INTERACTIONS OF ACTIN, MYOSIN, AND A NEW ACTIN-BINDING PROTEIN OF RABBIT PULMONARY MACROPHAGES

II. Role in Cytoplasmic Movement and Phagocytosis

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ABSTRACT

Actin and myosin of rabbit pulmonary macrophages are influenced by two other proteins. A protein cofactor is required for the actin activation of macrophage myosin Mg^2ATPase activity, and a high molecular weight actin-binding protein aggregates actin filaments (Stossel T. P., and J. H. Hartwig. 1975. *J. Biol. Chem.* 250:5706–5711). When warmed in 0.34 M sucrose solution containing Mg^2-ATP and dithiothreitol, these four proteins interact cooperatively. Actin-binding protein in the presence of actin causes the actin to form a gel, which liquifies when cooled. The myosin contracts the gel into an aggregate, and the rate of aggregation is accelerated by the cofactor. Therefore, we believe that these four proteins also effect the temperature-dependent gelation and aggregation of crude sucrose extracts of pulmonary macrophages containing Mg^2-ATP and dithiothreitol. The gelled extracts are composed of tangled filaments.

Relative to homogenates of resting macrophages, the distribution of actin-binding protein in homogenates of phagocytizing macrophages is altered such that 2–6 times more actin-binding protein is soluble. Sucrose extracts of phagocytizing macrophages gel more rapidly than extracts of resting macrophages. Phagocytosis by pulmonary macrophages involves the formation of peripheral pseudopods containing filaments. The findings suggest that the actin-binding protein initiates a cooperative interaction of contractile proteins to generate cytoplasmic gelation, and that phagocytosis influences the behavior of the actin-binding protein.

Phagocytosis is a fundamental biological process. Early microscopists observing phagocytosis by macrophages theorized that consistency changes or gel-sol transformations of the peripheral cytoplasm might explain the flow of pseudopods around particles that characterizes engulfment (16). The appreciation in recent years that phagocytosis depends on energy metabolism (21), the formal demonstration that microfilaments are prominent in the cell periphery adjacent to particles being engulfed and in pseudopods embracing particles (27), and the unequivocal demonstration of contractile proteins in many nonmuscle cells (26) all support the idea that contractile proteins are involved in phagocytosis. The observation that actin polymerized subnormally in extracts of hu-

man polymorphonuclear leukocytes which ingested particles at markedly impaired rates provided further evidence for this idea (1).

As part of our endeavor to understand the mechanism by which phagocytic cells ingest particulate objects, we have been studying their contractile proteins and have purified actin and myosin from rabbit pulmonary macrophages. The myosin had low Mg^2-ATPase activity which was activated by F-actin at low ionic strength only in the presence of an additional protein cofactor which was partially purified from the macrophages. We discovered and purified a new high molecular weight protein which coprecipitated with actin when extracts of rabbit pulmonary macrophages, prepared with ice-cold 0.34 M sucrose-1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.0, were made 0.075–0.1 M in KCl and warmed to room temperature with stirring. In 0.1 M KCl, the purified high molecular weight protein-bound macrophage F-actin into dense arrays of interconnecting filaments which led to our naming it "macrophage actin-binding protein." We hypothesized that an interaction between macrophage actin, actin-binding protein, myosin, and cofactor might explain the assembly and movement of macrophage pseudopods which characterize ingestion (11, 33).

Kane reported that actin filaments formed a gel when extracts of sea urchin eggs prepared with glycerol and ethyleneglycolbis[β-aminoethyl ether]N,N' tetraacetic acid (EGTA) were warmed and that the gelled actin was associated with other proteins, including a high molecular weight polypeptide similar in electrophoretic mobility in dodecyl sulfate to the macrophage actin-binding protein (14). Pollard subsequently discovered that if extracts of *Acanthamoeba castellanii* prepared with ice-cold 0.34 M sucrose containing 1 mM EGTA and 1 mM ATP were warmed to room temperature *without* stirring, the extracts gelled and subsequently underwent a rapid shrinkage resembling a contraction (22). In light of the possible relevance of cytoplasmic gelation in the phagocytic process, these phenomena appeared to provide a model for analyzing the mechanical interactions between macrophage contractile proteins and for determining whether phagocytosis influences these interactions.

We report here the results of our studies concerning the effects of phagocytosis on the gelation and contraction of extracts of rabbit pulmonary macrophages. We present evidence that this gelation and contraction is the result of a cooperative interaction between macrophage actin, actin-binding protein, cofactor, and myosin which correlates with enzymatic and binding interactions studied previously. The actin-binding protein, some further properties of which we describe, is the initiator of this interaction. Ingestion of particles increases the extractability of actin-binding protein from the macrophages which is the first example of a change in a contractile protein associated with phagocytosis. Some of this work has been presented in summary form (35).

MATERIALS AND METHODS

Preparation and Handling of Pulmonary Macrophages

Macrophages were obtained from the lungs of New Zealand albino rabbits by intratracheal lavage according to the technique of Myrvik et al. (20). The rabbits previously received complete Freund's adjuvant intravenously which increased cell yields (19). The macrophages were washed twice with 0.15 M NaCl at 4°C by centrifugation at 250 *g*-minute, and suspended at a final concentration of 5–10 mg cell protein/ml in Krebs-Ringer phosphate medium, pH 7.4.

The cells were then incubated for 20 min at 37°C with 0.2 vol of a suspension of *Escherichia coli* lipopolysaccharide-coated droplets of diisodecyl phthalate containing oil red O (31). These particles were treated with either heated (56°C, 30 min) or else fresh rabbit serum for 20 min at 37°C. Fresh but not heated serum opsonizes these particles by depositing a fragment of the third component of complement on their surfaces (32). The rate of ingestion of the particles by the macrophages was assayed spectrophotometrically (30). The macrophages were washed free of uningested particles twice with ice-cold 0.15 M NaCl with centrifugation at 250 *g*-min. The cells were then suspended in 15 vol of ice-cold distilled water, mixed by inversion, and packed by centrifugation at 250 *g*-min. The washed macrophages were suspended in 2 vol of an ice-cold homogenizing solution containing 0.34 M sucrose, 10 mM dithiothreitol, 5 mM ATP, 1 mM EDTA, 20 mM Tris-maleate buffer, pH 7.0 (hereafter called "sucrose solution"). ATP was omitted from the sucrose solution in some experiments. The cells were disrupted in this solution as reported previously (11). The homogenates were layered under an equal volume of 0.25 M sucrose, 10 mM Tris-maleate, pH 7.0 solution, and centrifuged at 100,000 *g*-h at 4°C. This step yielded the extract supernate, a pellet of membranes and organelles, and a pellicle of phagocytic vesicles which floated through the 0.25 M sucrose layer covering the extract supernate (34). The

presence of this layer facilitated complete removal of these structures from the supernatant fraction.

Isolation of Macrophage Actin, Myosin, Cofactor, and Actin-Binding Protein

Macrophage actin was purified from the sucrose extracts by procedures previously reported (11). As observed by Pollard concerning *Acanthamoeba* extracts, warming of macrophage extract supernates from 4°C to 25°C for 1 h resulted in their gelation (discussed in more detail below). Centrifugation of the gels in round-bottom test tubes at 12,000 *g*-min (25°C) compressed them, yielding an opaque pellet. Myosin was isolated from the supernatant fluids remaining after centrifugation of the gelled extract supernates.[1] The supernates were cooled to ice-bath temperature, and an equal volume of ice-cold saturated ammonium sulfate containing 0.01 M EDTA, pH 7.0, was slowly added. The resulting precipitates were dialyzed in 0.6 M KI, 20 mM sodium thiosulfate, 5 mM ATP, 5 mM dithiothreitol, 20 mM Tris maleate, pH 7.0, and applied to 2 × 85-cm columns of Bio-Gel A15, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.), with the upper sevenths equilibrated in the KI solution just defined. The rest of the columns were equilibrated and eluted with 0.6 M KCl, 5 mM dithiothreitol, 0.5 mM ATP, 10 mM Trismaleate solution by the method of Pollard et al. (25). Fractions with K^+- and EDTA-activated ATPase activity (myosin) and fractions which activated macrophage actomyosin Mg^{2+}-ATPase activity (cofactor) were pooled separately.

