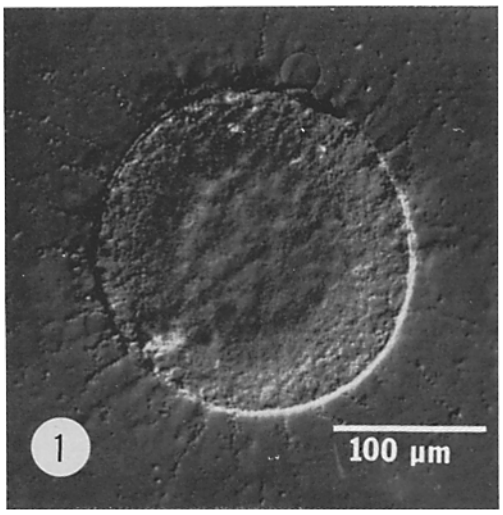

FIGURE 1 *Echinosphaerium nucleofilum* after incubation for 10 min in relaxation solution. The cytoplasm was freely permeable to the dye eosin Y. The axopodia have retracted and no motions of particles were evident. × 200.

[1] Edds, K. T. 1975. Motility in *Echinosphaerium nucleofilum*. I. An analysis of particle motions in the axopodia and a direct test of the involvement of the axoneme. *J. Cell Biol.* **66**:145–155.

The permeability of the membrane in both stabilization and relaxation solutions was tested with 0.5% eosin Y (mol wt 692). Within seconds after addition of the stain, the cytoplasm appeared pink and several cytoplasmic inclusions were stained dark red, thus indicating that the dye passed readily through the membrane or remnants thereof. In contrast, control specimens were not stained after 30 min in the dye.

When the cytoplasm, in relaxation solution, was stretched with a glass needle, long fibrils became visible (Fig. 2), which could be drawn out without breaking. When the cytoplasm was stretched in the same manner while in stabilization solution, similar fibrils were also seen, but these fractured readily under tension (Fig. 3). No saltations were observed in either the stabilized or relaxed cytoplasm.

When the contraction solution was introduced into the vicinity of the cytoplasm in stabilization or relaxation solution, the cytoplasm exhibited a vigorous contraction lasting 5–10 s. The entire cytosome contracted, including the remnants of the axopodia (Fig. 4). The threshold concentration of Ca^{++} for contraction was 2.4×10^{-7} M. The contracted cytoplasm could be stretched into strands that were positively birefringent (Fig. 5).

Only when the cytoplasm, in stabilization or relaxation solution, was stretched into fibrils (Figs. 2 and 3), and the contraction or flare solutions then added, were motions observed that were similar to saltations. Particles that were attached to the fibrils moved in both directions after addition of the contraction or flare solution, apparently as a result of the shortening of the fibrils. The fibrils eventually (after ca. 5 s) pulled themselves apart.

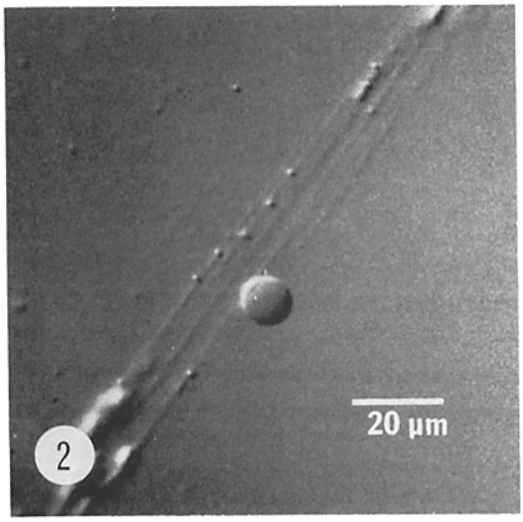

FIGURE 2 Long fibrils were stretched from the cytoplasm of *Echinosphaerium* that had been relaxed. × 600.

