ACTIN AND MYOSIN AND CELL MOVEMENT

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INTRODUCTION

Movement of living cells is one of the fundamental properties of life. Various biological movements have now been related to four general mechanisms. Bacteria are propelled by the mysterious beating of their simple flagella, which are composed of a single type of protein lacking any enzymatic activity.[1] Certain peritrichous ciliates possess a contractile stalk containing a rubberlike organelle, the spasmoneme, which contracts when exposed to Ca^{++}.[2] The beat of cilia and sperm tails (and perhaps some cytoplasmic movements as well) is apparently due to the interaction of microtubules and the ATPase dynein.[3,3a] In muscle, contractile force is generated by the sliding interaction of actin and myosin filaments, with the energy provided by the hydrolysis of ATP.[4]

Until recently, little or nothing was known about the molecular mechanism of a diverse group of biological movements, including amoeboid locomotion, cytoplasmic streaming, cytokinesis, and morphogenetic movements. The studies reviewed here establish that many motile cells which exhibit such movements, including protozoa and vertebrate cells, which lack the highly organized contractile apparatus found in muscle, possess the contractile proteins actin and myosin. Therefore, it is likely that, as in muscle, the interaction of force transmitting actin filaments with the energy transducing enzyme myosin is responsible for generating the force for movement in these cells.

The contractile proteins from cells other than muscle are sometimes referred to as "nonmuscle" actin and myosin. Because this seems somewhat awkward, and because we want to differentiate these proteins from their myofibrillar counterparts, we have chosen to call them *cytoplasmic* actin and myosin, as first suggested by Bray.[5] The cytoplasmic actins and myosins will be specifically named by using the name of the cell of origin, for example, *Acanthamoeba* actin or *Physarum* myosin. This practice seems preferable to coining new names, such as "thrombosthenin-M" for platelet myosin or "neurin" for brain actin.

This review is a comprehensive critical evaluation of biochemical studies on cytoplasmic actin and myosin. Selected physiological and cytological studies relating these contractile proteins to cell motility are considered in less detail. Thorough

coverage of early descriptive work on cell motility is found in the book *Primitive Motile Systems in Cell Biology*,[5a] and more recent studies are found in the report of the symposium *Motile Systems of Cells*.[5b] Komnick, Stockem, and Wohlfarth-Botterman[5c] have recently written an extensive review stressing cytological studies on motility of amoebae and myxomycetes, while two brief reviews by Pollard[5d] and Huxley[5e] cover some of the recent biochemical studies on cell motility.

HISTORICAL BACKGROUND

The pioneering work on the biochemical basis of cytoplasmic movement was reported by Loewy in 1952.[6] He investigated an extract from the plasmodium of the myxomycete *Physarum polycephalum* in the hope that it "might give insight into the mechanism by which unspecialized or primitive tissue is able to convert chemical energy into mechanical work." Quite remarkably, the extract shared several important properties with muscle actomyosin, including the effect of ATP upon its viscosity and the ability to hydrolyze ATP. This suggested that there might be a basic biochemical similarity between the streaming in *Physarum* and the contraction of muscle. The boldness of these experiments is emphasized when one recalls that muscle actin and myosin had only recently been separated and identified as the contractile proteins of muscle[7] and that only scant information was available on the ultrastructure or chemistry of cytoplasm.

Several years later, Ts'o and co-workers[8] studied *Physarum* actomyosin with the most sophisticated available techniques, including electron microscopy and analytical ultracentrifugation, but they were unable to resolve the components of the mixture. Nakajima also examined a crude preparation of *Physarum* actomyosin and concluded that it had many properties in common with muscle actomyosin.[8a] In 1959, Bettex-Galland and Luscher[9] extended these studies to a nonmuscle vertebrate cell, human platelets, from which they extracted a crude actomyosin, which they called thrombosthenin.

At about the same time, Hoffman-Berling (reviewed by Arronet[10]) carried out a number of experiments on contractile cell models. Inspired by reports that glycerinated muscle and isolated myofibrils contracted when treated with Mg^{++} and ATP, he extracted a number of different cells with glycerol and found that feeble contractions could be induced by exposure to Mg^{++} and ATP.

Consequently, by 1960 it was known that actomyosin was not confined to muscle but also could be extracted from motile vertebrate and protozoan cells, although there seems to have been limited interest in and acceptance of this work. The skepticism resulted, at least in part, from the heterogeneous nature of the protein mixtures that were used in these experiments and the difficulty in resolving the active components with available analytical and preparative techniques. Even the separation of thrombosthenin into two fractions having the properties of actin and myosin[11] failed to stimulate more widespread investigation.

The turning point in this field was the purification of actin from *Physarum* by Hatano and Oosawa in 1966.[12,13] The *Physarum* actin had physical, chemical and biological properties which established its close relation to muscle actin. Shortly thereafter, both Hatano and Tazawa[14] and Adelman and Taylor[15,16] developed methods for purifying *Physarum* myosin. Building on these pivotal investigations, recent biochemical work has moved forward quickly, aided by modern analytical and preparative methods such as gel electrophoresis and column chromatography on ion exchangers and gel filtration media.

The second major stimulus for further investigation was the development by Ishikawa, Bischoff, and Holtzer[17] of an ingenious technique for identifying actin filaments *in situ*. This procedure takes advantage of Huxley's observation that a soluble fragment of muscle myosin called heavy meromyosin will bind to actin filaments, forming distinctive complexes with the shape of periodically repeating arrowheads.[18] Ishikawa found that after glycerol extraction heavy meromyosin could enter cells and bind to the actin filaments, forming arrowhead complexes which he visualized using the electron microscope. This procedure has now been used to identify and localize actin filaments in a variety of cells and is helping to relate the biochemical findings to events taking place on the cellular level.

MECHANISM OF MUSCLE CONTRACTION

As it is now apparent that many cells share common contractile proteins with muscle, it seems

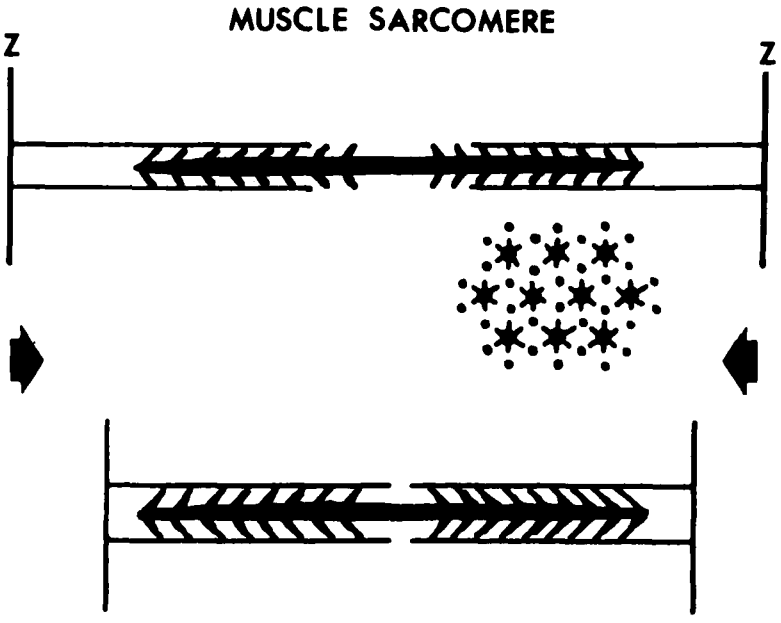

FIGURE 1. Line drawings of a striated muscle sarcomere. From the top, longitudinal section of an extended sarcomere, cross section through a region of thick and thin filament overlap, longitudinal section of a contracted sarcomere.

helpful to mention here for orientation some of the most important facts known about muscle contraction. It is these facts which have guided (and also prejudiced) investigations on the contractile molecules in other cells. More extensive accounts of the curent status of muscle research are found in the *Cold Spring Harbor Symposium on Quantitative Biology*, Volume 37[19] and in several recent reviews.[4,20-23]

Striated muscle consists of two types of interdigitating filaments, which in cross section are seen to be arranged in a double hexagonal array (Figure 1). The basic unit of the contractile apparatus is called a sarcomere and is repeated many times in series and in parallel in each muscle cell, accounting for the microscopic striations seen in skeletal and cardiac muscle cells. The thin filaments are polymers of the globular protein actin, associated with the control proteins troponin and tropomyosin. The thick filaments consist primarily of a bipolar assembly of the fibrous protein myosin. The bipolarity of the thick filament and the opposite polarity of the actin filaments at each end of the sarcomere make the whole sarcomere bipolar.[18]

Muscle contraction is caused by the synchronous shortening of its sarcomeres. As the sarcomeres shorten, the thin filaments slide past the thick filaments, pulling the ends of the sarcomere toward the middle.[24] The force for this sliding movement is generated by the cyclic interaction of myosin cross bridges with the actin filaments.[4,25] Each cross bridge has an actin binding site and ATPase activity and is free to swing out from the backbone of the thick filament to contact the actin filament.[26]

While the details of the force generating mechanism are still under investigation, it has been established that the energy for muscle contraction comes from the myosin-catalyzed hydrolysis of the terminal phosphate of ATP.[27] In resting muscle the myosin ATPase activity is very low, but during contraction the cyclic interaction of myosin with actin stimulates the myosin ATPase, providing the energy for movement.[28]

The interaction of myosin and actin (and hence muscle contraction) is regulated by a reversible inhibition of actin-myosin binding. This regulatory block is modulated by the concentration of free Ca^{++} in the muscle cytoplasm and arises in two ways: (1) In the muscles of higher organisms, the regulatory system is located on the thin, actin-containing filaments and consists of a complex of four proteins called troponin-tropomyosin.[29,30]

Tropomyosin is an alpha-helical coiled-coil[31] which lies along the length of the actin filament.[32,33] Troponin consists of three protein subunits called troponin-I (for inhibitory component), troponin-C (for Ca^{++} binding component), and troponin-T (for tropomyosin binding component).[30] The troponin complex binds to tropomyosin and is situated at intervals of about 40 nm along the actin filament.[29,32-33] Together, troponin-tropomyosin blocks actin-myosin binding in the absence of Ca^{++} [33a] (2) In the muscles of molluscs and some other invertebrates, the regulatory system is incorporated into the myosin molecule[34,35] and depends on the presence of a low molecular weight polypeptide attached to the myosin.[36] The molluscan thin filaments contain actin and tropomyosin but no troponin.[35]

The outcome is the same in both cases: In resting muscle the Ca^{++} concentration is 10^{-7} *M* or less, and the regulatory systems prevent actin-myosin interaction. Muscle contraction is activated by a complicated sequence of events, beginning with the arrival of a nerve impulse at the muscle and ending with the release of Ca^{++} into the cytoplasm[36a] from the membranes of the sarcoplasmic reticulum. This causes the Ca^{++} concentration in the muscle cytoplasm to increase to about 10^{-5} *M*. The Ca^{++} then binds to the regulatory component, either the troponin or the myosin, inhibition of actin-myosin binding is reversed, and contraction ensues.[29]

ACTIN

Numerous studies show clearly that actin is a major protein constituent of various cells, which lack a highly organized contractile apparatus, and closely resembles actin from muscle. As is developed in more detail below, these cytoplasmic actins share with muscle actin the ability to form double helical filaments from globular monomers, to form periodic arrowhead-shaped complexes with heavy meromyosin, to activate the Mg^{++} ATPase of myosin or heavy meromyosin, and to interact with the regulatory proteins troponin-tropomyosin. In view of these similarities, it is not surprising, therefore, that the amino acid composition and peptide maps of several of these actins, and the composition of certain peptides isolated from one of these actins, closely resemble the corresponding amino acid compositions for muscle actin.

Isolation

Cytoplasmic actins are generally prepared using a small repertory of procedures which take advantage of properties specific for actin.

In some cases cells are initially dried with acetone, which serves to denature myosin and some other, but not all, unwanted proteins.[37-39] The actin is then extracted from the acetone powder with a dilute solution of ATP and a reducing agent such as cysteine or 2-mercaptoethanol.[13,39] Yang and Perdue investigated the optimal extraction period and found that the purity of the actin extracted decreased after an initial 10 min extraction.[39] Other workers have extracted for various apparently arbitrary lengths of time. In favorable cases (actin from glycerinated fibroblasts[39] and brush borders[38]), homogeneous actin (judging by gel electrophoresis) can be isolated from extracts of acetone powders by simply carrying out one additional step consisting of a single cycle of polymerization and depolymerization. The fibroblast actin[39] was contaminated by a protein with the electrophoretic mobility of tropomyosin. This contaminant was removed by collecting the actin polymers formed in the presence of 0.6 *M* KCl, which is expected to dissociate contaminating tropomyosin from actin filaments.[40] In brush border[38] the concentration of actin is so high that contamination with other proteins may be a minor problem.

In other cases cells are initially extracted with concentrated solutions of KCl. Using *Dictyostelium*, Woolley found that actin was extracted after short periods but that actomyosin was extracted only after longer periods.[43] In other cases it is not known whether varying the period of extraction leads to preferential extraction of one or another protein. KCl extracts of cells have been further fractionated in several different ways. In the case of *Physarum*,[12,13] *Acanthamoeba*,[44] and *Dictyostelium*,[43] muscle myosin was added to the extract and the hybrid actomyosin precipitated in 0.05 *M* KCl. The precipitate was then dried with acetone, which denatures the myosin, and actin was extracted from the acetone powders using dilute buffers containing ATP and a reducing agent. Final purification was achieved by various combinations of polymerization and depolymerization, gel filtration, ammonium sulfate fractionation, and isoelectric precipitation. Only in the case of *Acanthamoeba* actin has the value of

each step been demonstrated by showing that each step led to actin of higher content of N^{τ}-methylhistidine and higher reduced viscosity.[44]

When actin is isolated as a hybrid actomyosin, it is necessary to show that the actin isolated is not a contaminant from the added myosin. This has been done by showing that the amount of protein isolated from the muscle myosin by an identical purification procedure could not account for the final yield of cytoplasmic actin actually attained,[43,44] or by demonstrating that the muscle myosin showed neither a viscosity drop[12,13,43,44] nor superprecipitation[12,13] in the presence of ATP, or by showing that the actin isolated from cells labeled with a radioactive amino acid has the same specific activity as the total cell protein.[44]

In the case of KCl extracts of platelets[45] and brain,[49,50] contractile proteins which behave like typical actomyosin are present in very high concentration, making it possible to isolate actomyosin directly. Platelet actin, which appears homogeneous by gel electrophoresis, has been isolated from platelet actomyosin[46-48] using slightly different combinations of the procedures discussed above. Actin is isolated from brain actomyosin by gel filtration on Sephadex® G-200 or by sucrose density gradient centrifugation in the presence of 0.6 *M* KI and 1 m*M* ATP, which separate the actin and myosin. The final actin preparation appears homogeneous on gel electrophoresis in the presence of urea (except for some material of unknown composition which fails to enter the gel).

Adelman and Taylor[15,16] purified *Physarum* actin from a high speed tris-maleate-pyrophosphate extract using ammonium sulfate fractionation and gel filtration in a dilute buffer.

All the cytoplasmic actins which have been examined appear homogeneous by polyacrylamide gel electrophoresis, and several also appear homogeneous by sedimentation velocity and equilibrium ultracentrifugation. However, all cytoplasmic actins contain somewhat less than the theoretical (1 mol/mol) amounts of bound nucleotide, and most contain less N^{τ}-methylhistidine (Table 1), raising the possibility that they are not yet completely pure. The data on brain actin present some puzzling inconsistencies. The apparent homogeneity by urea gel electrophoresis is in accord with one value for N^{τ}-methylhistidine content (0.9 mol/45,000) but disagrees with a second value for N^{τ}-methylhistidine content (0.3 mol/45,000) and the slow continued release of phosphate after polymerization (discussed below).

Physical-chemical Properties

All the physical-chemical parameters of cytoplasmic actins which have been investigated have been found to be almost identical with the same parameters for muscle actin. These include the monomer molecular weight, the amino acid composition of the protein and certain of its peptides, the content of bound nucleotide and divalent cation, and the ability to polymerize.

Molecular Weight

Most cytoplasmic actins have exactly the same subunit molecular weight as muscle actin as determined by gel electrophoresis in the detergent sodium dodecyl sulfate (Table 1), but the absolute value of the molecular weight is in doubt because the molecular weight of muscle actin is subject to some uncertainty. Muscle actin molecular weights in the range 43,000 to 48,000 daltons have been obtained by analytical ultracentrifugation,[60] by gel filtration, and from the content of nucleotide[54,60] and of N^{τ}-methylhistidine,[55-58] but the recently complete amino acid sequence of muscle actin gives a calculated molecular weight of 42,000 daltons.[61] Because of this inconsistency, we have arbitrarily used a molecular weight of 45,000 daltons for all of the actins in order to have a common basis for the comparative data presented in the tables.

Actins must consist of a single polypeptide because determinations of the native molecular weight of actin from muscle[54,60] and platelets[62] are approximately the same as the molecular weights in denaturing solvents.

Amino Acid Composition

Amino acid compositions are now available for actin from four different cells (three protozoa and sea urchin eggs) and human platelets (Table 2). The compositions of these actins are remarkably similar to one another and to muscle actin. No amino acid in the cytoplasmic actins differs from the corresponding residue in muscle actin by as many as ten residues, and only four differences of ten or more residues (aspartic acid, methionine, isoleucine, and leucine) are found among the cytoplasmic actins. Two of these latter differences may be caused by artifacts of hydrolysis. The

TABLE 1

Physical Chemical Properties of Various Actins

Source of actin	Monomer molecular weight (daltons)	Reduced viscosity of polymers (dl/g)		Bound nucleotide	Bound divalent cation	N^{τ}-Methyl-histidine content
		0.1 M KCl	0.1 M KCl + 1–2 m*M* $MgCl_2$	(moles/45,000 g protein)		
Acanthamoeba[44,51]	45,000	3.9				0.81
Brain (cat, cow or rat)[49,50]				0.69		0.3 or 0.9
Brush border (chicken intestine)[38]	46,000					
Dictyostelium[43]	48,000	3.5	2.7			0.86
Egg (sea urchin)[37,74,8]		2.1				
		0.8	0.27			
		1.8	0.1 ($MgCl_2$ only)			
Fibroblast (chick embryo)[39]	45,500					
Physarum	57,000[13]			0.79		
		5.6[52]	0.56			
	37,000–44,000[15,16]	3.6	3.4	0.71		
	45,000[55,8]				1 Ca^{++}[53]	
Platelet (cow)[47]	45,000					
(human)	44,000		12	1[62]		1
(pig)[47]	45,000					
Rabbit striated muscle	45,000[54]		11.9	1.0	1.1	1.05[55,56-58]
		6.7[59]	7.0			

Published molecular weights are cited except for actin from *Acanthamoeba* and cow and pig platelets, which are assigned the molecular weight 45,000 daltons because they coelectrophorese with rabbit actin. The reduced viscosity of *Acanthamoeba* actin was presented in Table 1 of Reference 44; the other reduced viscosities were calculated from the highest measurements of viscosity shown in the respective references. The data for content of nucleotide and N^{τ}-methylhistidine were normalized to the molecular weight 45,000 daltons using data given in the respective references.

methionine content of sea urchin egg actin and the isoleucine content of *Physarum* actin are unusually low, but it is not clear from the published reports whether the precautions used in the case of the *Acanthamoeba* actin[44,46] were taken to ensure complete recovery of these amino acids. Hence, these values may be spuriously low. Therefore, it is possible that the content of only two amino acids differs by as many as ten residues among the various cytoplasmic actins. In contrast, the amino acid composition of the microtubule subunit, tubulin, varies considerably from species to species.[3]

The unusual amino acid N^{τ}-methylhistidine has been identified in four cytoplasmic actins (Tables 1 and 2). Muscle and human platelet actins contain 1 mol, *Acanthamoeba* and *Dictyostelium* actins contain about 0.8 mol, and brain actin contains about 0.3 or 0.9 mol/mol of protein. All these actins appear homogeneous by gel electrophoresis, but, because some contain less than 1 mol of N^{τ}-methylhistidine/mol of protein, it is possible that they are incompletely methylated. However, the possibility that they are not completely purified cannot be ruled out. Because N^{τ}-methylhistidine is a component of these actins, we expect that it will be identified in other cytoplasmic actins.

About 1 mol of the unusual amino acid N^{ϵ}-dimethyllysine/mol of protein, together with smaller amounts of N^{ϵ}-monomethyllysine and traces of N^{ϵ}-trimethyllysine, has been identified in actin from *Acanthamoeba*.[51,63] These amino acids are not present in actin from *Dictyostelium*[43] or rabbit muscle,[64] but their identification has not been attempted in other actins.

TABLE 2

Amino Acid Composition of Various Actins

Residue	Acanthamoeba[44]	Dictyostelium[43]	Physarum[13]	Sea urchin egg[74a]	Human[62] platelet	Rabbit muscle[57]
			(moles/45,000 g protein)			
Lys	20.7	22.0	18.4	24.0	20.0	21.0
Me-Lys	0.27	0	?	?	?	0
$(Me)_2$-Lys	0.89	0	?	?	?	0
$(Me)_3$-Lys	trace	0	?	?	?	0
His	7.2	7.7	7.6	7.5	9.5	8.1
N^{τ}-Me-His	0.81	0.86	?	?	0.94	0.97
Arg	19.7	19.5	18.0	22.3	18.9	20.7
Asp	33.9	41.0	37.8	48.5	35.6	38.6
Thr	29.3	29.2	27.4	26.0	27.6	27.9
Ser	25.3	26.1	25.2	29.0	26.9	23.3
Glu	44.9	46.4	51.3	49.5	49.6	44.5
Pro	21.5	17.9	27.4	18.1	21.6	20.9
Gly	36.4	33.0	33.3	30.8	34.7	31.5
Ala	33.9	34.5	30.6	31.8	35.0	32.3
1/2Cys	5.6	?	5.0	3.7	4.2	5.3
Val	22.9	23.1	14.4	21.2	17.6	21.8
Met	16.8	15.3	12.6	5.0	10.4	16.9
Ile	28.7	28.2	15.3	22.3	24.4	28.6
Leu	37.4	28.4	27.4	30.8	32.3	29.2
Tyr	15.6	14.8	14.4	17.1	12.6	17.0
Phe	13.4	14.4	13.0	17.1	14.3	13.7
Trp	5.7	?	?	?	4.8	4.3

Isolated Peptides

Amino acid compositions are available for three cyanogen bromide fragments of *Acanthamoeba* actin.[51] These include a peptide which contains N^{τ}-methylhistidine (peptide CB-10), a peptide which contains N^{ϵ}-methyllysine (peptide CB-16), and a peptide which is distinguished by its high content of glutamic acid and the absence of proline (peptide CB-17). The compositions of these peptides resemble the compositions of similar peptides from rabbit muscle actin and bovine cardiac actin.[51,65] While the location of these peptides in the polypeptide chain of *Acanthamoeba* actin has not been established, it is likely from the similarities of amino acid composition and the other close resemblances between actin from *Acanthamoeba* and rabbit muscle that the amoeba and muscle peptides are located in the same region of the respective parent molecules and also that the amino acid sequences are similar.

Fragments of actin from human platelets and rabbit muscle prepared by cyanogen bromide cleavage have been compared by disc gel electrophoresis. Several bands with identical mobilities as well as bands with different mobilities were observed.[62] The relation of these bands to the well-characterized cyanogen bromide peptides from *Acanthamoeba* and rabbit muscle actin might be clarified by amino acid analysis.

