Isolation of an Actomyosin-like Protein Complex from Slime Mold Plasmodium and the Separation of the Complex into Actin- and Myosin-like Fractions*

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ABSTRACT: Extracts of the plasmodium of *Physarum polycephalum* prepared with various homogenization media contained a calcium-activated adenosine triphosphatase and a rapidly sedimenting actin-like protein. When plasmodial extracts prepared with 0.05 M sodium pyrophosphate–0.01 M Tris-maleate (pH 6.8) were fractionated by precipitation with ammonium sulfate, the adenosine triphosphatase and the rapidly sedimenting protein were isolated as a partially purified actomyosin-like complex. As judged by viscometry, boundary and zone sedimentation, and electron microscopy slime mold actomyosin was basically similar to muscle actomyosin but differed in having a greater solubility at low ionic strength and a lower myosin:actin ratio. After desalting on Sephadex G-25, slime mold actomyosin was resolved into partially purified actin- and myosin-like components by gel chromatography on Sephadex G-200 at low ionic strength.

It has often been suggested that the motile phenomena observed in most biological systems are caused by protoplasmic contractions and that such systems might contain "contractile" proteins similar to the actomyosin isolated from vertebrate skeletal muscle. Indeed numerous workers have claimed the isolation of actomyosin-like proteins from diverse nonmuscular tissues, organisms, and organelles, some of which are not even usually studied in connection with motility (Jahn and Bovee, 1967; Gibbons, 1968). The plasmodium of the myxomycete slime mold, *Physarum polycephalum*, is a logical choice for experiments on primitive motile system proteins. Extensive work has been carried out on the protoplasmic streaming in slime mold (Kamiya, 1959) which is of interest both because of its cyclic nature (shuttle streaming) and because of the high particle velocities—up to several millimeters per second—which are observed. Furthermore, it is easy to culture large quantities of slime mold plasmodium.

In 1952 Loewy reported the presence of an actomyosin-like protein in high ionic strength extracts of frozen plasmodium. Subsequently Ts'o and coworkers (Ts'o *et al.*, 1956a,b, 1957a-b) extracted frozen plasmodium with concentrated KCl solutions and used ammonium sulfate precipitation and differential centrifugation to prepare a protein fraction which they called myxomyosin. Like actomyosin, myxomyosin gave a viscometric response (reversible decrease in solution viscosity; Portzehl *et al.*, 1950) to ATP. Unlike the muscle protein, however, myxomyosin was soluble at low ionic strength and was judged, on the basis of physical–chemical measurements, to be a one-component system made up of rod-like molecules ~70 Å in diameter with varying lengths in the range of several thousand angstroms. Little data were given on the enzymatic properties of myxomyosin. Later Nakajima (1960, 1964) used alkaline-buffered KCl solutions to prepare extracts from fresh plasmodium. Employing repeated low ionic strength precipitations he obtained a protein fraction, called plasmodial myosin B, which exhibited viscometric response to ATP at high ionic strength and which showed the superprecipitation response (Szent-Györgyi, 1947) to addition of ATP at low ionic strength. Plasmodial myosin B had ATPase activity similar in several respects to the actomyosins of smooth muscles and Nakajima considered it to be a truly actomyosin-like protein; he did not, however, provide extensive data of a physical-chemical nature.

Since the above results left unresolved several questions as to the nature of the slime mold protein and since no detailed description of the presumptive actin- and myosin-like components of nonmuscle actomyosins had appeared, we undertook a reinvestigation of the slime mold system. In this paper we describe the preparation from *P. polycephalum* of an actomyosin-like protein complex and its resolution into actin- and myosin-like fractions. In the following paper we describe the further purification and characterization of slime mold actin and slime mold myosin. Brief reports of this work have already appeared (Adelman and Taylor, 1967; Adelman *et al.*, 1968). While our research was in progress Hatano and coworkers (Hatano and Oosawa, 1966a,b; Hatano *et al.*, 1967) described the isolation, from acetone powders of the slime mold, of an actin-like protein. Hatano and Tazawa (1968) recently prepared an actomyosin-like slime mold protein resolvable into myosin- and actin-like species.

Materials and Methods

Culture of P. polycephalum. Although a completely defined soluble medium is now available for culturing slime mold (Daniel *et al.*, 1963), the oatmeal culture technique described

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by Camp (1936) is more convenient when large quantities of material are needed. For all experiments described here the following modification of the Camp technique was used. One-half of a 6 × 1.5 cm petri dish was wrapped with a 12 × 16 cm piece of absorbant paper toweling, filled with oatmeal flakes ("old fashioned," Quaker Oats Co.), and placed in a covered 10 × 2 cm petri dish. Requisite numbers of such culture chambers were autoclaved (~20 min at 15–20 psi) and to the outer petri dish of each was added sufficient (~25 ml) autoclaved distilled water to cause wetting of the oatmeal. Frequently penicillin (1000 units/ml, "buffered potassium penicillin G," E. R. Squibb and Sons) and/or streptomycin (1 mg/ml; "streptomycin sulfate," Charles Pfizer and Co.) were added (to the previously autoclaved water) to suppress bacterial growth. A small sample of plasmodium was transferred to the moist oatmeal surface and the culture chambers were incubated in a light-tight cabinet at 18–22°. After 5–7 days the plasmodial growth had completely covered the oatmeal and was beginning to migrate out of the smaller dish. After samples were removed for subculturing, the main plasmodial mass was harvested by transferring the oatmeal-filled inner dishes (bottom down, 3–4/pan) to a corn meal agar surface (1–2%; Baltimore Biological Lab), poured in the bottom of an 8.5 × 12 × 1.5 in. deep Pyrex baking pan. These pans were covered with aluminum foil and stored in dim light at 18–22°. Within 12–24 hr the plasmodia migrated out onto the agar surface from which the material was gathered up using a bent glass rod Under these conditions each culture chamber yielded 2–3 g of plasmodia relatively free of slime and bacteria; harvestings of 100–150 g were routine. By serial subculture the original strain of plasmodium, kindly provided by Dr. R. W. Tuveson (Department of Botany, University of Illinois), was maintained for over 2 years. Recently plasmodia cultured from a sclerotial sample provided by Dr. J. W. Daniel (McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.) and from a plasmodial sample provided by Dr. Edmund Guttes (Department of Biology, Loyola University, Chicago, Ill.) have been used. No major differences, with regard to the results reported here or in the following paper, have been noted among the strains.