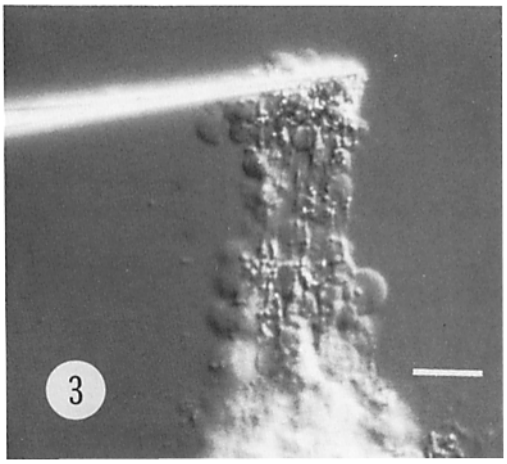

FIGURE 3 Fibrils could also be stretched from cytoplasm in stabilization medium; however, these fibrils readily fractured under increased tension. Marker, 20 µm. × 450.

The flare solution, which causes the formation of pseudopodial-like cytoplasmic loops in amoeba, did not cause any similar type of motility in *E. nucleofilum* cytoplasm; however, it did induce contraction in both stabilized and relaxed cytoplasm.

The cytoplasm could be "cycled" from the stabilized state to the relaxed state to the contracted state. Efforts to demonstrate transition from the contracted state to either the relaxed or stabilized condition were not successful.

Calcium-induced contraction was observed in the cytoplasm over a pH range between 6 and 8 and over an ionic strength range of $\mu = 0.02$–0.1.

The effects of drugs, believed to cause the depolymerization of microtubules (colchicine) and inhibit some types of motility associated with microfilaments (cytochalasin B), were tested on the contractile cytoplasm. Neither colchicine (2.5×10^{-2} M) nor cytochalasin B (10 µg/ml) had any inhibitory effect on contraction when used as a pretreatment before addition of the contraction solution.

Ultrastructure of Isolated Cytoplasm

Electron microscope examination of negatively stained preparations of *E. nucleofilum* cytoplasm

showed copious quantities of thin and thicker filaments (Fig. 6). The thinner filaments averaged 65 Å in diameter and were of variable lengths, from 0.5 to ca. 5.0 μm (Fig. 7). In some cases the appearance of the filament is as would be expected for a double helix with a cross-over repeat distance of ca. 360 Å. Upon treatment of these filaments with HMM from rabbit striated muscle, they were found to have been decorated with characteristic arrowhead-shaped arrays ca. 360 Å apart (Fig. 8). Control preparations, treated with HMM followed by several rinses with the 1 mM ATP-containing relaxation solution, lacked these arrays.

The thicker filaments ranged from 0.4 to 1.6 μm in length, and most of them exhibited a nonhelical, double-stranded appearance extending along their

