Journal of Supramolecular Structure 5:317 (269) – 334 (286) (1976)

Cytoskeletal Functions of Cytoplasmic Contractile Proteins

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This is a review of the evidence that the cytoplasmic contractile proteins function as a cytoskeletal system in the cytoplasmic matrix. Biochemical experiments show that cycoplasmic actin filaments can form a solid gel under conditions likely to exist in living cells. The actin filaments are associated with other proteins which may stabilize the gel and which are involved with motile force generation like myosin. Ultrastructural studies show that actin filaments are difficult to preserve, but that under stabilizing conditions networks of actin filaments are found throughout the cytoplasmic matrix.

Key words: actin, cytoskeleton, filament, gel

INTRODUCTION

I will argue for the revival of an idea first espoused by the French cytologist Dujardin in 1835 (1) – that cellular movements and cytoplasmic structure have a common basis. Recent experimental work shows that cytoplasmic contractile proteins do more than generate the forces for cellular movements; they also act as cytoskeletal elements by virtue of their ability to form a solid gel in the cytoplasmic matrix. This second role for contractile proteins appears to require high concentrations of actin, and may explain why nonmuscle cells have much more actin than needed for their motility.

ORIGIN OF THE IDEA

In the 1830s Dujardin studied a number of living protozoa by microscopic observation, micromanipulation, and crude chemical extraction procedures. He concluded that all of the creatures examined were composed of a substance which he called sarcode. He wrote:

> I will call sarcode this glutinous, diaphanous substance which is insoluble in water, which contracts into globular masses, which attaches itself to the dissecting needles and stretches like mucus, and which can be found in all inferior animals interposed with the other structural elements [(1) p. 367].

I believe that he was describing the cytoplasmic matrix, the substance interposed between the various organelles. His important insight was that the sarcode was involved with both cellular structure and motility. Regarding its chemical nature he wrote:

> Sarcode decomposes gradually in water, reduces its volume and finally leaves only an irregularly granular residue. Potash does not dissolve it suddenly like mucus or albumin; it only seems to hasten its decomposition in water; nitric acid and alcohol coagulate it suddenly and make it white and opaque [(1) p. 368].

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As described below, gels of cytoplasmic contractile proteins dissolve gradually in dilute buffers, leaving a residue of insoluble proteins and vesicles, which might be described as a "granular residue." In all likelihood, Dujardin was examining the properties of the cytoplasmic contractile proteins. His sarcode is probably actomyosin, and it was he who first realized that the motile machinery also contributes to cytoplasmic structure.

DEVELOPMENT OF THE IDEA TO 1950

During the 1930s and 40s the structure of living protoplasm was investigated in some detail, and it was generally accepted that cytoplasm is a dynamic thixotropic gel. The cell physiologists of that generation had no information about the molecular basis of cytoplasmic structure, but they devised ingenious experiments with living cells using the centrifuge microscope, micromanipulators, and intracellular magnetic particles to show that cytoplasm has a gel-like consistency.

The following points were established:

1) Cytoplasm is viscous: Efforts were made to measure cytoplasmic viscosity quantitatively. Although there were disagreements regarding the absolute value of cytoplasmic viscosity, there was agreement that it is high [see (2) for review].

2) Cytoplasmic consistency varies spatially: It was clear that the vicous material in the cytoplasm is not distributed uniformly inside cells. Typically there is a peripheral region of high viscosity and a central region of lower viscosity. In giant amoebae the viscous peripheral zone is called ectoplasm and the inner, more fluid region, endoplasm (2).

3) Structural elements in the cytoplasm are dynamic: High and low viscosity regions are interconvertable. For example, the giant amoebae cyclically convert high viscosity ectoplasm to low viscosity endoplasm and back again as they move (2).

4) The cytoplasmic gel is thixotropic: This was originally suspected when cells were found to round up when agitated. Later, Angerer (3) was able to measure a reduction in cytoplasmic viscosity proportional to the extent of mechanical stimulation.

5) The formation of cytoplasmic gels is endothermic: This is consistent with the common observation that cells round up in the cold and reestablish assymetrical shapes when returned to their natural temperature. The endothermic nature of the gel-forming reaction was established by Landau et al., (4), who studied the temperature dependence of cytoplasmic gel formation. They also found that high pressure liquifies the cytoplasm, showing that the gel formation involves a volume change.

Although virtually all of the work summarized above was carried out with giant amoebae or marine eggs, it could be shown that the cytoplasm of living vertebrate cells has similar properties. Crick and Hughes (5) examined the physical properties of cultured chick embryo cells by measuring the rate of displacement of ingested magnetic particles by external magnetic fields. They concluded that the cytoplasm is a thixotropic gel with feeble elastic properties. They were impressed that the gel was so weak that it would appear to be a liquid if viewed in bulk in a test tube. This apparent weakness of chick cytoplasmic gel relative to that of protozoa could be because the measurements on chick cytoplasm were limited to the region near the cell center, which may be a region of relatively low viscosity.

Cell movement depends upon the presence of gelled cytoplasm. Diverse treatments which destroy cytoplasmic structure, including low temperature, high pressure, and mechanical stimulation, stop movement. Before 1950 movement and cytoplasmic struc-

ture were thought to be so closely related that Mast's then popular sol-gel transformation theory of amoeboid movement (6) supposed that the consistency changes caused the movement.

ELECTRON MICROSCOPY AND THE DECLINE OF THE IDEA

Interest in the properties of the cytoplasmic matrix has declined since 1950. This change is attributable in part to the absence of matrix structure in early electron micrographs of cells fixed with osmium tetroxide. Rather than revealing a network of protein fibers in gelled regions of cytoplasm, the early electron micrographs showed membrane-bounded organelles suspended in vacant plastic! It was realized that the absence of structured cytoplasmic matrix was artifactual and probably due to extraction by osmium. This artifact seems to have discouraged further work on the cytoplasmic matrix.

The introduction of glutaraldehyde as an electron microscopy fixative in the mid-1960s improved the preservation of the cytoplasmic matrix and created the era when microtubules were accepted as *the* cellular cytoskeletal elements (7). Electron micrographs of cells fixed with glutaraldehyde and osmium showed the cytoplasmic matrix to have an unappealing fibrogranular appearance, and revealed the widespread occurrence of microtubules. In some cases the microtubules were shown to have cytoskeletal properties (7), and today some biochemists and cytologists have the impression that cells are bags of soluble enzymes supported by microtubule struts in which membrane-bounded organelles float about.