Preparation of Extracts. Freshly harvested plasmodium was washed by several cycles of suspension in two to three volumes of ice-cold distilled water followed by pelleting for several minutes at 500–1000*g* in an International centrifuge. The washing and all subsequent procedures, unless otherwise noted, were carried out in a 3–5° cold room; whenever possible homogenizers, dialysis flasks, etc., were kept packed in crushed ice. The mass of the plasmodium increased about 25% upon washing, presumably due to trapping of wash water in the pellets; the weight of mold used for each experiment was determined after washing. All homogenates were prepared using a ratio of 1 ml of extraction medium:1 g of washed plasmodium. The plasmodial mass was first slurried with the extraction solution by three passes in the motor-driven homogenizer described by Taylor (1963). The slurry was then further homogenized by ten passes in a 50-ml capacity hand tissue grinder (Arthur H. Thomas Co.) of the TenBroeck type (1931). Homogenates were stored ~1 hr with occasional gentle mixing and were centrifuged 45'-27K-No. 30 (85,000). The resulting supernatants (~1.5 ml/g of washed plasmodium) are referred to as extracts.

Centrifugation. Preparative centrifugations were carried out at 3° using Spinco equipment. For convenience the notation, for example, 30'-27K-No. 30 (85,000) is used to indicate a 30-min centrifugation at 27,000 rpm in the Spinco No. 30 rotor for which conditions the maximum *g* force is approximately 85,000*g*.

For zone sedimentation, linear 5–20% (w/v) sucrose gradients were prepared in nitrocellulose tubes; the gradient-making device (Adelman, 1969) was designed and constructed in the University of Chicago, Department of Biophysics, machine shop. Linearity of sample gradients was verified on several occasions by including ATP in the 20% sucrose solution and monitoring OD_{260nm} of collected fractions. Each gradient was overlayed with 0.2 ml of appropriate protein solution and centrifuged the indicated time at 3°, 50,000 rpm in the SW65 rotor which was decelerated without braking. After bottom puncture, 15-drop fractions (0.20–0.25 ml) were collected and assayed for protein, ATPase, or marker activity. Catalase (aqueous crystalline suspension; Worthington Biochemical Corp.) was run as an external marker and assayed essentially according to Martin and Ames (1961).

Boundary sedimentation in the model E was carried out under standard conditions (Schachman, 1959): 12-mm filled-Epon centerpieces were used. When corrections to $s_{20,w}$ were made, a value of $\bar{v} = 0.73$ was assumed. Sedimentation coefficients are expressed in svedbergs with the symbol S (= 10^{-13} sec) usually understood.

Viscometry was carried out at $T = 20.0 \pm 0.05°$ using 1-ml capacity Cannon-Manning semimicro viscometers (Cannon Instrument Co., Boalsburg, Pa) with solvent outflow times of 28–30 sec. Data are presented as relative outflow time (outflow time for solution/outflow time for same volume of buffer).

Flow birefringence was studied using a Rao apparatus (Rao Instrument Co., Brooklyn, N. Y.) thermostated by circulating water from a bath maintained at 20–22°.

Electron Microscopy. Samples were prepared by negative staining with uranyl acetate (1%, pH 4–4.3) or phosphotungstic acid (1%, pH 7.0). The protein stock solution was first diluted, with 0.10 M ammonium acetate, to give an appropriate concentration (0.01–0.05 mg/ml for uranyl acetate; 0.10–0.50 mg/ml for phosphotungstic acid). A drop of the diluted protein was placed on a carbon-coated, fenestrated formvar covered grid. After 2 min the grid was blotted, a drop of stain solution was added, and this was blotted off after 2 min. Grids were examined in an RCA EMU 3-G operated at 50 kV and micrographs were taken on 70-mm Kodalith L-R film, making use of the roll film magazine described by Tonaki *et al.* (1967). Magnification was determined with reference to a calibration grid (54,864 lines/in.; Ernest Fullan, Inc., Schenectady, N. Y.).

Radioactivity. Aliquots (0.1–0.2 ml) of solutions containing labeled compounds were mixed with 10 ml of Bray's solution (1960) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Salt fractionation was carried out by adding appropriate volumes of saturated $(NH_4)_2SO_4$ solution (pH adjusted with HCl or NH_4OH) to the protein solution. The percentages reported are nominal since no correction was made for volume changes. Precipitates were pelleted at relatively high *g* forces, to minimize salt occlusion, and were resuspended in the appropriate buffer by gentle homogenization with a Teflon–glass homogenizer of the type described by Pierce *et al.* (1953) (Arthur H. Thomas, or Glenco, Houston, Texas).

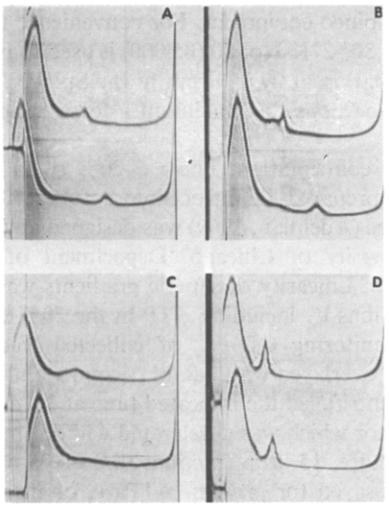

FIGURE 1: Schlieren patterns of crude plasmodial extracts. Extracts were prepared as described in Materials and Methods. For A–C the upper trace represents a 10% dilution of the lower trace with 0.1 M ATP to give 10 mM ATP; for D both traces are at the same protein concentration but the upper trace also contains 10 mM ATP. Each picture was taken at a phase-plate angle of 55°, approximately 32 min after maximum speed was reached, which was 44,770 rpm for A–C and 40,000 rpm for D. The s values given are for the major rapidly migrating boundaries and are not corrected for solvent conditions or temperature. The protein concentrations given were determined on separate aliquots after dialysis according to Table I. (A) Extraction with 1.4 M KCl; T = 22.1°; C = 12.3 mg/ml, s = 33 (lower) and 24 (upper). (B) Extraction with 0.90 M KCl, 0.015 M Na_2CO_3, and 0.06 M $NaHCO_3$; T = 21.1°; C = 14.6 mg/ml; s = 30 (lower) and 21 (upper). (C) Extraction with 0.01 M Tris-maleate, pH 6.8; T = 20°; C = 9.2 mg/ml; s = 23 (upper). A boundary with $s \sim 75$ (lower) had pelletted by the time this picture was taken. (D) Extraction with 0.01 M Tris-maleate–0.05 M sodium pyrophosphate, pH 6.8; T = 20.2°; C = 12.8 mg/ml; s = 20 (lower) and 19 (upper).

Sephadex Chromatography. Small columns (~1 × 15 cm) were allowed to flow freely under low-pressure heads. Larger ones were pumped (Holter Micro-Infusion Roller Pump; Extracorporeal Medical Specialties, Inc., Mt. Laurel Township, N. J.) at a rate approximately equal to that produced by a pressure head of 10 cm of buffer. For large columns eluted under downward flow the aliquot of protein solution was diluted ~5% with a 40% (w/v) sucrose solution and was layered under the column buffer. For upward flow elutions the sucrose was omitted and the protein solution was applied *via* a three-way valve from a separate reservoir. Void volumes of Sephadex columns were determined using the Blue Dextran 2000 (0.1–0.2%, Pharmacia). All marker runs involved an aliquot size equal to that of the protein samples chromatographed.