The pellets produced by centrifugation of gelled extract supernates were dissolved at a concentration of about 3 mg protein/ml in a solution of ice-cold 0.6 M KCl, 5 mM dithiothreitol, 20 mM Tris-maleate, pH 7.0. This solution was centrifuged for 3 h at 80,000 *g* at 4°C. Actin-binding protein was isolated from the supernatant fluid. All solutions were carefully kept at 0°–4°C. The supernatant fluids were concentrated with an Amicon pressure device (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) with an XM-50 membrane and dialyzed against 0.6 M KI, 20 mM sodium thiosulfate, 5 mM dithiothreitol, 20 mM Tris-maleate solution, pH 7.0. The concentrates were applied to 2 × 85-cm columns of Bio-Gel A15, 200–400 mesh, equilibrated in and eluted with the above solution. Peaks with absorbance at 290 nm were pooled, concentrated as described above, and analyzed for the presence of a high molecular weight polypeptide (actin-binding protein) by polyacrylamide gel electrophoresis with sodium dodecyl sulfate. Rabbit skeletal muscle actin was prepared by the technique of Spudich and Watt (29), and rabbit skeletal muscle heavy meromyosin by the method of Lowey et al. (17).

[1] As discussed below, myosin becomes incorporated into the gel with time and contracts it. Salt accelerates the contraction rate. After 1 h of incubation at room temperture in the absence of added salt, most of the myosin in the extract was in the supernate rather than in the gel.

Analytical Procedures

Proteins were negatively stained with uranyl acetate as reported (11). Intact macrophages were fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate, postfixed with Dalton's chrome osmium and 1% uranyl acetate, dehydrated with acetone, and embedded in Epon-Araldite. Thin sections were stained with lead citrate. These sections and negatively stained proteins were examined in a Philips 300 microscope with an accelerating voltage of 60 kV.

Assays of ATPase activity, protein concentration, polyacrylamide gel electrophoresis with sodium dodecyl sulfate, and quantitative densitometry of stained polyacrylamide gels were performed by means of methods cited previously (11). The amino acid composition of purified, reduced, and alkylated (5) actin-binding protein after 24-h acid hydrolysis was determined (AAA Laboratories, Seattle, Wash.). Changes in the turbidity of protein solutions or of supernatant extracts were continuously assayed using a recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 350 nm in 0.5-ml quartz cuvettes as described in an earlier report (36). The temperature of the solutions being analyzed was thermostatically controlled (Haake, Inc., Saddle Brook, N.J.) by circulating water through a jacketing device in the spectrophotometer.

RESULTS

The Contractile Proteins of Pulmonary Macrophages

The purity, subunit composition, and morphology of contractile proteins of macrophages were described previously (11) but are demonstrated in Figs. 1 and 2 for purposes of comparison with the morphology and protein composition of cytoplasmic extracts, gels, and contracted aggregates to be described. Macrophage actin migrated during electrophoresis as a single subunit of mol wt 42,000 on dodecyl sulfate polyacrylamide gels. Macrophage F-actin appeared as double helical filaments (Fig. 1). Macrophage myosin had heavy (mol wt 220,000) and light (mol wt 20,000 and 15,000) subunits, and in 0.1 M KCl at pH 7.0 formed bipolar filaments which were 300 nm long (Fig. 1). The actin-binding protein as originally purified co-migrated with one of the subunits of the erythrocyte protein spectrin during electrophoresis on dodecyl sulfate polyacrylamide gels. In 0.1 M KCl at pH 7.0, it appeared as beaded aggregates when negatively stained (11). When the protein was prepared by means of the modified procedure

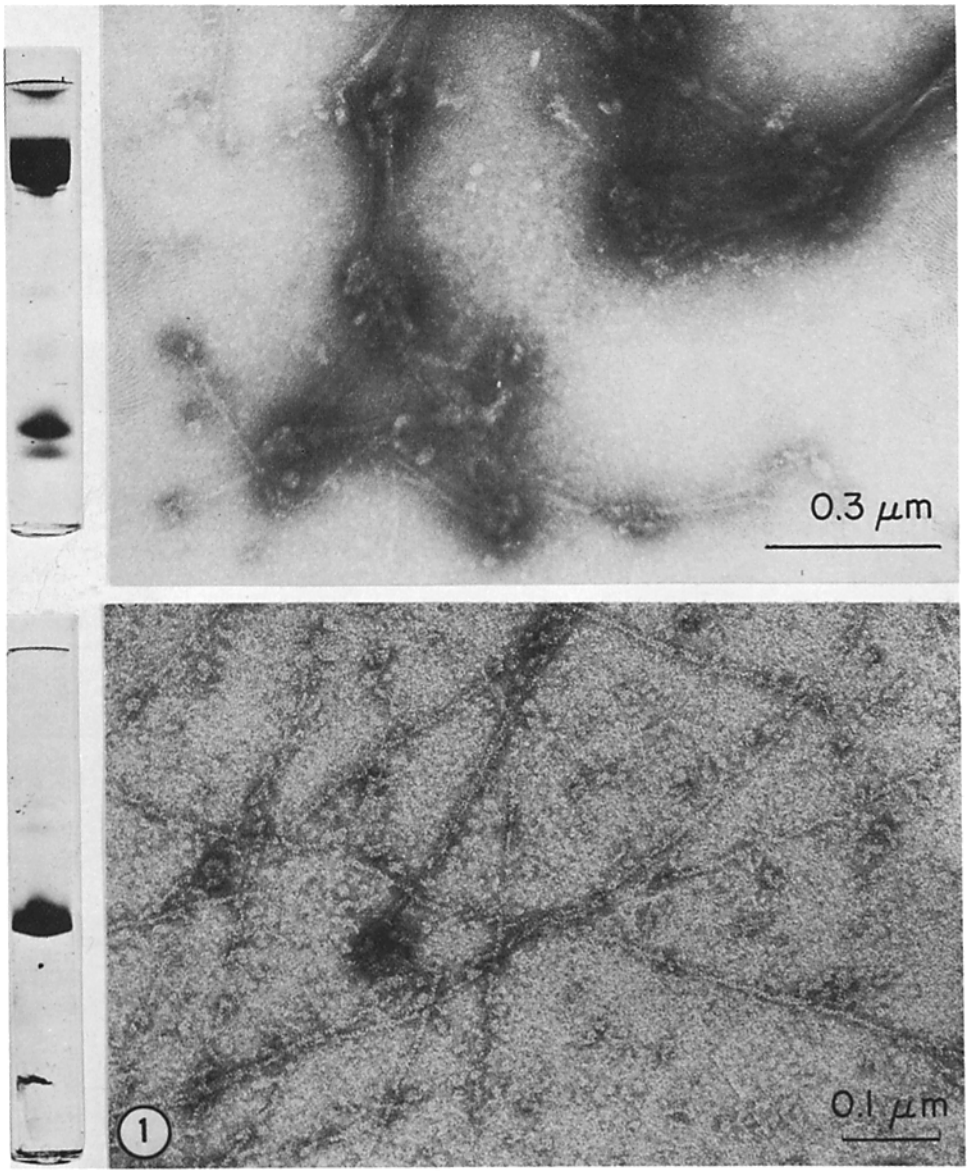


FIGURE 1 Morphology and subunit composition of the purified macrophage proteins, (top) myosin, and (bottom) actin. The reduced and denatured proteins are shown on Coomassie blue-stained dodecyl sulfate 5% polyacrylamide gels. Also shown is the morphology of macrophage myosin and actin negatively stained and photographed in the electron microscope.

described in this report, with careful attention paid to keeping the protein cold during purification, the purified protein migrated more slowly than the spectrin subunits during electrophoresis on polyacrylamide gels with dodecyl sulfate. Using other high molecular weight proteins for calibration, we now estimate that the actin-binding protein has a mol wt of approx. 270,000 (Fig. 2). We ascribe the faster mobility observed previously to proteolysis. Purified actin-binding protein warmed to 25°C for 20 min in 0.1 M KCl, 10 mM Tris-maleate buffer, pH 7.0, and negatively stained appeared as beaded hollow coils with diameters of 12 nm in the electron microscope (Fig. 2). The amino acid composition of macrophage actin-binding protein is indicated in Table I.