Tryptic peptide maps have been used to identify actin in several cells (described below in the section on Methods of Identifying Actin). The results show that actin from muscle and several nonmuscle tissues of one animal all have similar peptide maps, suggesting that there may be a common gene for all actins.[5]

Bound Nucleotide

Actin from muscle binds 1 mol of ATP/mol of monomer.[54,60] Bound nucleotide has been identified in actin from *Physarum*[13] and brain[49] after exposure to solutions containing ATP and is probably present in human platelet actin because phosphate is released (presumably from bound

ATP) during polymerization of this actin.[62] Because actin monomers undergo nucleotide exchange,[67] in order to identify which nucleotide is present in vivo, it will be necessary to isolate the actin in the absence of added nucleotide and to identify the nucleotide present in the actin.

In those cases where data were presented, the cytoplasmic actins bound less than 1 mol of nucleotide/45,000 of protein (Table 1). Because purified muscle actin binds 1 mol of ATP/mol of monomer, it is possible that the cytoplasmic actins as isolated are incompletely purified or partially denatured.

Bound Divalent Cation

Actin from muscle can bind 1 mol of divalent cation (Ca^{++} or Mg^{++})/mol of monomer.[54,68,69] While the binding constant for Ca^{++} is about four times that for Mg^{++},[70] probably only Mg^{++} is bound in vivo because of the vast excess of Mg^{++}. Among cytoplasmic actins, only *Physarum* actin has been investigated for the presence of divalent cation. About 1 mol of Ca^{++}/mol of monomer was identified, and the bound Ca^{++} could be exchanged for Mg^{++}.[53] Because this preparation of *Physarum* actin was isolated in the absence of added divalent cation, Ca^{++} is probably the cation which is bound in vivo.

Stability

Removal of bound ATP[71,72] or divalent cation[68,69] or blockage of certain sensitive sulfhydryl groups[79] causes muscle actin to lose polymerizability. The ability of these factors to stabilize cytoplasmic actins has not been investigated.

Polymerization

Muscle and cytoplasmic actins share the ability to polymerize into filaments. This is a key functional property for actin because only actin organized as filaments can transmit tension, and only actin filaments efficiently activate the myosin ATPase during the conversion of the chemical energy of ATP into mechanical work. It is important, therefore, to study the properties of polymerization in vitro in an attempt to identify factors which influence polymerization in vivo.

Polymerization of actin into ordinary actin filaments (usually referred to as "F-actin") is induced in vitro by adding KCl to a final concentration of 0.1 *M* or $MgCl_2$ to a final concentration of 1 to 2 m*M*.[71] In the case of actin from *Physarum*,[73] and possibly from *Dictyostelium*[43] and sea urchin eggs,[37,74a] however, the polymers formed in the presence of Mg^{++} have different properties from the polymers formed in the presence of KCl alone. This so-called "Mg-polymer" is discussed in more detail below, after the properties of ordinary actin filaments have been discussed.

Polymerization of actin has been studied in four ways, which are discussed below: analytical ultracentrifugation, flow birefringence, electron microscopy, and viscometry.

Analytical Ultracentrifugation

Upon polymerization, the 3S monomer of actin from *Acanthamoeba*,[44] *Dictyostelium*,[43] *Physarum*,[13] and sea urchin eggs[37,74,74a] is converted to a component of higher sedimentation coefficient. The faster sedimenting component generally shows self-sharpening of the sedimenting boundary, indicating the formation of a highly asymmetrical species. This behavior is consistent with filament formation. Most workers note that less than 100% of the 3S component is converted to the high S component, but there have been no quantitative studies of the factors involved with this incomplete polymerization.

Flow Birefringence

Simple observation of the development of flow birefringence by stirring a solution between crossed polaroids can be used as a rough and ready indication of the progress of polymerization during preparative procedures. Quantitative measurements of flow birefringence can be used to determine the average length of actin filaments in solution, but these measurements are infrequently made. Using this technique, Hatano and coworkers found that the average length of *Physarum* actin filaments polymerized in KCl was 2.2 μm, a value in good agreement with direct measurements by electron microscopy.[73]

Electron Microscopy

Actin filaments can be visualized directly by electron microscopy of negatively stained specimens (Figure 2), and all of the purified cytoplasmic actins have been studied in this way. Most cytoplasmic actin filaments have been reported to be about 5 to 8 nm wide with a mean around 6 nm (Table 3). The variation in these measurements

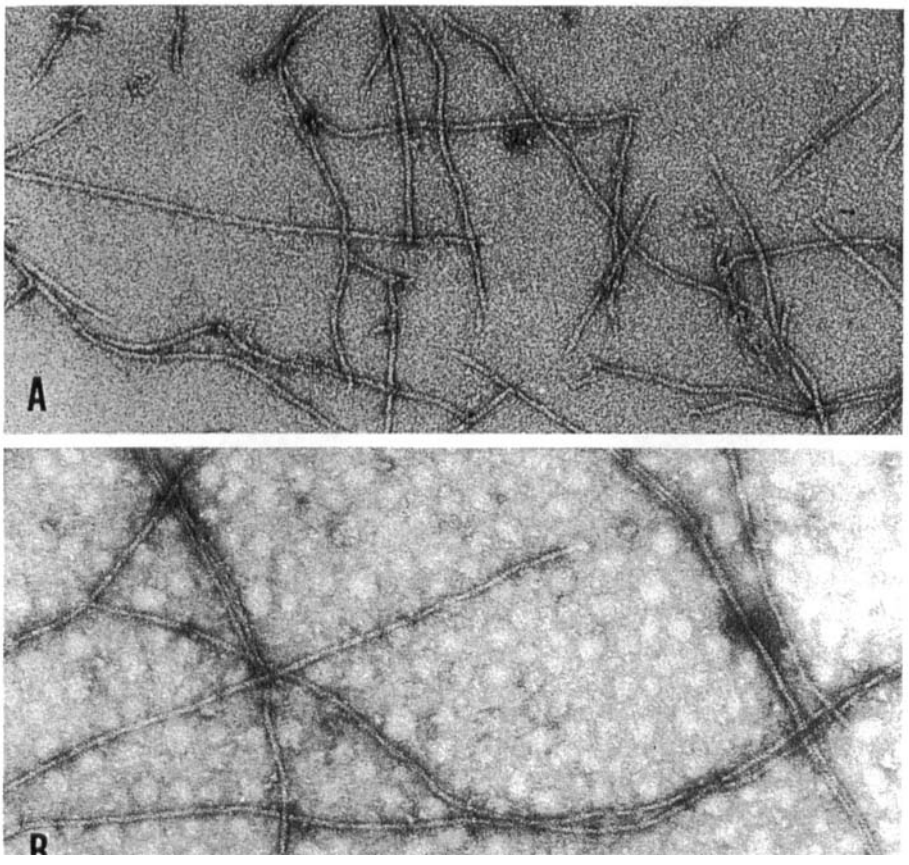

FIGURE 2. Actin filaments negatively stained with uranyl acetate. A. *Physarum* actin filaments in *Physarum* actomyosin treated with Mg^{++} ATP. (Micrograph by V. T. Nachmias.) B. *Acanthamoeba* actin filaments. Magnification x 94,000.

TABLE 3

Dimensions of Bare and Decorated Actin Filaments

Source of actin	Width of filament (nm)	Half pitch of double helix (nm)	Spacing of heavy meromyosin complexes (nm)
Acanthamoeba[75]	5.8 ± 1.1 (± 2 S.D.)	37	37
Dictyostelium[43]	6.0–7.5	35 ± 1.5	35
Egg (sea urchin)[74,74a]	6.0–8.0		
Fibroblast[39] (chick embryo)	8.0 ± 0.5		36.6 ± 4.3
Leukocyte (guinea pig)[76]			36
(horse)[77-78a]	8.0	35[78a]	
Platelet[100a]	7.0		
human	6.0		
Physarum	7.5[14]	35–42	
	5.0–6.0[80]		35–36
	5.0–8.0[16]		
	6.0[73]	29	
Rabbit muscle	6.0–7.0[18]	35–37	35–37
	8.0	35	

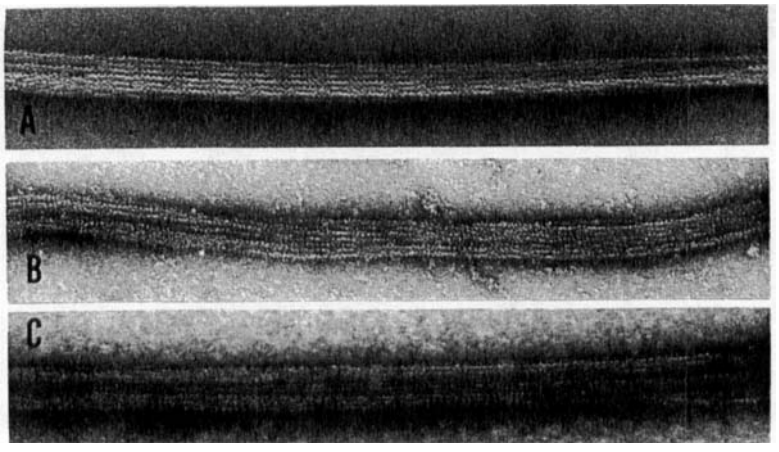

FIGURE 3. Actin paracrystals formed in the presence of 25 or 50 m*M* $MgCl_2$ and negatively stained with uranyl acetate. A. Muscle actin. B. *Dictyostelium* actin. C. Pig platelet actin. (Micrographs by J. A. Spudich.) Magnification x 137,000.

probably reflects the difficulty in measuring the width of the negatively stained filaments rather than differences in the actual width of the different actin filaments.

In favorable cases the substructure of these negatively stained actin filaments can be resolved. The best micrographs indicate that, like muscle actin filaments, the cytoplasmic actin filaments consist of 5 nm globular monomers arranged in a double helix with a half pitch of about 36 to 38 nm (Table 3).

The pitch of the actin helix can be measured more easily from the periodicity of actin paracrystals formed by the side to side association of actin filaments in high concentrations of Mg^{++}[82] (Figure 3). The helices of adjacent actin filaments are in register, thereby reinforcing their periodicity. These paracrystals have been made with actin from *Physarum*,[83] various types of platelets,[47] and *Dictyostelium* (Figure 3).

The half pitch of the actin helix can be measured most simply from the periodicity of the arrowhead-shaped complexes formed by "decorating" actin filaments with heavy meromyosin[18] (Figure 4). The heavy meromyosin binds to the actin molecules in a specific way, giving the arrowhead shape and amplifying the periodicity of the underlying actin filament.[84] Various muscle actins and five purified cytoplasmic actins have been decorated with heavy meromyosin or subfragment-1 from rabbit myosin,

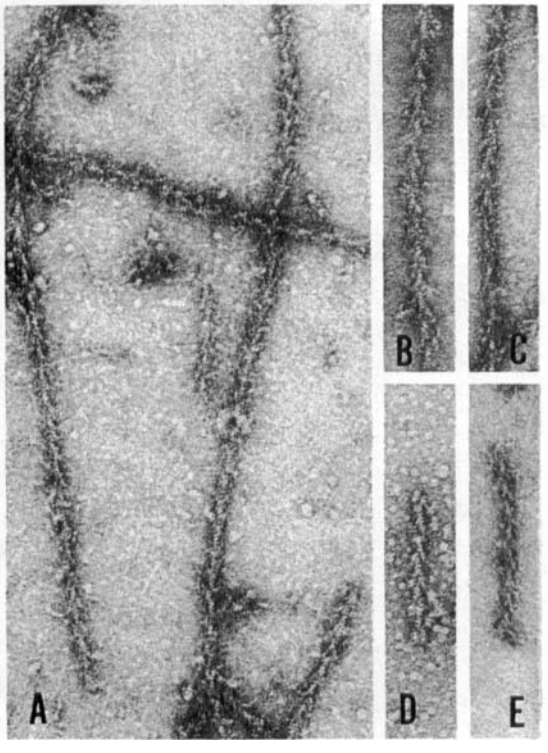

FIGURE 4. Decorated actin filaments from various sources negatively stained with uranyl acetate. A. Muscle actin with muscle heavy meromyosin. B. *Amoeba proteus* thin filament with muscle heavy meromyosin. C. Purified *Acanthamoeba* actin with muscle heavy meromyosin. D. Human platelet actin with platelet myosin head. E. Guinea pig granulocyte actin and myosin. Magnification x 86,000.

and there is close agreement that the resulting arrowheads have a periodicity of about 36 nm (Table 3; Figure 4).

Viscometry

The course of polymerization and the effects of various treatments on the ability of actin to polymerize can be most conveniently followed in vitro by measurements of viscosity. The measurement is easy and rapid, and the reduced viscosity of polymers formed from purified actin is well defined for a given set of conditions.

The reduced viscosity reported for purified cytoplasmic actins (Table 1) polymerized with 0.1 *M* KCl is generally somewhat lower than for muscle actin. The reason for this is not known at present because careful comparative studies using actin polymers shown to be free of denatured actin (e.g., by sedimentation of polymerized actin) or contaminating protein have not been carried out. If denatured actin or protein contaminants were present in a solution of actin polymers, they would lower the reduced viscosity by an amount directly related to their concentration. Alternatively, it is possible that the average polymer length and, therefore, the viscosity of cytoplasmic actins are lower than for actin from muscle.

Hydrolysis of Bound Nucleotide

Upon polymerization of muscle actin, the bound ATP is rapidly and stoichiometrically converted to bound ADP and free phosphate.[71,85] Completely polymerized muscle actin does not carry out further hydrolysis of ATP, although ATP hydrolysis can continue under certain abnormal conditions such as addition of insufficient polymerizing cation,[86] sonication,[87] pressure elevation,[88] or temperature elevation.[89]

Actin from *Physarum*,[73] brain,[49] and human platelets[62] releases phosphate from bound ATP upon polymerization. During polymerization of *Physarum* actin in KCl, both the viscosity increase and the liberation of phosphate are complete in 15 min, showing that the phosphate release is a consequence of polymerization. The amount of phosphate liberated by actin from *Physarum* and human platelets is close to the theoretical amount (1 mol/45,000 g protein).

In the case of brain actin,[49] 0.79 mol of phosphate/45,000 g protein was released after 16 hr. After 1 hr about half this amount was released. Because the rate of phosphate liberation and polymerization were not compared, we do not know if polymerization and phosphate release occurred in parallel. If polymerization required 16 hr for completion, this would be very slow in comparison with muscle actin, which would have been completely polymerized in 1 hr under the same conditions. If the amount polymerized in 1 hr is equivalent to the concentration of native brain actin, then the additional phosphate released after 16 hr may have been produced by the action of a contaminating ATPase (possibly brain myosin), or brain actin may form Mg-polymer, which would be expected to continue to hydrolyze ATP (see below). These possibilities cannot be distinguished without further experiments.

Mg-polymer

Both the Hatano[13] preparation of actin from *Physarum* and rabbit muscle actin mixed with the minor muscle protein beta-actinin[59] form an unusual polymer termed "Mg-polymer." This form of actin was discovered by Hatano and coworkers,[73] who observed that the viscosity of *Physarum* actin polymerized with 0.1 to 2.0 m*M* $MgCl_2$ was substantially lower than the viscosity of the same concentration of *Physarum* actin polymerized with KCl (Figure 5a). Magnesium has a similar effect on the polymerization of actin from *Dictyostelium*[43] and sea urchin eggs,[74a] but *Physarum* actin prepared by the method of Adelman and Taylor[16] and *Acanthamoeba* actin[90] have the same viscosity in KCl or $MgCl_2$ (Figure 5b).

Various physical measurements show that Mg^{++} causes *Physarum* actin to polymerize but that the resulting polymer is apparently shorter and more flexible than ordinary actin filaments. The sedimentation coefficient of Mg-polymer is about the same as that of actin polymerized in 0.1 *M* KCl, but the boundary shows less self-sharpening, indicating that the polymer is less asymmetric.[73] Compared with actin polymerized in KCl, Mg-polymer has a much lower viscosity,[73] less flow birefringence,[53] and shorter filaments are seen in electron micrographs.[53] Similar differences in length and flexibility have been identified by quasi-elastic scattering of laser light.[91]

In addition to these distinctive physical properties, Mg-polymer continues to hydrolyze ATP after the completion of polymerization.[73] This continued hydrolysis is accompanied by

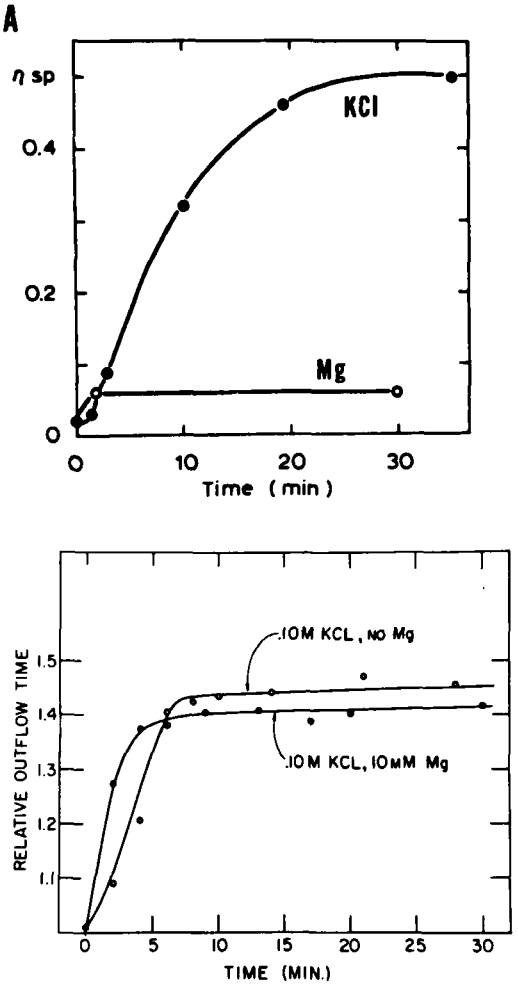

FIGURE 5. The polymerization of *Physarum* actin in the presence of KCl and Mg^{++} or KCl alone. A. Conditions are 0.1 *M* KCl or 0.1 *M* KCl + 2 m*M* $MgCl_2$. (From Totsuka, T. and Hatano, S., ATPase activity of plasmodium actin polymer formed in the presence of Mg^{2+}, *Biochim. Biophys. Acta*, 223, 189, 1970. With permission.) B. (From Adelman, M. R. and Taylor, E. W., Further purification and characterization of slime mold myosin and slime mold actin, *Biochemistry*, 8, 4976, 1969. With permission.)

exchange of free ATP into the Mg-polymer.[52] Because actin monomers and actin polymerized in KCl do not hydrolyze ATP, and because ATP does not exchange into ordinary actin filaments, these results imply that in Mg-polymer either (1) the filament remains intact but intrafilament actin-actin bonds are continually breaking and reforming with concomitant hydrolysis of ATP exchanged for ADP; or (2) ATP exchanges at the ends of filaments, and the filaments themselves are continually breaking and reforming with the hydrolysis of ATP exchanged for ADP. Kinetic analysis of the course of ATP exchange favors the first mechanism,[52] the intrapolymer cycle of breakage and reformation of actin-actin bonds.

Mg-polymer and ordinary actin filaments can be interconverted by dialyzing a solution of one form against a solution which favors the other form. This transformation is usually rather slow, but Mg-polymer can be transformed to ordinary actin filaments in 30 min by heating the Mg-polymer at 55°C in the presence of suitable concentrations of ATP and KCl.[92] After transformation at 55°C, "the reverse transformation of F-actin to Mg-polymer was not induced by simply changing the condition to that favorable to the Mg-polymer."[92] Data in support of this important result were not presented, but the statement suggests that Mg-polymer transformed into ordinary actin filaments by heating cannot be transformed back to Mg-polymer. It will be important to determine whether the heat treatment irreversibly alters the actin itself or some other protein component (such as beta-actinin), which recent results suggest is necessary for the formation of Mg-polymer.[59]

Recent experiments show that the treatment of Mg-polymer with KCl and ATP at room temperature induces a rapid increase in both the root mean square end to end distance (calculated from the quasi-elastic scattering of laser light)[91] and the viscosity, together with a decrease in ATPase activity.[93] Superficially, the results appear to contradict earlier statements that the interconversion of Mg-polymer and ordinary polymers is very slow at room temperature. However, the observed changes in these parameters indicate that there was an incomplete conversion of Mg-polymer to ordinary polymer in the recent experiments. These results could be accounted for if the conversion proceeds through a rapid initial step followed by a slower, rate-limiting step.

Which form of *Physarum* actin polymer exists in the living cell? There is no direct evidence on this point, but measurements of the total concentration of Mg^{++} (7 m*M*), Ca^{++} (1.2 m*M*), K^{+} (28 m*M*), Na^{+} (0.9 m*M*),[53] and ATP[94] (0.4 mmol/kg wet wt) indicate that Mg-polymer might be the type of *Physarum* actin polymer found in vivo. In addition, the experiments showing rapid changes in Mg-polymer at room temperature suggest that

the flexibility of actin polymers in vivo could be controlled by changes in the concentration of ions and ATP.

Recent data suggest that the formation of Mg-polymer may be caused by the presence of beta-actinin* contaminating certain preparations of actin from *Physarum*. When actin and beta-actinin from rabbit muscle are mixed together and the actin is polymerized with $MgCl_2$, then the polymers formed have the low viscoisty, the low birefringence, and the short particle lengths typical for Mg-polymer from *Physarum*.[59] The Mg-polymer of muscle actin also hydrolyzes ATP, but the rate is about one half that found for Mg-polymer from *Physarum*. The mixture of muscle proteins could be transformed into ordinary actin polymers by heating at 55°C, and ATP was necessary for this process as is the case for transformation of Mg-polymer from *Physarum*. However, KCl was not required for thermal transformation of the muscle proteins, although it is necessary for transformation of the *Physarum* actin. Unpublished data of Maruyama et al. (see Reference 9 of Kamiya et al.)[59] which are stated to show that a beta-actininlike protein is present in *Physarum* and data of Maruyama[95] which show that beta-actinin from muscle can be inactivated by heating between 50 to 60°C raise the possibilities that the actin from *Physarum* forms Mg-polymer because it is contaminated with low levels of a beta-actinin-like protein and that thermal transformation of Mg-polymer to ordinary polymer is caused by thermal denaturation of the possible beta-actinin contaminant. If beta-actinin is required for the formation of *Physarum* Mg-polymer and Adelman and Taylor's preparation of *Physarum* actin contains no beta-actinin, it is clear why they did not observe the formation of a low viscosity actin polymer in the presence of Mg^{++}. Clearly, more data on the beta-actinin content of these *Physarum* actin preparations are necessary.

Interaction with Myosin

As discussed above, muscle myosin or its proteolytic fragments bind to muscle or cytoplasmic actin filaments, giving the same distinctive appearance in electron micrographs of negatively stained specimens (Table 3; Figure 4). This observation is very important because it shows that all actin filaments have the same helical conformation and orientation of myosin binding sites. In all cases the binding of myosin to actin is blocked by ATP or pyrophosphate. (It is generally assumed that Mg^{++} is required for this dissociation, although this has not been proven in the case of the cytoplasmic actins.)

Although ATP appears to dissociate actin and myosin, in skeletal muscle, actin undergoes a cyclic interaction with myosin in the presence of ATP which activates the myosin ATPase and simultaneously generates the mechanical forces which cause movement. An in vitro correlate of this behavior is actin activation of the low ionic strength Mg^{++} ATPase of myosin or its proteolytic fragments, heavy meromyosin or subfragment-1.