Protein Concentrations were assayed essentially according to Lowry *et al.* (1951) using bovine plasma albumin (Armour) as a standard. For accurate assays a series of dilutions of the protein solution was made to obtain an OD_{750nm} of between 0.15 and 0.20. A proportionality constant of 3.25 optical density units/mg was assumed for computations. When large numbers of fractions were assayed, a standard curve was used to correct for nonlinearity but appropriate dilutions were assayed so that corrections greater than 25% were not necessary.

ATPase assays were carried out at room temperature (~22°) in 0.50 M KCl–0.01 M $CaCl_2$–0.002 M ATP–0.05 M Tris-HCl buffer (pH 8.0) unless otherwise specified. Conditions were chosen so that P_i liberation was proportional to protein concentration and four time points were taken to assure linearity of reaction. When large numbers of fractions were to be assayed, an appropriate single time point was chosen and a zero-time correction was made. Duplicate assays normally agreed to within 10%. The use of 10 mM Ca in the standard assay resulted in formation of a precipitate and a downward drift in pH when the less pure fractions were assayed at high protein concentrations. This was a problem only in the case of crude extracts and some ammonium sulfate fractions; by using low protein concentrations and short assay times it was possible to obtain fairly reliable activity figures even for these impure fractions. P_i was assayed essentially according to Martin and Doty (1949), with silicotungstic acid as the protein precipitant. Isobutyl alcohol–benzene was used 3 ml/1 ml of aqueous sample); the stock $SnCl_2$ reagent was diluted 1:50 and only used immediately thereafter. The color developed (λ 725 nm) was linearly related to the P_i concentration in the reaction sample (up to 1 μmole/ml) with a proportionality constant of 2.10 optical density units/μmole.

Miscellaneous. Spectrophotometry was carried out in a Cary Model 14 using quartz or glass cuvets with a 1-cm path length. All pH values given are those read at room temperature using a Sargeant Model LS meter equipped with a combination electrode and standardized at pH 7 and 10. Solutions were prepared with deionized distilled water (Bantam demineralizer, Barnstead Co., Boston, Mass.; or Continental demineralizer, Continental Co., Chicago, Ill.). Buffers containing cysteine or β-mercaptoethanol were flushed with nitrogen, stored under a nitrogen atmosphere, and used as soon as possible thereafter. Whenever possible, columns equilibrated with such buffers were flushed with fresh buffer immediately prior to use. Enzyme grade Na_2ATP, enzyme grade ammonium sulfate, and β-mercaptoethanol were purchased from Nutritional Biochemicals Corp., Mann Research Lab, and Eastman Kodak Co., respectively, while Tris and L-cysteine hydrochloride were Sigma products.

Results

A. Studies on Crude Extracts. Extracts of slime mold plasmodium prepared, according to Materials and Methods, with buffers of varying composition were assayed for protein content and ATPase activity and examined in the analytical ultracentrifuge. The protein and ATPase data for several types of extracts are summarized in Table I; Figure 1 shows schlieren profiles of selected extracts in the presence and absence of 10 mM ATP. The extracts prepared with unbuffered 1.4 M KCl (under these conditions the total homogenates have pH 6.5–7.0) were intended to be comparable with those of Ts'o *et al.* (1957b). Since Ts'o and coworkers actually used frozen plasmodium, parallel extractions were carried out on fresh and frozen material: the results were similar. Homogenization in alkaline 0.90 M KCl should have produced extracts comparable in ionic composition with those of Nakajima. When assayed at high ionic strength, both types of high salt extract showed considerable Ca^{2+}-activated ATPase activity (Table I, 1 and 2). Extraction with 0.01 M Tris-maleate (pH 6.8), in the presence (Table I, 4) but not in the absence (Table I, 3) of 0.05 M $Na_4P_2O_7$, also resulted in preparations having pronounced Ca^{2+}–ATPase activity. For simplicity extracts

TABLE I: Protein Content and ATPase Activity of Various Slime Mold Extracts.[a]

No. of Expt		Extraction Solution	Protein Content (mg/ml)	ATPase Activity (μM P_i/mg per 15 min)	
				No Me^{2+}	+10 mM Ca^{2+}
1	4	1.4 M KCl unbuffered	13 (10.8–14.6)	0.06 (0.051–0.065)	0.49 (0.451–0.602)
2	4	0.90 M KCl–0.015 M Na_2CO_3–0.06 M $NaHCO_3$	14 (11.4–15.6)	0.07 (0.050–0.086)	0.51 (0.440–0.661)
3	5	0.01 M Tris-maleate (pH 6.8)	10 (6.7–12.9)	0.02 (0.012–0.035)	0.09 (0.071–0.129)
4	9	0.01 M Tris-maleate–0.05 M $Na_4P_2O_7$ (pH 6.8, TM–PP_i)	11 (9.0–12.7)	0.06 (0.045–0.070)	0.45 (0.403–0.531)
5	3	0.01 M Tris-maleate–0.05 M EDTA (pH 6.8)	12 (11.5–12.9)	0.01 (0.007–0.017)	0.17 (0.140–0.192)
6	3	TM–PP_i + 0.05 M EDTA	12 (11.6–13.2)	0.04 (0.039–0.043)	0.52 (0.447–0.623)
7	3	0.30 M KCl–0.01 M Tris-maleate (pH 6.8)	11 (9.0–11.4)	0.03 (0.027–0.047)	0.29 (0.168–0.421)

[a] Extracts were prepared according to Materials and Methods and were dialyzed overnight *vs.* two changes of a large excess of 0.50 M KCl–0.01 M Tris (pH 7.8). ATPase activity was assayed in 0.50 M KCl–0.05 M Tris–0.002 M ATP (pH 8.0) with and without 0.01 M $CaCl_2$. Results are presented as mean (range) of the indicated number of experiments.

prepared under the above conditions are referred to as "Ts'o," "Nakajima," "TM–PP_i," and "TM" extracts, respectively.

Analytical ultracentrifugation revealed, in all plasmodial extracts, large amounts of heterogeneous slowly sedimenting material. Those extracts with pronounced Ca^{2+}–ATPase activity also showed small fairly discrete high s boundaries.[1] Ts'o and Nakajima extracts (Figure 1A,B) contained a small boundary with $s_{20} \sim 30$ or greater; when ATP was added this boundary was replaced by a sharper one with $s_{20} \sim 20$–25. TM extracts (Figure 1C), to which ATP had been added, showed a very small ~20–25S boundary. TM–PP_i extracts (Figure 1D), in the presence or absence of ATP, revealed a sharp $s \sim 20$ peak. Ribonuclease digestions (0.1 mg/ml, 1 hr, room temperature) did not remove these high s boundaries although the trailing shoulder on the ~20S peaks (especially obvious in Figure 1B, upper trace) was thereby reduced. The sedimentation of these high s species was very concentration dependent; for example, the "20S" peak in the TM–PP_i extracts was estimated, by dilution with the extraction solution, to have $s^0_{20,w} > 30$.