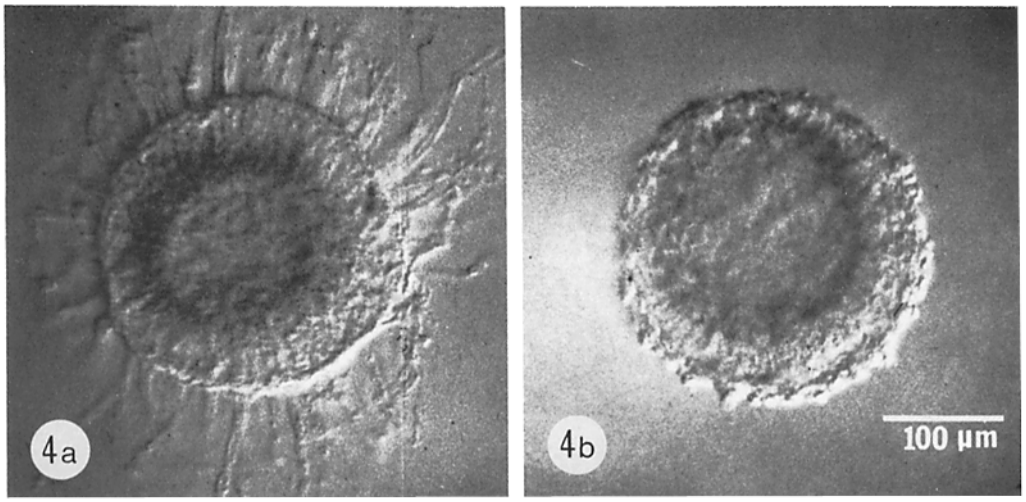

FIGURE 4 (*a*) *Echinosphaerium* in relaxation solution, (*b*) same organism after addition of contraction solution. The axopodial remnants have contracted toward the cytosome. × 160.

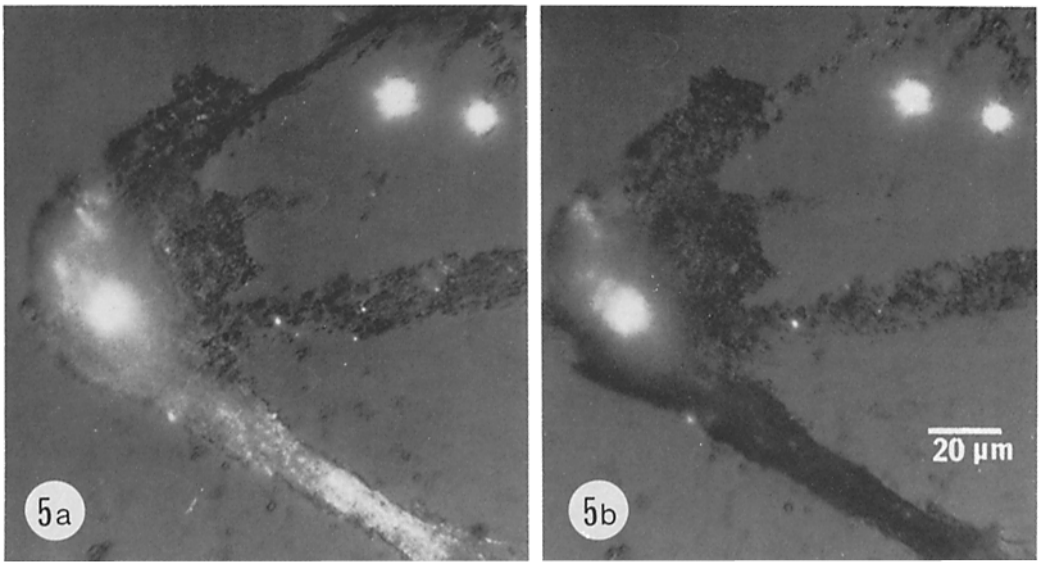

FIGURE 5 Contracted cytoplasm of *Echinosphaerium* that has been stretched into strands. The cytoplasm is positively birefringent. The two photomicrographs were taken at opposite compensator settings with polarized light optics. × 500.