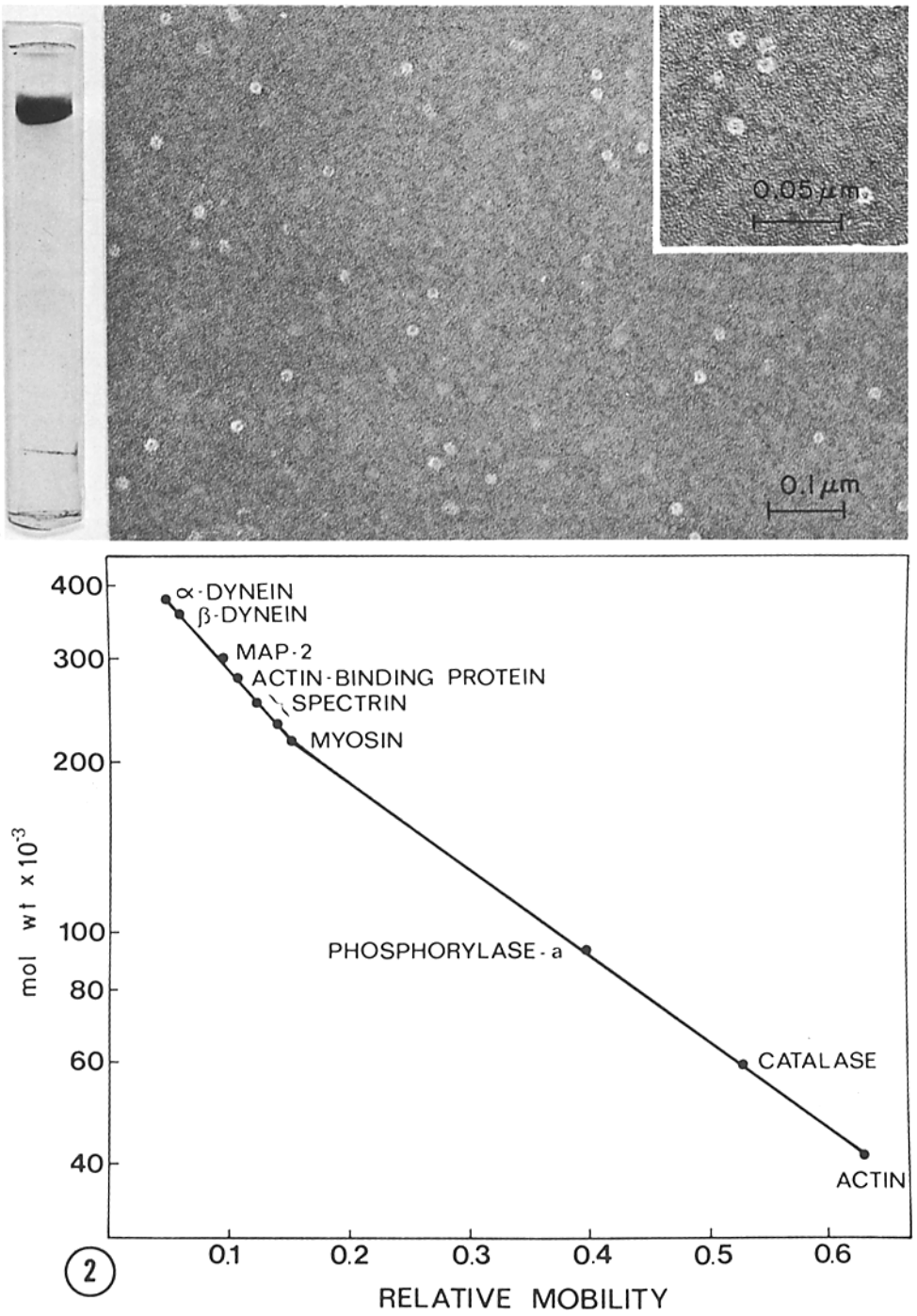


FIGURE 2 (Top) Morphology and subunit composition of macrophage actin-binding protein. A Coomassie Blue-stained dodecyl sulfate 5% polyacrylamide gel of purified macrophage actin-binding protein is shown. The micrographs show the morphology of purified macrophage actin-binding protein stained with uranyl acetate and viewed in the electron microscope. (Bottom) Comparison of the relative mobilities of reduced denatured proteins on dodecyl sulfate 5% polyacrylamide gells. Bands designated α-dynein, β-dynein and MAP-2 were observed in purified tubulin preparations (a gift of Dr. Michael Shelanski). The molecular weights of these proteins are those reported by Burns and Pollard (3) and Sloboda et al. (28). Rabbit erythrocyte spectrin and pulmonary macrophage actin-binding protein, myosin, and actin were purified as described by Hartwig and Stossel (11). Rabbit muscle phosphorylase-*a* and beef liver catalase were purchased from Sigma Chemical Co., St. Louis, Mo.

Morphology and Quantitation of Phagocytosis by Macrophages

Rabbit pulmonary macrophages observed with the phase-contrast microscope extended glassy pseudopods from the cell periphery when warmed to room temperature (Fig. 3). These pseudopods lacked the refractile bodies which were abundant in the interior cytoplasm. The pseudopods surrounded opsonized lipopolysaccharide-coated oil droplets and internalized them. Thin section electron micrographs of macrophages fixed while ingesting particles revealed that the pseudopods were composed of ill-defined arrays of randomly oriented interconnecting thin filaments and dense debris which excluded other recognizable intracellular organelles from the pseudopods. As shown in Fig. 4, pulmonary macrophages ingested opsonized particles rapidly but did not ingest unopsonized control particles.

Gelation and Contraction of Macrophage Extracts: Effect of Phagocytosis

Rabbit pulmonary macrophage extracts in 0.34 M sucrose containing dithiothreitol, ATP and EDTA (sucrose solution) gelled when warmed from ice-bath temperature to room temperature, and the gel was not disrupted by inversion (Fig. 5 *a*). Subsequently, strands formed a net in the gelled extract. This net then slowly pulled away from the vertical sides of the container. When the net was completely free of the sides of the vessel, it released from the bottom and underwent a rapid vertical contraction. This event produced a condensed aggregate which, because of trapped air bubbles, floated to the top of clear liquid squeezed out of the gel (Fig. 5 *b*).

TABLE I

Amino Acid Composition of Macrophage Actin-Binding Protein

Amino acid	Composition
	mol/100,000 g
Asp	76
Thr	54
Ser	59
Glu	99
Pro	62
Gly	103
Ala	64
Cys*	4
Val	74
Met	11
Ile	38
Leu	54
Tyr	27
Phe	28
His	19
Lys	52
Arg	36
Trp	10
Total	870

* Analyzed as *S*-carboxymethylcysteine after reduction and alkylation.

Semiquantitative measurements of gelation were achieved by determining the time required for the supernatant extract to agglutinate and partially to immobilize latex particles on a microscope slide tilted back and forth, which gave an estimate of the strength of the gel. Viscometry was not suitable for quantifying gelation more precisely since the gel broke into chunks if disrupted. The optical density of extract supernates was assayed at a wavelength to maximize detection of light scattering, since light scattering has been used to monitor polymerization reactions (7). During warming and gelation, the turbidity of extract supernates increased (Fig. 6). Often an initial decrease in turbidity occurred which was clearly not due to condensation clearing from the cuvettes. If the gelled extracts (1 h, 25°C) were cooled, the gels liquified within 10 min (Fig. 6), and could be gelled again by warming. The total protein content of the liquified extract was not diminished by ultracentrifugation at 80,000 *g* for 3 h (4°C). During contraction of the gels, the net that formed in the gelled supernates was pulled out of the light path. Therefore, turbidity diminished markedly, and this diminution provided a quantitative assay for the final phase of contraction (hereafter called "final contraction").

Resting macrophage extracts prepared in sucrose solution gelled in 20–40 min. The final contraction of the extract gels assayed by clearing of the light path was very slow and required 3–12 h for completion. Both the gelation rate and the very gradual onset of final contraction were greatly increased by the addition of an excess of $MgCl_2$ (5 mM). The effect of $CaCl_2$ on the behavior of extract gels in the presence of excess $MgCl_2$ is shown in Fig. 7. $CaCl_2$ (either 1.3 mM or a buffered calcium solution 1 mM $CaCl_2$–0.6 mM EGTA) caused a flocculent precipitate to form immediately in the supernate, and gross gelation did not occur. Rapid gelation and final contraction occurred in either the absence of added $CaCl_2$ or in