To test the possibility that the material in the ~20S boundary was actin-like, TM–PP_i extracts were dialyzed to low ionic strength. Upon prolonged (20–30 hr) dialysis *vs.* 1 mM Tris–0.5 mM ATP (pH 8.1), the peak disappeared—apparently by a time-dependent breakdown to more slowly sedimenting material. When the salt concentration was raised again (final conditions: 0.10–0.15 M KCl, pH 7), the high s boundary reappeared, although it was no longer as sharp as in the untreated extract. After such dialysis to low ionic strength, TM–PP_i extracts showed no signs of precipitate formation and retained most (~80%) of their Ca^{2+}–ATPase activity.

Extracts prepared with the TM–PP_i buffer showed intense flow birefringence: the presence of orientable material was easily detectable even after a three- to fivefold dilution. All crude extracts were heavily contaminated with the yellow plasmodial pigment and were slightly turbid; therefore measurements of retardation were not made. Some difficulties were encountered in measuring the extinction angle, χ, but reasonably reproducible values were obtained: χ for undiluted TM–PP_i extracts ranged from ~40° at a shear gradient of 600 sec^{-1} to ~22° at a shear gradient of 4500 sec^{-1}. In distinct contrast, extracts prepared with the TM buffer without pyrophosphate showed no flow birefringence even at shear gradients of ~5000 sec^{-1}.

The flow birefringence studies and the results of low ionic strength dialyses suggested that most, but not all, of the material in the ~20S boundary was actin like. It was tentatively assumed that the high ionic strength Ca^{2+}–ATPase activity of the various extracts was a rough measure of their content of myosin-like protein. The ~30S boundary seen in Ts'o and Nakajima extracts was presumed to represent an actomyosin-

[1] In general we use the term high s to indicate species for which $20 < s < 50$. The term low s is used for species with $S < 10$.

TABLE II: Protein and ATPase Recoveries During the Preparation, by $(NH_4)_2SO_4$ Fractionation, of Slime Mold Actomyosin.[a]

A

Stage	Protein		ATPase	
	mg/ml	mg/100 g of Mold	μM P_i/mg per 15 min	μM P_i/15 min per 100 g of Mold
Crude extract	13.7	1950	0.41	795
Salt fraction I	20.5	720	1.06	763
Crude actomyosin (= salt fraction II)	11.1	582	1.20	695

B

	Vol (ml)	Protein (mg/ml)	ATPase (μM P_i/mg per 15 min)
Crude extract	~150	10–15	0.40–0.50
Salt fraction I	~37	16–24	0.70–1.1
Crude actomyosin (= salt fraction II)	~55	9–14	1.0–1.4

[a] TM–PP_i extracts were prepared according to Materials and Methods: the volume (= 'E' ml) was noted. The extract was brought to 45% saturation with ammonium sulfate at pH 6.5 and, after 30 min, the precipitate was centrifuged down [30'-27K-No. 30 (85,000)]. The pellets were resuspended in 0.20 × 'E' ml of 0.01 M Tris-maleate (pH 6.2) and the solution was clarified [30'-27K-No. 30 (85,000)]. The supernatant (= salt fraction I) was brought to 35% saturation with ammonium sulfate at pH 6.0 and the precipitate formed after 30 min was spun down [30'-50K-No. 65 (218,000)]. The pellets were resuspended in 0.33 × 'E' ml of 0.01 M Tris (pH 7.8) and clarified [30'-27K-No. 30 (85,000)] to give crude actomyosin. Samples were dialyzed (see footnote to Table I) prior to protein and ATPase assay. Table IIA gives data for a fairly representative experiment. Table IIB summarizes the range of values in a large number (>20) of such fractionations, all figures having been normalized to a hypothetical initial mass of 100 g of washed plasmodium.

like complex which was dissociated by ATP (Figure 1 A, B, upper traces) or pyrophosphate[2] into its component species: an ~20S actin-like boundary and a more slowly sedimenting myosin-like component obscured by the large amount of low *s* material.

Further studies on crude extracts (some data are included in Table I) indicated that the extraction by PP_i was not due to its chelating properties since EDTA neither substituted for (Table I, 5) nor interfered with (Table I, 6) it. The enhanced extraction of actin and myosin by TM–PP_i over that observed with TM was partially but not completely due to the increased ionic strength (Table I, compare 4, 5, and 7, all of which should have comparable ionic strength). ATP could be substituted for PP_i suggesting that the effect was at least partially due to polyanion binding. Addition of KCl (0.50 or 1.0 M) or $MgCl_2$ (2 mM) to the TM–PP_i buffer, adjustment of the pH to 9, or prolonged extraction (up to 6 hr) did not significantly increase the yield of the presumptive actin- or myosin-like species.

Several attempts were made to effect differential extraction of either the myosin- or actin-like protein. Extraction with ~10 mM ATP seemed to enrich slightly for the high *s* boundary relative to the Ca^{2+}–ATPase. On the other hand, when the pellets from a TM extract were washed with TM and then homogenized in TM–PP_i plus 2 mM Mg^{2+}, the resulting extract had appreciable Ca^{2+}–ATPase activity but relatively little high *s* material. In no case, however, was a truly clean separation of ATPase from high *s* peak achieved by such manipulations. Since we wished to study both components, an extraction procedure was chosen which gave high yield of each. The TM–PP_i extraction solution was adopted for routine use because it appeared to maximize the amount of high *s* material without giving significantly different ATPase values from those obtained with Ts'o or Nakajima extracts and because it was hoped that the relatively low ionic strength and pH would minimize extraction of other components.

B. Partial Purification and Characterization of Slime Mold Actomyosin. When TM–PP_i extracts were subjected to salt fractionation with ammonium sulfate, little material precipitated until the salt concentration reached ~20% saturation. Sequential salt fractions obtained between 20 and 50% saturation contained appreciable amounts of ATPase activity and high *s* peak, in approximately constant ratio; material precipitating above ~45–50% was essentially free of both components. Several variations of ammonium sulfate fractionation (*e.g.*, pH, presence of ATP) were examined in attempts to separate Ca^{2+}–ATPase from high *s* material; however all such attempts were essentially unsuccessful. Therefore salt fractionation was adopted as a means of achieving a partial purification of the actomyosin-like complex and broad saturation limits were chosen so as to minimize losses of either component.

Crude TM–PP_i extracts were first fractionated at pH 6.5 (see footnote to Table II); the material precipitating between 0 and 45% saturation was redissolved in 0.01 M Tris-maleate

[2] In the case of muscle actomyosin, the PP_i-induced dissociation has a strong requirement for Mg^{2+} (Granicher and Portzehl, 1964); also unpublished experiments by R. W. Lymn, B. Finlayson, and M. R. Adelman). The Mg^{2+} content of the plasmodium is presumably sufficient to satisfy this requirement in our crude extracts.