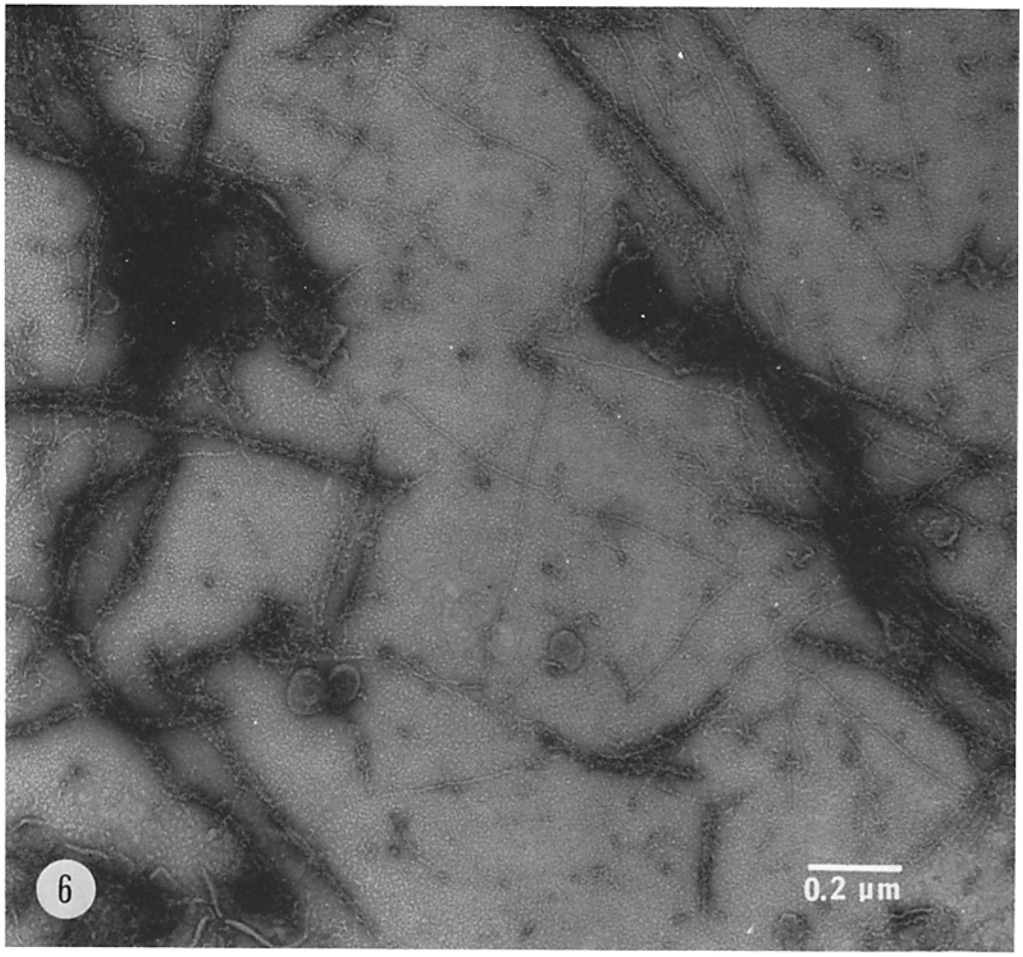

FIGURE 6 Thin and thicker filaments as observed in negatively stained preparations of *Echinosphaerium* cytoplasm isolated in stabilization medium. × 59,800.

length (Fig. 9). The thicker filaments varied in diameter from 100 to 180 Å. In some cases the core appeared to contain a single filament ca. 85 Å in diameter which could have represented a side view of the double strand described above. The thicker filaments all had surface projections some of which were less distinct than others, which measured ca. 200 Å in length (Figs. 9 and 10). The morphology of the thicker filaments was not changed by varying the pH from 6 to 8, or varying the ionic strength from $\mu = 0.02$ to $\mu = 0.1$. However, ionic strengths of $\mu = 0.6$ caused them to disappear. Neither ATP (1 mM), calcium ions (10 mM), nor the presence of colchicine altered the morphology of the thicker filaments. They did not bind HMM.

DISCUSSION

Three kinds of motility are observed in *Echinosphaerium*: axopodial locomotory movements (19), the formation of food-cup pseudopodia from the cortical cytoplasm (18), and saltatory motion of cytoplasmic particles in both axopodia and the cortical cytoplasm.[1]

Motility is generally believed to be associated with the presence of linear elements. Available evidence suggests that at least two basic types of force-producing systems involving linear elements have evolved among the eucaryotes: those associated with the microtubular structures derived from centrioles and basal bodies (cilia, flagella, axostyles, spindles, etc.) and the contractile systems

whose cytoplasm contains cytoplasmic actins and, at least in some cases, cytoplasmic myosins. The progress of investigation of the molecular basis of nonmuscular motility has been hampered by the technical problems of isolating and purifying the proteins from single cells or from the small homogeneous populations of cells usually available (see reference 12).