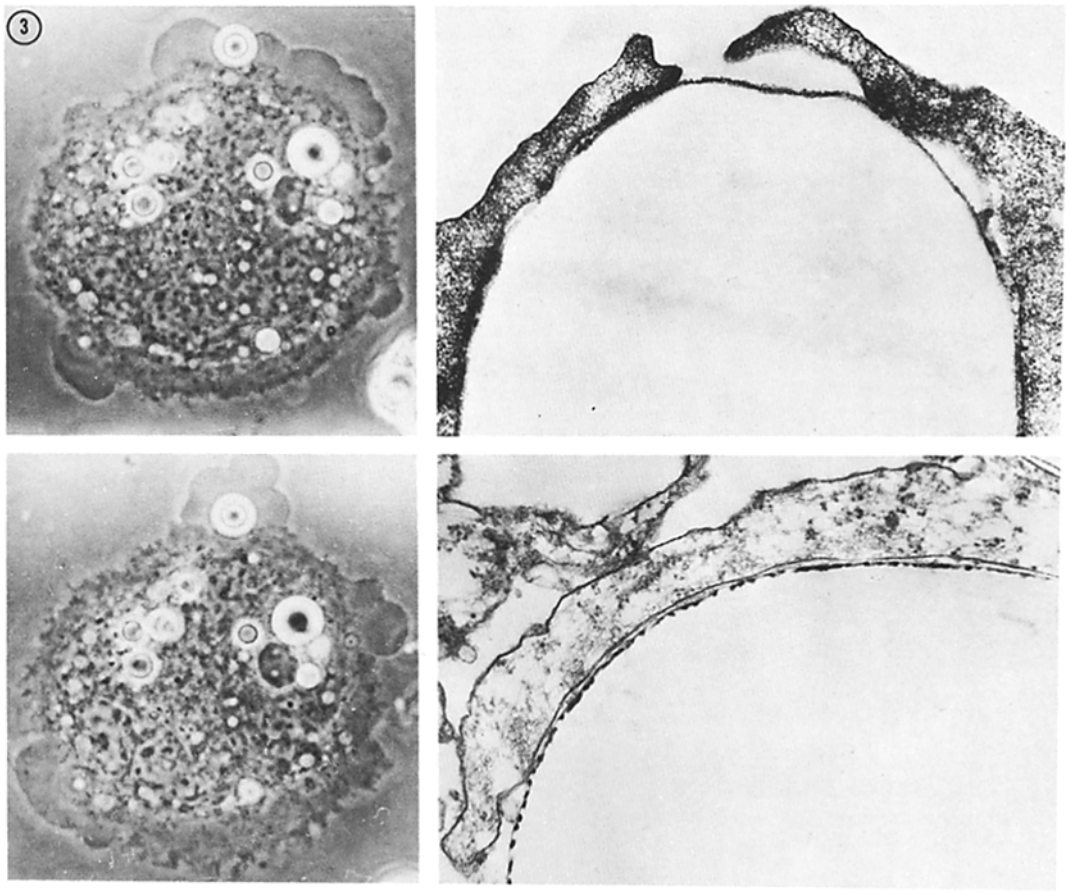


FIGURE 3 Morphology of rabbit pulmonary macrophages. (Left) Macrophages ingesting lipopolysaccharide-coated oil particles, phase contrast. × 1,000. (Right) Thin sections show macrophage pseudopods surrounding oil droplets. (Upper) × 42,500. (Lower) × 61,200.

the presence of 2.7 mM EGTA. Extracts prepared with sucrose solution minus ATP gelled but did not contract even after addition of $MgCl_2$.

Extracts of ingesting macrophages gelled more rapidly during warming than extracts of resting cells, as determined by the latex immobilization assay. Extracts from ingesting cells immobilized particles (gelled) in 10–20 min or half the time required for gelation of resting extracts, and the gels were stronger. Extracts of ingesting cells also scattered more light than extracts of resting cells during warming (Fig. 8, top). Extracts of ingesting cells containing $MgCl_2$ also gelled more rapidly than extracts of resting cells containing $MgCl_2$. On the other hand, the onset of the $MgCl_2$-stimulated final contraction was not accelerated in ingesting cells. In fact, resting cell extract gels began their final contraction sooner than ingesting cell extract gels (Fig. 8, bottom), possibly because more gel or a stronger gel had to be contracted.

Protein Composition of Intact Macrophages, Extract Supernates, and Gels: Effect of Phagocytosis

The concentration of proteins in macrophages, extract supernatants, and gels collected by low-speed centrifugation after 1 h of standing at 25°C was determined by quantitative densitometry of Coomassie Blue-stained dodecyl sulfate polyacrylamide gels after electrophoresis. The results are shown in Table II. Extracts prepared from cells that had ingested opsonized particles contained 58% more actin-binding protein than resting cells. The amounts of actin and myosin in the extract

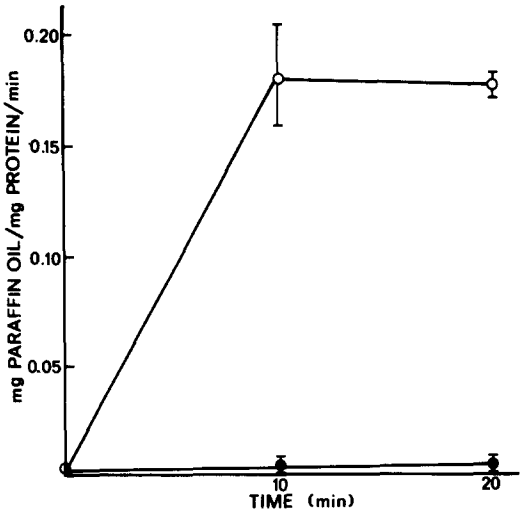


FIGURE 4 Ingestion of *E. coli* lipopolysaccharide-coated particles by rabbit pulmonary macrophages. Macrophages, 10 mg/ml cell protein, were incubated in 4 ml of Krebs-Ringer phosphate medium, pH 7.4, for 10 min at 37°C. At zero time, 1 ml of particles treated with fresh (O) or heat-inactivated (●) rabbit serum was added. Samples were removed from incubation flasks for washing and oil red O analysis at zero, 10, and 20 min.

supernates of resting and ingesting cells were not measurably different.

The quantities of actin, myosin, and actin-binding protein in whole cells did not differ whether the cells were resting or ingesting. Therefore, the increased amounts of actin-binding protein in extracts of ingesting macrophages relative to resting cells was the result of either redistribution, increased solubility, or increased extractability of the protein during phagocytosis.

An example of the protein composition of an extract gel (from resting cells) collected by low-speed centrifugation is shown in Fig. 9. Although there was some variation in the quantitative distribution of proteins in these gels, the major band was invariably actin. Actin-binding protein and myosin heavy chain were other prominent components. A polypeptide of approximately 90,000 mol wt, close to the molecular weight previously assigned to macrophage cofactor, was also present. Actin-binding protein and myosin were purified from these extract gels, which verified that the bands identified as these proteins on polyacrylamide gels in fact were these contractile proteins. Extract gels from ingesting cells contained 110% more actin-binding protein than extract gels from resting cells. The findings suggested that the increased rate of gelation of extracts of ingesting cells could be explained by the increased concentration of actin-binding protein in these extracts. This conclusion would mean that the acting-binding protein was responsible for the gelation of actin under these conditions. The validity of this idea was established by experiments with purified macrophage proteins.

Gelation and Contraction of Purified Macrophage and Muscle Proteins

During the warming of purified actin-binding protein from 0° to 25°C in 0.34 M sucrose solution, the turbidity of the solution increased (Fig. 10). Cooling reversed this turbidity and returned the absorbance to the initial basal measurement before warming. Purified rabbit skeletal muscle or macrophage G-actins alone in the 0.34 M sucrose solutions at the concentrations used did not gel or develop significant turbidity during warming. Purified actins plus purified macrophage myosin did not gel but slowly precipitated and settled such that turbidity decreased (Fig. 11). The addition of rabbit skeletal muscle or macrophage G-actin in sucrose solution at 0°C to actin-binding protein reconstructed the gelation phenomenon observed in the crude supernatant extracts. When the mixture of purified proteins was warmed to 25°C, the mixture gelled very rapidly (within minutes). But the increase in turbidity of the combined proteins with time was invariably less than that of actin-binding protein alone (Fig. 11). In repeated experiments the strength of the gel formed was roughly proportional to the quantity of actin-binding protein added. For example, at a weight ratio of actin-binding protein: rabbit muscle F-actin of 1:2 or 1:5, a solid gel that did not move at all on a tilted microscope slide formed within 15 min at room temperature in constant volumes of sucrose solution. At ratios of 1:10 and 1:20, the mixture was weakly mobile but fractured into chunks when perturbed. Actin alone (1 mg/ml) moved freely. A combination of purified actin, actin-binding protein, and myosin also gelled during warming; but marked increases in turbidity gradually occurred as observed in crude extract supernates. When the combination of actin, actin-binding protein, and myosin were left overnight at 25°C, final contraction occurred but only if myosin was present (Fig. 11). As reported previously, the presence of a macrophage cofactor increases markedly the Mg^{2+}-ATPase activity of macrophage actomyosin (Table III). Partially