TABLE III: ATPase Activity of Crude Slime Mold Actomyosin.[a]

	(Specific Activity, μM P_i/mg per 15 min)			
	No Me^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+} + Mg^{2+}
High ionic strength				
Expt 1	0.177	1.29	0.108	0.134
Expt 2	0.105	1.12	0.063	0.088
Low ionic strength				
Expt 1	0.065	0.385	0.048	0.032
Expt 2	0.042	0.295	0.041	0.020

[a] The results of assays on two different actomyosin preparations are included: both were produced as indicated in Table II and dialyzed overnight *vs.* 0.50 M KCl–0.01 M Tris (pH 7.8) prior to assay. Assays were at room temperature in 0.025 M Tris (pH 8.0) with 2 mM ATP. Divalent ions, as indicated, were present at final concentrations of 10 mM each. The KCl concentrations, including salt introduced along with the protein aliquots, were: high ionic strength, 0.525 M KCl; low ionic strength 0.075 M KCl.

at pH 6.2, clarified, and reprecipitated at pH 6 and 35% saturation. The material obtained in this manner will be referred to as crude slime mold actomyosin or simply as actomyosin: the term crude is included to emphasize that the content of impurities was quite high because fractionation limits had been chosen to maximize recovery of enzymatic and high *s* components.

The recoveries of protein and Ca^{2+}–ATPase for one such salt fractionation are presented in Table IIA; the range of data for a large number of such purifications is indicated in Table IIB. Recovery of high ionic strength Ca^{2+}–ATPase was normally 70–90% with an increase in specific activity of 2.5 to 3. Recovery of the high *s* material was more difficult to estimate; examination of schlieren patterns of material precipitating above 45% ammonium sulfate saturation in the first fractionation step (or 35% in step II) and comparison of the schlieren profiles of TM–PP_i, extracts and crude actomyosin solutions suggested that losses were small.

Crude actomyosin solutions were bright yellow and faintly turbid. Electron microscopy revealed fibrous material, in varying states of aggregation, with minimum strand diameters of 50–80 Å (Figure 2A). The dependence on ionic strength and divalent ions of the actomyosin–ATPase activity is indicated in Table III. The enzyme was Ca^{2+} activated at both low and high ionic strength and the Ca^{2+} activity was higher at high ionic strength than at low ionic strength. Under the conditions used in these assays the enzyme was not activated by Mg^{2+}; instead a small and variable inhibition was observed. As shown in Figure 3, the addition of ATP to high ionic strength solutions of crude slime mold actomyosin produced a rapid and reversible decrease in solution viscosity. Return to the high viscosity state was accelerated by Ca^{2+}, presumably reflecting hydrolysis of the ATP by the Ca^{2+}-activated enzyme. The viscosity recovery was inhibited by Mg^{2+}. The magnitude of the ATP-induced change was small: ATP sensitivity, computed according to Portzehl *et al.* (1950), ranged from 30 to

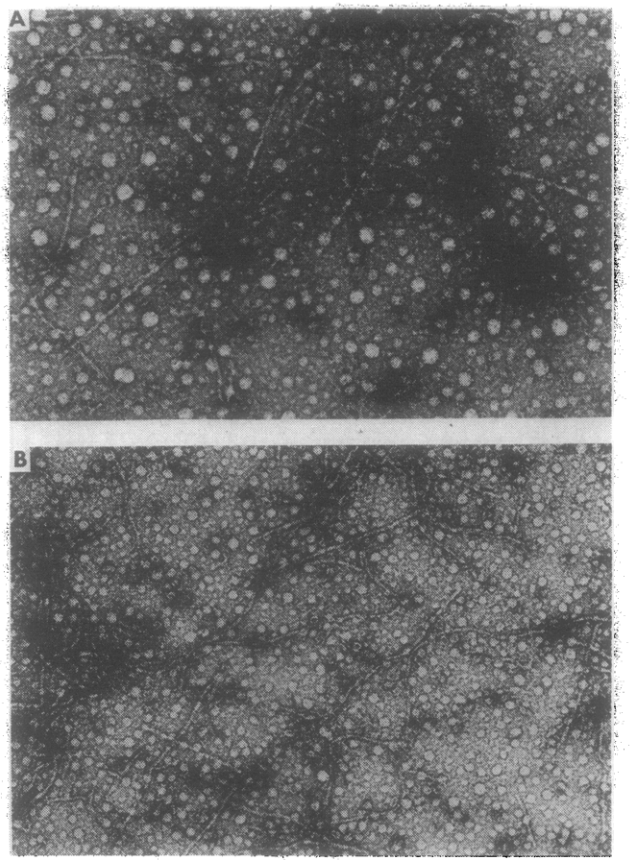

FIGURE 2: Electron micrographs demonstrating fibrous material in crude slime mold actomyosin. The negative stain was 1% uranyl acetate. Both micrographs were taken at a direct magnification of 21,000 and are printed at a total magnification of 62,000. (A) Crude slime mold actomyosin. (B) An aliquot of fraction 10 of the sucrose gradient in Figure 5D. See text for discussion.

50%. Furthermore, the viscometric studies revealed several complexities. Solutions of actomyosin exhibited "work softening" such as described for myxomyosin (Ts'o *et al.*, 1956a). Often the solutions "recovered" to a viscosity level higher than that observed before addition of ATP. AMP and ADP produced a slow rise in viscosity to an appreciably higher level, the effect of the former being somewhat more pronounced. After pretreatment with AMP, solutions of actomyosin still responded to ATP, giving a final viscosity comparable with that observed without the intermediate AMP addition. Thus the measured ATP sensitivity values were increased and, in some cases, nearly doubled. Since crude slime mold actomyosin was soluble at low ionic strength (see below), some preliminary viscometry studies were made with material in 0.05 M KCl–0.01 M Tris (pH 7.8). ATP produced a sharp drop in solution viscosity; the values of η_{rel} before and after addition of ATP were very similar to those for the same preparation at high ionic strength. At low ionic strength the addition of ADP produced a slow decrease in viscosity whereas AMP had essentially no effect.

Sedimentation analyses of the slime mold protein provided the best evidence of its actomyosin-like nature. Boundary centrifugation in 0.50 M KCl revealed, in addition to considerable slowly sedimenting material, a major rapidly spreading

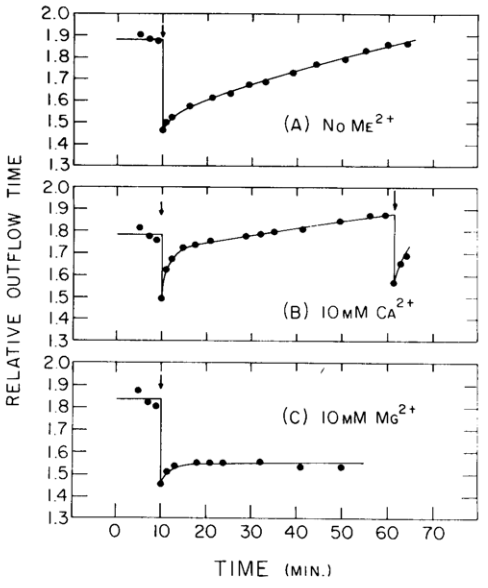

FIGURE 3: Viscometric response of crude actomyosin to addition of ATP. Each viscometer contained 1 ml of crude actomyosin (4.5 mg/ml; specific Ca^{2+}–ATPase = 0.92 μM P_i/mg per 15 min) in 0.50 M KCl–0.01 M Tris, pH 7.8. The solutions also contained (final concentrations) (A) no added divalent ion, (B) 10 mM $CaCl_2$, (C) 10 mM $MgCl_2$. Arrows indicate the addition of 0.1 ml of the KCl–Tris buffer containing 0.2 μmole of ATP.