All the cytoplasmic actins which have been tested activate rabbit myosin or heavy meromyosin ATPase (Table 4), and human platelet actin activates human platelet myosin.[62] As expected from experiments with muscle actin and myosin (or heavy meromyosin), the cytoplasmic actins show greater activation when the concentration of actin is increased (Table 4). Furthermore, the activation decreases when the ionic strength is increased (Table 4), presumably owing to greater relative dissociation of actin filaments from myosin or heavy meromyosin at higher ionic strengths.[28,95a]

Actin from *Acanthamoeba*, *Dictyostelium*, and human platelets activates rabbit myosin or heavy meromyosin less efficiently than equal amounts of rabbit muscle actin (Table 4). In contrast, actins from chick embryo fibroblasts and rabbit muscle are equally efficient activators of rabbit myosin ATPase (Table 4). Of course, the results with the protozoan actins could be explained by the presence of contaminating proteins or denatured actin, but a more interesting possibility is raised by experiments with tropomyosin and troponin-tropomyosin. Eisenberg and Weihing found that when troponin-tropomyosin[96] or tropomyosin alone[97] is added to a mixture of *Acanthamoeba* actin and rabbit heavy meromyosin, the activation more nearly reaches the activation caused by rabbit actin alone. More experiments will clarify these interactions, but it appears that some actins

*Beta-actinin is a protein isolated in low yield from muscle which may be related to actin because its amino acid composition is very similar to that of actin. Digestion of actin filaments with Nagarse causes them to behave like actin filaments containing beta-actinin, raising the possibility that beta-actinin may be denatured actin.[95]

TABLE 4

Activation of Mg-ATPase of Myosin or Heavy Meromyosin by Various Cytoplasmic Actins

Source of actin	Rabbit myosin or heavy meromyosin?	Concentration of actin (mg/ml)	Concentration of KCl (m*M*)	Mg^{++} ATPase: No actin (μmol/min mg myosin or heavy meromyosin)	Mg^{++} ATPase: Cytoplasmic actin (μmol/min mg myosin or heavy meromyosin)	Activation factor: Cytoplasmic actin	Activation factor: Rabbit muscle actin
Acanthamoeba[44]	HMM	0.2	7.7	0.03	0.20	6.7	
		0.2	10.7		0.16	5.3	21.6
		0.2	12.7		0.14	4.6	
		0.4	12.7		0.21	7.0	
Brain[49]	Myosin	Between 0.01–0.05	60	0.022	0.10	4.7	
Dictyostelium[43]	Myosin	0.05	50	0.015	0.078	5.2	23
Fibroblast (chick embryo)[39]	Myosin	0.023	50	0.008	0.067	8.0	9.3
					0.087	10.4	
Platelet (human)	Myosin[46]	Between 0.2–0.4	60	0.086	0.426	5.0	
	HMM[48]	0.2	13	0.10	1.49	14.9	
	Myosin[62]	0.056	50	0.02	0.024	1.2	2.2
		0.112			0.028	1.4	3.8
		0.164			0.031	1.5	5.1
		0.221			0.034	1.7	6.5
Physarum	Myosin[12]		50	0.019	0.28	14.7	
			100	0.013	0.11	8.5	
			600	0.006	0.013	2.2	
	Myosin[16]	0.14	24	0.015	0.185	12	
		0.14	39	0.011	0.046	4.1	

may require tropomyosin for efficient activation of myosin ATPase. (There is a precedent for a tropomyosin requirement for actin activation of myosin ATPase in muscle from the horseshoe crab, *Limulus*, but there the tropomyosin-dependence is a property of *Limulus* myosin, not *Limulus* actin.[98])

A further discussion of the interaction of cytoplasmic actin with the regulatory proteins, troponin-tropomyosin, is found below in the section on control mechanisms.

Other Methods of Identifying Actin

The preceding discussion has illustrated in detail how actin can be identified in nonmuscle cells by direct isolation and characterization. Often, however, it is not practical to carry out direct isolation, and so it seems useful to discuss several less direct, small-scale methods of identifying actins. These are (1) heavy meromyosin binding, (2) ultrastructural observation of depolymerization, (3) formation of paracrystals, (4) peptide mapping, (5) coelectrophoresis with authentic actin, and (6) content of N^{τ}-methylhistidine. Recent information about a naturally occurring antibody which may be specific for actin is also discussed.

Heavy Meromyosin Binding

Because the complex of muscle heavy meromyosin (or subfragment-1) with all purified actin filaments is so distinctive (Figure 4), the formation of these "arrowheads" can be used for the tentative identification of other actin filaments. Three-dimensional reconstruction of the complex is now well understood and illustrates how the arrowhead shape is the consequence of very specific interactions between actin and the myosin head.[84] It appears extremely unlikely that a complex with similar morphology might arise from the interaction of myosin with any type of filament except actin, making this the most specific "histochemical" technique yet devised. Although heavy meromyosin does not visibly bind to membranes or filamentous structures such as microtubules,[17,99-101] neurofilaments,[17,99] tonofilaments,[17] or bacterial flagella,[99] we recommend that the specificity of the reaction between heavy meromyosin and a presumed actin filament be confirmed by showing that inhibitors of actin-myosin interaction like Mg^{++} ATP or Mg^{++} pyrophosphate block the binding of heavy meromyosin to the filament.

The complex between heavy meromyosin and actin is visualized most easily by negative staining, but this procedure is not applicable to intact cells, and the information about the location of the actin filaments within the cells is lost. This problem was overcome when Ishikawa and coworkers[17] discovered that muscle heavy meromyosin will enter glycerinated cells and bind to 6 nm filaments in a manner identical to the binding of heavy meromyosin to muscle actin filaments. Their technique has been widely used to identify actin filaments within cells (Table 5). The initial report described the reaction of heavy meromyosin with cytoplasmic filaments in cells where biochemical proof for the existence of actin was lacking, but the validity of the procedure is now firmly established by experiments with *Acanthamoeba*,[75] intestinal brush border,[38] platelets,[62,100-101] and *Physarum*[80,116] which show that filaments of purified cytoplasmic actin and 6 nm filaments *in situ* react with heavy meromyosin in the same way.

The distinctive morphology of the heavy meromyosin-actin filament complex, on which the specificity of this technique is based, is much more difficult to resolve in thin sections than by negative staining, making the controls with ATP or pyrophosphate even more important than when negative staining is used. The problem with visualizing the arrowheads is apparently related to two properties of these preparations: There may be some shrinkage and distortion of the complex during fixation, embedding, and sectioning; but, more importantly, when decorated filaments are viewed in a random thin section of a cell, most of the filaments will be oriented at some angle other than 90° to the electron beam. Because of this tilting, the apparent periodicity of most of the arrowheads will be less than the true value of about 36 nm, and many of the decorated filaments will appear fuzzy rather than having definite polarized arrowheads. Experiments using an electron microscope with a tilting stage show that this fuzziness can be due to the overlapping of adjacent arrowheads on filaments tilted more than 20 to 30° away from perpendicular to the beam.[117]

It may also be possible to identify actin by fluorescence microscopy of cells treated with heavy meromyosin tagged with fluorescein,[117a]

TABLE 5

Thin Filaments of Nonmuscle Cells Decorated *in situ* with Heavy Meromyosin

Type of cells	Spacing of heavy meromyosin (arrowhead) complexes	Complexes dissociated by ATP or pyrophosphate	Localization in cell	
			Seen with or without heavy meromyosin	Seen only after treatment with heavy meromyosin
1. Blood cells				
Macrophages (guinea pig)[102]				
Platelets (human)[100,101]	36	Yes	Cortical	
2. Connective tissue				
Chondrogenic cell (chick embryo)[17]	37.8 ± 3.1	Yes		Cortical in metaphase arrested cells
Fibroblasts (chick embryo)[17]				As preceding
(chick embryo heart)[103]			Sheath; possibly network filaments	
(mouse, Balb/c 3T3)[104]			Anterior expansion	
3. Epithelial cells				
Epidermis (chick embryo)[17]				
Intestine (chick embryo)[17]	34.6 ± 3.2		Brush border	
(chicken)[38]			Brush border	
Lung (mouse embryo)[103]			Base and apex	
Renal tubule (rat)[105]		Yes	Base	
Salivary gland (mouse embryo)[103]			Base and apex	
Trachea (chick embryo)[17]			Brush border	
4. Nervous tissue				
Glia (chick embryo)[106]		Yes	Cortical sheath	
Neurones (chick embryo)[106]				
Neuroblastoma (mouse)[107,108]	35	Yes	Beneath plasma membrane in axon and cell body; more abundant after heavy meromyosin	
5. Reproductive tissue				
Egg (newt)[109]			Contractile ring	
Spermatocyte[110,111] (crane fly)		Yes		Spindle and cortex
Spermatozoa (crane fly)[112]	36			Sperm tail
(starfish)[113]			Acrosomal process	
(sea urchin)[113a]	36			

TABLE 5 (continued)

Thin Filaments of Nonmuscle Cells Decorated *in situ* with Heavy Meromyosin

Type of cells	Spacing of heavy meromyosin (arrowhead) complexes	Complexes dissociated by ATP or pyrophosphate	Localization in cell: Seen with or without heavy meromyosin	Localization in cell: Seen only after treatment with heavy meromyosin
Testis (locust)[114]			Spindle and cortex; not stated if presence of filaments requires heavy meromyosin	
6. Protozoa				
Acanthamoeba[75,118]	30–35	Yes	Cortical; attached to isolated plasma membrane	
Amoeba proteus[115]	38	Yes	Seen in motile extracts	
Chaos carolinensis[231]	34	Yes	Plasma membrane, cortex, central region	
Physarum[116]	20–29 a few with 33			
7. Cultured cell lines				
HeLa[268]	27–35	Yes	Contractile ring	
Erlich ascites[268a]	36	Yes	Microvilli, cortex, near nucleus	
8. Plants				
Nitella[118a]	37	Yes		

although this approach has not been extensively investigated.

Depolymerization

Because actin filaments depolymerize in dilute buffers, presumed actin filaments can be tested for this property and the results assessed by electron microscopy or gel electrophoresis.[127] This approach recently has been used by Pollard and Korn[118] to selectively remove actin filaments from purified *Acanthamoeba* plasma membranes and could theoretically be used on glycerinated cells.

Paracrystals

Formation of the distinctive actin paracrystals (Figure 3) in 50 m*M* $MgCl_2$ has been used to identify actin in isolated endoplasm from *Physarum.*[83] This approach should be applicable to other small samples of material, providing that they contain high enough actin concentrations to allow paracrystal formation.

Antibody

Although it has been difficult or impossible to experimentally produce antibodies to pure actin (perhaps because of the ubiquity and similarity of the actins), it now appears that some patients with an inflammatory liver disease known as chronic active hepatitis have, for unknown reasons, a naturally occurring antibody which may react specifically with actin. Originally it was shown by fluorescence microscopy that the antibody bound to smooth muscle cells,[119] and recently Gabbiani

et al.[120] found that these sera react with a number of cells known to contain actin, including platelets, cultured fibroblasts, and intestinal epithelium. Three observations support, but do not prove, the idea that the antibody specifically binds to actin: (1) The serum precipitates pure platelet actin; (2) the binding to cells is blocked by absorption of the serum with pure platelet actin; and (3) by immunofluorescence the antibody binds to specific parts of cells known to contain actin, such as intestinal and renal tubular microvilli and the "stress fibers" in cultured fibroblasts. The technique has now been used to tentatively identify actin in hepatocytes, renal glomeruli, certain lymphocytes, some endothelial cells, and in granulation tissue fibroblasts of healing wounds.[120]

Peptide Mapping

This is another method which should be nearly as specific as heavy meromyosin binding for identifying actin in cells in which it is impractical to identify actin by direct isolation. Three factors contribute to the success of this approach. First, it is already known that peptides having similar compositions are present in actin from *Acanthamoeba*, rabbit skeletal muscle, and bovine myocardium[51,65] and that actins from various mammals, chicken, frog, perch, and scallop all have many tryptic peptides with identical mobilities on peptide maps.[121] Thus, it is likely that authentic actin and other as yet unidentified actins will have similar peptide maps. Second, it is expected both on theoretical and experimental grounds that a pure protein will produce a specific peptide map. Third, various methods are available by which peptide maps can be produced from very small amounts of material, even as small as the amount of protein found in a single band on a polyacrylamide gel.

In the studies available so far, radioactive peptides from cytoplasmic actins were identified on radioautograms of the peptide maps by comigration with peptides derived from muscle actin. Radioactivity was introduced in two ways. In the case of actin from neurones of chick embryo sympathetic ganglia,[66] dissociated neurones were grown in the presence of ^{35}S-methionine. In the case of actin from chicken epithelial cells, fibroblasts, and neurones,[5] a presumed actin band was eluted from a polyacrylamide gel, and the protein was iodinated with ^{125}I in the presence of chloramine T (method of Hunter and Greenwood).[122] In both procedures the presumed actin was shown to have the same molecular weight as authentic actin by electrophoresis, and tryptic digestion and electrophoresis in two dimensions at two different pH's were carried out on the small amounts of protein eluted from polyacrylamide gels.

In the case of neuronal actin labeled with ^{35}S-methionine, 10 of 14 radioactive spots coincided with ninhydrin spots derived from added carrier muscle actin. The number of radioactive spots produced (total of 14) was close to the expected number of tryptic peptides which should contain methionine (11 or 12) (derived from the sequence data for muscle actin[61,65]). Four radioactive spots did not coincide with ninhydrin spots, suggesting either that (1) the actins from neurones and from muscle contain different peptides and, therefore, differ slightly in sequence or that (2) the actin eluted from the gels is impure. These possibilities cannot be distinguished without further experiments.

In the case of the ^{125}I labeling procedure, actin from chicken epithelial cells, fibroblasts, and neurones gave the same peptide map as chicken actin. Similarly, the actin from scallop gill gave the same peptide map as actin from scallop muscle. However, there were differences between the scallop and chicken actin; one peptide was absent, and another was shifted in the maps of scallop actin. This degree of species difference is consistent with the degree of difference observed by Carsten and Katz[121] for peptide maps of muscle actin from these two species.

Again using the sequence data for muscle actin,[61,65] if one assumes that both tyrosine and histidine (the major expected sites for iodination) are fully iodinated or that only tyrosine is fully iodinated, then either 14 or 12 radioactive peptides should be produced. Somewhat fewer than this number were found, and, while it is known that iodination is frequently incomplete,[123] the meaning of the observation of fewer than the theoretical number of peptides is difficult to evaluate because some spots were very faint and combined data from several maps were not presented, nor were analytical data showing the degree of iodination of tyrosine and histidine presented.

In both these methods, label is introduced into only a limited number of the 40 or so peptides

which are expected in a tryptic digest of actin, and, thus, all the information which is potentially available in peptide maps of actin has not yet been considered. It should be possible in principle to identify all the peptides by reacting them with sensitive fluorescent reagents such as dansyl chloride, as described by Gerday et al.[124] for bovine carotid actin or a newly developed fluorescent reagent, fluorescamine.[125] Combining the approach of identifying all peptides with the approach of identifying peptides which are specifically labeled in vivo with a radioactive amino acid such as ^{35}S-methionine should provide still more discrimination. In contrast, the use of ^{125}I labeled peptides seems fraught with difficulties because of uncertainties about the degree and sites of iodination alluded to earlier.

Coelectrophoresis and Presence of N^{τ}-methylhistidine

Two easy methods are available for tentative identification of actin in a cell: coelectrophoresis with authentic actin and identification of N^{τ}-methylhistidine. A band which comigrates with authentic actin is prominent in the electrophoretic pattern of unfractionated *Acanthamoeba*[126,127] and chicken intestine brush border.[38] In both cases actin has been identified in these cells by direct isolation, but, if this had been impractical, it would have been possible to verify the identification by peptide mapping.

N^{τ}-methylhistidine is an unusual amino acid which to date has been identified only in the contractile proteins actin and myosin and certain nuclear proteins.[128-130] Its identification in a cell such as *Acanthamoeba*[44] provides suggestive evidence for the presence of the contractile proteins actin and myosin.

Content of Actin in Cells

To understand the function of cytoplasmic actin, it will be necessary to know how much actin a cell contains so that this can be related to the content of myosin and other contractile proteins. Estimates of actin content are available for several cells, but, as discussed bélow, it should be possible to refine the experiments to obtain more precise estimates.

The maximum and minimum actin content of any cell can be established by determining the cell's content of N^{τ}-methylhistidine and the yield of purified actin, respectively. For example, *Acanthamoeba* contains enough N^{τ}-methylhistidine that if it were present only in actin, then 20% of the cell's protein would be actin, but only 0.2% of the cell's protein is recovered as purified actin.[44] The true value lies somewhere between these limits, because N^{τ}-methylhistidine may be present in other proteins and there are significant losses during purification. Purified actin accounts for about 2 to 4% of *Physarum*[16] and 10 to 13% of sea urchin egg protein.[37a]

Quantitative densitometry of a gel electrophoretic pattern can also be used to estimate the amount of actin in a cell or cell fraction. In *Acanthamoeba*,[126] guinea pig granulocytes,[76] and human platelets,[130a] a protein with the mobility of actin accounts for 10 to 15% of the stained protein. In sympathetic neurones grown in tissue culture in the presence of ^{35}S-methionine, quantitative densitometry of the radioautogram of the gel electrophoretic pattern of a soluble fraction showed that a protein with the mobility of actin accounts for 20% of the radioactivity.[66] These experiments can provide reliable estimates of the amount of actin (and other proteins) in whole cells and cell fractions, provided all the following information is available: (1) the relation of staining intensity (or film blackening) to the amount of protein applied to the gel, (2) the absence of gross contamination with proteins of similar mobility as shown by peptide mapping[66] or N-terminal amino acid analysis, and (3) in the case of radioautography, the cells are labeled for long enough to assure that the proteins are uniformly labeled.

An independent measurement of actin content might be made by immunological methods, provided it can be shown that the antibody present in sera of patients with chronic active hepatitis is specific for actin.

A simple calculation can be made to decide whether these estimates of the amount of actin in a cell are consistent with the appearance of the actin filaments in a thin section. In *Acanthamoeba*, which contains 0.43×10^{-6} mg of protein per cell,[131] 0.2% of the cell protein was isolated as actin.[44] Assuming a molecular weight of 45,000 daltons, then one cell contains at least 11.5×10^{6} molecules of actin. Assuming that all the actin is polymerized into filaments with a pitch of 74 nm and that there are 28 monomers per turn, then we can use the number of molecules per cell to calculate that one cell should contain 30,000

filaments 1 μm long. If the cell is a sphere 26 μm in diameter,[132] then a thin section 60 nm thick taken at the equator will account for 0.35% of the volume of the cell. Thus, if the actin filaments are distributed uniformly throughout the cell, then 0.35% of them or about 100 filaments should be visible in such a thin section. If the estimate of actin content based on the amount of N^{τ}-methylhistidine is correct, then 20% of the cellular protein is actin, and the parameters should be revised upward 100-fold, i.e., to 11.5×10^8 molecules of actin per cell and about 10,000 filaments in a thin section. This range is in rough agreement with the number of thin filaments seen in fixed amoebas,[75,133] and this is gratifying because we used assumptions which are either clearly incorrect (uniform distribution of actin filaments) or about which there is no quantitative information (degree of polymerization, length of filaments).

MYOSIN

It is generally accepted that neither actin nor myosin is capable, by itself, of generating force for movement in muscle. This implies that if the cytoplasmic actins discussed above are involved in cell movement, then myosin must coexist with actin in the cytoplasm. Although the studies on cytoplasmic myosins are less extensive than the studies on cytoplasmic actins, myosin has been found in several actin-containing cells, and it is likely that further investigation will reveal other examples.

Two striking generalities have already emerged from these studies: (1) All of the myosins share with muscle myosin two features thought to be essential for force generation in muscle, namely, the ability to bind reversibly to actin filaments and the possession of an actin-activated ATPase activity; and (2) in contrast to the actins, muscle and cytoplasmic myosins vary considerably in their physical, chemical, and enzymatic properties.

The diversity of the myosins has led to the question of which enzymes should be called myosins. We suggest that the term myosin be used to designate a class of enzymes with actin-activated ATPase activity that binds reversibly to actin filaments. This definition which is based on the properties most important to the function of myosin during contraction is likely to include all myosins and still allow for considerable variation in other properties such as size and shape.

To date, five classes of myosin have been discovered, and their properties are summarized in Tables 6, 7, 8, and 9. While this classification is admittedly arbitrary, it is useful for organizing the discussion. Each class will be considered in turn and the general conclusions discussed thereafter.

Striated Muscle Myosin

Because of the wealth of experimental data on the properties of myosin from vertebrate striated muscles (especially rabbits and chickens) (see Lowey, 1971, for a review),[26] these myosins are generally thought of as "typical" myosins with which all other myosins are inevitably compared. Since more is known about the contractile process in striated muscle than elsewhere, these comparisons have been extremely useful in studying the mechanism of force generation in other cells. On the other hand, it is well to remember that striated muscles and their myosins are highly specialized for a stereotyped contractile movement, so these myosins may not be "typical" myosins in some respects.

Striated muscle myosin is a large molecule with a molecular weight of about 470,000 daltons.[134,135] It is composed of two large polypeptide "heavy" chains (200,000 daltons each) and two or more small polypeptide "light" chains with molecular weights of 16,000 to 27,000[26] daltons (Table 6). The number and size of these light chains vary from one muscle type to the next.[136-138] In addition, limited amino acid sequence data have proven that cardiac and skeletal muscle myosins have similar but not identical heavy chains.[139]

Biochemical analysis and electron microscopy revealed that each myosin molecule is composed of two globular "heads" attached to a long "tail."[140,141] (Figure 6). The tail (also known as the "rod") is about 140 nm long and is composed of the C-terminal half of the two heavy chains.[142] The two polypeptides are almost completely alpha-helical and are wound around each other in a coiled-coil. About 90 nm from the tip of the tail is a section which is particularly susceptible to cleavage by proteolytic enzymes (such as trypsin)[141] or by cyanogen bromide.[142] It has been postulated,[4] but not proven,[26] that this region may serve as a "hinge" in the tail of the molecule. Each of the two globular heads consists

TABLE 6

Physical Properties of Various Myosins

Type of myosin	Native molecular weight (daltons)	Subunit composition (# x daltons)	Stokes radius (Å)	Sedimentation coefficient (S)	Physical state in 0.6 *M* KCl	Physical state in 0.1 *M* KCl
1. Striated muscle						
Rabbit skeletal[26]	460,000	2 x 200,000 1 x 20,000 2 x 18,000 1 x 16,000	192	6.4	Monomer	Large bipolar filament
2. Smooth muscle						
Human uterus		?x 200,000[117] ? x ∿19,000 ? x ∿16,000	∿190[117]		Monomer	Bipolar[146] filaments
3. Vertebrate cytoplasmic						
Guinea pig granulocyte[76]	?	? x 200,000 ? x ∿20,000	∿190	?	? Monomer	Small bipolar filaments with Mg^{++}
Human platelet	∿540,000[147]	2 x 200,000[148] 2 x 19,000[48,130a] 2 x 16,000	∿190[117]	6.8[147]	Monomer	Bipolar[148] filaments
Mouse fibroblast[149]	?	? x 200,000 ? x ∿20,000	?	?	? Monomer	Small bipolar filaments
Rat brain[150]	?	? x ∿240,000	?	?	?	?
4. *Physarum*	∿460,000[16]	? 2 x 240,000[151] ? x ∿12,000	171[16]	6.38	Monomer	Small[152] bipolar filaments with Ca^{++}
5. *Acanthamoeba*[126,153,154]	∿180,000	1 x 140,000 ? 1 x 16,000 ? 1 x 14,000		∿8[117]	Monomer	Monomer

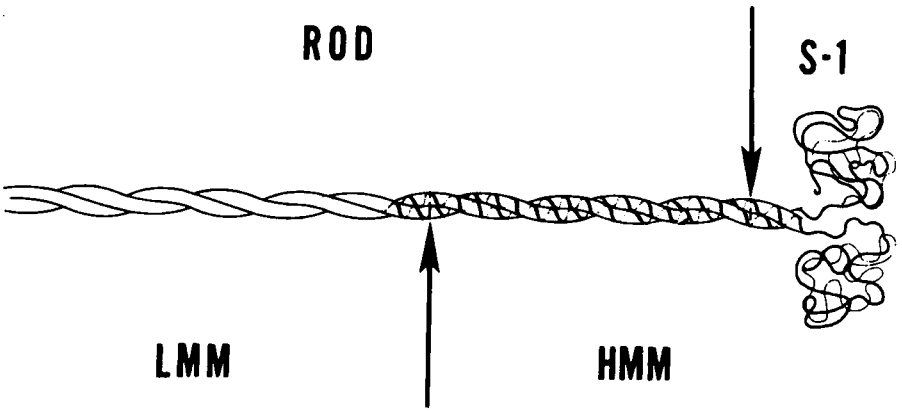

FIGURE 6. An artist's conception of the structure of a muscle myosin molecule. The heavy lines represent the myosin heavy chains, and the light lines represent the myosin light chains. The upper arrow shows the position where papain cleaves the molecule, and the lower arrow shows the position where trypsin cleaves the molecule. LMM: light meromyosin; HMM; heavy meromyosin; S-1; subfragment-1, the heads of the molecule; Rod: the tail of the myosin. (From Lehninger, A. L., *Biochemistry*, Worth Publishers, New York, 1970, 589. With permission.)