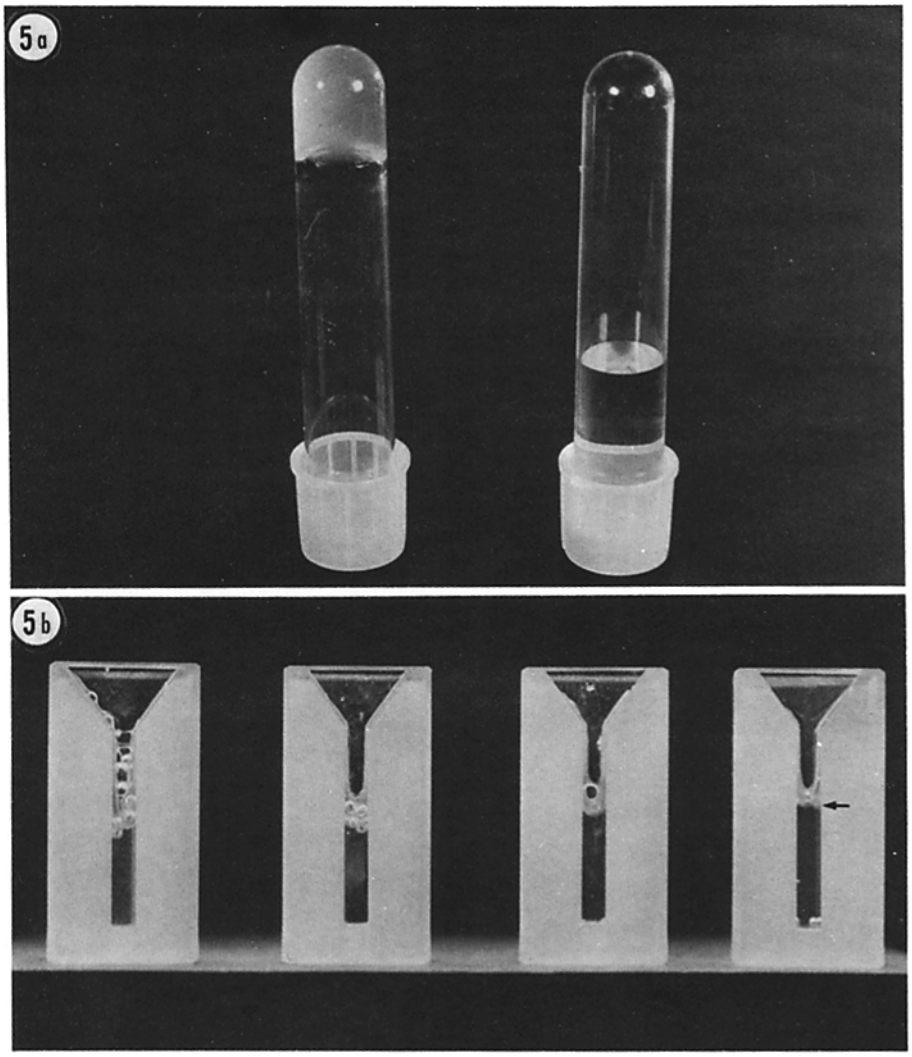


FIGURE 5 Gelation and contraction of rabbit pulmonary macrophage supernatant extracts in sucrose solution. (*a*) Macrophage supernate gelled by warming to 25°C for 60 min. Shown are inverted tubes of (left) gelled supernate and (right) water for comparison. (*b*) Stages in contraction of macrophage supernatant gels in cuvettes. From left to right, diffuse reticular filaments which progressively contract into the center of the cuvette, resulting in the final aggregate which has floated to the surface of the cuvette (arrow).

purified cofactor was added to purified actin, actin-binding protein, and myosin to determine whether this increase in enzymatic activity correlated with the mechanical events. The addition of the cofactor alone did not greatly increase the amount of turbidity generated or hasten the onset of final contraction. However, when cofactor was added in the presence of excess $MgCl_2$ (5 mM), turbidity increased rapidly and final contraction was accelerated (Fig. 12). The amount of turbidity formed was greater than that arising in the absence of cofactor plus Mg^{2+} (compare with Fig. 11). The effect of $CaCl_2$ on the behavior of the reconstituted system is shown in Fig. 13. Both the rate of increase in turbidity and the onset of final contraction occurred slightly sooner in EGTA than in the presence of free Ca^{2+}.

Analysis by polyacrylamide gel electrophoresis with dodecyl sulfate of the aggregate formed by final contraction of the gel formed from purified

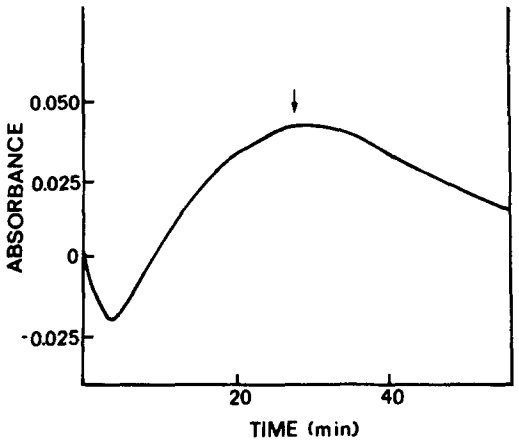


FIGURE 6 Effect of temperature on the turbidity (A_{350}) of a macrophage supernatant extract in sucrose solution. At zero time the extract was warmed to 25°C. The temperature of water circulating through the cuvette holder was lowered to 0°C at the time indicated by the arrow. Delay in cooling of the cuvette caused the fall in turbidity to occur more slowly than liquefaction of gelled extracts placed directly on ice.

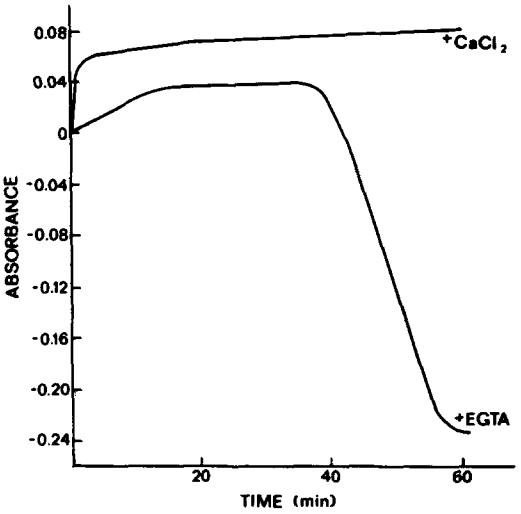


FIGURE 7 Effect of $CaCl_2$ or EGTA on the temperature-dependent changes in turbidity of macrophage supernate. At zero time, the supernates in sucrose solution containing added 5 mM $MgCl_2$ and either 1.3 mM $CaCl_2$ or 2.7 mM EGTA as indicated were warmed to 25°C. The abrupt decrease in absorbance was associated with contraction of the gel. Since protein was removed from the extract by the contraction, the absorbance was less than the starting optical density (explaining the negative readings).

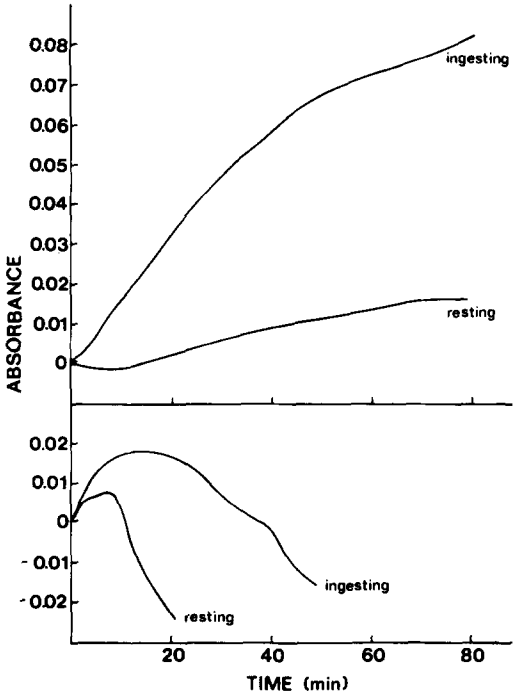


FIGURE 8 Temperature-dependent changes in turbidity of macrophage extracts prepared in sucrose solution from resting or ingesting cells. (Top) The ingesting extract was solidly gelled in 15 min; the resting in 30 min. The bottom panel shows turbidity of extracts to which 5 mM $MgCl_2$ was added. The abrupt decrease in absorbance was associated with final contraction of the gels. At zero time the extracts were warmed to 25°C. Total protein concentrations of ingesting and resting extracts were 9.1 mg/ml and 8.7 mg/ml, respectively.

proteins revealed five polypeptide bands, three of which corresponded to the macrophage proteins, actin, actin-binding protein, and myosin heavy chain, and two of which, with apparent mol wt of 155,000 and 90,000, have not been definitively identified (Fig. 14). These proteins were quantitatively removed from the supernatant fluid surrounding the aggregate. The 90,000 mol wt band was a polypeptide tentatively thought to represent macrophage cofactor (33).

The Morphology of Contracting Gels

Macrophage extracts were allowed to gel for 120 min and collected by low-speed centrifugation. The gelatinous pellet was gently disrupted at 25°C with 100 vol of 0.1 M KCl, 10 mM Tris-maleate, pH 7.0, in a Dounce homogenizer (Kontes Glass Co.,

TABLE II

Comparison of Actin-Binding Protein, Myosin and Actin Concentrations between Resting and Ingesting Macrophages, Extracts, and Gelled Extracts Collected by Centrifugation

	Resting*	Ingesting	P‡
	%	%	
Whole cells			
Actin-binding protein	1.82 ± 0.48	1.72 ± 0.45	NS
Myosin	2.06 ± 0.19	1.91 ± 0.67	NS
Actin	10.38 ± 2.01	12.58 ± 1.90	NS
Supernate			
Actin-binding protein	1.72 ± 1.04	2.72 ± 1.00	<0.01
Myosin	4.28 ± 0.68	4.69 ± 1.05	NS
Actin	12.56 ± 2.01	14.81 ± 4.45	NS
Gel			
Actin-binding protein	2.26 ± 0.56	4.77 ± 1.54	<0.01
Myosin	3.47 ± 0.32	3.08 ± 0.62	NS
Actin	31.43 ± 2.60	32.45 ± 1.87	NS

* Numbers express percent ± standard deviation of the total stained protein by densitometry of 5% polyacrylamide gels with dodecyl sulfate where $n = 5$.