The microtubule-supported, fluid-filled bag concept of cytoplasmic structure is obviously wrong on 2 counts: 1) the whole cytoplasm is viscoelastic rather than fluid; and 2) microtubules cannot be the only cytoskeletal elements, because many cells (including the giant amoebae whose cytoplasm was studied in detail by the previous generation) have few or no microtubules in highly structured regions of the cytoplasm.

Thus there must be a second cytoskeletal system. In the following sections I will summarize the evidence that actin, myosin, and their associated proteins are the components of this second system. Some of the evidence supporting this idea comes from biochemical experiments, some from electron microscopy, and some from recent work with the drug cytochalasin-B.

CONTEMPORARY BIOCHEMICAL STUDIES

New data concerning the molecular basis of cytoplasmic structure has come from recent biochemical work with cytoplasmic extracts capable of self-assembly of the cytoskeletal elements. The study of microtubules advanced rapidly after Weisenberg's success (8) in polymerizing tubulin in crude brain extracts. The in vitro assembly of a cytoplasmic acto-myosin system actually preceded the work on microtubules, but this was not exploited experimentally for a number of years following its discovery by Thompson and Wolpert (9, 10). A reason for the neglect of this important discovery was the contemporary lack of convincing evidence for the existence of cytoplasmic actin and myosin (11).

Thompson and Wolpert (9) found that crude extracts of Amoeba proteus appeared to gel and exhibited streaming movements when warmed from 4°C to room temperature in the presence of ATP. When confined to a capillary tube, the extract contracted into an opaque thread which could be removed intact. Electron micrographs of the contracted

material fixed with osmium tetroxide alone revealed some filaments 9–12 nm wide, and some disorganized spongy material (10). They thought that the spongy material and the 2 types of filaments might be interconvertable, and that the formation of the filaments might be responsible for the gelation of the extract upon warming with ATP (10). The molecular nature of the filaments was not known.

Their observations on Amoeba proteus extracts were confirmed and extended some years later by Ito, Korn and myself (12, 13). We showed that there is a dramatic consistency change brought about by warming the cold extracts, and that this change is accompanied by the assembly of a high concentration of actin filaments from soluble subunits in the cold extract. The actin filaments are associated with thicker filaments having the morphology of myosin filaments. Movement occurred only when the 2 types of filaments were present together. We concluded that the temperature-dependent polymerization of actin filaments caused the consistency change in the extract, and therefore suggested that the assembly and disassembly of actin filaments might control the consistency of cytoplasm. Since the experiments also implicated the actin filaments with motile force generation, they provided the first link, on the molecular level, between elements of the cytoskeleton and the cell's motility apparatus.

A few years later, Taylor et al., (14) suggested a second way that actin and myosin might regulate cytoplasmic consistency. They showed that cytoplasm isolated from Chaos carolinensis is viscoelastic, and that its physical properties are influenced by calcium and ATP in such a way that the observed consistency changes could be caused by the association and disassociation of stable actin and myosin filaments. More recent work, described below, indicates that both mechanisms may be used by cells to control cytoplasmic structure.

In the past 2 years at least 5 different laboratories have studied cytoskeletal functions of cytoplasmic contractile proteins from a variety of cells. Much of this work was inspired by Bob Kane's success in making cold extracts of sea urchin eggs gel when heated to 37°C (15). Isolation of the components of the gel showed that actin was the major structural element. I have done some new biochemical and structural experiments with crude extracts of Acanthamoeba looking at the cytoskeletal functions of its contractile proteins (16). Similar experiments were carried out by Stossel and Hartwig (17) with rabbit macrophages, and by Weihing using Hela cells (18). Taylor and his colleagues (19) have continued their work on extracts of giant amoebae and Dictyostelium. Most of us have been in close contact and have exchanged preliminary results, so progress has been rapid and many interesting interspecies comparisons can be made. The general picture is one of broad similarities among species, with some important differences in molecular details. Actin emerges as a major cytoskeletal element, and is found associated with a variety of proteins in different species.

The general design of my Acanthamoeba experiments is very simple. Cells are homogenized in cold ATP-EGTA-sucrose buffer and centrifuged at 140,000 g for 1 hr. The cold supernatant forms a solid gel when warmed to room temperature (Fig. 1). This must be done without stirring. The gel is so strong that it will not pour from an inverted breaker. It is thixotropic and shatters when strongly agitated. If returned to the cold the newly formed gel liquifies. If kept in the warm the gel slowly contracts to form a miniture opaque cast of its container (Fig. 1).

The strength of the gels formed by the extract is very impressive, since the concentration of gel-forming molecules in the extract is only one-third to one-sixth of their concentration inside the cell. Inside the cell even stronger gels may be formed.

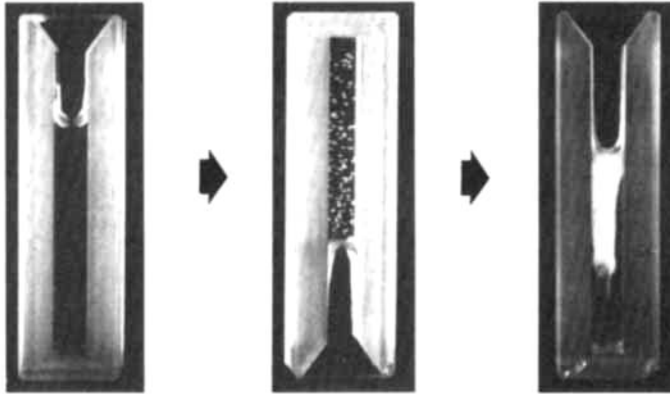

Fig. 1. Gelation and contraction of Acanthamoeba extract. An extract of soluble constituents is prepared in a sucrose-ATP-EGTA buffer at 0°C (left cuvette). Upon warming to 24°C the extract first solidifies after a few min (center cuvette) and then slowly contracts over several hr (right cuvette).

Contractile proteins are the chief components of the gel (Fig. 2). The gel is separated from soluble components by ultracentrifugation, and it's composition determined by electrophoresis on polyacrylamide gels. The newly formed gel consists of a high concentration of actin associated with myosin, and a 280,000 dalton polypeptide tentatively identified as the actin-binding protein originally described in macrophages by Stossel and Hartwig (20). After contraction, all of the actin, myosin, and actin-binding protein of the extract pellets with the gel (Fig. 2). There are also a number of minor components in the gel, including a polypeptide with a molecular weight of 93,000 daltons. It is though to be the cofactor protein required for actin-myosin ATPase activity in Acanthamoeba (16).