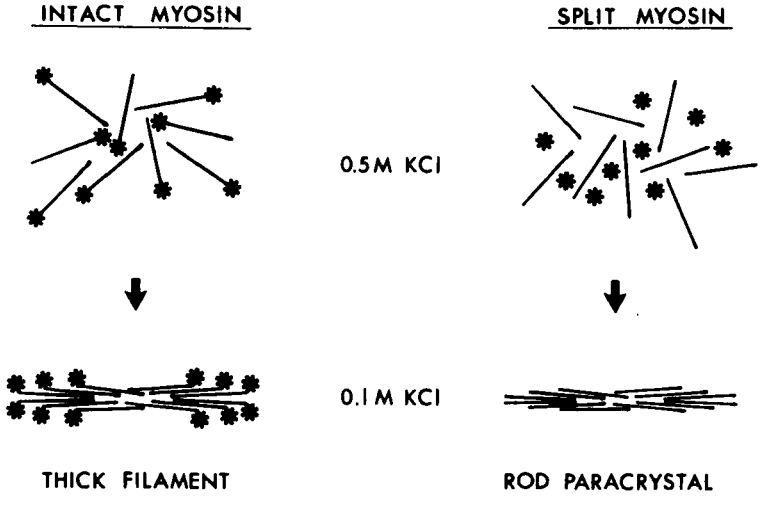

FIGURE 7. Line drawings showing the assembly of myosin into bipolar filaments and the assembly of myosin rod into paracrystals in 0.1 *M* KCl.

of the N-terminal half of one of the heavy chains with associated light chains. Each head contains an actin binding site and an active site for ATP hydrolysis.[141]

The physical properties of the various regions of the molecule are closely related to their function. The C-terminal 90 nm of the tail can be isolated by limited proteolytic digestion, and the fragment is known as light meromyosin. Under physiological conditions this portion of the tail is insoluble and aggregates with other myosin tails to form the backbone of the thick filaments found in striated muscle (Figure 7).[18] The part of the molecule on the N-terminal side of the "hinge" region shows no tendency to aggregate under physiological conditions,[141] so it may be free to swing out from the backbone of the thick filaments to interact with actin and thereby form a crossbridge between the two filaments.[4]

Myosin has the property, apparently unique among ATPases, that its enzyme activity is strongly inhibited by Mg^{++} and activated either by Ca^{++} or by K^{+} in the presence of EDTA.[143] (The EDTA is required to chelate Mg^{++} contaminating

the ATP.)[144] The Ca^{++} and EDTA activations are useful for following myosin during purification procedures, but only the activity in the presence of Mg^{++} is physiologically relevant, because the other conditions are not found in muscle.

As a consequence of the Mg^{++} content of muscle (> 8 mmol/kg),[20] myosin ATPase activity would be too low to account for the transduction of the chemical energy of ATP into force for movement, if it were not for the unique ability of actin filaments to activate the Mg^{++} ATPase activity of myosin.[28] This effect of actin seems to be due to its ability to facilitate the dissociation of the products of ATP hydrolysis from the myosin, the step which is thought to be rate limiting in the ATPase reaction.[22,145] Consequently, ATP hydrolysis is tightly coupled to actin-myosin interaction, which in turn utilizes, in some unknown way, the energy from ATP hydrolysis to drive the force generating mechanism, causing the actin filaments to slide past the myosin. In this complicated reaction, ATP plays a dual role: It is the source of energy which drives the reaction, but it also dissociates actin from myosin[145a] to start each new cycle of interaction consisting of (1) ATP hydrolysis by myosin, (2) dissociation of products of ATP hydrolysis upon binding of actin to myosin, (3) sliding movement, and (4) dissociation of actin from myosin by the rebinding of ATP to the myosin.[145] Recent work[145b-d] suggests that the actual mechanism may be more complicated than this simple four-step model suggests.

Smooth Muscle Myosin

In spite of the importance of smooth muscle for visceral and vascular function in higher organisms, relatively little is known about smooth muscle myosin (less, in fact, than is known about some of the cytoplasmic myosins discussed below). In general, smooth muscle myosins resemble striated muscle myosins from the same species. They are large molecules composed of heavy chains with a molecular weight of about 200,000 daltons and two classes of light chains,[130a] one of which is not essential for ATPase activity.[138] Actin activated ATPase activity is lower than that of striated muscle myosins (Table 7), presumably accounting for the slow contractions typical of smooth muscle.[157] Under more or less physiological conditions, purified smooth muscle myosin will form thick filaments which have been variously described as having typical bipolar symmetry with a bare central zone[146,158] or an asymmetrical distribution of projections on the two sides of the filament.[159] Several laboratories have now observed thick filaments in electron micrographs of thin sections of fixed smooth muscle,[160-163a] but there is a lively debate as to whether these filaments occur in the living muscle.[160-163c]

Vertebrate Cytoplasmic Myosin

It is now conclusively proven that myosin is present in a number of vertebrate cells or tissues which are clearly not muscle, including platelets, granulocytes, fibroblasts, and brain (Tables 6, 7, and 8). The identification of these myosins is based in each case on purification of the protein and demonstration that it has ATPase activity and will interact with actin filaments in the expected manner. As there are no major differences among these proteins, they will be considered together and are likely to be found in virtually all motile cells of higher organisms. It has been noted in the original reports that these myosins resemble smooth muscle myosin in certain respects, but there is no definitive data showing that they are, in fact, identical.

Several schemes have been developed for the purification of these myosins, most of which use high ionic strength extraction of the tissue followed by precipitation of actomyosin at low ionic strength. Granulocyte myosin can also be extracted in 0.34 *M* sucrose.[76] Ultracentrifugation of the resulting actomyosin in concentrated KCl with Mg^{++} and ATP separates some of the actin from the myosin, but there is inevitably some contamination with actin, possibly because small oligomers of actin do not sediment rapidly enough to be separated from the myosin. Ammonium sulfate fractionation or sucrose gradient ultracentrifugation has achieved further purification in some cases, but the most successful procedure has been gel filtration,[76,148,149] which takes advantage of the large Stokes radius of the myosin to separate it from contaminating proteins. In the case of fibroblast myosin, this results in a homogenous protein.[149] Even with gel filtration, contamination with actin or proteolytic fragments of the myosin has been a problem with platelet and granulocyte myosins. This has now been overcome by first depolymerizing the actin oligomers with

TABLE 7

Enzymatic Activity of Various Myosins

Type of myosin	ATPase activity (μmol P_i/min/mg protein) K⁺EDTA	Ca^{++}	Mg^{++}	Temperature (°C) of assay	Energy of activation (Kcal/mol)	Substrate specificity
1. Striated muscle						
Rabbit skeletal	1.51[155]	0.19	0.002	25	12	ITP, UTP, GTP[20]
	5.5[173]	1.0	0.01	37		>ATP, CTP
2. Smooth muscle						
Horse esophagus[157a]	0.61	0.28	0.004	25		
	1.37	0.80	0.03	37		
3. Vertebrate cytoplasmic						
Guinea pig granulocyte[76]	0.186	0.208	0.020	25	?	?
Human platelets[148]	0.55	0.44	0.02	37	?	?
Mouse fibroblast[149]	0.43	0.50	<0.01	37	?	?
Rat brain[150]	?	0.27	0.03	37	?	?
4. *Physarum*	0.03[16]	0.87	0.03	20	12	ATP>ITP>GTP
	0.007[156]	0.537	0.030	25		
5. *Acanthamoeba*[126,153]	3.5	0.4	<0.05	29	9	ATP>GTP, CTP, ITP

TABLE 8

Actin-Myosin Interaction

Type of myosin	Actin Binding		Mg^{++} ATPase				
	−ATP	+ATP	− actin	+ actin[a]	Actin concentration (mg/ml)	Ionic strength (M)	Temp. (°C)
1. Striated muscle							
Rabbit skeletal (heavy meromyosin)[28]	Yes	No	0.02	1.0	0.9	∿0.066	25
			0.02	0.17	0.9	∿0.106	25
2. Smooth muscle							
Horse esophagus[157a]	Yes	No	0.004	0.015	0.07	0.074	25
3. Vertebrate cytoplasmic							
Guinea pig granulocyte[76]	Yes	No	0.0025	0.0075	0.68	0.10	25
Human platelet (head)[48]	Yes	No	0.02	0.07	0.8	0.039	37
Mouse fibroblast[149]	Yes	No	0.01	0.09	0.3	0.035	37
Rat brain[150]	?	?	0.037	0.11	?	0.096	37
4. *Physarum*[156]	Yes	No	0.027	0.057	?	0.046	25
				0.047 *Physarum* actin			
5. *Acanthamoeba*[154]	Yes	No	0.05	1.5	1.0	0.016	29

[a]Rabbit muscle actin unless noted otherwise.

KI[49] and separating the myosin from the actin by gel filtration in a buffer containing KI, ATP, and a reducing agent.[76,130a]

While the myosin content of these cells is not known precisely, estimates based on enzyme activity of purified myosin and crude homogenates or on densitometry of SDS-polyacrylamide gels of whole cells show that the amount of myosin is variable, being as high as about 2% in platelets[130a,148] or less than 1% in granulocytes.[76] In all cases, though, there is a clear excess of actin over myosin.

Like muscle myosin, these cytoplasmic myosins have a sedimentation coefficient of about 6S and a large Stokes radius (derived from gel filtration), which together are indicative of a large asymmetrical molecule (Table 6). Estimation of the native molecular weight of platelet myosin from the sedimentation coefficient and diffusion coefficient gave a value of 540,000,[147] and, while this value is undoubtedly close to the true value, the electron micrographs of these myosin preparations[147] and the experience of other investigators indicate that this platelet myosin must have been contaminated with some platelet actin. Gel electrophoresis in SDS shows that these myosins all consist of heavy chains with molecular weights of 200,000 daltons and usually two classes of light chains with molecular weights of 16,000 and 19,000 daltons,[48,130a,164a] present in a molar ratio of roughly 1:1:1. Presumably, these myosins consist of two heavy chains and one or more of each of the light chains, although the exact stoichiometry is not known.

The amino acid composition is known only for human platelet myosin, and it appears to be very close to the composition of rabbit muscle myosin. Only four residues differed by more than 10% according to the analysis of Booyse, et al.,[147] and the similarities may be even more striking in samples free of actin contamination. Adelstein and colleagues[164,164a] have isolated the two light chains from human platelet myosin. One of the platelet myosin light chains contains a phosphorylated amino acid, and the amino acid composition of these light chains differs clearly from rabbit skeletal muscle myosin light chains.[165] This difference in light chains is not unexpected as it has been shown by amino acid sequence that one of the rabbit cardiac muscle myosin light chains is unrelated to the skeletal muscle myosin light chains from the same animal.[138] In the same manner, it would be surprising if the amino acid sequence in the heavy chains of the cytoplasmic myosins was the same as the muscle myosin heavy chain sequence because of the known differences in the sequence of myosins from skeletal and cardiac muscle.[139]

Individual myosin molecules in this class have not been directly visualized by electron microscopy, but is is clear in the case of the platelet myosin that the molecule consists of two distinct regions as does muscle myosin. This assertion is based on the isolation of two fragments of the platelet myosin molecule corresponding to the head region and the tail region of the molecule.[148] The mechanism of this cleavage is not known, but it is thought to be due to proteolytic digestion of the myosin in the intact platelet or during the isolation procedure. The tail region consists of polypeptides with a molecular weight of about 130,000 daltons, contains little proline (as expected for a highly alpha-helical polypeptide),[165] and lacks actin binding sites or ATPase activity. The isolated head is usually contaminated with platelet actin or tropomyosin but has been obtained in sufficient purity to show that it consists of a polypeptide of about 80,000 daltons* associated with two light chains[117] and that it has the ATPase activity and actin binding site found in the intact myosin. The cleavage of the native platelet myosin must be limited and very specific as these fragments account for the mass of the entire molecule and electrophoresis in SDS reveals little size heterogeneity of the fragments (although, like muscle myosin subfragment-1,[26] platelet myosin head may run as a closely spaced doublet on these gels).[117]

All of these cytoplasmic myosins have ATPase activity which is stimulated by Ca^{++} and inhibited by Mg^{++} (Table 7). There are some differences in the rates reported, but, in highly purified preparations isolated with careful protection of sulfhydryl groups, the Ca^{++} ATPase rates are comparable to those of smooth and striated muscle myosins. These preparations also show high activity in the presence of KCl and EDTA, but here the rates are clearly lower than those of skeletal muscle myosin. As the activity of these myosins may be labile, detailed comparisons of ATPase rates with either

*The original estimate of 100,000 daltons[148] for the molecular weight of the head is probably too high.[117]

smooth or striated muscle myosin do not seem to be particularly informative.

Where investigated, actin activates the low ionic strength Mg^{++} ATPase of the cytoplasmic myosins (Table 8), but neither the amount of activation relative to the rate without actin nor the absolute rate of hydrolysis is very great, and both appear to be lower than that of skeletal muscle myosins. We suspect that improved preparative techniques or assay conditions will result in higher rates of actin activation.

Like other myosins, this group of myosins binds to actin filaments and can be dissociated from them by ATP (Table 8). This was demonstrated by sedimenting the myosin with actin in the ultracentrifuge[76,148,149] and by direct visualization of the actin-myosin complex by negative staining electron microscopy (Figure 8B and C).[76,148] The morphology of the complex between platelet or granulocyte myosin and actin filaments is identical to the muscle actin-myosin complex, showing that the head of these myosins must have the bent configuration characteristic of the head of muscle myosin.

The myosins from platelets, granulocytes, and fibroblasts all spontaneously form insoluble bipolar "thick" filaments in dilute buffers (Table 6; Figure 9C-E). Electron microscopy shows that the tail portion of the myosin makes up the fibrous backbone of the filament (Figures 7 and 9F). The lateral projections found at both ends of the filament are the globular head portion of the molecule containing the actin binding site and the ATPase activity.[148] Because these thick filaments have actin binding sites at both ends, they are capable of cross-linking two or more actin filaments (Figure 8B and C). The conditions necessary for myosin filament assembly have not been thoroughly examined, although it is clear that filament formation is promoted at slightly acid pH and by the presence of divalent cations.[166]

One of the principal unresolved mysteries about these myosins is the form which they take in the living cell. In the case of platelets, for example, myosin is known to comprise about 2 to 3% of the total platelet protein, and the purified myosin rapidly forms thick filaments in "physiological" salt solutions.[148] A simple calculation* reveals that there is enough myosin in one platelet to make 1,300 thick filaments 0.25 μm long and that 40 of these filaments would be visible in a 60 nm thin section of a platelet. These thick filaments are rarely visualized in electron micrographs of the intact resting platelets.[100,167] It is not known whether this disparity is due to an artifact in the preparation of intact platelets for electron microscopy or whether the myosin is prevented from forming these thick filaments within the cell. Variations in the pH, ionic strength, and divalent cation concentration affect the size of the thick filaments but it is not yet known if these factors account for the absence of thick filaments from the platelet's cytoplasm.[166]

In the case of fibroblasts and granulocytes, the apparent absence of thick filaments in the cells may be due, in part, to the low concentration of myosin. In addition, it has recently been shown that granulocyte myosin will not form thick filaments in dilute buffers in the absence of divalent cations,[76] so intracellular Ca^{++} and Mg^{++} concentrations may play a role in their scarcity in electron micrographs of intact cells.

Physarum Myosin

At the present time, *Physarum* myosin is the only representative of its class and is distinguished by its unusually large heavy chain and its solubility in dilute buffers. Presumably, further exploration will reveal similar myosins in related species (although apparently not in a cellular slime mold, *Dictyostelium discoideum*, which appears to have a myosin with a heavy chain molecular weight of 200,000, according to preliminary experiments by Clarke and Spudich[168]).

It could be inferred from the original experiments on slime mold actomyosin by Loewy in 1952[6] that *Physarum* did indeed contain myosin, but the definitive demonstration that this is true came over 15 years later when two groups, working independently, devised two quite different procedures for purifying myosin from *Physarum*.

*Platelet myosin filaments: A platelet 3 μm in diameter has a volume of 14.2×10^{-2} cm^3 and contains about 14.2×10^{-13} g of protein of which about 35×10^{-15} g (assuming 2.5% myosin) is myosin. One thick filament 0.3 μm long with a 0.2 μm central bare area and four myosin molecules/every 14.3 nm on each side of the bare area could be assembled from 32 myosin molecules. There are about 42,000 myosin molecules/platelet, so up to 1,300 filaments could be formed in a single platelet. A section 60 nm thick passing through the equator of a 3 μm platelet contains about 3% of the platelet's volume and, therefore, might contain 40 complete myosin filaments.

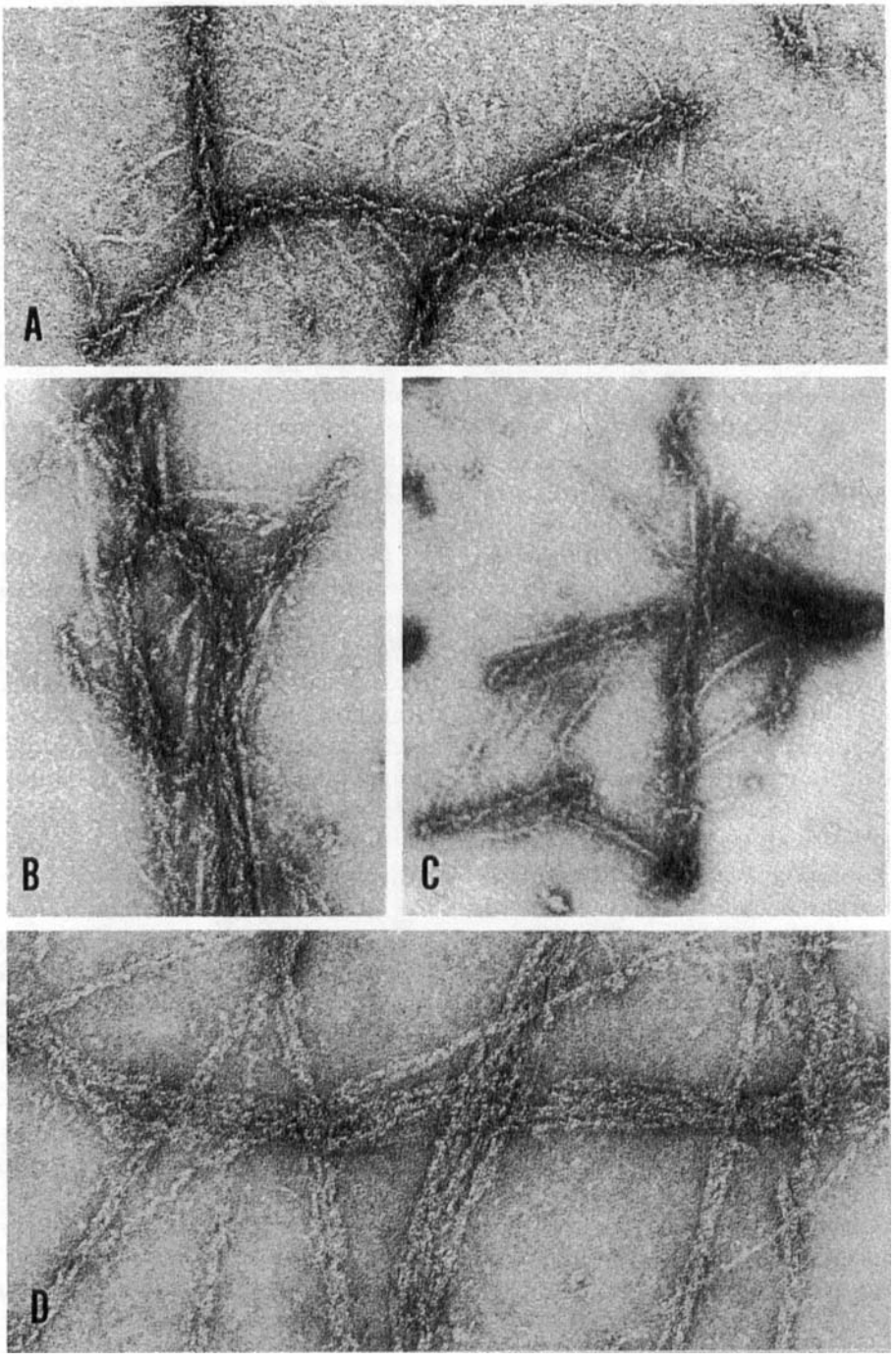

FIGURE 8. Complexes of various myosins with actin negatively stained with uranyl acetate. A. *Physarum* myosin and actin. The long tails of the myosin are seen trailing out from the decorated filaments. (Micrograph by V. T. Nachmias.) B. Human platelet myosin and actin. In several places thick filaments of myosin cross-link decorated actin filaments. C. Guinea pig granulocyte myosin and actin. Note the myosin cross-links. D. *Acanthamoeba* myosin and muscle actin. The filaments bind the myosin, but no arrowheads are visible. In the presence of *Acanthamoeba* myosin, the actin filaments are held in bundles. Magnification x 94,000.