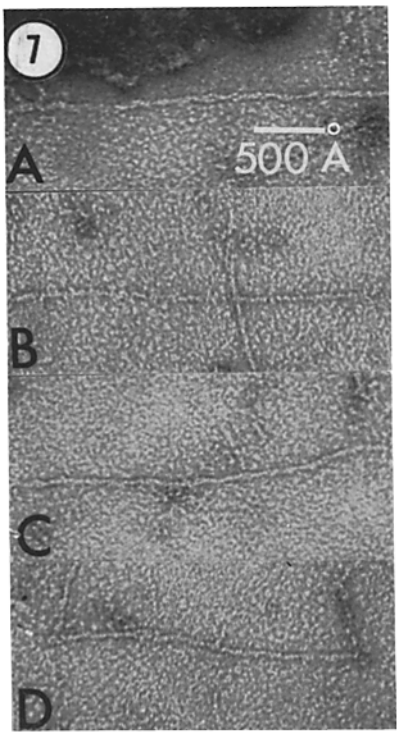

FIGURE 7 Thin filaments from *Echinosphaerium* cytoplasm in relaxation solution. In some cases a double helical pitch can be observed. The cross-over repeat distance is ca. 360 Å. × 200,000.

Until recently it would have been reasonable to suppose that cytoplasmic motility in *Echinosphaerium* might be attributable to some action of the microtubules alone, because only one unconfirmed report claimed the presence of any linear structures other than microtubules (3). The motions of particles might especially be suspected to depend upon some action of microtubules which are present within one or two micrometers of many of the most striking motions.

Two separate lines of evidence now seem to establish that the microtubular axoneme does not serve a force-generating role. First, a glass needle can be substituted for the axoneme in an artificial axopodium that exhibits particle motions indistinguishable from those in normal axopodia. Second, colchicine, which causes normal axopodia to collapse, has no effect on movements in artificial axopodia supported by a glass microneedle.[1] These results suggest strongly that microtubules serve no function in motive force production in this system and that their role is mainly supportive.

The present experiments, in which the cytoplasm of *E. nucleofilum* has been isolated, have shown that the cytoplasm is contractile, and that its contractility can be controlled in the same way that myofibrils of vertebrate striated muscle and amoeba cytoplasm can be controlled. The cytoplasm can be made to pass into any of three physiological states, rigor, relaxed, or contracted, by manipulating the concentrations of Ca^{++} and ATP.

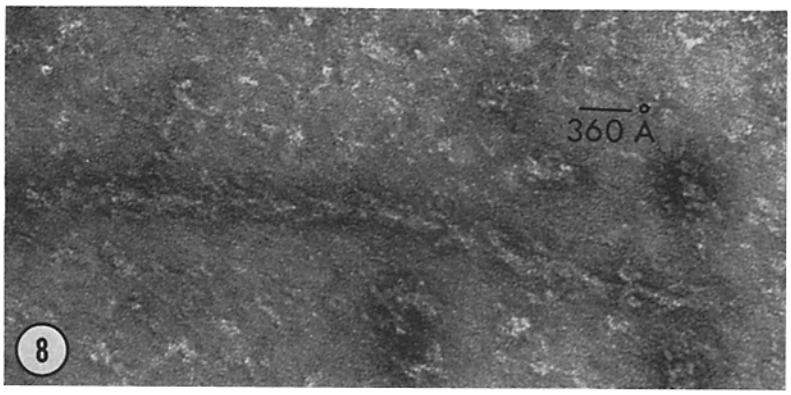

FIGURE 8 A thin filament that has been treated with rabbit heavy meromyosin. The arrowheads were dissociated upon treatment with ATP. × 194,000.