Light and electron microscope observations confirm the biochemical conclusion that actin is the major component of the gel. In the phase contrast microscope, fragments of the gel are homogeneous, slightly dense masses. Vesicles from the extract are frequently trapped in the gel. By polarized light microscopy the gel is birefringent (16), indicative of ordered substructure. Scanning electron micrographs of fractured pieces of contracted gel reveal a fibrous matrix surrounding trapped vesicles (Fig. 3). Masses of actin filaments woven together with a few microtubules, vesicles, and other subcellular particles can be seen in transmission electron micrographs (Fig. 4). As discussed in more detail below, special efforts must be made to prevent actin filament destruction during fixation. Further ultrastructural studies are needed to establish how the actin filaments are bound together to form the solid gel.

Since the gel scatters more light than its soluble precursors, absorbance changes can be used to estimate the rates of gel formation and contraction (16). The rates of both reactions are highly temperature-dependent; the activation energies for both gelation and contraction are about 20 kcal/mole.

The ion and nucleotide requirements for gelation and contraction were examined in extracts desalted by gel filtration to remove endogenous ions and nucleotides. In the desalting buffer, which consists of EGTA, sucrose, and imidazole, the extract gels very slowly or not at all. The addition of 1 mM Mg-ATP stimulates gelation (16). Recently, contraction has also been observed under these same completely defined conditions (Fig. 5). The reaction rates are much slower under these defined conditions than in the extract,

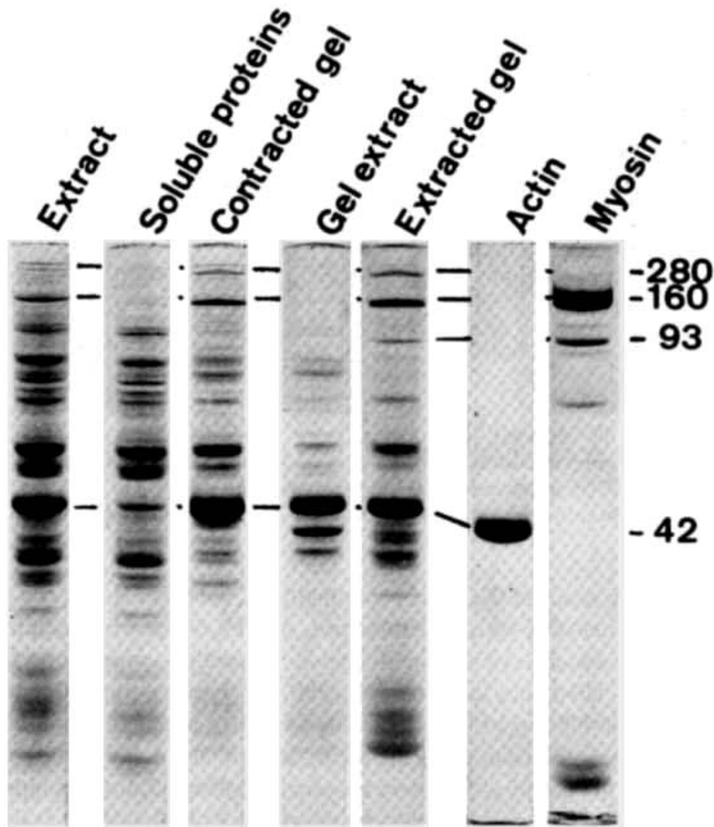

Fig. 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of protein fractions obtained from Acanthamoeba. Extract is the soluble fraction obtained in sucrose-ATP-EGTA buffer which gels and contracts upon warming. The soluble proteins are those remaining in the supernatant after pelleting the contracted gel by centrifugation. Actin (42,000 daltons) can be selectively extracted at low ionic strength from the contracted gel and this gel extract separated from the insoluble extracted gel by centrifugation. The extracted gel is enriched in the 280,000 dalton actin-binding protein, the 160,000 dalton myosin heavy chain, and the 93,000 dalton cofactor protein. Actin and myosin are further purified by gel filtration. Details of these experiments are described in (16). Reproduced with permission of the Cold Spring Harbor Laboratory from (26).

which contains a complex mixture of low molecular weight components. The nucleotide specificity of the gelation reaction is shown in Table I. The various purine nucleotide triphosphates all support gel formation, with ITP being the most effective, followed by ATP and GTP. Adenosine diphosphate did not support gelation. The only pyrimidine nucleotide triphosphate tested, CTP, did not stimulate gel formation. Since it is likely that cells maintain millimolar concentrations of ATP in their cytoplasm, the nucleotides are not attractive candidates for regulators of gel formation and dissolution. The ion requirement is minimal – only millimolar concentrations of $MgCl_2$ are necessary.

What macromolecular interactions are necessary for gel formation? Since the actin concentration in the gel is only about 1 mg/ml, the actin filaments must be cross-linked to each other in some way to form a solid gel. The mechanism of the cross-linking is not

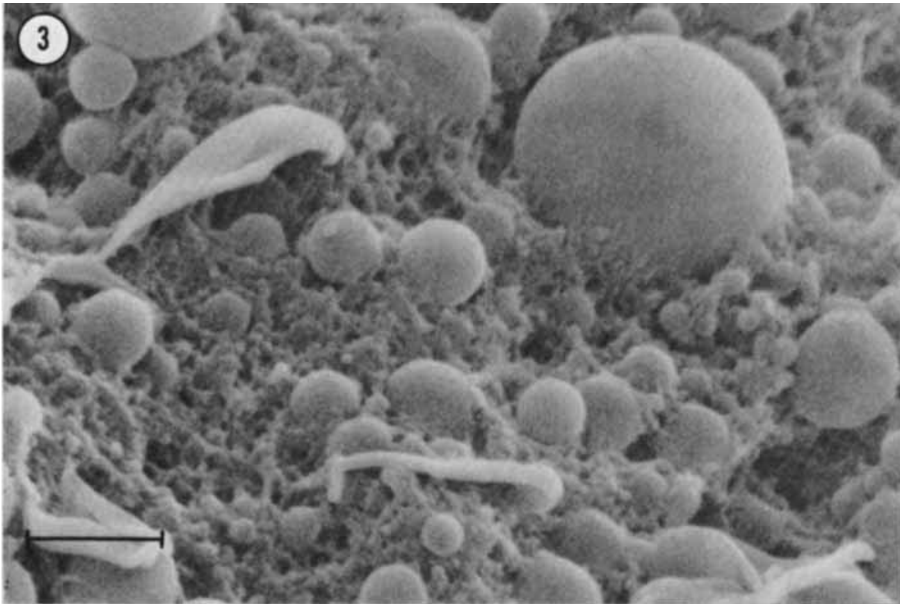

Fig. 3. Scanning electron micrograph of contracted gelled extract from Acanthamoeba. Prepared by fixation, critical point drying, fracturing, and metal coating. Bar is 1 μm. Magnification 18,000 ×.