‡ P indicates the significance of the difference between resting and ingesting means.

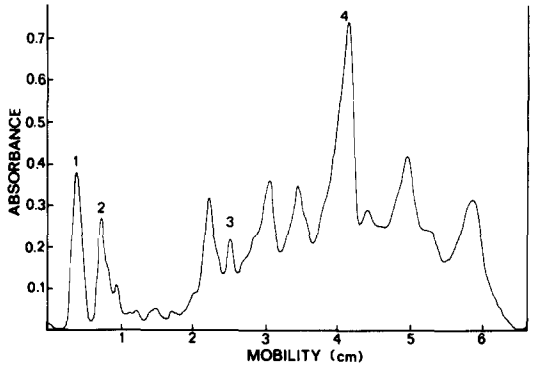


FIGURE 9 Densitometric scan of Coomassie Blue-stained dodecyl sulfate 5% polyacrylamide gel of a macrophage extracts gel collected by centrifugation after 60 min. The numbers label peaks corresponding to *1*, actin-binding protein; *2*, myosin heavy chain; *3*, cofactor; and *4*, actin.

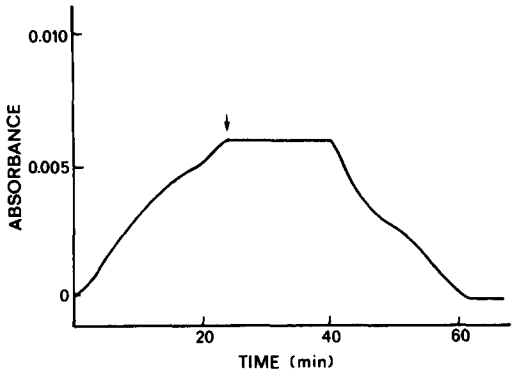


FIGURE 10 Effect of temperature on purified actin-binding protein. Purified actin-binding protein was dialyzed in sucrose solution and warmed to 25°C at zero time. The temperature of water circulating through the cuvette holder was lowered to 0°C at the time indicated by the arrow. Total protein concentration was 0.4 mg/ml.

Vineland, N.J.), and a sample was negatively stained and examined in the electron microscope (Fig. 15). The overall appearance was that of arrays of filaments and background debris (Fig. 15, top). Single and paired thin filaments (6–7 nm wide) were evident as were thicker (12–14 nm wide) filaments (middle). The pellet suspended in 0.1 M KCl solution was incubated with rabbit skeletal muscle heavy meromyosin, negatively stained, and examined (bottom). Most of the filaments observed had acquired arrowhead projections.

DISCUSSION

In a previous study, we showed that when cold sucrose extracts of pulmonary macrophages were warmed with stirring, a macrophage actin binding protein linked actin filaments into dense arrays of interconnecting cables (33). We have now demonstrated that cold sucrose extracts of pulmonary macrophages containing EDTA, dithiothreitol, and ATP gel and aggregate when warmed without stirring, and we have reproduced these phenomena with purified macrophage proteins. Identical re-

sults with extracts of human chronic myelogenous leukemic granulocytes (2) suggest that this may be a widespread cytological phenomenon. We have also shown that phagocytosis produces a change in the distribution of a macrophage actin-binding protein which secondarily results in enhanced gelation of macrophage extracts.

Gelation

Actin is the major component of macrophage gels, as it is of gels formed in extracts of other cells (14, 22). It has long been known that rabbit skeletal muscle F-actin is thixotropic (6), and recent studies on the state of rabbit skeletal muscle F-actin in solution have suggested that it exists as a weak gel (4). Whether rabbit macrophage F-actin differs from rabbit skeletal muscle F-actin with respect to the strength of a gel that it can form on its own (as has been suggested from work with *Acanthamoeba* actin [22]) is unknown. However, at the concentrations examined, purified rabbit macrophage or muscle G- or F-actin formed a gel (actually a rubbery solid which fractured when disrupted) only in the presence of purified actin-binding protein, and extracts of ingesting macrophages, containing more actin-binding protein than extracts of resting macrophages, gelled more rapidly than extracts of resting macrophages. Therefore, we were able to demonstrate that the actin-binding protein was responsible for the gelation of macrophage or muscle actin as it was for the precipitation of stirred actin filaments. While stirring does not prevent cross-linking of actin filaments by actin-binding protein, it must fracture the gel.

The macrophage actin-binding protein itself underwent a change during warming as evidenced by increases in turbidity. These alterations were reversed by cooling. The results were consistent with the actin-binding protein's initiation of gelation by undergoing a temperature-dependent aggregation, and the solubilization of crude macrophage extract gels by cooling supported this con-

TABLE III

Interaction of Macrophage Myosin, Cofactor, Actin-Binding Protein, and Rabbit Skeletal Muscle F-Actin

	Mg^{2+}-ATPase activity	
	nmol Pi/mg myosin protein/min	%
Complete system	380	100
−Actin-binding protein	387	102
−Myosin	12	3.1
−Cofactor	10	2.9
−Actin	9	2.3

The complete system contained, in 0.5 ml, 0.03 mg of macrophage myosin, 0.3 mg of rabbit skeletal muscle F-actin, 0.13 mg of crude cofactor, and 0.02 mg of actin-binding protein in 5 mM $MgCl_2$, 1.0 mM ATP, 40 mM KCl, and 10 mM Tris-maleate, pH 7.0. Incubations were carried out for 30 min at 37°C.

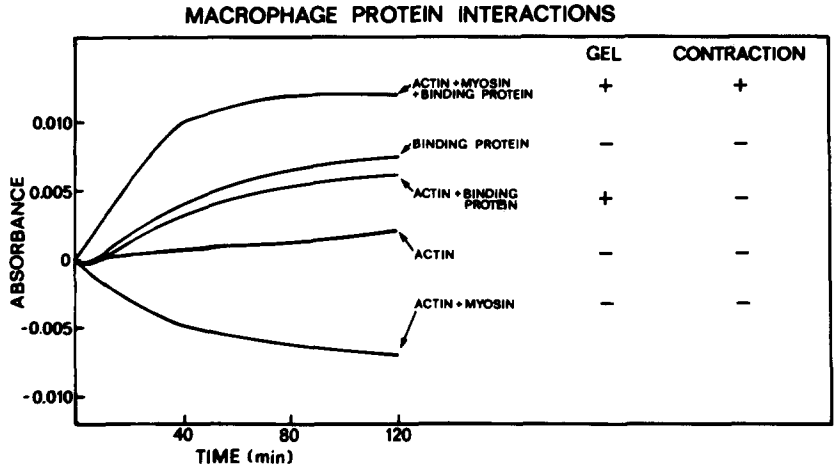


FIGURE 11 Effect of temperature on the turbidity of purified macrophage proteins in sucrose solution. At zero time the proteins in a total volume of 0.3 ml of sucrose solution were warmed to 25°C in cuvettes. Final protein concentrations were: actin, 2.0 mg/ml; actin-binding protein, 0.12 mg/ml; and myosin, 0.12 mg/ml. Gelation and/or final contraction occurred where indicated.

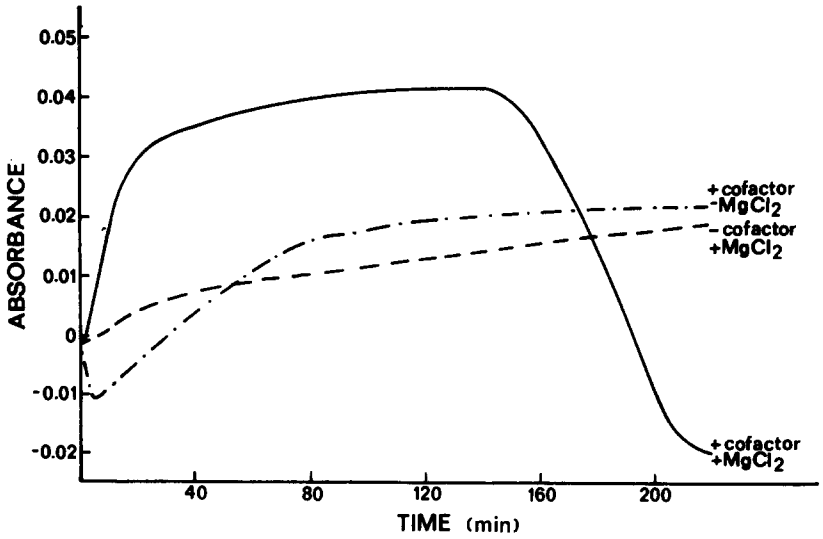


FIGURE 12 Effect of macrophage cofactor and $MgCl_2$ on the turbidity of actin-binding protein, myosin, and actin warmed to 25°C in sucrose solution. The rapid decrease in absorbance indicates final contraction. Total protein concentrations in a total volume of 0.3 ml of sucrose solution were: actin, 2.0 mg/ml; actin-binding protein, 0.12 mg/ml; myosin, 0.12 mg/ml; and cofactor, 0.5 mg/ml. The final $MgCl_2$ concentration was 5 mM.