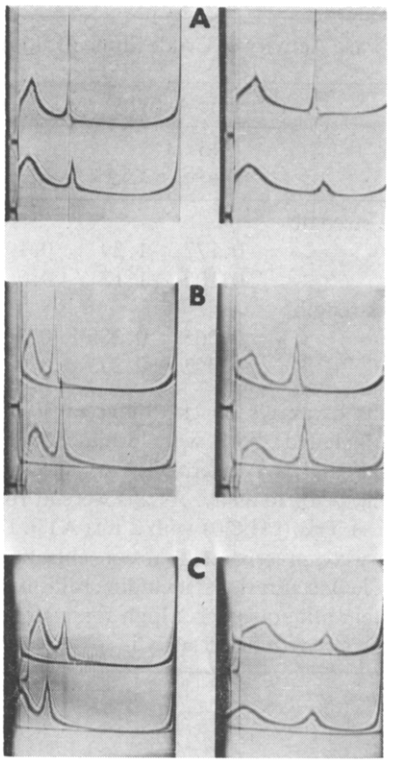

FIGURE 4: Boundary centrifugation of slime mold actomyosin A, B, and C show different preparations of crude slime mold actomyosin (= salt fraction II) prepared as in Table II and dialyzed *vs.* the indicated buffers. Sedimentation was at 20°, 40,000 rpm. (A) Actomyosin in 0.50 M KCl–0.01 M Tris, pH 7.8. Pictures taken 24 and 40 min after maximum speed was reached, at phase-plate angles of 60 and 55°, respectively. Protein concentration was ~7.6 mg/ml in each cell; the upper cell also contained 1 mM MgATP. For the major high S peaks, $s_{20,w} \sim 24$ (upper) and 26 (lower). The slower peak in the upper trace had $s_{20,w} \sim 6.6$. (B) Actomyosin in 0.15 M KCl–0.01 M Tris-maleate, pH 6.8. Pictures were taken at a phase-plate angle of 55°, 16 and 32 min after maximum speed was reached. The protein concentration in the lower cell was ~9.8 mg/ml; the material in the upper cell had been diluted 10% to give a final concentration of ~10 mM ATP. For the major high S peaks, $s_{20,w} \sim 22$ (upper) and 23 (lower). (C) Actomyosin in 0.05 M KCl–0.01 M Tris-maleate, pH 6.8. Pictures were taken 16 and 48 min after maximum speed was reached at phase-plate angles of 60 and 55°, respectively. The protein concentration in the lower cell was ~8.9 mg/ml; the material in the upper cell had been diluted 10% to give a final concentration of ~10 mM ATP. For the major high S peaks, $s_{20,w} \sim 19$ (upper) and 21 (lower).

high *s* peak (Figure 4A, lower trace). Addition of ATP (10 mM) or Mg–ATP) (1–2 mM) resulted in a sharpening of the high *s* boundary and a decrease in its sedimentation rate. ATP (or Mg–ATP) also produced a sharpening of the broad low *s* peak, best described as the super position of a small sharp boundary on a larger more heterogeneous one. That these changes in the schlieren profiles reflected dissociation from the high *s* species of a low *s* myosin-like enzyme was verified by zone sedimentation in linear sucrose gradients.

When crude actomyosin was subjected to zone sedimentation at high ionic strength (Figure 5A, lower gradient), the Ca^{2+}–ATPase activity migrated as a reasonably discrete peak whose apparent *s* value was 20–30. If the protein zone was first treated with ATP or Mg–ATP and sedimented under otherwise identical conditions (Figure 5A, upper gradient), a striking shift in the ATPase profile was observed, with most of the enzyme activity sedimenting as a sharp peak with $s = 6$–8. Thus the enzyme distribution in these gradients was consistent with the hypothesis of an ATP-induced actomyosin dissociation. Contrary to what was expected on the basis of the schlieren patterns, no high *s* protein peak appeared in the gradients (Figure 5A). It was found, however, that a high *s* protein peak was demonstrable in gradients run at a lower ionic strength (compare Figure 5B, lower and upper gradients). When the experiment represented by Figure 5A was repeated in 0.15 M KCl (Figure 5C), a rapidly sedimenting protein species was demonstrable in both gradients, while the Ca^{2+}–ATPase activity profiles were similar to those obtained at high ionic strength. Finally, when Mg–ATP was included in the gradient (at 0.15 M KCl) as well as the zone it was possible to demonstrate that virtually all ATPase activity sedimented at ~6–8 S, well resolved from a pronounced high *s* protein peak which was nearly devoid of enzymatic activity (Figure 5D). Electron microscopic examination of fractions corresponding to the enzyme and high *s* protein peaks (see footnote to Figure 5D) demonstrated 50–80-Å fibers restricted to the latter fractions (Figure 2B).[3]

It was possible to carry out the gradient analysis of Figure 5C (lower) because crude slime mold actomyosin was truly soluble at low ionic strength. When the pellets from the second salt fractionation were dissolved in and dialyzed against 0.15 M KCl or 0.05 M KCl at pH 6.8 the resulting solutions showed only the faint turbidity observed in high ionic strength solutions of the actomyosin; upon centrifugation (15 to 30′-27K-

[3] The globules in Figure 2A,B are discussed in the following paper.