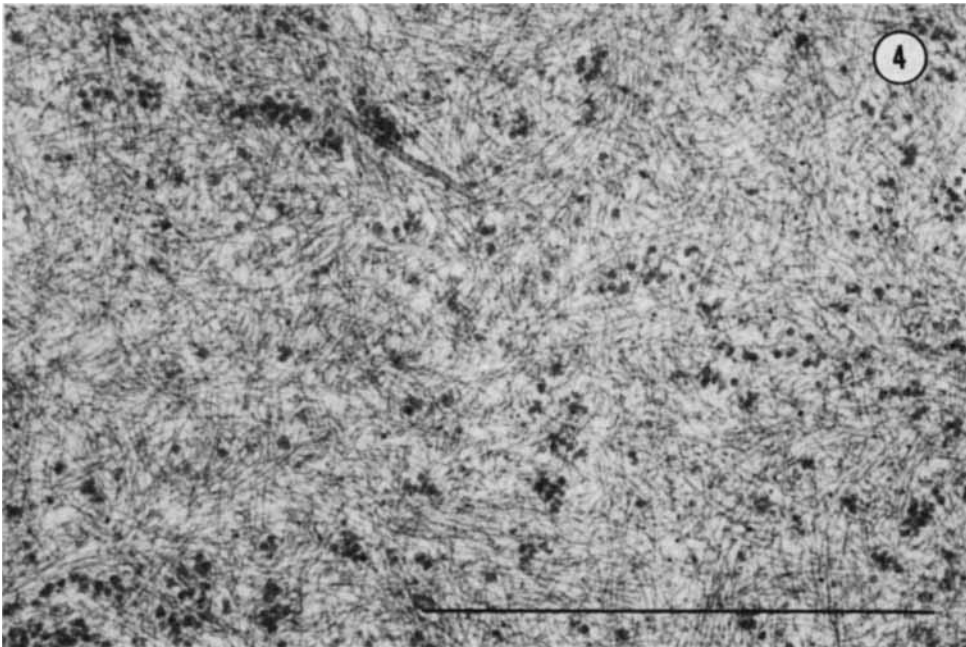

Fig. 4. Transmission electron micrograph of a thin section of a pellet of gelled Acanthamoeba extract containing 1.5 mg/ml of muscle tropomyosin to stabilize the actin filaments during fixation. Bar is 1 μm. Magnification 67,000 ×.

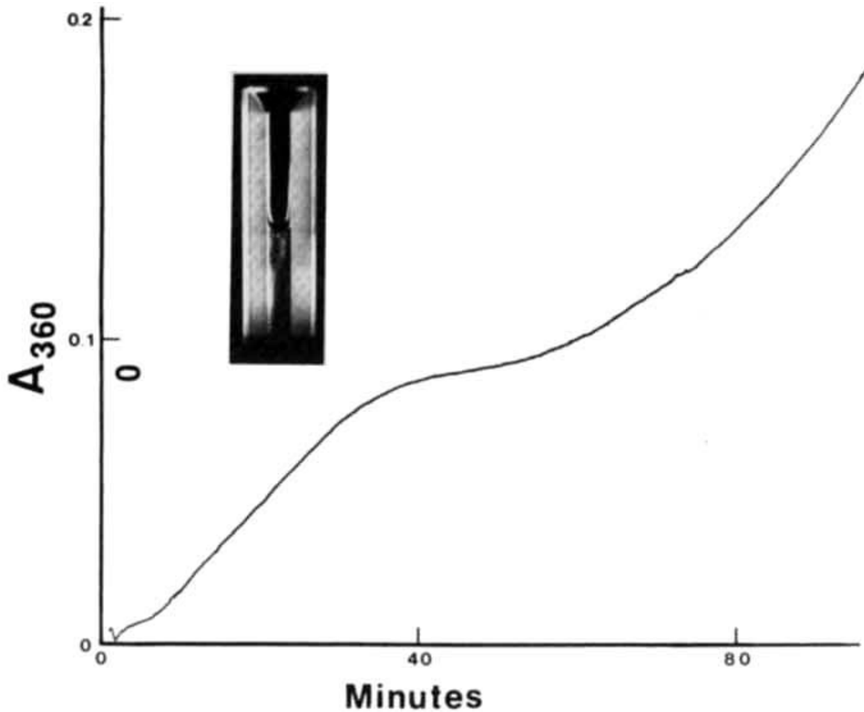

Fig. 5. Time course of gelation and contraction of Acanthamoeba in a defined medium. The extract was desalted on a column of Sephadex G-25 equilibrated with a buffer consisting of 0.34 M sucrose, 0.2 mM EGTA, 0.5 mM dithiothreitol, 10 mM imidazole, pH 7. At zero time Mg-ATP was added to a concentration of 1 mM and the sample warmed to 26°C. Gelation and contraction were monitored by recording absorbance at 360 nm vs time. Gelation was completed by about 30 min. The contraction is responsible for the second phase of absorbance increase, commencing at about 60 min. The inset shows a photograph of the cuvette containing the contracted gel.

settled and may differ from one cell type to another as evidenced by a comparison of Kane's work on sea urchin eggs, that of Stossel and Hartwig on rabbit macrophages and my own on Acanthamoeba.

In the case of Acanthamoeba, I proceeded by purifying actin from the gelled extract by two different methods (16) and tested the ability of the purified actin to form a gel. Neither of the preparations of Acanthamoeba actin is absolutely pure, but neither contains any detectable actin-binding protein. Actin purified from the gelled extract by low ionic strength extraction and gel filtration is contaminated by about 1% of a 50,000 dalton polypeptide and a variable amount of a 38,000 dalton polypeptide which is probably a breakdown product of actin. Actin prepared from the gel by high ionic strength extraction and gel filtration has less of these two contaminants, but it sometimes has other low molecular weight impurities.

Both types of purified Acanthamoeba actin will form a solid gel in the presence of Mg-ATP (16). No additional proteins are required. As in the crude Acanthamoeba extract, the rate of gel formation by purified Acanthamoeba actin is temperature dependent. In contrast to the gelled crude extract which liquifies upon cooling, the gel of purified actin is stable in the cold.

TABLE I. Nucleotide Requirement for Gel Formation in Desalted Acanthamoeba Extract

Nucleotide	Gel formation
ATP	+1
GTP	+1
ITP	+2
CTP	0
ADP	0

The cold extract was desalted as described in Fig. 5 to remove endogenous nucleotides. Gel formation was assessed as described in Fig. 6 after warming samples to 25°C for 10 min with 1 mM $MgCl_2$ and 1 mM nucleotide.