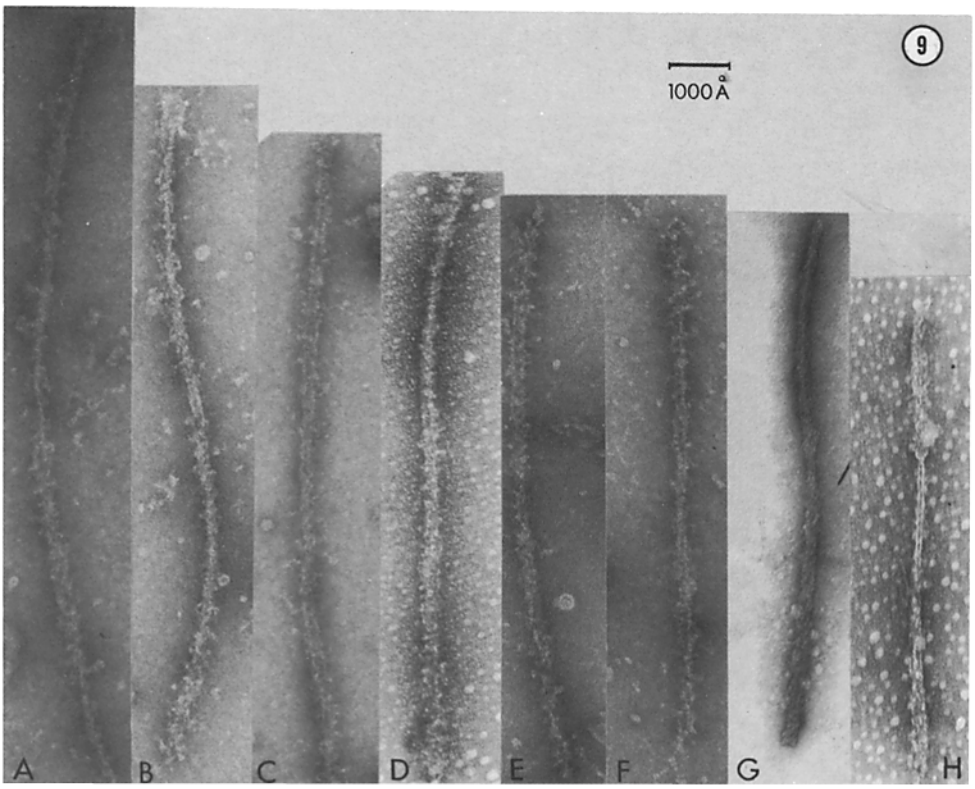

FIGURE 9 Eight examples of negatively stained thicker filaments from different preparations of cytoplasm isolated from *Echinosphaerium* in relaxation solution. × 80,000.

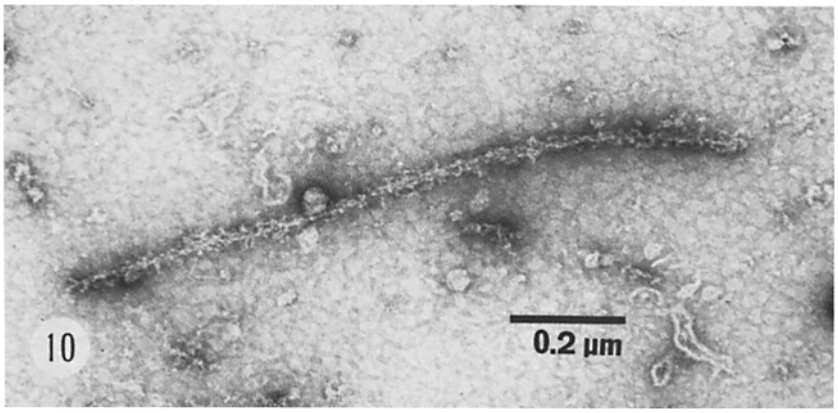

FIGURE 10 A thicker filament from *Echinosphaerium* cytoplasm. Note the apparent regularity of some of the surface projections. × 75,000.