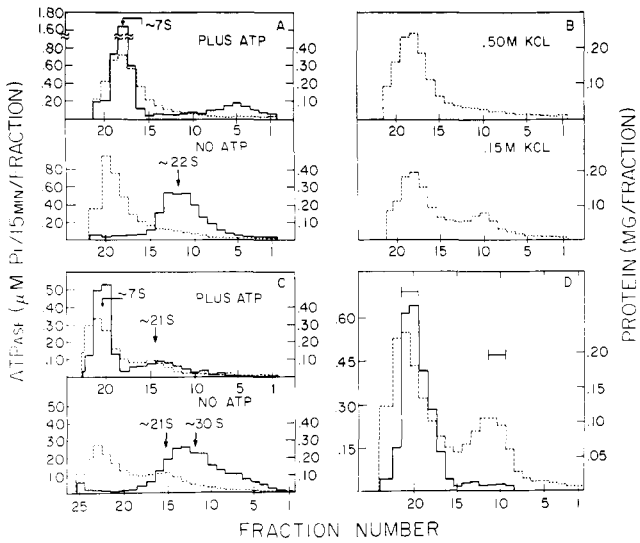

FIGURE 5: Zone sedimentation of crude slime mold actomyosin in sucrose gradients. See Materials and Methods for general techniques. Sedimentation is from left to right in all figures. The left and right ordinates refer to Ca^{2+}–ATPase activity (———) and protein content (– – –), respectively. (A) Both gradients were prepared in 0.50 M KCl–0.01 M Tris, pH 7.8. The actomyosin (dialyzed overnight *vs.* 0.50 M KCl–0.01 M Tris, pH 7.8) was applied to each gradient at a concentration of 11 mg/ml (specific activity ~1.6 μM P_i/mg per 15 min). The zone applied to the lower gradient contained 1.6 mM Mg^{2+}; the zone for the upper gradient contained 1.6 mM Mg^{2+} and 1.6 mM ATP. Sedimentation was for 3 hr. (B) The lower gradient was prepared in 0.15 M KCl–1 mM Tris-maleate, pH 6.8. The upper gradient was prepared in 0.50 M KCl–1 mM Tris-maleate, pH 6.8. The actomyosin (dialyzed 6 hr *vs.* 0.15 M KCl–0.01 M Tris-maleate, pH 6.8) was applied to each gradient at a concentration of 8.9 mg/ml; each zone also contained ATP at a final concentration of 10 mM. Sedimentation was for 3 hr. (C) Both gradients were prepared in 0.15 M KCl–0.01 M Tris-maleate, pH 6.8. Actomyosin at a concentration of 12 mg/ml (specific activity ~1.2 μM P_i/mg per 15 min) was applied to the lower gradient. The zone for the upper gradient was the actomyosin stock diluted 10% to give a final concentration of 10 mM ATP. Sedimentation was for 2.5 hr. (D) The gradient was prepared in 0.15 M KCl–0.01 M Tris-maleate–1 mM $MgCl_2$–1 mM ATP, pH 6.8. Actomyosin solution (12 mg/ml; 1.3 μM P_i/mg per 15 min) containing 10 mM ATP was applied. Sedimentation was for 3 hr. Heavy bars indicate fractions which were sampled for electron microscopy (see text and Figure 2B). The actomyosin preparations used in C and D had been dissolved in 0.01 M Tris (pH 7.8) but were not dialyzed prior to use. In all cases the protein solutions were centrifuged 15 or 30′ -35K-No. 40 (110,000) prior to use. The ATPase profile in the upper part of Figure 5A is uncorrected for zero time phosphate. This correction, applied in the case of 5C (upper) and 5D, was normally 10–25% of the peak fractions; hence recovery in 5A was overestimated. The protein recoveries in 5B (upper and lower) were identical and equaled ~78%. Generally the protein and ATPase recovered from gradients accounted for 70–90% of the input.

No. 30 [85,000]) the loss of protein and ATPase was small and not significantly greater at low ionic strength than at high ionic strength. Furthermore, schlieren patterns of actomyosin in 0.15 and 0.05 M KCl (Figure 4B,C) did not reveal extensive aggregation: the high *s* peak was fairly sharp, although some asymmetry to the high *s* side was detectable. Addition of ATP eliminated much of the leading shoulder on the high *s* peak and produced a sharpening of the slowly sedimenting boundary indicative of actomyosin-like dissociation.

C. Separation of Crude Slime Mold Actomyosin into Fractions Containing Actin- and Myosin-like Proteins. The observation (Results, A) that the Ca^{2+}–ATPase did not precipitate when TM–PP_i extracts were dialyzed to an ionic strength low enough to "depolymerize" the presumptive actin suggested the separation described here. The basis of the fractionation is the resolution, by gel chromatography at low ionic strength, of a high molecular weight myosin-like enzyme from a low molecular weight G-actin-like subunit.

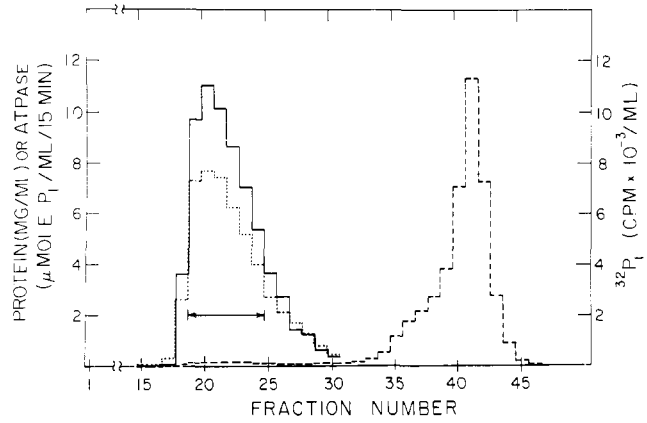

FIGURE 6: Desalting of crude actomyosin by chromatography on Sephadex G-25. The column (4.4 × ~47 cm) was equilibrated and eluted with 1 mM Tris–1 mM ATP– 5 mM cysteine, pH 7.8. The input zone (63 ml) contained 11.1 mg of protein/ml with an ATPase activity of 1.22 μM P_i/mg per 15 min. The zone also contained 2% sucrose, 5 mM ATP, and [^{32}P]P_i (~11,750 cpm/ml). The column was eluted, under downward flow, at ~105 ml/hr and fractions (16.6 ml) were assayed for protein (· · · ·), ATPase (——), and [^{32}P]P_i (– – –). Fractions pooled for subsequent use are indicated by arrows.

To assure that the actin-like component was fully depolymerized, crude slime mold actomyosin solutions were desalted by passage over Sephadex G-25. The results of one such experiment are shown in Figure 6. Elution buffers were maintained at a slightly elevated pH (by analogy with standard techniques for depolymerizing muscle actin) and contained 1 mM ATP, which was expected to protect the actin subunit and minimize myosin–actin and myosin–myosin interactions. It was found necessary to include a mild reducing agent to protect the actin: 5 mM cysteine was used in many experiments but the most satisfactory actin preparations were obtained using 5 mM β-mercaptoethanol.

The actomyosin solutions applied to the G-25 columns were quite viscous, despite the addition of 5 mM ATP to guarantee dissociation, and desalting sufficient to allow actin depolymerization (as judged by analytical ultracentrifugation, Figure 7) was reproducibly achieved only with certain precautions. The second salt precipitate was pelleted at high speed (see footnote to Table II) to minimize occlusion of ammonium sulfate, and the pellets were dissolved in 1 ml of 0.01 M Tris (pH 7.8)/3 ml of original crude extract. After clarification[4] these solutions (see footnote to Table II) had a protein concentration of ~10–12 mg/ml. When applied to G-25 at a

[4] When the actomyosin was to be used for G-25 chromatography (as opposed to viscometry, sucrose gradients, etc.), sucrose (2% w/v final) and ATP (5 mM final) were added to the resuspended material prior to clarification. Clarification in the presence of sucrose and ATP gave actomyosin solutions with slightly (5–10%) more protein and ATPase per milliliter than those indicated in Table II.