I concluded from these experiments that Acanthamoeba actin alone is sufficient for solid gel formation, although one or more of the minor low molecular weight contaminants might also be involved. In this respect Acanthamoeba actin differs from other actins which have been studied.

Pure muscle actin will form but a weak network in solution; this network is destroyed by very small shear forces (21). Muscle actin combined with heavy meromyosin (22) or alpha-actinin (21) will form a solid gel. Similarly, pure macrophage actin does not form a solid gel unless combined with high molecular weight actin-binding protein (17). Sea urchin egg actin appears to require the presence of both a 58,000 dalton protein and a high molecular weight protein for solid gel formation (15). I suspect that subtle differences in the actin molecules and the nature of any contaminants are responsible for their different requirements for associated proteins during gel formation. Acanthamoeba actin may have a stronger tendency for self-association than do the other actins. Alternatively, minor contaminants present in the purified Acanthamoeba actin may act as cross-linkers of some sort. In all systems studied, actin-associated proteins could either cross-link the actin filaments and stabilize the gel, serve in a regulatory capacity or, like myosin, be part of the force-generating mechanism.

These biochemical experiments with crude extracts from a variety of cell types show that actin and its associated contractile proteins are capable of forming a strong, cross-linked gel which is a reasonable candidate for the structural framework of the cytoplasmic matrix. Since there is also good evidence (11) that these contractile proteins are responsible for powering cellular movements, Dujardin's link between motility and structure is now well-supported at the molecular level.

A major unresolved question is the nature of the cellular mechanism controlling the assembly and disassembly of the dynamic cytoplasmic gel. Clearly, cells do not control their cytoplasmic consistency by changing their temperature like we do in the extract experiments. There is evidence from Taylor's work (19) that variation of the ionic calcium concentration may regulate gel formation in Amoeba proteus, but I have not yet been able to demonstrate a similar calcium dependence in Acanthamoeba.

STUDIES WITH CYTOCHALASIN-B

The drug cytochalasin-B is known to alter a variety of cellular activities, including many types of motility, but its mode of action has been debated [see (11) for review]. Early ultrastructural studies linked its action to an effect on microfilaments, but there is

no convincing evidence that the concentrations of the drug which are effective in vivo have any effect on isolated contractile proteins. In contrast, good evidence is available showing that the drug has a high affinity for transport systems in membranes.

Recently, Hartwig and Stossel (23) and Weihing (18) have independently discovered that low concentrations of cytochalasin-B inhibit gel formation in extracts of macrophages and Hela cells. Their data indicates that the drug inhibits actin-binding protein-actin filament interactions.

Since Acanthamoeba actin does not seem to require actin-binding protein for gel formation and because this organism is widely separated on the phylogenetic tree from the vertebrate cells initially studied, I examined the effects of cytochalasin-B upon the gelation of Acanthamoeba extracts. As shown in Figs. 6 and 7, very low concentrations inhibit gelation. Although no gel forms in the presence of cytochalasin-B concentrations greater than 0.6 ug/ml, a white precipitate forms after 0.5 hr at 25°C. By electrophoretic analysis, it was found that this precipitate consists mainly of actin and other components found in the gel formed in the absence of cytochalasin-B. The absence of a viscosity rise in the cytochalasin-treated sample argues against this precipitated actin being in the form of filaments.

Additional work is necessary to link these effects on actin gel formation to the diverse effects of cytochalasin-B on cellular activity. It is conceivable that the drug acts to disrupt a major structural component in the cytoplasm, and that this in turn leads non-specifically to a variety of cellular dysfunctions. If this is true, the drug has been very aptly named "cytochalasin for cell-relaxation" (24).

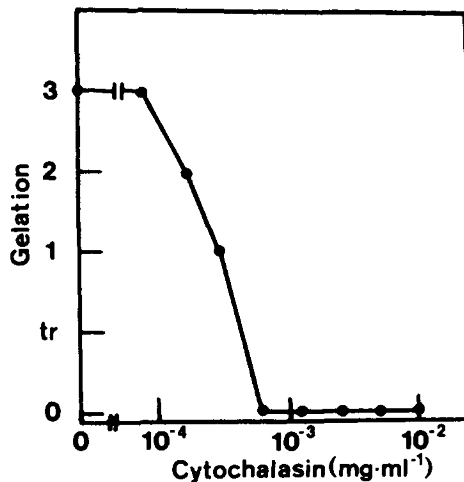

Fig. 6. Inhibition of gelation of Acanthamoeba extract by cytochalasin-B. The drug was added to the cold extract and the extent of gel formation after 20 min at 25°C assessed using the following scale: 0 = liquid; tr = viscous liquid; +1 = semisolid gel which pours from an inverted tube; +2 = solid gel which pours from an inverted tube; +3 = solid gel which does not pour from an inverted tube. All samples contained 0.2% DMSO.

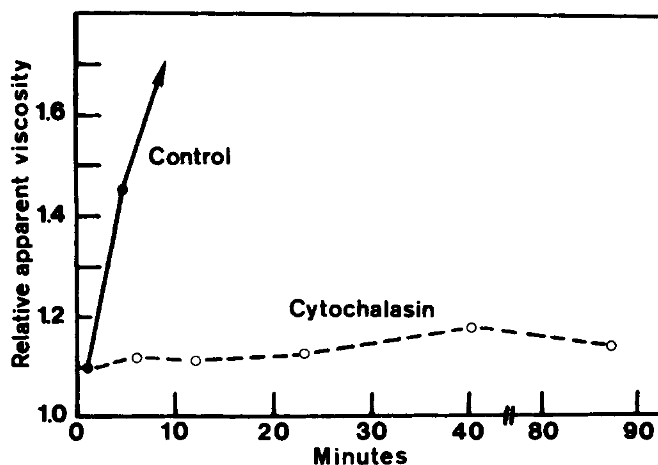

Fig. 7. Inhibition of apparent viscosity changes in warmed Acanthamoeba extract by 2 μg/ml of cytochalasin-B. At zero time a control sample with DMSO alone and the experimental sample with the drug and DMSO were warmed to 25°C by adding them to Ostwald viscometers. Flow times were recorded at intervals. By 10 min the control sample gelled and would not flow through the viscometer.

STRUCTURAL STUDIES

What is the structure of the cytoplasmic matrix? Can the distribution of actin filaments be related to the consistency of living cells? There are many studies of filament distribution in cells, but there are not definitive answers to these questions because of difficulty in preserving actin filaments for electron microscopy. In the following paragraphs, I will review our evidence that actin filaments may, in fact, be destroyed by osmium tetroxide as conventionally used for fixation, and the progress we have made in solving this problem (25, 26).