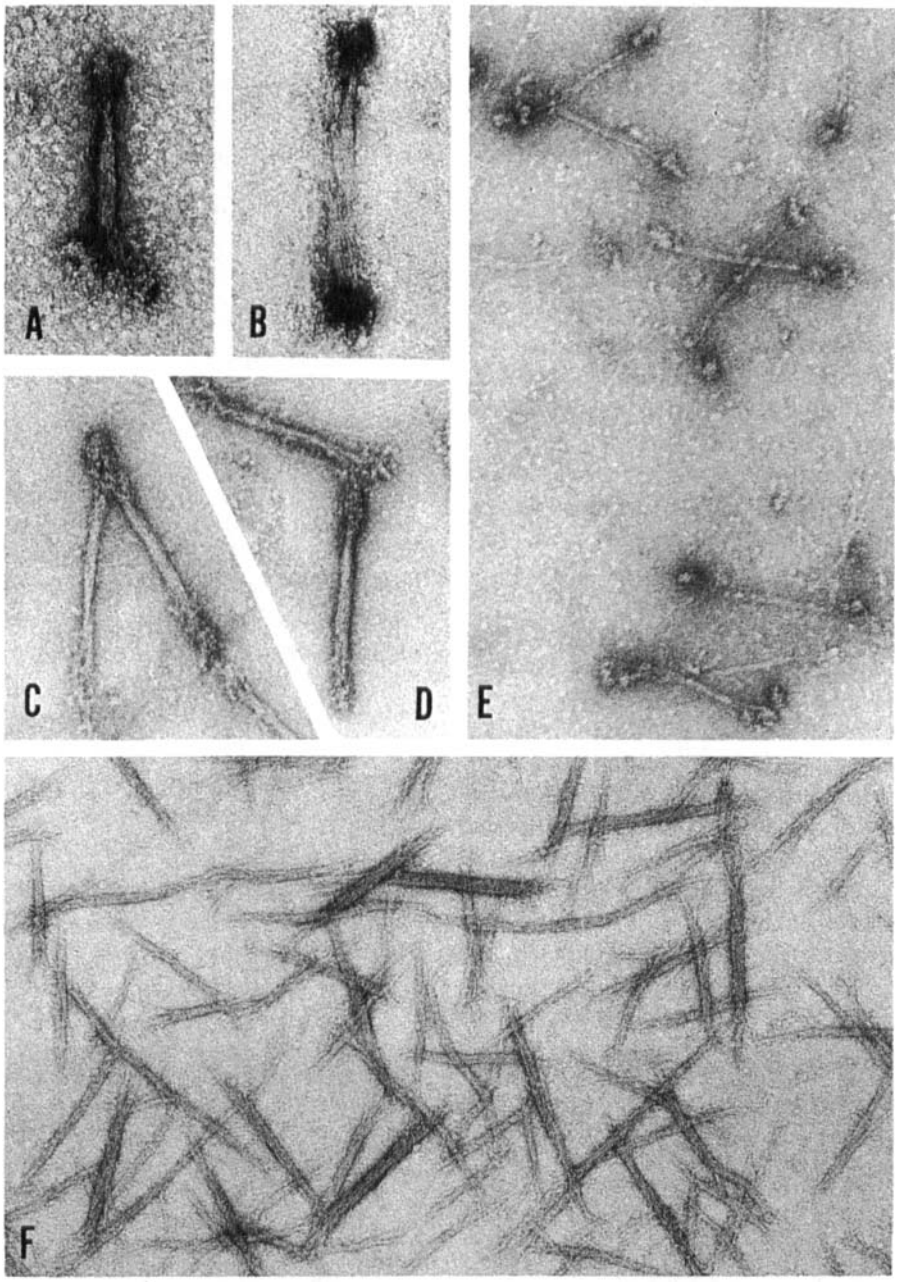

FIGURE 9. Myosin filaments negatively stained with uranyl acetate. A and B. *Physarum* myosin filaments formed in the presence of calcium. (Micrographs by V. T. Nachmias.) C and D. Human platelet myosin filaments. E. Guinea pig granulocyte myosin filaments formed in the presence of calcium. F. Short filamentous aggregates of human platelet myosin rod. Note the absence of the terminal projections compared with the filaments of the intact platelet myosin in C and D. (Micrograph by R. Niederman.) Magnification x 91, 000.

Hatano and Tazawa[14] prepared *Physarum* actomyosin by high ionic strength extraction and low ionic strength precipitation, then removed part of the actin from the myosin by ultracentrifugation in 0.5 *M* KCl with Mg^{++} and ATP. The myosin remained in the supernatant but was contaminated by actin. Later, Hatano and Ohnuma[156] found that the contaminating actin could be precipitated with part of the myosin by dialysis at low ionic strength, and, although much of the myosin was lost in the precipitate, the purified *Physarum* myosin remaining in the supernatant appeared homogeneous by sedimentation velocity ultracentrifugation. Using gel electrophoresis, Nachmias found that *Physarum* myosin prepared by a modification of Hatano's method contains several impurities that could be separated from the myosin by gel filtration on 4% agarose.[152]

Independently, Adelman and Taylor[15,16] devised another method for preparing slime mold actomyosin using pyrophosphate extraction and ammonium sulfate fractionation. By column chromatography of the actomyosin on Sephadex G-200 and DEAE-cellulose, they obtained a highly purified preparation of *Physarum* myosin which was free of nucleic acid contamination and at least 75% pure by analytical ultracentrifugation. More rigorous analysis by gel electrophoresis has not been reported.

Both methods yield 5 to 10 mg of purified *Physarum* myosin/100 g of starting material, corresponding to about 0.3% of the cell's protein. This is the lower limit of the quantity of myosin in *Physarum*, while estimates based on data of Adelman and Taylor[16] for specific activities of the enzyme in crude homogenates and of purified enzyme suggest the true value is 8 to 10 times higher, or about 2 to 3% of the slime mold's total protein. Both groups estimate that there is at least a twofold weight excess of actin, corresponding to a 20-fold molar excess of actin over myosin in *Physarum*.

Using the sedimentation coefficient of 6.4S and a diffusion coefficient estimated by gel filtration, Adelman and Taylor[16] calculated that the native molecular weight of *Physarum* myosin is about 460,000. By SDS gel electrophoresis of agarose purified *Physarum* myosin, Nachmias determined that the molecule consists of three classes of polypeptide chains: one of 240,000 daltons and two in the 12,000 to 15,000 dalton range.[151,169] While this follows the same general pattern of light and heavy chains found in other myosins, the *Physarum* myosin heavy chain is distinctly larger, and the light chains may be smaller than the corresponding polypeptides from other myosins.

Direct visualization of individual *Physarum* myosin molecules by electron microscopy[170] (Figure 10) shows that they resemble muscle

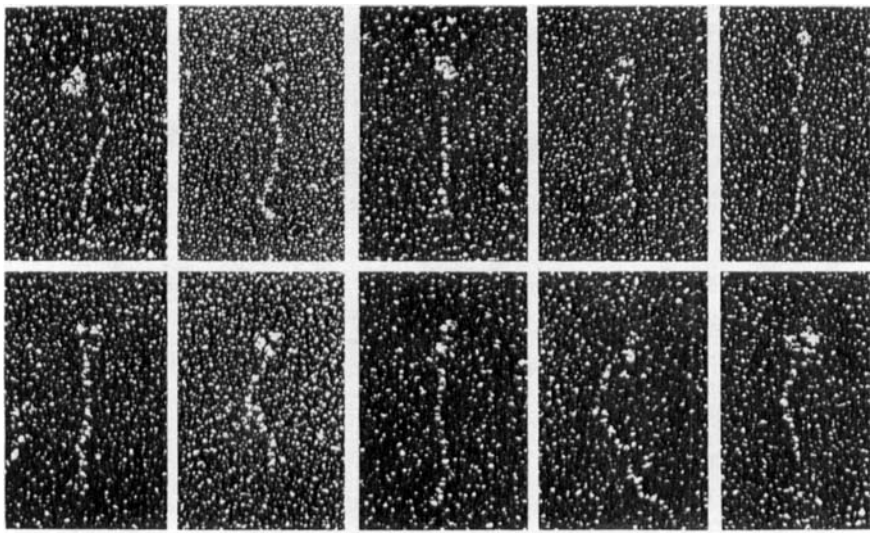

FIGURE 10. Individual *Physarum* myosin molecules contrasted by rotary shadowing. Magnification x 160,000. (From Hatano, S. and Takahashi, K., Structure of myosin A from the myxomycete plasmodium and its aggregation at low salt concentrations, *J. Mechanochem. Cell Motility*, 1, 7, 1971. With permission.)

myosin in size and shape, having an extended tail about 120 nm long with a globular region at one end. In some of the molecules, the globular region can be resolved into two separate "heads" about 11 nm in diameter.

The available data, therefore, are consistent with a two heavy chain model for the *Physarum* myosin molecule, although certain inconsistencies raise some doubts about this conclusion. If the molecule consists of two heavy chains with molecular weights of 240,000 and at least two light chains with molecular weights of 12,000 to 15,000, the native molecular weight must be over 500,000, which is greater than the value of 460,000 determined by Adelman and Taylor.

In spite of its overall physical similarity to striated muscle myosin, *Physarum* myosin differs from it significantly in being soluble in certain dilute buffers in which muscle myosin aggregates into insoluble bipolar filaments. Ultracentrifugation of purified *Physarum* myosin in dilute KCl solutions at neutral pH reveals only monomer and some small aggregates with sedimentation coefficients up to 15S.[16,156,170] In agreement with these solution studies, electron microscopy of *Physarum* mysoin in 0.05 *M* KCl alone reveals no visible aggregates.

Bipolar thick filaments of *Physarum* myosin were first observed by Hinssen[171] in preparations of *Physarum* actomyosin aged in a "relaxing solution" containing Mg^{++} and ATP. Later, Nachmias found that addition of 1 m*M* $CaCl_2$ or 10 m*M* $MgCl_2$ to purified *Physarum* myosin in 0.05 *M* KCl results in the formation of short bipolar filaments up to 25 nm wide and 450 nm long (Figure 9).[152] In recent experiments with more highly purified *Physarum* myosin, filaments up to 2,000 nm long have been observed.[169] Like muscle myosin filaments formed in vitro, these aggregates of *Physarum* myosin have a bare central region with a fibrous substructure (undoubtedly due to the longitudinal alignment of the myosin tails) and tufted ends (corresponding to the globular head regions of the myosin). The actin binding sites are in the projections at the ends of the filaments, judging by the way the myosin aggregates can cross-link actin filaments in vitro[151,172] (Figure 8A). Although *Physarum* contains a total of 1.2 m*M* Ca^{++},[53] we do not know the free Ca^{++} concentration in the cytoplasm, making it difficult to relate these properties of the myosin to its state of aggregation in the living cell.

The amino acid composition of homogeneous *Physarum* myosin has not been reported, but Hatano and Ohnuma[156] found that their purified *Physarum* myosin lacked the amino acid cysteine. This finding is particularly remarkable because muscle myosin contains a number of cysteine residues, and two of these have been implicated in the active site of the ATPase.[173,174] The apparent absence of cysteine in *Physarum* myosin is hard to reconcile with the fact that sulfhydryl reagents (*p*-chloromercuribenzoate and *N*-ethyl maleimide) inhibit the ATPase activity of the enzyme.[156] Of course, neither of these reagents is completely specific for cysteine, so other groups could have been affected in these experiments.

Physarum myosin catalyzes the hydrolysis of the terminal phosphate from ATP and at lower rates from ITP and GTP.[16] ATPase activity is activated by Ca^{++} and inhibited by Mg^{++}, but, unlike some other myosins, EDTA inhibits activity even in the presence of high concentrations of KCl.[16,156] This pattern of ATPase activity is shared by muscle myosin blocked in the S-1 sulfhydryl group.[173]

In the original work on *Physarum* myosin, little[156] or no actin activation of low ionic strength Mg^{++} ATPase was found, but Nachmias[169] has now prepared *Physarum* myosin which is activated 4- to 6-fold by muscle actin. No quantitative details about this actin-myosin interaction are available, but, in contrast to *Acanthamoeba*, a cofactor protein (see below) may not be required for actin activation of this protozoal myosin.

Acanthamoeba Myosin[126,153,154]

Acanthamoeba castellanii is a small, free-living soil amoeba which has been adapted to grow in liquid cultures. It contains the most unusual myosin yet discovered. This myosin is unique because it is a relatively small, globular ATPase which, nonetheless, is capable of cross-linking actin filaments and having its enzyme activity stimulated by actin. Because of its size and solubility at low ionic strength, it was initially missed in searches for myosin in this cell, but it was eventually identified by gel filtration of amoeba extracts. Similar small myosins have not been described in other cells but could have been missed because of their unusual properties.

Because the enzyme comprises only 0.3% of the amoeba's total protein, an elaborate procedure is necessary to obtain purified *Acanthamoeba* myosin. This involves ion exchange chromatography, ammonium sulfate fractionation, reversible binding to agarose beads, and hydroxyapatite chromatography. The purified enzyme consists of an approximately 1:1:1 molar ratio of three polypeptide chains with molecular weights of 140,000, 16,000, and 14,000 daltons. They account completely for the native molecular weight of the enzyme, about 180,000 daltons, estimated by gel filtration, which also shows that the native molecule is globular. The sedimentation coefficient of about 8S[117] is also appropriate for a globular protein of this size. *Acanthamoeba* myosin is soluble and monomeric in both 0.5 and 0.1 *M* KCl, although at very low ionic strength it can aggregate or bind to some other component in the cell homogenate.[117]

The small size of *Acanthamoeba* myosin raises the question of whether the isolated enzyme is the product of the degradation of a larger native myosin. Although it is impossible to rule out absolutely such a possibility, a number of control experiments are all consistent with 180,000 daltons being the size of the native *Acanthamoeba* myosin.

The amino acid composition of the protein is remarkable because it bears no general resemblance to that of any other myosin or to that of any fragment of muscle myosin that has been examined. Like *Physarum* myosin, the amoeba myosin apparently lacks cysteine, an amino acid known to be important in the active site of skeletal muscle myosin. The amoeba myosin also lacks the methylated lysines and histidine found in some muscle myosins.

In spite of these major differences in physical and chemical properties, the *Acanthamoeba* myosin and muscle myosin have almost identical ATPase activities under a wide variety of conditions, showing that the active site may be quite similar in spite of the overall dissimilarities. Specifically, activity is inhibited by Mg^{++}, stimulated slightly by Ca^{++} irrespective of the monovalent cations present, and stimulated maximally by EDTA in the presence of high concentrations of KCl.

Acanthamoeba myosin binds reversibly to muscle actin filaments. Electron microscopy shows that the bound myosin accentuates the periodicity of the underlying actin filament (Figure 8D), but (probably due to its globular shape) the complex does not have any obvious polarity as do the typical arrowhead-shaped complexes formed by actin and other myosins. In the presence of *Acanthamoeba* myosin, the actin filaments are often aligned (in register) in parallel arrays. These clusters of actin filaments break down when the *Acanthamoeba* myosin is dissociated from the actin filaments by treatment with ATP, suggesting that the *Acanthamoeba* myosin forms cross-links between adjacent actin filaments.

Actin alone will not activate the Mg^{++} ATPase of the purified *Acanthamoeba* myosin, but, in the presence of another *Acanthamoeba* protein, called the cofactor, actin strongly activates ATPase activity. The cofactor is a new protein not previously described in muscle or other cells. Available data show that it is a globular protein with a molecular weight of about 100,000 daltons. The cofactor lacks ATPase activity and does not resemble any of the known components of the control system found in muscle or any fragment of muscle myosin. Preliminary experiments show that the maximal effect of the cofactor occurs when it is present in approximately equimolar amounts with the *Acanthamoeba* myosin. The concentration of actin also affects the resulting ATPase activity in a complicated manner, while the time course of the ATP hydrolysis suggests that the interaction of ATP with the three proteins is a cooperative process.

Using a figure of about 10% as an estimate of the fraction of the cell's protein which is actin, it is clear that there is a large molar excess of actin over myosin in *Acanthamoeba*, on the order of 100 to 1. If all the actin was polymerized into filaments, there would be about one myosin molecule for every 0.3 μm of actin filament.

Myosin in Other Cells

The only convincing method now available for identifying myosin in nonmuscle cells is the direct isolation and characterization of the protein. Less satisfactory approaches are to isolate actomyosin, to identify thick myosin-like filaments by electron microscopy, or to demonstrate cross reaction of tissues with antibodies to myosin or actomyosin.

Actomyosin

Protein mixtures with certain properties of actomyosin from muscle (reviewed by

TABLE 9

Actomyosin-like Proteins

A. Proven to contain actin or myosin	Purified actin	Purified myosin
Brain (cat, rat)[177]	+[49]	+[150]
Dictyostelium[42]	+[43]	
Leukocytes[77,78] (horse, human, guinea pig)	+[78a]	+[76]
Physarum[6,8,14,15,178]	+[13,16]	+[16,156]
Platelets[9,179]	+[46,62]	+[147,148]

B. Not proven to contain actin or myosin	Separation of actin- and myosin-like fractions	Precipitation in dilute buffer	ATP reduces viscosity	Superprecipitation	Contractile threads	Characteristic ATPase	Antibody
Endothelial cells[120,180] (human)							+
Erythrocyte[181] membrane	+	+	+				
Mitochondria[182] (liver)	+		+	+	+	+	
Naegleria[183]		+		+		+	
Nitella[184]			+				
Nuclei[185] (calf thymus)	+		+	+	+	+	
Sarcoma cells[187]		+	+	+		+	
Spermatozoa[188] (human)		+	+				
Leaf vascular[175,176] bundles (*Hydrilla*, pumpkin, tobacco)			+				

Poglazov)[186] have been extracted from a number of cells (Table 9). Most frequently, it is found that ATP lowers the viscosity of these preparations (presumably due to dissociation of myosin and actin). Other properties shared with actin and myosin include ability to be separated into actin- and myosin-like fractions, precipitation in dilute buffer, superprecipitation, formation of contractile threads, and ATPase activity responding to divalent cations, like actomyosin from muscle. All these properties together provide presumptive evidence for actomyosin, and in 5 cases cells which yield actomyosin-like protein mixtures have been proven to contain actin and myosin (Table 9). On the other hand, the properties may not be specific for actomyosin, and until the remaining preparations (Table 9) can be shown to contain actin or myosin by more rigorous criteria, such as direct isolation, peptide mapping, etc., these experiments must be interpreted cautiously. For example, ATP lowers the viscosity of plant extracts,[175] but the ATPase in these preparations also hydrolyzed ADP (unlike myosin) and had a very low molecular weight by gel filtration,[176] suggesting that the enzyme is not myosin. Furthermore, later investigators were unable to confirm the original isolation of actomyosin from mitochondria.[176a]

Myosin-like Thick Filaments

Filaments with the size and shape of myosin filaments have been observed in electron micrographs of several cells: *Amoeba proteus*,[189,189a] *Chaos carolinensis*,[190] platelets,[100,167,191] and *Saccamoeba* sp.,[192] as well as tunicate epidermal cells[193] and cultured fibroblasts[193a] treated with cytochalasin B.[194] In the case of platelets and fibroblasts, these filaments are undoubtedly myosin, because myosin has been purified from these cells and shown to form similar filaments in vitro. In the other cases, their identity as myosin filaments is much less certain. Besides the simple observation of the morphology of these filaments,

two revealing tests would aid in their identification as myosin: (1) examination of their solubility in various concentrations of KCl[195] and (2) testing for their ability to bind to actin filaments.[196]

Antibodies

Antibodies have been prepared to myosin from striated muscle,[197] platelets,[147] and granulocytes,[76] and to actomyosin from smooth muscle[180] and platelets.[198] Providing that these antibodies to myosin can be shown to be specific (and this appears to be true in the case of antimuscle myosin, antigranulocyte myosin, and possibly some of the other antibodies), they can be used, in principle, to demonstrate the presence of myosin in other cells, either by immunohistochemical procedures or by more quantitative methods such as radioimmunoassay.[198a] The antiplatelet myosin has been used in this way to demonstrate a possible deficiency of myosin in platelets from patients with the hereditary disease Glanzman's thrombasthenia.[199]

General Conclusions

The foregoing discussion and the data summarized in Tables 6, 7, 8 and 9 prove beyond any doubt that myosin exists in many motile cells. All these cytoplasmic myosins share with muscle myosin the ability to cross-link actin filaments (Figure 8) and to have their Mg^{++} ATPase activity stimulated by actin. Therefore, strong arguments can be made for their role in the transduction of chemical energy stored in ATP into force for movement (see section on Relation of Cytoplasmic Actin and Myosin to Cell Movement).

Is there any physiological significance to the wide variation in the measured rates of ATP hydrolysis by the various myosins (Tables 7 and 8)? In muscles, Barany has shown that both Ca^{++} ATPase and actin-activated Mg^{++} ATPase are more active in the myosins from more rapidly contracting muscles.[157] With the nonmuscle cells, no such correlation is possible now because the effects of factors such as assay conditions, enzyme impurity, or denaturation on the enzyme rate are not yet well investigated, and no standard assay for "contractility" in a nonmuscle cell is available.

Assay conditions are particularly crucial for measurements of actin activation (Table 8) because the rate of ATP hydrolysis is a sensitive function of actin concentration, ionic strength, and temperature. These parameters have been examined carefully for the actin-muscle heavy meromyosin Mg^{++} ATPase, and it was found that (1) the dependence of the rate on actin concentration can be described by simple Michaelis-Menten kinetics,[28] suggesting a direct physical interaction between the actin and myosin; (2) increasing the ionic strength inhibits the rate because of greater dissociation of the actin and myosin;[28] and (3) the energy of activation is very high (28 kcal/mol).[200] Two of these parameters have been investigated for a single cytoplasmic myosin. The actin activation of *Acanthamoeba* myosin Mg^{++} ATPase is strongly inhibited by increasing the ionic strength, but the rate is dependent on the actin concentration in a complicated way.[154] The influence of these factors (the actin concentration, ionic strength, and temperature) is illustrated in Table 8, which shows that some of the differences in the actin activated myosin ATPases can be accounted for by differences in assay conditions.

There is more uniformity in the interaction of these cytoplasmic myosins with actin in the absence of ATP, where they all bind to and can form cross-links between actin filaments. Usually the complex of myosin with actin has the appearance of polarized arrowheads, although no polarity is obvious in the *Acanthamoeba* myosin-actin complex.

In most cases the myosin self-assembles into bipolar thick filaments in dilute buffers, although some require divalent cations for assembly (Table 6; Figure 9A, B, and E). The bipolarity of these filaments is important because in vitro the cross-linked actin filaments are usually attached to opposite ends of the myosin filament (Figure 8B and C). This type of cross-link may also predominate in vivo, and it is probably not coincidental that the polarity of this type of actin-myosin cross-link is appropriate for a sliding filament mechanism of movement similar to that in striated muscle. In the case of the globular myosin from *Acanthamoeba*, a different type of cross-link is formed without the intervention of a long connecting piece composed of a myosin filament backbone, so adjacent actin filaments are held close together (Figure 8D).

Beyond these important functional properties, there are interesting differences and similarities in the physical and chemical properties of the myosins. All the myosins are large proteins composed of extraordinarily large polypeptides with

molecular weights of 140,000 to 240,000 daltons, and all have associated polypeptides of much lower molecular weight, known as light chains (Table 6). Although it is not known why myosins contain such large polypeptides, it may be because a large polypeptide is necessary to accommodate the functions of filament formation, hinge, and ATPase in a single molecule.[26] *Acanthamoeba* myosin, which lacks a tail, has a correspondingly smaller heavy chain.

Acanthamoeba myosin is also unique in being composed of only a single heavy chain. Muscle myosin (and probably the other myosins listed in Table 6) consists of two heavy chains. While this bipartite structure is necessary for the formation of the coiled-coil tail of the myosin, it is not clear whether the two heads are necessary for contraction. However, it is known that one-headed myosin (prepared by limited proteolytic digestion) is capable of superprecipitation.[201]

It is indeed striking that all of the myosins have light chains. In vertebrate muscles some of the light chains are necessary for ATPase activity, and in molluscan muscle one light chain is necessary for Ca^{++} modulated regulation of actin-myosin interaction. It has not yet been determined whether the light chains of cytoplasmic myosins are necessary for activity, although the absence of intrinsic regulatory activity indicates that none of them are comparable to the regulatory light chain of the molluscan myosins. Their universal presence even in the myosins from protozoa suggests that they must have some essential role in the force generating mechanism which is not presently appreciated.