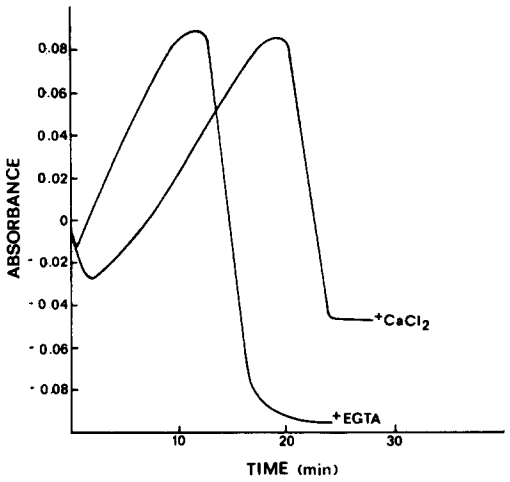


FIGURE 13 Temperature-induced turbidity changes of macrophage mixtures of actin-binding protein, myosin, actin, and cofactor in sucrose solution (minus EDTA) with added $MgCl_2$ and either $CaCl_2$ or EGTA. The decrease in absorbance indicates final contraction. Total protein concentrations in a total volume of 0.3 ml of sucrose solution were: actin-binding protein, 0.12 mg/ml; myosin, 0.12 mg/ml; actin, 2.0 mg/ml; and cofactor, 0.5 mg/ml. The final concentrations of $MgCl_2$, $CaCl_2$, and EGTA were 5 mM, 1.3 mM, and 2.7 mM, respectively.

cept. The increase in turbidity associated with warmed, purified actin-binding protein was diminished by the presence of actin undergoing gelation despite the presence of more protein in the system. This paradoxical finding suggested that the structure of actin-binding protein differed in the presence of actin. The optical phenomena could be explained if actin-binding protein, instead of aggregating, interacted with actin. Our earlier results indicated that 1 mol of actin-binding protein could bind as many as 100 G-actin monomers, although the maximal binding capacity of the actin-binding protein has not been formally determined (33). The actin-binding protein could promote filament assembly by binding actin monomers and bringing them into apposition. Sedimentation studies indicated that actin in sucrose extracts polymerized when warmed and depolymerized when cooled. Actin was sedimentable in extracts gelled by warming. Ultracentrifugation of gelled extracts, liquified by cooling, removed less than 10% of the actin and 5% of the total protein from the supernate. On the other hand, purified rabbit skeletal muscle actin polymerized by warming in sucrose remained completely sedimentable after cooling. While it is not clear at this time whether tempera-

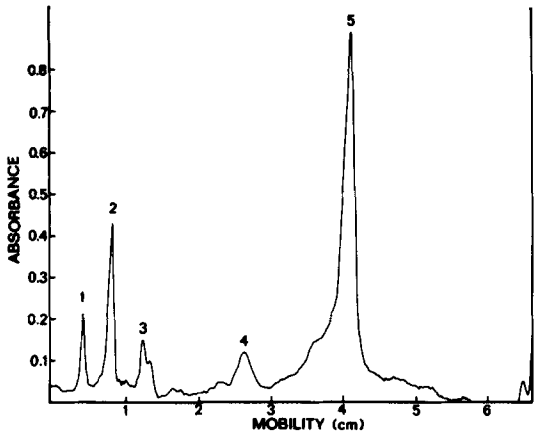


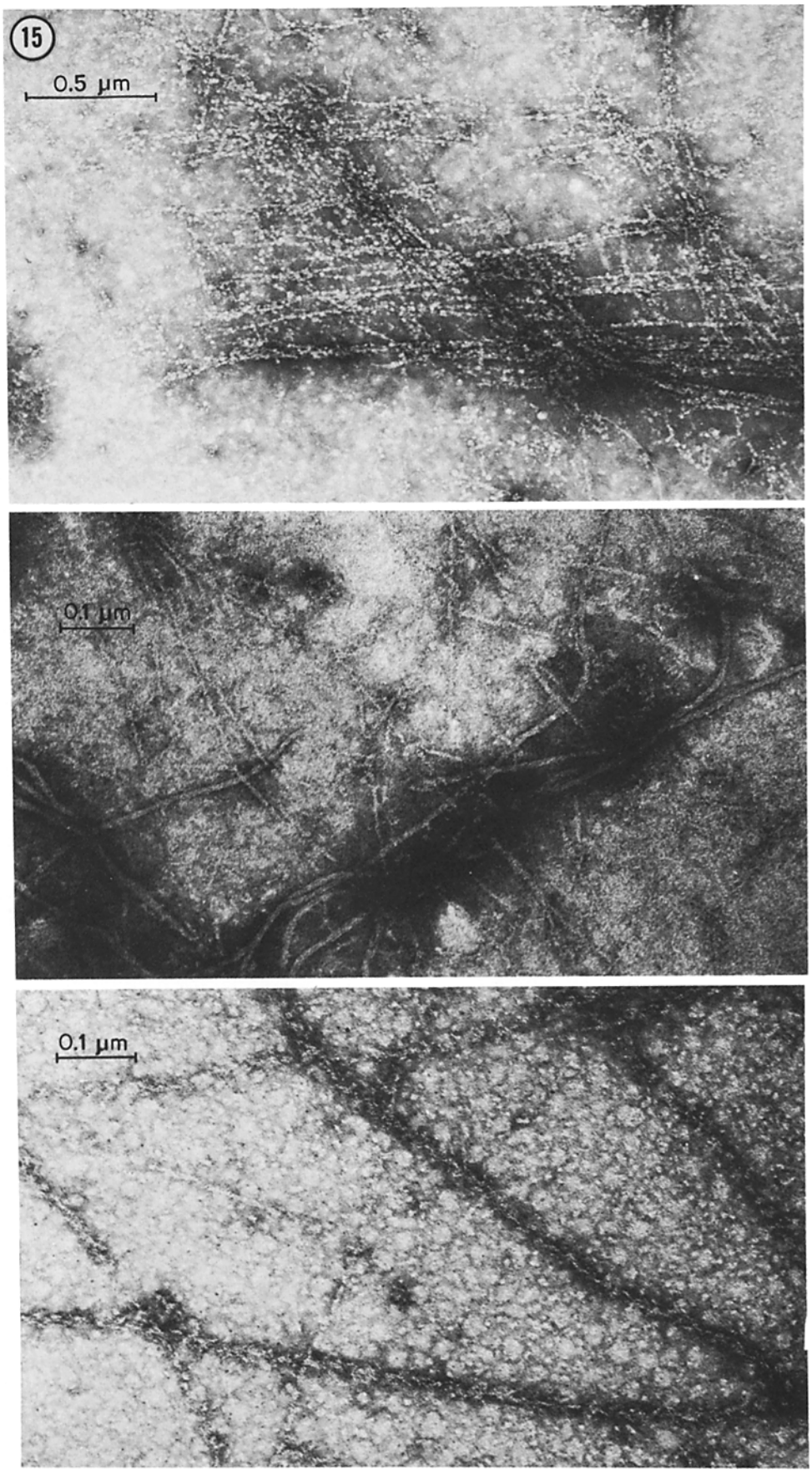
FIGURE 14 Densitometric scan of Coomassie Blue-stained dodecyl sulfate 5% polyacrylamide gel of the contracted aggregate made with purified macrophage proteins. The contracted aggregate formed when 0.03 mg of actin-binding protein, 0.03 mg of myosin, 0.13 mg of crude cofactor, and 0.5 mg of actin were warmed in 0.3 ml of sucrose solution containing added 5 mM $MgCl_2$ and 2.7 mM EGTA. The numbers label peaks corresponding to *1*, actin-binding protein; *2*, myosin heavy chain; *3*, 150,000 mol wt band; *4*, cofactor; and *5*, actin.

ture-dependent reversibility of actin assembly is a property of macrophage actin or is conferred by other proteins such as actin-binding protein, it appears that the actin polymers formed in macrophage extracts show unusual behavior relative to muscle F-actin alone.