Electron micrographs of actin filaments prepared by negative staining show that the filaments are relatively straight and unbranched (27). The actin filaments inside cells prepared for electron microscopy by negative staining have a similar appearance (28). In contrast, the actin filaments seen in most fixed cells appear as "microfilament networks"; individual filaments are bent, broken and branched (Fig. 8A). Even when cytoplasmic actin filaments are clustered in bundles where they appear relatively straight, individual filaments are frequently damaged. Our model studies (25, 26) show that straight actin filaments can be transformed into microfilament networks by the destructive action of osmium tetroxide and dehydration, so we suspect that similar artifacts are found in fixed cells.

The destructive effect of osmium tetroxide on actin filaments can be shown by polarized light microscopy (26) and by viscometric measurements (Fig. 9). In this experiment actin filaments were fixed with glutaraldehyde and then separated from the glutaraldehyde by gel filtration. After measuring the viscosity of the fixed filaments, osmium tetroxide was added to a concentration of 1% (40 mM), and the loss of actin filament integrity followed by further viscosity measurements. The destruction of the

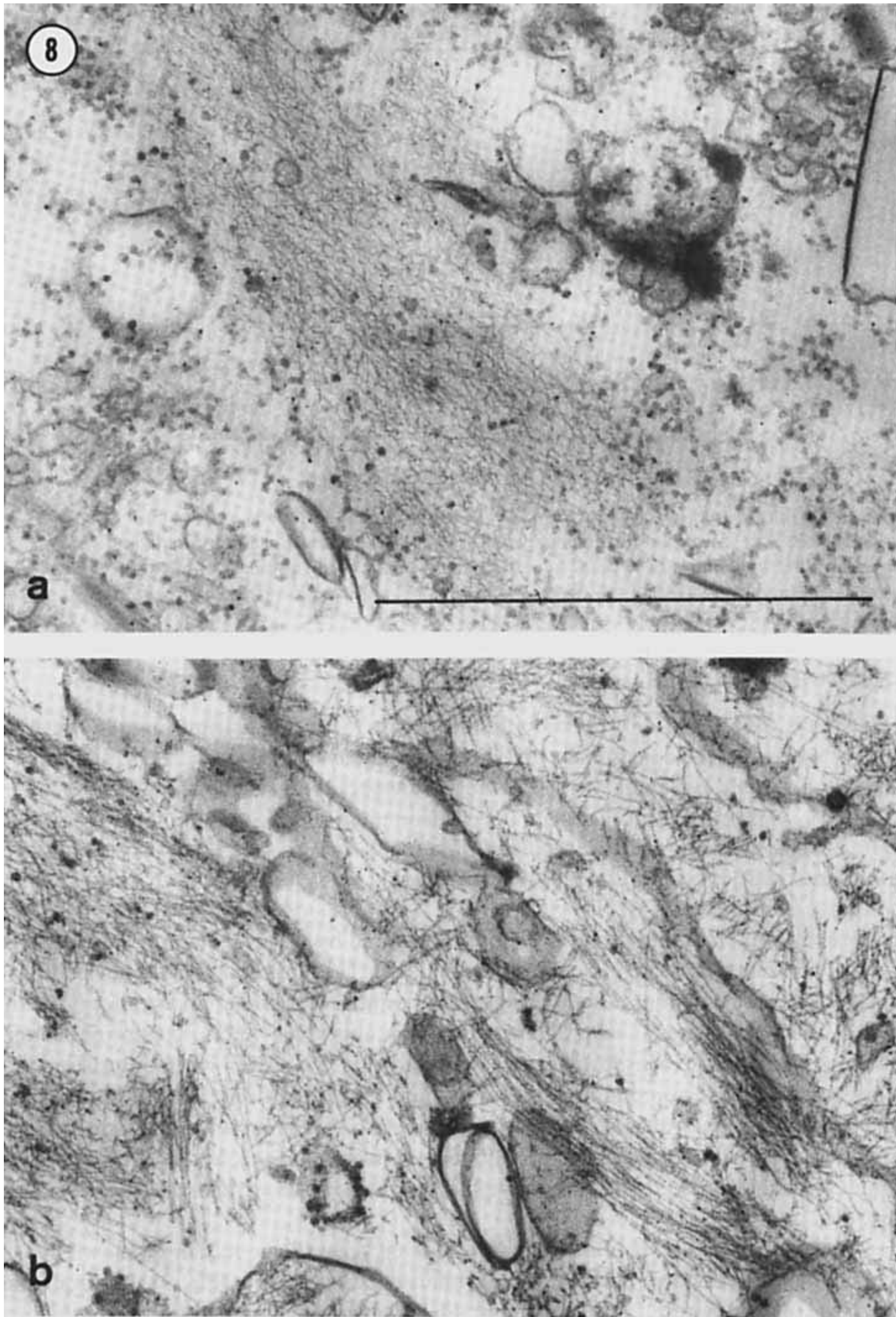

Fig. 8. Transmission electron micrographs of thin sections of Acanthamoeba extracted with 50% glycerol: 50% 50 mM KCl, 5 mM $MgCl_2$, 20 mM phosphate buffer, pH 7.3 containing either (a) no added protein or (b) 3 mg/ml of muscle tropomyosin. Fixation with glutaraldehyde followed by 1% osmium tetroxide in 20 mM phosphate buffer, pH 7.3. Part (a) shows the best preserved actin filaments observed without tropomyosin. Part (b) shows the straight, slightly thicker filaments seen after treatment with tropomyosin. Bar is 1 μm. Magnification 67,000 ×.

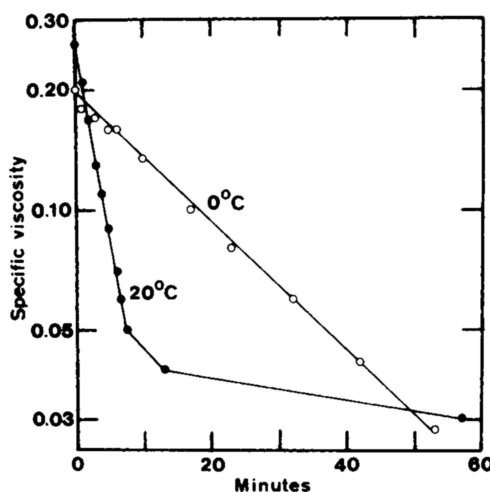

Fig. 9. Viscometric analysis of actin filament destruction by 1% osmium tetroxide. Muscle actin filaments were fixed in 1% glutaraldehyde for 30 min at 24°C and then separated from the glutaraldehyde by gel filtration in 100 mM phosphate buffer, pH 7. By absorbance at 290 nm the concentration of actin was 0.4 mg/ml. Viscosity (dl/gm) was measured in Ostwald viscometers. At zero time osmium tetroxide was added and the viscosity measured at intervals.

filaments is a first-order process with a half-time or a few min at 20°C. The destruction rate is about one-fifth as large at 0°C. The rate of destruction of unfixed actin filaments is similar. Using this quantitative assay we found that the rate of destruction is a function of osmium concentration, buffer type, buffer concentration, pH, and temperature. Under the least favorable conditions, as few as 15 osmium molecules per actin molecule can reduce the actin viscosity by 80% in less than 10 min. Under favorable conditions (Fig. 9), the rate of destruction can be reduced by a factor of at least 500.