LOCALIZATION OF CYTOPLASMIC ACTIN AND MYOSIN WITHIN CELLS

There is now an extensive morphological literature on various types of filaments within cells. In some cases these filaments have been identified as actin or myosin, but in most cases identification has not been reported. A detailed evaluation of these studies on cytoplasmic filaments is beyond the scope of this review, so we will concentrate on those cases where the filaments are known to be actin or myosin. Some of the material on this topic is discussed in the sections Other Methods of Identifying Actin, Myosin in Other Cells, and Relation of Cytoplasmic Actin and Myosin to Cell Movement.

Actin

Ishikawa's heavy meromyosin labeling technique[117] has now been widely used for the identification of actin filaments *in situ* (Table 5). The major drawback of this procedure is the need to glycerinate the cells. In some cells, including *Acanthamoeba*[75] and the intestinal epithelium,[17,38] the actin filaments are well preserved in their natural location after glycerol extraction, but in other cases the filaments do not survive glycerination. A rapid glycerination procedure has recently been described which may lessen this problem in some cases.[108] A second problem has been that in some cells more filaments are seen after treatment with heavy meromyosin than before (Table 5), raising the possibility that the heavy meromyosin is somehow influencing the extent and possibly the position of filament polymerization. This is, of course, possible, because studies with pure muscle actin have shown that myosin or heavy meromyosin promotes polymerization.[202] Alternatively, the heavy meromyosin may simply stabilize preexisting actin filaments so that they are preserved by the fixation procedure used to prepare these specimens for electron microscopy. Regardless of the explanation for this effect of heavy meromyosin, the problem makes it mandatory that the presumed actin filaments be localized in routinely fixed cells and in glycerinated cells both with and without heavy meromyosin to be certain that their natural position has been identified. Once the actin filaments have been identified by heavy meromyosin binding in glycerinated cells, it is clearly advisable to establish their interrelationship with the other cytoplasmic components in unextracted cells where these features are preserved.

The immunofluorescent[120] and fluorescein heavy meromyosin[117a] techniques discussed above are useful for determining the general distribution of actin within cells, although the resolution in the light micrographs is inferior to that in electron micrographs. A possible advantage of these approaches is that they potentially could reveal the localization of monomers or small oligomers of actin which would be missed in electron micrographs. A serious problem with the antibodies is the uncertainty about their specificity.

A third approach is the isolation of various cell fractions under mild conditions and the determination of their actin (or myosin) content by direct isolation of the protein,[150] gel electrophoresis,[66,127] electron microscopy,[118] or enzyme assays.[150] The disadvantage of this approach is the possibility that the natural distribution of actin might be altered during cell disruption or fractionation.

The results obtained with these three approaches show that although nonmuscle cells lack a highly organized contractile apparatus like that of striated muscle, their actin filaments are localized in certain regions. Except for one report suggesting the presence of actin in the nuclei of *Physarum*,[203] actin filaments are usually found in the cytoplasmic matrix or "ground substance" which surrounds the various membrane-bounded organelles. Although there are exceptions, most cells have some actin filaments in the "cortical" region just inside the plasma membrane (Table 5). These cortical filaments are sometimes extensive, and, because they exclude membranous organelles, this region frequently appears clear in the light microscope, giving rise to terms such as "hyaline zone" or "hyaline ectoplasm." A bundle of these cortical actin filaments usually extends into microvilli on the surface of cells, where they appear to support these thin processes.

The close proximity of these cortical actin filaments to the plasma membrane has suggested that they are attached to the inner surface of the plasma membrane. This appears to be true in the intestinal brush border[38] and in *Acanthamoeba*,[118,127] where it is possible to isolate the plasma membrane with actin filaments still attached (Figure 11). In stereo electron micrographs of isolated *Acanthamoeba* plasma membranes, the actin filaments appear to make direct physical contact with the cytoplasmic surface of the plasma membrane without a visible specialization at the attachment site.[118] In the brush border is a dense plaque on the inner surface of the plasma membrane where the actin filaments attach.[204] The actin filaments of brush border have been decorated *in situ* with heavy meromyosin, and all the actin filaments appear to have the same polarity, with the arrowheads pointing away from the plasma membrane.[17] This may also be true in *Acanthamoeba*, although it has been difficult to show unambiguously.[117] This polarity is, of course, the same as the polarity of muscle actin relative to the Z-line[18] and could have the same significance. Because the actin filaments can be removed from the *Acanthamoeba* plasma membranes by the gentle procedure of dialyzing against dilute buffer to depolymerize the actin,[118,127] it is clear that the actin is attached to, but not an integral part of, the membrane. Perdue has presented ultrastructural evidence for the interesting idea that actin filaments may penetrate the plasma membrane of fibroblasts.[204a]

By morphological criteria alone, actin filaments (or filaments having the appearance of actin) appear to be attached to the plasma membrane in many other cells.[104,205,206] These membrane attachments undoubtedly account for the concentration of the actin filaments in the cell cortex. Although their function has not been proven, it is reasonable to suppose that at sites where the plasma membrane makes contact with other cells or with a substrate, the filaments associated with the membrane would serve as an anchor against which the contractile apparatus could exert force for movement. An exaggerated example of this arrangement is found in tissue culture cells where "stress fibers"[207] or a "sheath"[106] of actin filaments is found near the base of the cells and extends into cell processes.

It has recently been claimed that actomyosin is a component of red blood cell plasma membranes,[208] but we think that this is an improper use of the term "actomyosin." The idea arose because proteins (originally called either "spectrin"[209] or "tektin"[210]) with molecular weights similar to those of muscle actin and myosin can be extracted from these membranes with dilute buffers. Some of these preparations can form filaments resembling actin,[211,212] and others are reported to have ATPase activity.[212] In these superficial ways, the mixtures are similar to actomyosin, but they have never been shown to have the properties specific for actin and myosin. Certain other properties, such as their solubility *only* in very dilute buffers[212a] and the molecular weights of the large polypeptides (about 250,000 daltons), are also inconsistent with the properties of the well characterized vertebrate actins and myosins.

In contrast to the work with the plasma membrane, little information is available about the possible interactions of actin and myosin with other cell fractions. Berl, Puszkin, and Niklas[150] have presented preliminary evidence that the actin

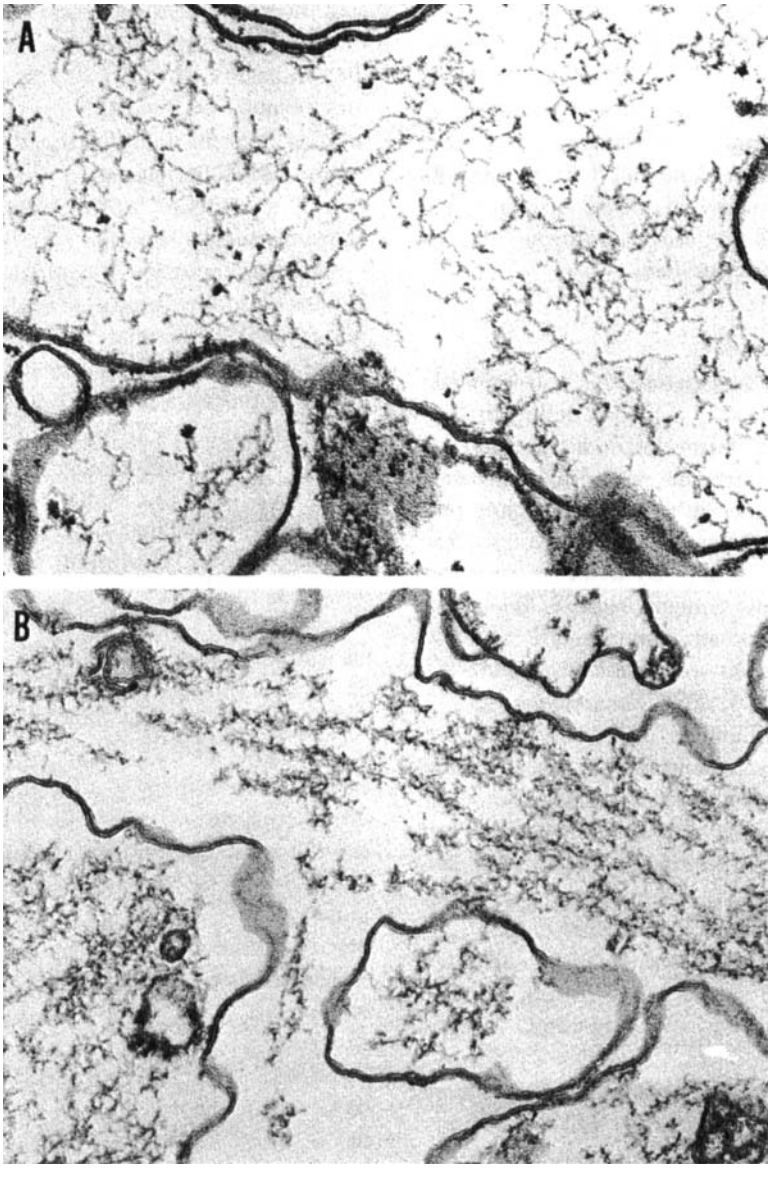

FIGURE 11. Micrographs of thin sections of isolated *Acanthamoeba* plasma membranes. A. Bare actin filaments are visible in this preparation treated with heavy meromyosin and pyrophosphate. B. Actin filaments decorated with heavy meromyosin. Polarized arrowheads are visible in some places. Magnification x 86,000.

of synaptosomes is associated with the synaptosomal membrane and that the myosin is associated with the synaptic vesicles. While this work cannot be evaluated in a definitive way until the experiments are described more completely, still the data presented are the first experimental support for the intriguing possibility (apparently first enunciated by Schmitt)[213] that intracellular transport could arise from interaction of complementary contractile proteins having different localizations.

In many cells bundles of parallel actin filaments can be found. In some cases the orientation of these bundles is clearly related to simple shortening movements, as in the contractile ring, and certain morphogenetic movements (which are discussed below).

In addition to these cortical filaments and

filament bundles, other actin filaments can be found throughout the cytoplasm of many cells, but their distribution and function have not been completely analyzed. Numerous ultrastructural studies on thin cytoplasmic filaments (sometimes referred to as microfilaments) have been published. Although many of these filaments are probably actin, this has not been proven, and the work will not be reviewed here.

Myosin Localization

Compared with the progress that is being made on the localization of cytoplasmic actin, little is known about the intracellular distribution of myosin in any nonmuscle cell. In most cells myosin filaments are either absent or not preserved by the fixation (as discussed above), so only actin filaments are usually seen. There are some apparent exceptions, though, because thick filaments similar to myosin filaments are regularly seen in thin sections of *Amoeba proteus*,[189] *Chaos carolinesis*,[190] and *Saccamoeba* sp.[192] These myosin-like filaments are usually associated with actin filaments (identified in the first two cells by heavy meromyosin binding), but their identity is still in question because myosin has not been isolated from these amoebas. In *Amoeba* and *Chaos*, difficulties with fixation have prevented correlation of the thick filament distribution with the streaming of the amoeba. In *Saccamoeba* the thick filaments are arranged in a pattern which is similar to the pattern of cytoplasmic streaming.[192]

Both *Physarum* and human platelets contain substantial amounts of myosin which can form thick filaments under suitable conditions (discussed above), although thick filaments are not usually seen in the cytoplasm. After glycerination, however, numerous filaments appear, some of which are similar to typical myosin filaments.[100,196] These thick filaments also have been observed in platelets after osmotic lysis[101] or other treatments.[214] In both cases the thick filaments are usually enmeshed by numerous thin filaments which have been identified as actin by reaction with heavy meromyosin. The heavy meromyosin decoration reveals an additional fact, however, which is that a number of thin filaments with widths between 2 and 10 nm do not react with heavy meromyosin.[100,196] These undecorated thin filaments can cross connect actin filaments and, on the basis of these connections and their failure to bind heavy meromyosin, are thought to be small myosin aggregates. This is the first indication that intracellular myosin filaments may be much smaller than those made in vitro from purified myosin. If this is true, these small myosin aggregates will have to be identified using immunological techniques because they are otherwise indistinguishable from the actin filaments.

Another approach used to localize myosin is the use of antisera prepared against crude platelet actomyosin[147] or smooth muscle actomyosin.[180] These antisera are specific for some component in the actomyosins, although it has not been proven to be myosin. By light[215] or electron microscopy,[198] the antisera appear to bind to some component on the outer surfaces of platelets[198,215] and other cells.[216] This binding can be blocked by absorption of the antismooth muscle actomyosin with muscle myosin or heavy meromyosin[217] or by absorption of the antiplatelet actomyosin with platelet actomyosin.[198] The platelet actomyosin antibodies also react with some component in the cytoplasm of platelets[198] (presumably the actin and myosin which have been identified there by other methods) as well as the cytoplasm of a variety of other cells.[217a] Smooth muscle actomyosin antibodies also bind to a component in the mesangial cells of the renal glomerulus,[218] where actin and myosin have not been identified by other means.

The binding of these antibodies to cytoplasmic components is expected from other results, but the binding to the outer surfaces of cells comes as a surprise. Independent proof that the outer surface component is actin or myosin will have to be obtained by other means, such as direct isolation, and the possibility that the apparent localization is an artifact caused by damage to the cells will have to be ruled out.[120]

These approaches still leave us with little detailed information about the distribution of myosin within cells. This information will have to be obtained before we have a clear picture of how actin-myosin interaction develops the motile force.

CONTROL MECHANISMS

The complex and varied movements of living cells obviously must be regulated carefully, presumably by factors controlling the interaction of their contractile proteins. Extensive informa-

TABLE 11

Interaction of Troponin-tropomyosin with Various Myosins

Type of myosin	Muscle actin activated Mg^{++} ATPase (μmol/min/mg)			
	Without troponin-tropomyosin		With troponin-tropomyosin	
	EGTA	Ca^{++}	EGTA	Ca^{++}
Acanthamoeba[221]	1.13	1.21	0.23	0.35
Guinea pig granulocyte[76]	0.0075	0.0074	0.0028	0.0088
Human platelet[48]	0.07	0.07	0.03	0.08
Rabbit muscle subfragment-1[221]	3.70	4.52	0.39	5.50

weight ratio of 1 to 2 when muscle actin was substituted for *Acanthamoeba* actin. Although direct tests for the binding of troponin-tropomyosin to *Acanthamoeba* actin were not done, it is likely that the troponin-tropomyosin acts by binding to *Acanthamoeba* actin, as it is known to bind muscle actin,[220] and does not interact directly with myosin.

Similar experiments have been reported with two other cytoplasmic actins (Table 10). Muscle troponin-tropomyosin makes the platelet actin activation of the Mg^{++} ATPase of muscle heavy meromyosin dependent on Ca^{++}.[48] The complex of *Physarum* actin and muscle myosin superprecipitates at low ionic strength with Mg^{++} and ATP in the presence or absence of Ca^{++}, but addition of muscle troponin-tropomyosin makes superprecipitation dependent on the presence of Ca^{++}.[219] Tanaka and Hatano[219] also showed by viscometry, flow birefringence, and analytical ultracentrifugation that the muscle troponin-tropomyosin binds to the actin. Together, these experiments show that cytoplasmic actin filaments have binding sites for muscle troponin-tropomyosin and that Ca^{++} can regulate the interaction of these hybrid filaments with muscle myosin.

Cytoplasmic myosins are also influenced by muscle troponin-tropomyosin (Table 11). The Mg^{++} ATPase of platelet and granulocyte myosin is stimulated by muscle actin, and the rate is the same with or without Ca^{++},[48,76] showing that these purified cytoplasmic myosins lack intrinsic Ca^{++}-sensitive regulatory activity such as that found in molluscan myosins. When muscle troponin-tropomyosin is added to the assay, actin-activation occurs only with Ca^{++}, showing that these myosins recognize the Ca^{++} modulated inhibition to actin-myosin interaction produced by troponin-tropomyosin.

Acanthamoeba myosin requires the presence of the cofactor protein for actin to activate its Mg^{++} ATPase, and the resulting activity is not affected by Ca^{++}.[154] Thus, neither the myosin nor the cofactor is influenced by Ca^{++}. When muscle troponin-tropomyosin is added to the assay, it inhibits, as expected, ATPase activity in the absence of Ca^{++}, but unexpectedly it also inhibits ATPase activity in the presence of Ca^{++}. Inhibition of actomyosin ATPase activity by troponin-tropomyosin in the presence of Ca^{++} is unique among myosins and suggests that troponin-tropomyosin of the muscle type must not exist in the amoeba, or it would block actin-myosin activity permanently. To investigate this phenomenon further, Pollard and co-workers[221] tested combinations of purified tropomyosin and purified troponin components on the ATPase activity of the hybrid system consisting of muscle actin with *Acanthamoeba* myosin and cofactor and found that tropomyosin, troponin-I, and troponin-C all behave normally, but troponin-T does not. The relation of these results to the control of amoeboid movement is not understood.

Calcium Dependent Movements

Even before it was known that actin and myosin are present in nonmuscle cells, it was thought that Ca^{++} might participate in the regulation of cell motility. Early experimental support for this idea came from the injection of various salts into giant amoebas or eggs of marine invertebrates. Dilute Ca^{++} solutions caused localized "precipitation" (contraction?) of the cytoplasm at the site of injection.[222]

Hoffman-Berling and co-workers[223,224] tested

tion (briefly reviewed above) is now available about the Ca^{++} modulated control of actin-myosin interaction in muscle. However, the data available on the factors controlling cell motility and the activity of cytoplasmic actin and myosin are so fragmentary that one cannot be certain of even the *general* aspects of the control mechanism in any nonmuscle cell. Several experiments suggest that some cells have Ca^{++} modulated regulatory systems as does muscle, while other experiments suggest that different mechanisms might be involved.

The relevant experiments fall into five categories:

1. Tests showing that the cytoplasmic contractile proteins can interact normally (or sometimes abnormally) with the regulatory proteins troponin-tropomyosin from muscle.

2. Tests for Ca^{++} dependence in various motile processes. In at least two organisms, *Physarum* and *Chaos*, motile processes which may well be caused by actin and myosin are dependent on Ca^{++}.

3. Direct search for control proteins. Tropomyosin has been isolated from platelets, electric organ, and brain, and extracts of *Physarum* and platelets contain factors which confer Ca^{++} dependence on the superprecipitation or Mg^{++} ATPase of actomyosin.

4. Investigation of intracellular localization and movements of Ca^{++}. In analogy with muscle, the guiding hypothesis is that movement is dependent on Ca^{++} and that Ca^{++} levels are regulated by Ca^{++} sequestering membranes.

5. Investigation of control mechanisms unique to cytoplasmic contractility. These may include regulatory proteins different from troponin-tropomyosin, such as cofactor protein from *Acanthamoeba*, and regulation by the cell of the disposition of contractile proteins.

Interaction of Muscle Regulatory Proteins with Cytoplasmic Actin and Myosin

Once purified preparations of cytoplasmic contractile proteins were available and it was known that they would form functional hybrids with complementary muscle proteins, it was interesting to know whether they could also interact with the control proteins from muscle. The first experiments were carried out by Eisenberg and Weihing,[96] who examined hybrid complexes of *Acanthamoeba* actin with muscle troponin-tropomyosin. Like pure muscle actin, *Acanthamoeba* actin by itself activates the Mg^{++} ATPase of muscle heavy meromyosin in both the presence and absence of Ca^{++}. Addition of muscle troponin-tropomyosin to a mixture of *Acanthamoeba* actin and muscle heavy meromyosin made the Mg^{++} ATPase dependent on the presence of trace amounts of Ca^{++} (Table 10). A similar effect is noted with muscle actin and corresponds, in a test tube, to the regulatory function of troponin-tropomyosin in muscle, where it allows actin-myosin interaction only in the presence of Ca^{++}. The maximum effect of troponin-tropomyosin occurs at a weight ratio of 1 mg troponin-tropomyosin to 3 mg of *Acanthamoeba* actin, which is similar to the

TABLE 10

Interaction of Troponin-tropomyosin with Various Actins

		Muscle heavy meromyosin Mg^{++} ATPase (μmol/min/mg)			
		Without troponin-tropomyosin		With troponin-tropomyosin	
Type of actin	Temp. (°C)	EGTA	Ca^{++}	EGTA	Ca^{++}
None[96]	25	0.03	0.03	–	–
Acanthamoeba[96] (0.2 mg/ml)	25	0.15	0.15	0.04	0.44
Human platelet[48] (0.2 mg/ml)	37	1.40	1.49	0.32	1.13
Rabbit muscle[96] (0.2 mg/ml)	25	0.67	0.64	0.04	0.54
Physarum[219] (+ muscle myosin)		Rapid superprecipitation	Rapid superprecipitation	Slow superprecipitation	Rapid superprecipitation

the effect of "relaxing grana" (vesicles isolated from muscle sarcoplasmic reticulum or from cultured fibroblasts by identical methods) on ATP-induced contraction of glycerinated cell models. The relaxing grana blocked contraction. Because it was known that the muscle-relaxing grana actively sequester Ca^{++} and that their effect on cell model contraction could be overcome by added Ca^{++}, it was reasonable to postulate, as Hoffman-Berling did, that changes in Ca^{++} concentration help regulate movement in the intact cell. However, as there was no direct measurement of the free Ca^{++} concentration in these experiments, the interpretation is uncertain.

More recent work has implicated Ca^{++} in the control of movement in *Physarum*, in which actin, myosin, and factors resembling troponin-tropomyosin are undoubtedly present. Hatano[225] treated *Physarum* with caffeine, which fragmented the plasmodium into 100 μm wide spheres limited by a plasma membrane. Streaming of the cytoplasm, assessed directly by light microscopy, occurred only when the external Ca^{++} concentration was greater than 10^{-7} *M*. The simplest interpretation of the experiment is that caffeine renders the plasma membrane permeable to Ca^{++} so that changes in the external Ca^{++} concentration can regulate the interaction of the contractile proteins. This interpretation has not been proven; however, there is a precedent for caffeine affecting membranes. In muscle caffeine appears to influence the influx and efflux of Ca^{++}[227] and the release of Ca^{++} from the sarcoplasmic reticulum.[228] Caffeine may also inhibit phosphodiesterase (as it does in many other cells[226]), which should increase the concentration of cyclic AMP. In an unrelated cellular slime mold, cyclic AMP stimulates the release of Ca^{++} from the organism.[229]

In a series of important experiments, Taylor and co-workers have shown that streaming in cell-free extracts of the giant amoeba, *Chaos carolinensis*, is dependent on traces of Ca^{++}.[230] The absence of a plasma membrane makes the manipulation of the ionic environment of the cytoplasm simple, and the lifelike movement of the extracts reassures one that normal cytoplasmic movements are being tested. The threshold Ca^{++} concentration for movement is 7×10^{-7} *M*, and, as the Ca^{++} concentration is increased up to about 5×10^{-3} *M*, the average rate of contraction increases in parallel (Figure 12). At the threshold of Ca^{++} concentration, with Mg^{++} ATP in the bathing medium, the cell extract extends pseudopods of naked cytoplasm closely resembling the membrane-limited pseudopods of the intact cell. Actin[231] and myosin-like thick filaments[190,231] have been identified in *Chaos*, but nothing is known about its control proteins or the mechanism by which Ca^{++} regulates the movement.

Certain movements of intact cells are dependent on Ca^{++} in the external medium. Thus, fibroblast locomotion[231a] and leukocyte locomotion and phagocytosis[232,233] can be reversibly inhibited by removal of external Ca^{++}. However, it is not clear whether changes in the external Ca^{++} concentration are affecting the contractile apparatus. Furthermore, these effects are not entirely specific for Ca^{++} because they can often be duplicated by removal of Mg^{++}.