Although the flare solution, which Taylor et al. (15) showed would cause naked amoeba cytoplasm to form looplike pseudopodial extensions of the streaming cytoplasm, would not induce similar movements in *E. nucleofilum* cytoplasm, it did cause a bidirectional contraction of stretched fibrils, i.e., particles attached to the fibrils would move first in one direction and then in the opposite

direction. It appeared as though it was the individual fibrils that were moving and not the particles along the fibrils. The fibrils ultimately pulled themselves apart. The conditions under which this was observed are compatible with the hypothesis that saltation in this system may result from localized contractions in the cytoplasm mediated by a local release of Ca ions.

An interesting but enigmatic observation from these experiments, in terms of in vitro polymerization of microtubules (see references 9 and 10), was that the microtubular axoneme "dissolved" when the intact organism was placed in either the stabilization or relaxation solution. Also, the membrane allowed the passage of the dye eosin Y and thus the constituents of the stabilization and relaxation solutions into the cytoplasm after incubation in either of these solutions. The question remains as to whether the dissolution of the axoneme and the increase in permeability after incubation in these solutions are related? If so, one explanation may be related to the disruption of an equilibrium between dimeric tubulin and its polymeric form (2). If the dimer "pool" is diluted when the permeability of the membrane is changed, then the equilibrium would shift to favor the depolymerization of the microtubules.

Since the cytoplasm can exhibit contractility, it is important to know what linear elements can be detected in freshly ruptured cells. Two classes of filaments that are present have been described. Thin filaments, identical in diameter, double-helical structure, and HMM-binding capacity to the thin filaments of striated muscle, are present. By currently accepted criteria these thin filaments must represent another example in a growing list of cytoplasmic (nonmuscular) actins.

The thicker filaments are not morphologically similar to thick filaments of muscle or to the myosin aggregates observed in other primitive motile systems already investigated (12). They are sufficiently plentiful to suggest that they may be part of a contractile system, perhaps an unfamiliar aggregated form of myosin (see reference 5) which may be artifactual, or a product of the aggregation of a globular myosin (see reference 12) with actin filaments. Alternatively, they may be related to the contractile protein fibers in *Stentor*, which are thought to shorten by a change in conformation (4). The dimensions of the surface projections do not exclude the possibility that they might be the "head" of myosin monomers (Figs. 9 and 10). Filaments very similar to the thicker filaments described here have been reported in *E. nucleofilum* that has been treated with high pressure. Tilney et al. (17) linked their appearance with the disappearance of the microtubular axoneme and therefore suggested that they may be a stage in the disintegration of the microtubules. These workers also reported that they were present, although in much smaller numbers, in unpressurized, control specimens that were assumed to be "poorly fixed." If the filaments described are, in fact, the same structures reported here, the possibility that they represent a stage in microtubular disassembly seems unlikely not only because of their respective dimensions, but also because the thicker filaments were found in preparations that had been treated with either colchicine or Ca ions, both of which depolymerize microtubules (10).

The solubility of these thicker filaments at high ionic strength provides a potentially useful lead in the efforts now in progress to identify the molecular components of these filaments.

It seems entirely possible that the contractile system demonstrated to be present in the cytoplasm of *E. nucleofilum* may be responsible for all types of motility of which the organism is capable. If so, it is probable that the contractile system (of which actin is a part) works to bring about locomotory movements of the axopodia by bending the axopodia against the resistive force of the microtubular axoneme. Saltatory motions of particles in the cortical and axopodial regions may result from highly localized contractions in the cytoplasm. The formation of food-cup pseudopodia may involve two phenomena, both of which are regulated by Ca ions. If, for example, a food organism becomes attached to one of the axopodia and somehow stimulates food-cup formation by that axopodium, the increased cytoplasmic motility involved in producing the food cup presumably requires the release of Ca ions. In addition to stimulating motility, the Ca^{++} release may also cause the dissolution of the axoneme and allow the food organism to be drawn toward the cytosome where it is ultimately engulfed.

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