What does osmium tetroxide do to the actin to cause the reduction in viscosity? Osmium reacts rapidly with some amino acid side chains, especially cysteine, methionine, arginine, and asparagine. As a consequence of these or other reactions of this powerful oxidizing agent, the actin polypeptide is consistently cleaved at a number of specific places (Fig. 10). If the actin is separated from the osmium by rapid gel filtration, the destruction stops and the viscosity remains constant thereafter. At the point where the viscosity is reduced to 60% of its original value, about 5% of the actin has been cleaved into peptides; these include 2 prominent species with molecular weights of about 40,000 and 20,000 daltons. This indicates that destruction of about 1 in 20 actin molecules is sufficient to cause the filaments to fragment. More extensive reaction of osmium with actin, under conditions like those sometimes used for ultrastructural preservation, results in the degradation of all of the actin molecules into small peptides. Pretreatment of actin filaments with glutaraldehyde, to cross-link the actin molecules (29), does not prevent osmium from cleaving individual actin molecules and destroying the filaments.

Actin-tropomyosin filaments are more resistant to osmium degradation than are pure actin filaments. The thin filaments of skeletal muscle, which are composed of actin, tropomyosin, and troponin, and actin filaments combined with tropomyosin in vitro, are relatively easy to fix for electron microscopy (Fig. 11). Pure actin filaments are reduced to a microfilament network by the same treatment (Fig. 11).

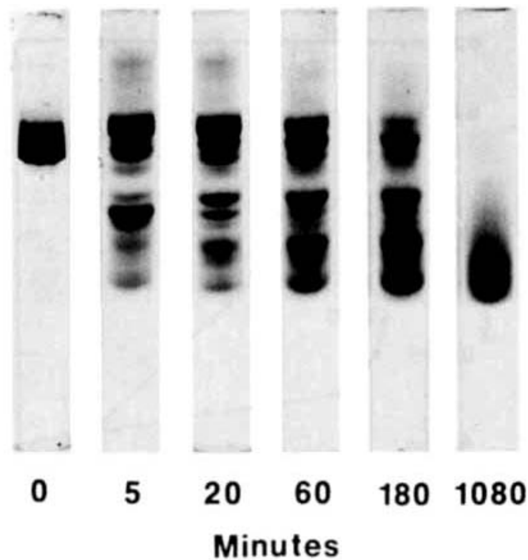

Fig. 10. Gel electrophoretic analysis of actin molecule destruction by 1% osmium tetroxide. Actin filaments in 50 mM phosphate buffer, pH 7, were treated with 1% osmium tetroxide for various times. The reaction was stopped by dialyzing small samples against phosphate buffer at 0°C. The resulting peptides were separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Twenty-five micrograms protein were loaded on each gel.

The actin filaments in Acanthamoeba can also be stabilized for fixation by combining them with tropomyosin. The muscle tropomyosin is introduced into the cytoplasm by extracting the cells in a glycerol solution containing the protein. After this treatment there are networks of interlacing straight actin-tropomyosin filaments concentrated in the cortex and extending throughout the cytoplasm (Fig. 8B). There are numerous contacts of these filaments with the plasma membrane and organelle membranes.

Since the stabilized actin filaments in situ (Fig. 8B) looked similar to the stabilized actin filaments in the gelled extract (Fig. 4), it seems likely that the cortical region of the Acanthamoeba has a gel-like consistency. Morphological proof of this idea will have to await the perfection of fixation protocols which preserve the structure of the actin filaments directly in situ, since the introduction of stabilizing tropomyosin molecules by glycerination must alter the natural arrangement of the cytoplasmic actin filaments.

These model studies suggest that the ease of filament preservation for electron microscopy in a particular cell type is directly related to the association of tropomyosin or other proteins with the actin filaments. In those cases where actin filament preservation is difficult or impossible, it is likely that the protein is bare. Examples include the actin filaments in Acanthamoeba and in the cortex of many other cells. In those cases where tropomyosin is associated with cytoplasmic actin filaments, such as the intestinal microvilli (30) and the stress fibers of cultured cells (31), the filaments are relatively easy to preserve. If this pattern holds true in other cases one can predict that the easily preserved actin filaments in the retinal cells described by Burnside [this volume, pp. 257 (209) – 275 (227)] are actin-tropomyosin filaments and the poorly preserved actin filaments in the same cells are bare.