As an aside, it is important to note that the simple observation of a Ca^{++}-sensitive movement is insufficient evidence to conclude that the movement is caused by actomyosin. Calcium-sensitive movement is observed in the spasmoneme of certain peritrichous ciliates.[2,234,235] The organelle is contracted in 10^{-6} *M* Ca^{++} and relaxes in 10^{-8} *M* Ca^{++}. These changes can be repeated over and over again by simple changes of the Ca^{++} concentration without the need for any source of energy such as ATP, although addition of Ca^{++} ATP causes repetitive cycles of contraction and relaxation. The solubility and elastic properties of the spasmoneme, which resemble those of rubber instead of muscle, show that the only property this system shares with actomyosin is its sensitivity to Ca^{++}.[2]

Regulatory Proteins from Nonmuscle Cells

If nonmuscle cells have a Ca^{++} regulated contractile mechanism, whether in the form of troponin-tropomyosin (or other new proteins) or integrated into the myosin as in mollusc muscle, one should be able to demonstrate that crude cytoplasmic actomyosin has low ionic strength Mg^{++} ATPase activity (or superprecipitation) influenced by Ca^{++}. This has been done in a few cases, as described below. Once this regulatory activity has been identified, the factors involved must be isolated and then recombined with purified actin and myosin to demonstrate their function. Experiments of this type have not been completed, although several cytoplasmic tropomyosins have been purified.

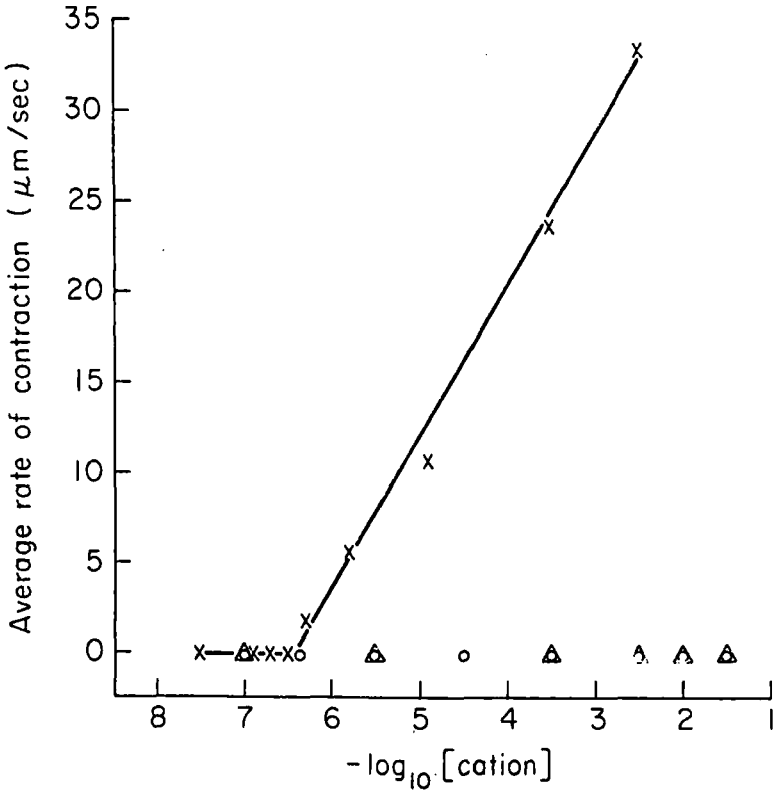

FIGURE 12. The effect of various calcium concentrations on the rate of contraction of isolated cytoplasm from *Chaos carolinensis*. (Unpublished data of Taylor, D. L., Condeelis, J. S., Moore, P. L., and Allen, R. D.)

Calcium-regulated Crude Actomyosin

It has apparently been difficult in many cases to show that the activity of crude cytoplasmic actomyosins is regulated by Ca^{++},[78] although Cohen and Cohen[236] have convincing data on a preparation of platelet actomyosin whose Mg^{++} ATPase is stimulated by increasing the free Ca^{++} concentration from 10^{-7} to 10^{-6} *M* (Figure 13). The regulatory components are not located on the myosin because the Mg^{++} ATPase of purified platelet myosin activated by actin free of regulatory proteins is not dependent on Ca^{++}.[48,236a] The regulatory proteins are presumably on the actin filament because addition of excess purified actin to the crude actomyosin eliminates Ca^{++} dependence of the actomyosin ATPase, presumably because the purified actin competes[35] with the endogenous actin which contains the regulatory components. The nature of these regulatory components is discussed below.

Shibata and co-workers[78] found that calcium stimulates the superprecipitation of crude actomyosin from horse leucocytes. Half the maximal

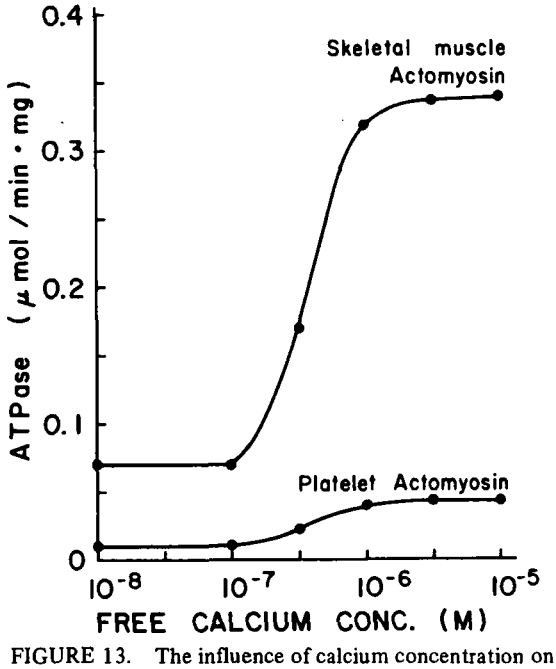

FIGURE 13. The influence of calcium concentration on the Mg^{++} ATPase activity of muscle and human platelet actomyosin. (Drawn from Cohn, I. and Cohen, C., A tropomyosin-like protein from platelets, *J. Mol. Biol.*, 68, 383, 1972.)

TABLE 12

Properties of Tropomyosins

Muscle tropomyosins	Subunit composition	Paracrystal period	% α-helix
1. Vertebrate skeletal (rabbit, chicken)[237]	2 x 35,000	39.5 nm (Mg^{++})	∼100%
2. Vertebrate cardiac (sheep, ox)[238,239]	2 x 35,000	40 nm(Mg^{++})	>95%
3. Vertebrate smooth (chicken)[238,239]	2 x 33,000	40 nm (Mg^{++})	>95%
4. Crustacean striated (crayfish)[238,239]	? x 33,000	40 nm (isoelectric precipitation)	>95%
5. Molluscan adductor (oyster)[238]	?	40 nm (isoelectric precipitation)	>95%
Cytoplasmic tropomyosins			
1. Vertebrate platelet (human)[236]	? x 30,000	34.3 nm (Mg^{++})	∼90%
2. Vertebrate brain (chick embryo)[240]	? x 30,000	34 nm (Mg^{++})	∼90%
3. Electric organ (*Torpedo* and *Electrophorus electricus*)[240a]	? x 35,000	40 nm (Mg^{++})	>90%

effect occurred at a Ca^{++} concentration of 10^{-6} *M*. These results are consistent with a Ca^{++} modulated control mechanism, but no experiments on leucocytes have been reported which reveal the factors involved.

Isolation of Regulatory Proteins

Tropomyosin has been purified from platelets, electric organs, and brain and investigated in some detail (Table 12). Proteins with some properties of troponin have been found in *Physarum* and platelets, but they have not been extensively studied to date, leaving major questions to be answered in this area.

Tropomyosin

Cohen and Cohen[236] isolated tropomyosin from an alcohol-ether extract of platelets by KCl extraction, boiling (which denatures most other proteins), isoelectric precipitation, and preparative gel electrophoresis in concentrated urea. The resulting platelet tropomyosin is homogeneous by analytical gel electrophoresis. Like muscle tropomyosin, platelet tropomyosin is over 90% alpha-helical and contains many acidic amino acids and no proline. The subunit molecular weight of platelet tropomyosin is 30,000 daltons, and it forms paracrystals in the presence of Mg^{++} which have a periodicity of 34 nm (Figure 14). Both the molecular weight and paracrystal periodicity are about 15% less than those of muscle tropomyosins isolated from various sources (Table 12). Given the lower molecular weight, the shorter periodicity of the paracrystals is expected, because in muscle tropomyosin paracrystals, the spacing is determined by the length of the tropomyosin molecule. A 5,000 dalton reduction in subunit molecular weight should lead to the observed 6 nm reduction in paracrystal periodicity. The morphology of these paracrystals and the other physical and chemical data are all consistent with a two stranded coiled-coil structure (like muscle tropomyosin) for the native platelet tropomyosin. Studies on the function of platelet tropomyosin were not carried out, but it was observed that the tropomyosin was present in the platelet actomyosin, presumably because it binds to the platelet actin.

Fine and co-workers[240] have isolated tropomyosin from embryonic chick brain using a modification of the platelet tropomyosin purification procedure. Gel electrophoresis in sodium dodecyl sulfate showed that the brain tropomyosin is at least 90% pure and has a molecular weight of 30,000 daltons. As expected, the brain tropomyosin paracrystals have a periodicity of about 34 nm (Figure 14), and the molecule is largely alpha-helical. Tryptic peptide maps of chick brain tropomyosin labeled with ^{125}I show an overall similarity to, but some differences from, chick muscle tropomyosin. Brain tropomyosin binds to muscle actin filaments and sediments with them in the ultracentrifuge. It interacts with muscle actin and troponin to form a complex whose activation of muscle myosin Mg^{++} ATPase is regulated by Ca^{++}. The apparently normal function of this hybrid complex is particularly remarkable because the brain tropomyosin, due to its

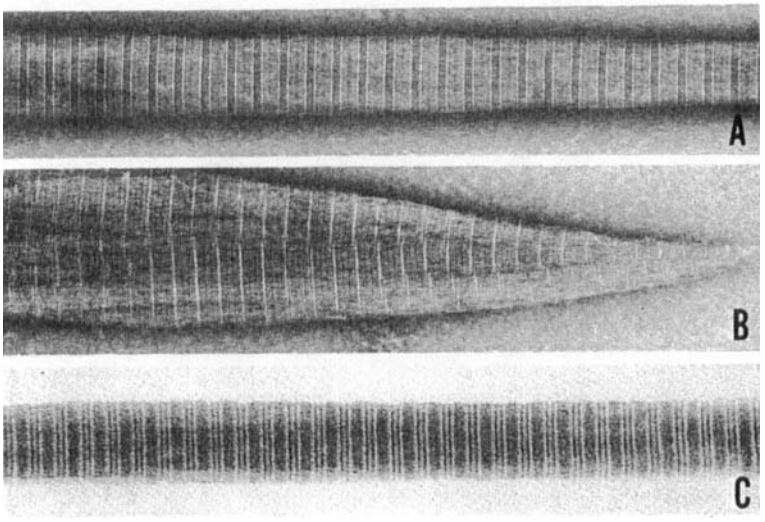

FIGURE 14. Electron micrographs of magnesium paracrystals of tropomyosins, negatively stained with uranyl acetate. Magnification x 86,000. A. Muscle tropomyosin (micrograph provided by I. Cohen and C. Cohen). B. Platelet tropomyosin (micrograph provided by I. Cohen and C. Cohen). C. Chick brain tropomyosin (micrograph provided by R. Fine).

shorter length, must interact with six rather than the usual seven actin monomers in the actin filament.

Tropomyosin isolated from electric organ of *Torpedo* and *Electrophorus electricus* has a molecular weight of 35,000 daltons and a paracrystal period of 40 nm,[240a] setting it apart from platelet and brain tropomyosin (Table 12). This resemblance to muscle tropomyosin may be related to the development of the electric organ from modified muscle cells.

Physarum Troponin-tropomyosin-like Activity

Experiments by Tanaka and Hatano[219] show that extracts of *Physarum* contain factors which behave like muscle troponin-tropomyosin. The slime mold was extracted with 0.05 *M* KCl and particulate material removed by centrifugation. Muscle actin and myosin (free of troponin-tropomyosin) were then added to the extract, in which they precipitated due to the low ionic strength. The precipitated muscle actomyosin and adhering material were collected by centrifugation. Prior to this treatment, the muscle actomyosin could superprecipitate in both the presence and absence of Ca^{++}. After exposure to the *Physarum* extract, superprecipitation became dependent on the presence of Ca^{++}. Addition of muscle troponin-tropomyosin to actomyosin superprecipitation is a specific test for troponin-tropomyosin or whether the effect of the *Physarum* extract was due to other causes.

myosin would have the same effect on superprecipitation of pure muscle actin and myosin, so it seems that the slime mold must contain the functional equivalent of troponin-tropomyosin, although the molecular basis of this activity is not yet known. One perplexing aspect of these experiments is that *Physarum* actomyosin lacks any Ca^{++} dependence for superprecipitation, raising the question of whether Ca^{++} dependence of muscle

Platelet Troponin-like Components

As discussed above, it appears that platelet actomyosin contains regulatory components which are bound to the actin filaments. These have not been proven to be troponin, but Cohen, Kaminski, and deVries[236a] have extracted actin from an acetone powder of platelet actomyosin and found that in addition to actin it contains tropomyosin and three polypeptides with molecular weights of 36,000, 18,000, and 14,000 daltons. Activation of muscle myosin Mg^{++} ATPase by this mixture of proteins is inhibited 50% by addition of EGTA, suggesting that these peptides are related to troponin. Thorens, Schaub, and Lüscher[241] used completely different procedures to isolate a mixture of proteins from platelets which can inhibit the Mg^{++} ATPase of platelet or muscle actomyosin

up to 40% in the absence of Ca^{++}. Further fractionation and characterization of the proteins present in these preparations will be necessary to determine whether any of them is a component of the regulatory system.

Investigation of Intracellular Localization and Movements of Ca^{++}

If regulation of contraction in nonmuscle cells is analogous to regulation in muscle, then we would expect to find intracellular sequestration of Ca^{++} analogous to sequestration by the sarcoplasmic reticulum, and, furthermore, we should expect to find that Ca^{++} is released just before contraction occurs. Both of these expectations have been realized. Calcium sequestering vesicles have been identified in several cells known to contain actin and myosin, but Ca^{++} release has been studied only in *Spirostomum*, which may not have an actomyosin contractile apparatus.

In the case of giant amoebas, which contain thin filaments which bind heavy meromyosin[115, 231] and thick filaments which look like myosin,[189-190] direct injection of sodium alizarin sulfonate (a Ca^{++} precipitant which turns red when combined with Ca^{++})[222] or glyoxal-bis-(2-hydroxyanil)[242] (a dye which turns red or purple with a number of cations including Ca^{++}, but whose Ca^{++} complex specifically resists decolorization by alcoholic Na_2CO_3-KCN) results either in the localized formation of red precipitates in the cytoplasm, where the cells attempt to extend a pseudopod (alizarin), or in the formation of red granules or bands in the anterior and middle region of the cell (glyoxal). In the case of the latter dye, only randomly oriented dye granules were seen in rounded cells, but in moving cells the dye is found in regions which are usually active in pseudopod extension. The results suggest that Ca^{++} could be released into the cytoplasm at the site of pseudopod formation.

Ettienne[243] demonstrated directly that release of Ca^{++} precedes the onset of contraction in the ciliate *Spirostomum*. The dye aequorin, which emits light when it binds to Ca^{++}, was injected into the cell, which was then induced to contract by an electric current. Contraction begins when the light intensity has peaked, showing that an increase in the concentration of free Ca^{++} precedes the onset of contraction. Relaxation occurs when the emitted light intensity (and presumably the Ca^{++} concentration) drops. The Ca^{++} released into the cytoplasm comes from an internal store, because placing the cell in a Ca^{++}-free medium does not alter the contractile response or the emission of light from aequorin. Calcium oxalate precipitates enclosed in vesicles were identified in electron micrographs of the organism,[243] and these are close to a cortical layer of thin filaments. Osborn[244] applied the electron microprobe with signal averager to 2-μ thick sections of the cortical region of the organism and found that the calcium signal was most intense over the thin filaments, a result which is in general agreement with Ettienne's electron microscope studies. Lehman and Rebhun concluded from ultrastructural investigations of the organism that it was most likely the thin filaments (rather than the microtubules) whose orientation could be correlated best with contraction of the organism.[245] All these data suggest that in *Spirostomum* the release of Ca^{++} from intracellular stores activates a filamentous contractile apparatus, but further experiments are necessary to determine whether the contractile apparatus is actomyosin or related to the *Vorticella* spasmoneme.[246]

Intracellular membranous vesicles which may sequester Ca^{++} are present in cells besides *Spirostomum*. In *Physarum*, these were identified by incubating the organism in oxalate and showing that precipitates, presumably composed of calcium oxalate, accumulated within the vesicles.[247] Microprobe analysis of the precipitates directly demonstrates that the precipitates contain Ca (and small amounts of K).[248] The formation of precipitate requires the presence of ATP and is inhibited by Salyrgan, a sulfhydryl inhibitor.[247] Similar vesicles can be identified in *Amoeba proteus*, *Chaos carolinensis*,[249] and *Carchesium*.[250]

A few studies are available which seem to show that membranes which accumulate Ca^{++} in vitro can be prepared from various nonmuscle cells. As mentioned earlier, Hoffman-Berling and co-workers[223,224] prepared "relaxing grana" from cultured fibroblasts by the same method which they used to prepare fragments of sarcoplasmic reticulum from muscle. The fibroblast preparation inhibited ATP-induced contraction of glycerinated cells, and this inhibition could be reversed by addition of Ca^{++}. Hence, the inhibition was presumed to be due to removal of free Ca^{++} from the medium. However, direct measurements of Ca^{++} uptake were not performed, and, therefore, the data are difficult to interpret unambiguously.

Calcium uptake has been measured directly using membranes isolated from platelets. A vesicular fraction, free of mitochrondria, granules, and dense bodies, was first isolated by Statland et al.[251] by sucrose density gradient centrifugation of the 14,000 to 100,000 x g fraction. More recently Robblee et al.[252] obtained vesicles of similar purity simply by collecting the fraction sedimenting between 14,000 to 40,000 x g. Under optimal conditions (in the presence of ATP, oxalate, Mg^{++}, pH 7), the vesicles accumulate up to 0.2 to 0.4 μmol Ca^{++}/mg of protein, corresponding to a 500-fold greater concentration of Ca^{++} within the vesicles as compared to the medium. Electron-dense precipitates, presumably corresponding to precipitates of calcium oxalate, can be seen in electron micrographs of the vesicles. Based on comparision with cytochemical and biochemical studies of platelets, Statland et al.[251] postulated that their preparations were fragments of the surface and canalicular membranes of the platelet "which function as a platelet sarcoplasmic reticulum." Both laboratories observed that ADP inhibited Ca^{++} uptake, which is particularly interesting because ADP is one of many factors which induces platelet aggregation.

The uptake of Ca^{++} by mitochondria is well known,[253] and reports have appeared showing that intact Ehrlich ascites tumor cells accumulate Ca^{++} and that this uptake is dependent on the presence of substrates which can be oxidized by mitochondria.[254] It is not clear that these effects can be related to contraction in these cells. Indeed, Ca^{++} affects so many processes[255] that it is clear that very careful work will be required to disentangle its possible direct effects on contractility in motile cells from other effects unrelated to movement.

Unique Control Mechanisms

Despite the growing evidence that Ca^{++} together with troponin-tropomyosin- like proteins may regulate cell motility, two lines of experimental work and some theoretical arguments suggest that additional factors may be involved. The fact that myosin from molluscs regulates its own interaction with actin[34] establishes a precedent for a similar control mechanism in other cells. Myosin-based Ca^{++}-sensitive regulation of actin-myosin interaction has been looked for with purified myosins from *Acanthoamoeba*, human platelets, and granulocytes (Table 11), but none was found. Either these myosins normally lack this regulatory activity, or the regulatory activity was lost during the purification of the myosins, or the assay conditions were inappropriate. Nonmuscle cells of molluscs are the obvious place to look for a self-regulated cytoplasmic myosin, although it might also be present in other systems.

The presence of the cofactor protein in *Acanthamoeba* shows that in some cells proteins different from troponin-tropomyosin may affect interaction of actin and myosin.[154] The limited data on the cofactor protein show that actin activation of the *Acanthoamoeba* myosin Mg^{++} ATPase requires the presence of the cofactor. Because this is the ATPase activity most likely to be related to cell movement, the cofactor is in a position to turn the system on or off. Unfortunately, there is no information on how the cofactor might be added to or removed from the actin and myosin in the cell or, alternatively, how the activity of the cofactor might be turned on or off. At least Ca^{++} does not seem to play a role.[221]

It is likely that factors besides Ca^{++}-sensitive control proteins must operate in control of cell motility. In striated muscle the regular array of actin and myosin filaments keeps the actin and myosin close together and in a favorable geometric relationship for interaction so that a simple Ca^{++}-controlled block to actin-myosin- interaction is sufficient to regulate the system. In cells with less organized contractile elements, presumably consisting of actin filaments and associated myosin, additional controls must also be exerted over the spatial and temporal assembly of the contractile elements. Essentially nothing is known about the factors influencing the disposition of the cytoplasmic contractile proteins within any cell, although there are striking examples that such controls must exist.

One example of the control which cells have over the disposition of their actin is the rapid formation, contraction, and dissolution of the "contractile ring" in the cleavage furrow of dividing cells (described in greater detail below).[256] Another example is the formation of an "acrosomal process" on starfish sperm[113] when they contact the egg. This process is up to 90 μm long and contains a bundle of actin filaments which are assembled from some precursor (possibly actin monomers) within a few seconds. Similarly in platelets, actin filaments do not appear until platelets have aggregated.[167] In

these examples it seems that actin is poised on the verge of polymerization but is restrained by unknown factors until its polymerization is triggered. However, recognition that the presence of mitotic apparatus, the presence of an egg, and platelet aggregation somehow influence actin polymerization has not revealed the molecular events regulating the polymerization.

RELATION OF CYTOPLASMIC ACTIN AND MYOSIN TO CELL MOVEMENT

At the present time, there is no direct experimental proof that actin and myosin generate the force for cell motility, although a large body of circumstantial evidence supports this appealing idea.

Comparative Arguments

In the first place, the numerous properties shared by muscle and cytoplasmic actins and myosins argue for a common force generating mechanism. Recall that the cycle of actin and myosin interaction which generates force in muscle is thought to consist of at least four steps:[22,145] (1) ATP hydrolysis by myosin, (2) dissociation of the products of ATP hydrolysis upon binding actin to myosin, (3) sliding movement, and (4) dissociation of actin from myosin by the rebinding of ATP to the myosin. It is striking that the cytoplasmic actins and myosins can carry out these four steps, although it is not known whether such a sequence actually occurs in any nonmuscle cell. (1) Cytoplasmic myosins can hydrolyze ATP. (2) Actin stimulates ATP hydrolysis by cytoplasmic myosin (although it is not proven that the mechanism of this stimulation involves the acceleration of product dissociation from myosin). (3) Threads of cytoplasmic actomyosins (discussed below) contract in the presence of ATP and Mg^{++} (although it is not proven that a sliding mechanism is involved). (4) ATP dissociates cytoplasmic actin and myosin. These striking similarities cannot reasonably be accounted for by chance alone. Rather, it seems likely that muscle and various other cells have a common mechanism for generating force for movement.