The ability of the actin-binding protein to create a strong actin gel is unique but has counterparts in other muscle and nonmuscle proteins which bind to actin. Alpha-actinin cross-links actin filaments, but this process is more efficient in the cold than at high temperatures (8). Furthermore, this protein increases the Mg^{2+}-ATPase activity of actomyosin whereas the macrophage actin-binding protein has no influence on this activity. Tropomyosin alters the rheological properties of actin, but does not cause the type of temperature-dependent gelation observed here (13). The erythrocyte protein, spectrin, is associated with actin in the red cell (39) and has solubility and other properties which resemble somewhat those of the macrophage actin-binding protein (11, 18, 33). An erythrocyte protein isolated by sucrose density gradient centrifugation, possibly spectrin, was found to form ring structures of about 14-nm diam (9, 10), and a recent report showed negatively stained spectrin preparations to contain C-shaped structures (39). However, macrophage actin-binding protein and spectrin differ in several respects. The amino acid composition of macrophage actin-binding protein varies from that of spectrin as published (18). Reduced macrophage actin-binding protein yields one band on dodecyl sulfate polyacrylamide gels whereas spectrin yields two. The macrophage subunit has now been shown to be larger than either of the spectrin subunits. In one study, spectrin did not cause the gelation of rabbit skeletal muscle actin, although it influenced its viscosity (39). Nevertheless, the possibility remains that these two proteins are related in some way. Proteins with high molecular weight subunits and associated with actin have been subsequently discovered in other cell types. A protein purified from human leukemic granulocytes co-migrates, during electrophoresis on dodecyl sulfate polyacrylamide gels, with the macrophage protein, and it causes the gelation of human granulocyte or rabbit skeletal muscle actins (2). A protein in *Acanthamoeba* co-migrates, during electrophoresis on dodecyl sulfate polyacrylamide gels, with the human granulocyte and rabbit macrophage actin-binding proteins and is found in *Acanthamoeba* cytoplasmic actin gels (22). A protein with a high molecular weight subunit was also observed in cytoplasmic actin gels of sea urchin eggs (14) and of fibroblasts (40). These observations suggest that cytoplasmic actin-binding proteins may be widely distributed.[2]

A detailed study of the requirements and optimal conditions for gelation of macrophage extracts or of macrophage actin plus actin-binding protein has not yet been done. The gelation of *Acanthamoeba* extracts has been examined, and the general features were quire similar to those of the macrophage system presented here (22). The process in macrophages was temperature dependent and was accelerated by salt. $MgCl_2$ was an efficient accelerator. In crude macrophage extracts gelation occurred in the absence of added calcium ions and in the presence of EGTA. However, the

[2] Isolation of a high molecular weight subunit protein from chicken gizzard smooth muscle which cross reacts immunologically with non-muscle cells has recently been reported (Wang, K., J. F. Ash, and S. J. Singer. 1975. Filamin, a new high molecular-weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. U.S.A.* 72:4483–4486).


15
0.5 µm
0.1 µm
0.1 µm

actual free calcium content of these extracts even in the presence of EDTA is not known. The addition of various concentrations of calcium or calcium-EGTA caused marked precipitation of the extracts, and gelation did not occur. Gelation occurred when purified actin was combined with purified actin-binding protein in the presence of EGTA. In these reconstitution experiments, it is unlikely that free calcium was present. We conclude, therefore, that gelation does not require calcium, although it remains possible that calcium regulates gelation.

Various cytoplasmic extract preparations capable of undergoing movement have been studied by morphologic techniques in attempts to fathom the mechanisms of cytoplasmic motility (23, 24, 38, 41). Although some of these extracts were found to have temperature-dependent consistency changes (23), actual gelation has been described only recently, and only in extracts prepared with sucrose or glycerol (14, 22). Sucrose stabilizes actin, permitting it to polymerize after the removal of its bound divalent cations and nucleotides (15). The relevance of actin-binding protein-induced actin gelation in sucrose to cytoplasmic consistency changes in vivo remains to be proven. We speculate that high intracellular protein concentrations could have the same stabilizing effects as sucrose does in vitro.

Contraction

The properties of the aggregation of the macrophage-extract gel (final contraction) indicate that it is equivalent to the contraction or superprecipitation of skeletal muscle actomyosin and is a model of contractile force generation (37). The contraction of the crude macrophage extract gel requires added ATP and is accelerated by $MgCl_2$. Myosin is needed for aggregation of the actin-actin-binding protein gel, and the activity of the myosin is increased by macrophage cofactor and by $MgCl_2$. The process therefore parallels the Mg^{2+}-ATPase activity of macrophage actomyosin which is activated by a cofactor (33). The phenomenon differs from classic actomyosin superprecipitation with respect to the starting material, a solid actin-actin-binding protein gel rather than a thixotropic actomyosin gel, and with respect to the cofactor requirement. It is of interest that final contraction of the actin-actin-binding protein gel does occur, albeit very slowly, in the presence of myosin alone, which suggests that the low but finite Mg^{2+}-ATPase activity of macrophage actomyosin in the absence of cofactor is the transducer of the mechanical activity.

The precipitation of crude macrophage extracts in the presence of added calcium ions and their lack of contraction cannot be readily interpreted. The fact that the complete system of purified proteins contracts in EGTA solution suggests strongly that the reaction does not require calcium, but it leaves open the question as to whether the reaction can be regulated by calcium. Factors capable of conferring calcium regulation upon the system could easily have been lost or inactivated, although it was shown previously that the Mg^{2+}-ATPase activity of cofactor-activated macrophage actomyosin in relatively crude fractions was not influenced by calcium ions (33). It is notable that controlled reversible actin filament assembly, if it exists in cells, obviates the absolute need for a separate regulatory system. Contraction could be controlled merely by the availability of actin filaments. In essence, contraction could be controlled by gelation. The effect of myosin on mixtures of actin and actin-binding protein followed by warming reveals that myosin is responsible for the large changes in light scattering induced by warming of crude macrophage extracts. The measured turbidity therefore reflects the total cooperative interaction of the component proteins undergoing gelation and contraction. Presumably, the formation of the actin-actin-binding protein lattice provides binding sites for myosin. Turbidity equivalent to that observed in the crude extract occurs only when the myosin is activated by cofactor, $MgCl_2$, and ATP. Therefore, contraction must begin immediately in the complete system, and it culminates in the final aggregation of the lattice. Myosin filaments were not definitely iden-

FIGURE 15 Morphology of contracting macrophage extract gel warmed in sucrose solution, disrupted with 0.1 M KCl–10 mM Tris-maleate buffer, pH 7.0, and negatively stained with uranyl acetate. (Top) Tangled filaments with associated beaded debris; (Middle) Thick and thin filaments; (Bottom) Bare and decorated thin filaments after addition of rabbit skeletal muscle heavy meromyosin to the disrupted extract gel.

tified in contracting macrophage extract gels. As reported in a microscope study of superprecipitation of rabbit skeletal muscle actomyosin, when actin is present greatly in excess of myosin, myosin filaments are not easily identified (12).

Macrophage Contractile Proteins and Phagocytosis

The gelation and contraction phenomena described may be relevant to the mechanism of certain cytoplasmic motions including phagocytosis. The increased extractability of actin-binding protein following ingestion by macrophages is the first example of a change in a contractile protein in response to phagocytosis. Hypothetically, contact of an ingestible particle with the plasma membrane could effect release of some actin-binding protein from sites on the plasmalemma or other membranes, or else alter the nature of its membrane-association. The activated or released actin-binding protein would then promote the assembly of G-actin to F-actin and cross link F-actin filaments to form a gel. The existence of an agent other than myosin for cross-linking actin filaments allows myosin to move randomly oriented filaments without elaborate mechanical arrangements or without restrictions on the polarity of the actin filaments. In whatever direction actin filaments cross-linked by actin-binding protein are moved by myosin, the net effect will be an internal collapse of the filament network, i.e., centripetal contraction. The total volume of the contracting system in vitro remains the same during contraction, because liquid is squeezed out of the interstices of the gel. If this occurs in the intact cell, a simple and plausible system exists for creating localized compression and for transmitting force to other regions of the cell. In an elongated system, contraction would become complete first in the shortest dimension. This would explain why the gels in rectangular cuvettes first contracted in a horizontal direction. This would also explain how contraction of membrane-associated filaments could form long narrow pseudopods surrounding particles. At the end of the reaction, the actin filaments will tend to orient in parallel, which is what the electron micrographs have revealed in negatively stained gels and in thin sections of pseudopods of intact cells. If this is true, then the parallel filament bundles are the *result* and not the *cause* of cytoplasmic movement. If the randomly oriented filament networks are the generators of mechanical force, the parallel filament bundles must disassemble in order for movement to continue. The mechanisms for such reversibility, other than cooling, are now unknown.

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