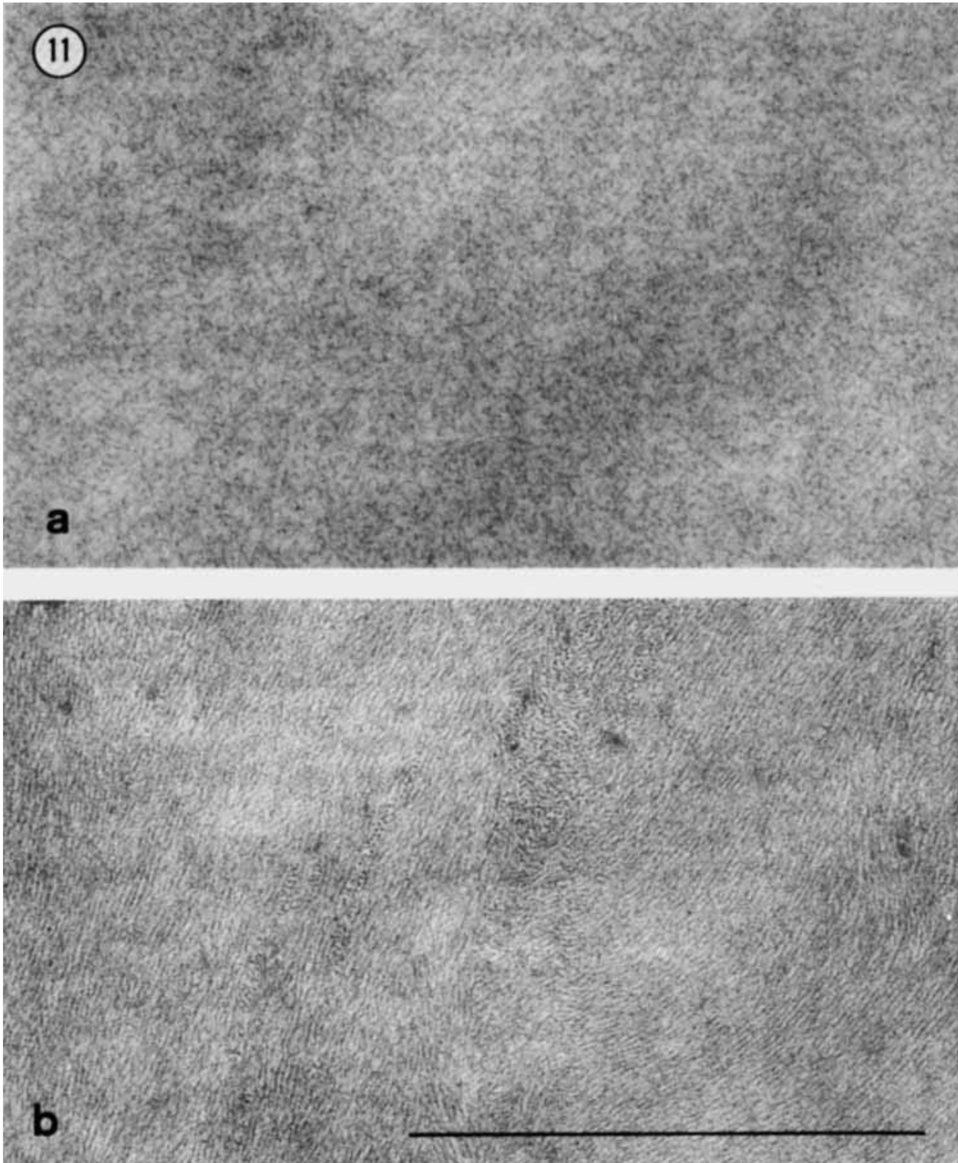

Fig. 11. Electron micrographs of thin sections of fixed-embedded pellets of (a) actin and (b) actin-tropomyosin. Fixation with 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.3, followed by 1% osmium tetroxide in the same buffer for 1 hr at room temperature. Bar is 1 μm. Magnification 67,000 ×.

Buckley (32) has taken another approach which has revealed important morphological features of the cytoplasmic matrix. Rather than striving for methods to preserve filament structure for analysis in thin sections, he devised methods to critical point dry whole cells for examination in the high voltage electron microscope. His micrographs show that the cytoplasmic matrix is filled with short, thin filaments forming a branched network, which are referred to as "trabecular networks." It is not yet clear how

the trabecular networks relate to the well-preserved actin filament networks shown in Figs. 4 and 8, but the presence of high concentrations of actin in his cells, and the similarity of actin filament and trabecular filament dimensions, argue that the trabecular networks are composed, at least in part, of actin. If so, I suspect that the filaments suffer some damage during preparation for electron microscopy. Nonetheless, this work strongly supports the idea that the cytoplasmic matrix is a cross-linked filamentous gel.

RELATION OF CONTRACTILE PROTEIN GELS TO OTHER CYTOSKELETAL ELEMENTS AND THE CELL SURFACE

There is now evidence for at least 3 different cytoplasmic fiber systems with cytoskeletal properties. These are microtubules, actin filaments, and intermediate (10 nm) filaments. How do these cytoskeletal elements relate to one another? There are good examples of each fiber system acting alone to perform a structural role: microtubules in the axostyles of heliozoans (7), actomyosin in the giant amoebae (14), and intermediate filaments in smooth muscle (33). In addition, there are numerous examples of these fiber systems coexisting in the cytoplasm. It is attractive to think that they are interacting in some way, but there is little convincing evidence for such interactions. For example, actin filaments (34, 35) and myosin molecules (26, 36) are found along with microtubules in the mitotic spindle, but there is no direct evidence that they interact in meaningful ways to move chromosomes. In fact, in Hela cells stained with antibodies against myosin and antibodies against tubulin labeled with contrasting fluorochromes, it is clear that the contractile protein and the microtubules have entirely different distributions in the cytoplasm (37). This leads to the suggestion that the dynamic contractile protein gel and the microtubules are complimentary cytoskeletal systems. Their relationships to the intermediate filaments is unknown.

There is widespread belief that the actomyosin system is connected to the plasma membrane, and even more speculation regarding the functions of such interactions. The evidence supporting these ideas is still very weak, although actin filaments have been found associated with isolated plasma membranes (38,39), and numerous electron microscope studies have shown actin filaments apparently in contact with the plasma membrane (30,40,41). It has been possible to remove the actin filaments from isolated membranes (38), but there has been no success in linking them back. Nonetheless, it seems likely that the structural elements in the cytoplasmic matrix are connected directly or indirectly to the plasma membrane, making it possible for them to influence the activities of the cell surface.

CONCLUSIONS

The biochemical and morphological data reviewed here convince me that Dujardin's ideas about the interrelation of the contractile machinery and cytoskeletal elements is correct. His sarcode is now recognized as actomyosin. The contractile proteins are capable of forming a fairly rigid structural matrix. These cytoskeletal properties of the cytoplasmic contractile proteins provide a molecular explanation for the classic observations on cytoplasmic structure from the 1930s. Given this new insight, it might be worth reinvestigating some of the physical properties of living cytoplasm. I suspect that some regions of the cytoplasm are essentially solid state environments with the organelles held rigidly in place by the structural elements in the matrix. The absence of Brownian motion in parts

of most living cells supports this idea. The actin filaments in the matrix may even provide a scaffolding for some enzyme systems, as a few enzymes have been shown to bind to actin filaments (42).

If these ideas are correct, they explain why nonmuscle cells have such high concentrations of actin. Actin accounts for 10–15% of the total protein in some cells like Acanthamoeba; this is probably much more than is needed for motile force generation (43). The excess actin probably serves in a structural role.

ACKNOWLEDGMENTS

The original work reported here was supported by U.S. Public Health Service Research Grant GM-19654 and Research Career Development Award GM-70755. The author is indebted to Chatal Fujiwara for translating the quotations from Dujardin's work, to Pam Maupin-Szamier for help with the electron microscopy, and to Linda Griffith, Dan Kiehart, and Mark Mooseker for their critical comments on the text.

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