This argument is strengthened by the ease with which the actins and myosins from various sources can cross species boundaries to interact with one another. Although there are some quantitative differences in the reactions (whose meaning is yet to be discovered), all cytoplasmic myosins can form active hybrids with muscle actin, and all the cytoplasmic actins form active hybrids with muscle myosin. Thus, the cytoplasmic proteins can interact with muscle proteins which are known to generate force for movement, making it likely the cytoplasmic actin and myosin themselves interact in vivo to cause movement.

Finally, the structural features of the muscle contractile proteins, which are so vital to the contractile mechanism in muscle, are shared by all of the cytoplasmic actins and most of the cytoplasmic myosins. All the actins form the same polarized double helical filaments and bind myosin in the same way. All the myosins, except *Acanthamoeba* myosin, form bipolar thick filaments with active sites for ATP hydrolysis and actin binding located at both ends. Bipolar myosin filaments interacting with unipolar actin filaments could generate force by a sliding filament mechanism in nonmuscle cells (Figure 15). All the measured biochemical properties of the cytoplasmic actins and myosins are also in accord with this suggestion. Force generation in *Acanthamoeba* could also be caused by sliding actin filaments linked by individual myosin molecules or small oligomers rather than by filamentous myosin aggregates (Figure 15).

We should point out that others have made similar suggestions on theoretical grounds[257] or based on the properties of the cytoplasmic contractile proteins they were studying.[126,151,171] Unfortunately, these comparative arguments will never prove that this sliding filament theory is correct; the theory must be proven experimentally.[16]

Contraction Analogues

Although it is difficult to design experiments to assess directly the role of cytoplasmic contractile proteins in cell motility, certain contraction analogues are available which show the contractile nature of cytoplasmic actin and myosin. Like muscle actomyosin, platelet[258] and *Physarum*[259] actomyosins dissolved in concentrated buffers form threads when squirted into low ionic strength buffers in which the proteins precipitate. When these actomyosin threads are exposed to Mg^{++} and ATP, they contract. Although the mechanism of this contraction is not known, even for muscle actomyosin threads, the results prove that these

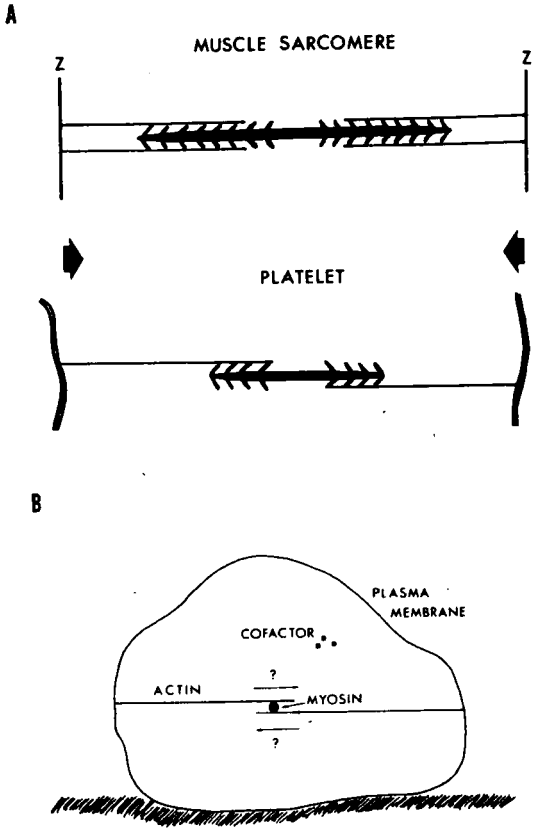

FIGURE 15. Structural similarities between the contractile protein of muscle and other cells. A. Comparison of a muscle sarcomere with one possible arrangement of contractile proteins in a platelet which is consistent with the properties of these proteins. B. A possible arrangement of the contractile proteins in *Acanthamoeba*. (From Pollard, T. D. and Korn, E. D., The "contractile" proteins of *Acanthamoeba castellanii, Cold Spring Harbor Symp. Quant. Biol.*, 37, 573, 1973. With permission.)

two cytoplasmic actomyosins can form a structure which is capable of at least some type of contraction.

A related contraction analogue used for years in experiments with muscle actomyosin is "superprecipitation." Here actomyosin is suspended as a fine precipitate in a low ionic strength buffer; upon treatment with Mg^{++} and ATP, the precipitate condenses and expels fluid. Once again, the mechanism is not understood, although it must be related to muscle contraction. The ability of cytoplasmic actomyosin from platelets,[9] *Physarum*,[178] leucocytes,[77,78] brain,[260] and *Dictyostelium*[42] to superprecipitate supports their role in cell movement.

Superprecipitation and contractile threads illustrate the potential for force generation by cytoplasmic actin and myosin, but these macroscopic movements are difficult to relate to microscopic cellular movements.

Motile Cell Extracts

Another experimental approach relating cytoplasmic contractile proteins to movement is the study of motile extracts of amoebas. Here the movements can be much more lifelike, although the composition of these preparations is much more complex than the contraction analogues. Cytoplasmic streaming outside a living cell was first described by Allen and co-workers in 1960.[261] They observed that cytoplasm from the giant amoeba *Chaos carolinensis* can stream in a normal pattern in a capillary tube in the absence of a limiting plasma membrane. This important experiment proved that the mechanism for developing force for cytoplasmic streaming is present in the cytoplasm itself and can operate outside a living cell.

Later, Thompson and Wolpert[262] developed methods for making larger motile extracts from mass cultures of another giant amoeba, *Amoeba proteus*. ATP stimulated the streaming and the contractions which occur when these crude cytoplasmic extracts (essentially a 1,000 x g supernatant of an amoeba homogenate) are warmed to room temperature. Electron micrographs of these extracts showed several types of filaments. Pollard and co-workers[115,189] confirmed these observations and showed that the motile extracts contain actin filaments and associated thick filaments which morphologically resemble myosin filaments. (Note that *Amoeba proteus* is *not* related to *Acanthamoeba*, which contains no myosin thick filaments.) No actin filaments are visble in cold nonmotile extracts, but, when movement is induced by raising the temperature to 22°C, the viscosity of the extract increases and actin filaments form concomitantly from precursors in the extract. These crude extracts can be fractionated by further centrifugation at 10,000 x g, yielding a nonmotile pellet containing the thick filaments, various membrane fragments, mitochondria, and a nonmotile supernatant containing the precursors of the actin filaments.[189] According to Wolpert, Thompson, and O'Neill,[263] recombining the pellet and supernatant restores motility. An attractive explanation for these observations is that the actin filaments interact with the myosin-like thick filaments and ATP to produce movement. Clearly,

further experiments are needed to substantiate this idea.

Recently, a third type of motile extract has been developed by Taylor, Condeelis, Moore, and Allen.[230] They simply rupture a single amoeba with a micropipet in one of several special buffers designed to mimic intracellular ionic conditions. In a "stabilization buffer" containing no ATP or Ca^{++}, the extract is stationary. Stretching portions of the stabilized cytoplasm reveals that it is elastic and contains birefringent fibrils. Addition of ATP causes the extract to lose its elasticity, suggesting that the cytoplasm has "relaxed" in some way. Addition of ATP and Ca^{++} results in various types of streaming and contraction which are sometimes remarkably lifelike. Electron microscopy shows that the extract contains both thin filaments (undoubtedly actin) and myosin-like thick filaments, which could easily account for the observed birefringence, streaming, and physical properties of these extracts. Perhaps the most important property of this system is the ease with which one can change the ionic composition of the medium, making it particularly useful to study control mechanisms (see section on Control Mechanisms).

Contractile Cell Models

As mentioned in the Historical Background, Hoffman-Berling discovered that glycerol-extracted nonmuscle cells could contract when exposed to Mg^{++} and ATP.[9a] Similar experiments have now been done with several cells including *Amoeba proteus*,[264] leucocytes,[265] *Physarum*,[195] and many other cells.[10] Treatment with ATP usually results in small isodiametric contractions which have little relationship to the movement of living cells, but more lifelike movements have been observed with briefly glycerinated leucocytes.[265]

Several of these glycerinated cells have been examined by electron microscopy and found to contain networks of filaments.[195,266] In the case of the giant ameobas, these filaments are probably actin, for they bind heavy meromyosin.[115,231] In addition to these thin filaments, thicker filaments resembling myosin have been observed in these glycerinated amoebas.[266] Micrographs taken after ATP treatment show some "condensation" of the filament networks.[195,266] The association of these filaments with the contraction of cell models strengthens the arguments for their role in cell movement.

Morphological Studies

Morphological studies are necessary to prove the relation of the cytoplasmic contractile proteins to cell movement, for this is the only way to determine how the proteins are organized to generate the forces for movement. Already there is a large literature, from which we will select examples, relating various cell movements to the presence of "microfilaments." In no case have these "microfilaments" been identified biochemically as actin, but decoration with heavy meromyosin shows that some of them are probably actin. In many other cases, the size and appearance of these microfilaments are consistent with their being actin.

The contractile ring of dividing cells (Figure 16) provides the strongest morphological support for a relation between cytoplasmic movement and actin filaments. The contractile ring is an ephemeral structure composed of 6 nm wide filaments found in the cleavage furrow during cell division.[267] In newt eggs[109] and HeLa cells,[268] these filaments bind heavy meromyosin tentatively identifying them as actin. Observations on contractile rings detached from the plasma membrane[269] show that the structure actually contracts, and measurements of the force exerted by the contractile rings of echinoderm eggs (about 2.5×10^{-3} dyne) prove that they develop enough tension to account for the formation of the cleavage furrow.[270] No myosin has been identified in the contractile ring,

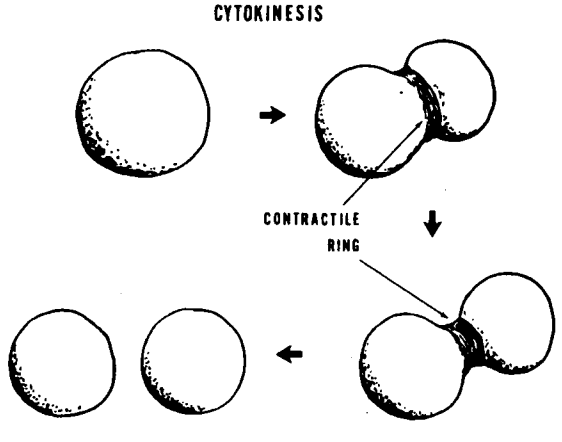

FIGURE 16. The role of the contractile ring in cytokinesis.

although careful measurements of the contractile ring volume during cleavage suggest that the actin filaments are sliding past one another during at least part of the contraction.[256]

In a similar manner, many different morphogenetic movements have been linked to appropriately placed bundles of microfilaments.[271-279] Recently, some of these filaments have been labeled with heavy meromyosin,[103] tentatively identifying them as actin. Again, no myosin-like filaments are seen with the actin.

One of the most dramatic correlations of filaments with movement is in tail resorption during metamorphosis of tunicate larvae.[272,276] At the time of metamorphosis of *Amaroucium* and *Distaplia*, bundles of thin filaments suddenly appear in the epithelial cells of the tail.[272] The filaments are oriented so as to be able to transmit the tension which these cells exert to pull the internal structures of the tail (including the rigid notochord and the musculature, which do not participate in this movement) into the body of the animal. The epithelial cells actively shorten during tail resorption, and, concomitantly, the filament bundles also become shorter, while maintaining their orientation along the tail axis. These results strongly suggest that filaments participate in tail resorption. After treatment of *Distaplia* with the drug cytochalasin B, "fusiform filaments 200 A in diameter and up to 0.14 μM in length were also found in many cells."[193] These dimensions are appropriate for myosin thick filaments, but such filaments are not detected in cells which have not been treated with cytochalasin B. Unfortunately, in this and many other cases, we know nothing further about the molecules making up these filamentous contractile structures.

As important as these ultrastructural studies are for the localization of the contractile proteins within cells, they have the obvious drawback that the cells are dead and that there is no assurance that the observed distribution of the proteins is normal. For this reason, new efforts are being made to resolve the contractile molecules by light microscopy of living cells. The use of phase randomized laser illumination has significantly increased the sensitivity of measurements of birefringence made with polarizing optics, so it is now possible to detect small amounts of anisotropic material in living cells and to correlate its distribution with the pattern of movement.[280]

Inhibition of Movement by Cytochalasins

The cytochalasins are a related group of mold metabolites which are of interest because they inhibit a variety of cellular movements, including locomotion,[206,281-290] phagocytosis,[290-292] pinocytosis,[102,293] cytokinesis,[267,283,294-299] cytoplasmic streaming,[300-306] chick embryo heartbeat,[307] and a variety of morphogenetic movements associated with thin filaments.[193, 206,278,279,308] The effects of the most widely used form, cytochalasin B, were reviewed two years ago by Wessells and co-workers.[310] Hoping that these drugs represented a specific inhibitor for microfilament-based motility, the authors wrote: "... *the prediction is that sensitivity to the drug implies presence of some kind of contractile microfilaments system.* Only further work will define the limits of confidence to be placed upon such diagnoses." Many new experiments, reviewed below, are difficult to reconcile with this prediction, but, as pointed out by Carter,[311] who originally described the effects of cytochalasins: "Unfortunately this proposal has been adopted as a basis for the design and interpretation of cytochalasin experiments instead of being regarded as one of several hypotheses which deserve to be tested." We shall now review experiments which fit the prediction of Wessells et al., then turn to certain other effects which are difficult to interpret in terms of effects on microfilaments, and conclude with a summary of currently unavailable information which must be obtained to understand the effects of cytochalasins.

In numerous instances effects on movement have been correlated with the disruption or disorganization of arrays of thin filaments. In at least one case (disruption of filaments associated with tail resorption of tunicate larvae),[193] the effects are rapid (seen within 0.5 to 1 min), are caused by low concentrations (2 μM), and are rapidly (1.7 min after washout) and completely reversible. Our interpretation of these observations is that the drug may rapidly and reversibly interact with a specific component(s) of the tail resorption system. In other cases (e.g., chick embryo oviduct),[278] concentrations of at least 60 μM applied over a span of hours were used to disrupt microfilaments and stop morphogenesis. The apparent need for higher concentration in these cells suggests that the drug penetrates poorly or that it is rapidly inactivated or that the target(s) of the drug is less sensitive. In the developing chick

heart, the filaments disrupted by rather high doses (100 μM) of cytochalasin B are thin filaments attached to Z lines and, therefore, are undoubtedly actin.[307] In other cases the cytoplasmic filaments which are disrupted by cytochalasin B also bind heavy meromyosin (filaments in glial cells, salivary epithelium, oviduct, and probably neurones from chick embryos)[103,106] and, hence, are presumably actin. In the remaining cases, the diameter of the filaments is near that of actin. It is usually easy to see how the observed arrangement of filaments which are affected by cytochalasin B could develop force for movement.

Cytochalasin B also lowers the viscosity of polymerized actin from platelets and muscle[312,313] and disrupts the organization of negatively stained actin filaments visualized by electron microscopy.[313] In addition, it lowers the viscosity of actomyosin prepared by mixing purified actin and myosin and decreases the actin activation of the Mg-ATPase of muscle heavy meromyosin.[312,313] However, it does not affect the ATPase of heavy meromyosin alone,[313] and all these effects, therefore, can presumably be explained by its effect on actin.

The drug does not lower the viscosity of the actin-troponin-tropomyosin complex, and the filaments formed by this complex appear prefectly normal by electron microscopy.[313] Therefore, reports that cytochalasin B does not affect actin filaments decorated with heavy meromyosin[314] or does not block spontaneous contractions of developing muscle cells[287,315] could be explained by postulating that these systems contain troponin-tropomyosin which protects the actin from the effects of cytochalasin B.

These experiments do not show that cytochalasin B affects actin in vivo; they only show that cytochalasin B has the *capability* of affecting actin. Furthermore, substantial effects in vitro were observed only at rather high (50 to 100 μM) concentrations of drug.[312,313] Therefore, the effects in vitro are difficult to relate to effects in vivo, which are frequently caused by 100-fold lower concentrations of drug.

Effects which suggest that the drug interacts with membranes rather than filaments have also been reported. In a variety of cells, cytochalasin B inhibits uptake of small molecules such as glucose,[320-322] deoxyglucose,[320,322-324] methylglucose,[321,326] glucosamine,[320,322,325,326] uridine,[327] and thymidine[327] at concentrations of the drug which range from a low of 0.08 μM to a high of 4.1 μM. Cytochalasin B also alters the properties of the action potential of cardiac muscle cells[327a] and changes the adhesion and electrophoretic mobility of amphibian gastrula cells.[327b]

Dose dependent effects suggesting that the drug attacks more than one site have been reported. In chick embryo fibroblasts,[323] the effects on uptake of glucose and deoxyglucose were stated to be observable at concentrations of cytochalasin B which were too low to alter the morphology of the cells. In human polymorphonuclear leukocytes, less than 2 μM cytochalasin B inhibits motility but *stimulates* chemotaxis, but above this concentration the drug inhibits both chemotaxis and motility.[282] In Earle's L mouse fibroblasts, 1 to 2 μM cytochalasin B inhibits locomotion, movements of the ruffled membrane, and cytokinesis, but nuclear extrusion is rare. Only at about 20 μM cytochalasin B are frequent nuclear extrusions noted.[283] The opposite response to concentration was noted for nuclear extrusion in cloned, pigmented retinal cells; frequent extrusions were noted at 2 μM cytochalasin B, but extrusion was inhibited at 20 μM cytochalasin B.[316] In the same cells, a web of thin filaments just beneath the apical protrusions of the cells is sensitive to 2 μM cytochalasin B, but a peripheral ring of thin filaments is disrupted only at 20 μM cytochalasin B.[316]

For unknown reasons, certain movements are not always inhibited by cytochalasin B. For example, the drug reportedly inhibits formation of a cleavage furrow in HeLa cells[267] and Arbacia egg,[256] but formation and regression of the furrow have been reported for L cells[298] and HeLa cells.[328] In addition, the thin filaments of the microvilli of C-4II cells,[308] the contractile ring filaments of *Xenopus* eggs,[294] and the filaments in certain algae[301] are not disrupted by cytochalasin B. Exocytosis can be either inhibited[329-333] or stimulated.[317,334,335] Still more paradoxical are the observations that cytochalasin *causes* certain movements. These include nuclear extrusion observed in many cells[283,287,311,316,336] and local contraction of cultured myotubes caused by cytochalasin D.[337] Certain effects such as inhibition of oxidation of glucose phosphates[290,335] or inhibition of incorporation of glucose and glucosamine into mucopolysaccharides[315] seem related to the effects on

transport.[338] Certain other effects seem difficult to relate either to effects on thin filaments or to effects on transport. These include induction of complex double membranes in Schwann cells,[339] inhibition of induction of tyrosine amino transferase by insulin and cortisol in Reuber hepatoma cells,[340] formation of crystalloids in mouse ova,[341] formation of leptomeric bodies in cultured myotubes[337] and possibly BHK-21 cells,[194] formation of nuclear filament arrays in cultured myotubes,[337] and potentiation of DNA synthesis in lymph node cells stimulated with phytohemagglutinin or concanavalin A.[342] and inhibition of mitochondrial contraction.[342a]

Interpretation of the effects of cytochalasins will require information which is not yet available. We do not know if cytochalasin even enters cells, although experiments of DeLaat et al.,[343] which show that the cleavage furrow of *Xenopus* eggs becomes sensitive to cytochalasin B at the same time that the membrane resistance decreases, do suggest that a permeability barrier to the drug has been removed and, hence, that the drug enters these cells. Assuming that cytochalasins enter cells, we still do not know whether we administer the active form of the drug or whether the drug must be metabolized to the active form or whether it is degraded to an inactive derivative. Such effects could explain certain cases of drug insensitivity. We do not know whether the drug attacks a single target or whether, as seems somewhat more likely in view of the currently available information, it attacks several sensitive targets. We do not know whether the target(s) is a protein(s) or whether, owing to the hydrophobic structure of the cytochalasins, it is the lipid portion of membranes. Finally, little or no information is available about the purity of any cytochalasin, and, hence, we do not know whether the active compound is the drug itself or a contaminant. These points can easily be investigated using radioactive cytochalasins, which can be prepared by biosynthesis from radioactive precursor[344] or by chemical introduction of tritium.[345-347] Early results with radioactive cytochalasins suggest that cytochalasin B binds to high and low affinity sites in platelets[345] and that cytochalasin D interacts with platelet myosin rather than platelet actin.[347]

Reconstitution Experiments

In the end, the most convincing evidence that cytoplasmic actin and myosin can interact to cause cell movements will be the reconstitution of a motile preparation from purified components. This reconstitution might most easily be made using procedures already available for making cell-free motile extracts which are described above, although it would be more elegant to enclose the proteins within a plasma membrane. Observation of streaming of *Physarum* or muscle actomyosin in thin capillary tubes provides preliminary evidence that this approach is feasible.[348]

CONCLUDING REMARKS

The biochemical studies reviewed above prove that actin and myosin are found in nonmuscle cells from phyla as widely separated as Protozoa, Echinoderms, and Chordates. The properties of the cytoplasmic actins and myosins, together with the ultrastructural and physiological observations on their localization and activity within cells, provide compelling evidence for the idea that many cells have the same force generating system that is found in muscle. Without the extensive information available on muscle contraction, we would know little about the function of cytoplasmic contractile proteins, but comparative arguments and the study of various contractile models leave little doubt that the actin-myosin contractile mechanism is much the same in all cells.

Compared with our knowledge of cytoplasmic actin and myosin, little information is available on the factors controlling the actin-myosin interaction or other steps in the activation of the contractile apparatus in nonmuscle cells. Further information is also needed on the intracellular localization of the contractile proteins. Both of these areas are under active investigation, so additional data will be forthcoming.

The identification of actin and myosin in "primitive" cells such as protozoa suggests that the mechanism for developing contractile force in muscle is not new but must have originated in primitive unicellular organisms long ago, where it was used for cytokinesis, locomotion, and phagocytosis. Comparisons of the properties of these "primitive" contractile proteins with those of muscle suggest how the actin-myosin contractile system may have evolved.

The first and perhaps most startling finding is that certain key functional properties of these proteins are the same. The actins all form

filaments which activate the Mg^{++} ATPase of the myosins. The properties of the active sites of all the actins and myosins are very similar, judging from the ease with which active hybrids are formed between proteins from distantly related organisms. Therefore, a satisfactory molecular mechanism for contraction involving actin and myosin must have evolved long ago, and strong constraints have prevented any major alteration in the functional properties of the active sites since that time.

Secondly, it appears that the only significant evolutionary alterations in the contractile apparatus have occurred in the various proteins which interact with actin, while actin has remained essentially unchanged. The reason for this conservation of actin is a matter of speculation, but it seems reasonable that changes have been prevented, because actin must interact with several other proteins: with itself to form an actin filament, with myosin to activate the Mg^{++} ATPase, with tropomyosin and troponin or other control proteins to regulate force generation. This explanation has been suggested previously for the evolutionary restraints on cytochrome-C, which must interact with only two other proteins.[349]

Finally, it is clear that different regions of the myosin molecule have changed much more than others. The properties of the active site for actin binding and ATP hydrolysis appear to vary little in different types of myosin, while the fibrous tail of various myosins has different solubility or (in the case of *Acanthamoeba* myosin) is absent. Such major variation in the tail of the myosin can be related to its organization in the cell, because different cells with different types of movement might easily require contractile proteins organized differently. The soluble myosins found in *Acanthamoeba* and *Physarium* seem adapted to the fluid cytoplasm of these cells, while the insoluble myosin of striated muscle is adapted to form part of a stable lattice of contractile filaments specialized for powerful contraction in one dimension.

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