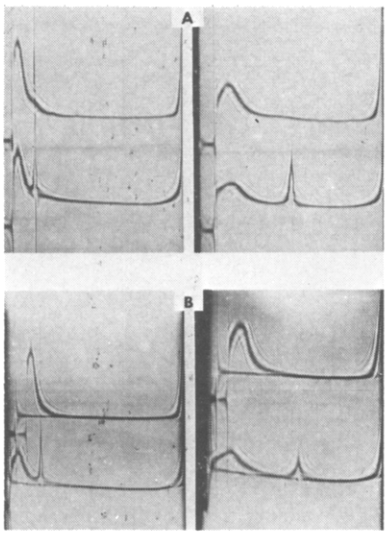

FIGURE 7: Boundary centrifugation of desalted slime mold actomyosin. In each case the upper trace represents the pooled effluent fractions from a Sephadex G-25 desalting column; the lower trace shows the same material after 10% dilution with a concentrated buffer solution giving, as final conditions, 0.15 M KCl–0.01 M Tris-maleate, pH 7.0. Centrifugation was at 40,000 rpm, $T = 20°$. (A) The desalting column was eluted with 1 mM Tris–1 mM ATP–5 mM cysteine, pH 7.8. Pictures were taken 8 and 32 min after maximum speed was reached, at a phase-plate angle of 55°. Protein concentration in the upper cell was 5.85 mg/ml. For the rapidly moving boundary in the lower cell $s_{20,w} = 25.5$. (B) The desalting column was eluted with 1 mM Tris–1 mM ATP–5 mM β-mercaptoethanol, pH 7.8. Pictures were taken 12 and 36 min after maximum speed was reached, at phase-plate angles of 60 and 50°, respectively. Protein concentration in the upper cell (not measured) was estimated as 4–5 mg/ml. For the rapidly moving boundary in the lower cell $s_{20,w} = 27.0$.

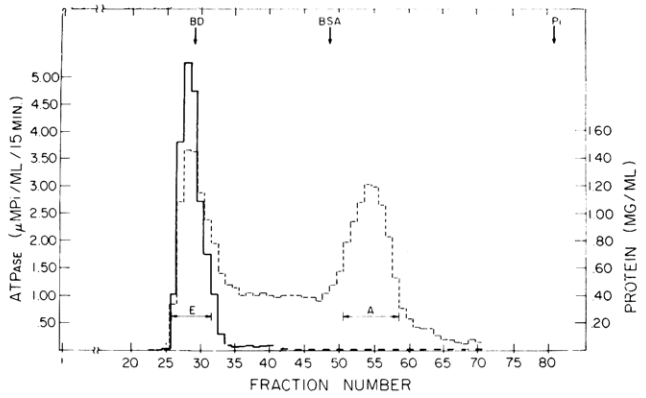

FIGURE 8: Chromatography of desalted actomyosin on Sephadex G-200. The column (5 × 91 cm) was equilibrated and eluted with 1 mM Tris–1 mM ATP–5 mM cysteine, pH 7.8. A zone (90 ml) containing desalted actomyosin at a protein concentration of 5.85 mg/ml (ATPase activity of 1.08 μM P_i/mg per 15 min) was applied to the column. Fractions of 22.5 ml were collected at an upward flow rate of 52 ml/hr and were assayed for protein content (· · · ·) and ATPase activity (——). Bars labeled E and A indicate fractions pooled as G-200 enzyme and G-200 actin, respectively. The arrows labeled BD, BSA, and P_i indicate the elution positions of Blue Dextran, bovine plasma albumin, and inorganic phosphate, respectively.

sample volume–bed volume ratio of ~1:10 and eluted at a relatively low flow rate, such actomyosin solutions were rapidly and efficiently desalted. Based on the distribution of [^{32}P]P_i counts (included as a marker in the experiment of Figure 6) the salt concentration in the effluent zone was only 1–2% of the estimated input concentration of ~0.05–0.10 M due to occluded ammonium sulfate. Protein and ATPase were quantitatively recovered from these columns; peak fractions, accounting for ~75% of the input material (a typical zone is indicated in Figure 6), were pooled for subsequent use. These solutions, designated desalted actomyosin, had a protein concentration of 4–6 mg/ml and were pale yellow[5] and very faintly turbid.

When desalted actomyosin solutions were examined in the analytical ultracentrifuge (Figure 7, upper traces), little high *s* material was detected even when centrifugation was carried out as soon as the protein emerged from the column. Occasionally, when the protein concentration was fairly high (Figure 7A, upper) a small amount of residual high *s* material was seen. Addition of salt to the G-25 column effluent resulted in immediate reappearance of the sharp high *s* boundary (Figure 7, lower traces). That this change represented the repolymerization of an actin-like species was strongly suggested by electron microscopic observations: desalted actomyosin contained no fibrous material, but, when samples of the same solution were examined after addition of salt, fibers similar to those in Figure 2 were again detectable.

Attempts to concentrate the desalted actomyosin prior to G-200 chromatography, for example, by exposure to dry Sephadex G-25, always resulted in the formation of some high *s* material. Therefore, the pooled G-25 effluent was applied directly to the G-200 column. Chromatography on Sephadex G-200 was routinely carried out using a sample volume–bed volume ratio of 1:20 and the column was eluted with the buffer used for the preceding desalting step. The results of a typical experiment are shown in Figure 8. Many of the large-scale separations which provided material for the experiments described in the following paper were carried out in TAM buffer[6] using a G-200 column (7.6 × 43 cm) with a much smaller height to diameter ratio, thus allowing considerably faster elution than was possible with the column in Figure 8. These modifications, which were necessary to protect the actin subunit, had little effect on the G-200 elution profiles other than to produce a slight broadening of the main peaks. Within experimental error the protein and ATPase activity applied to the G-200 columns were completely recovered.

Virtually all of the Ca^{2+}–ATPase activity was eluted as a sharp peak coincident with the void volume of the column. Zone sedimentation of the desalted actomyosin in a sucrose gradient made up in the Tris–ATP–cysteine column buffer showed all enzymatic activity sedimenting as a sharp 6S to 8S peak. The voiding from G-200 of a protein with this low an *s*

[5] The yellow coloring of the input actomyosin solutions was resolved into two components on Sephadex G-25. A bright lemon yellow zone was tightly bound to the gel and required several bed volumes of buffer for elution. A more brownish-yellow component migrated with the protein; since this coloration also migrated with the voided protein in the subsequent G-200 chromatography, it served as a convenient visual marker during both stages of column chromatography.

[6] TAM buffer is composed of 1 mM Tris–1 mM ATP–5 mM β-mercaptoethanol, pH 7.8.

value suggested considerable particle asymmetry and constituted further evidence of the myosin-like nature of the enzyme. The bar labeled "E" in Figure 8 indicates the fractions pooled as "G-200 enzyme" for further work. Use of the 7.6 × 43 cm column resulted in a somewhat less concentrated effluent; therefore the volume pooled was correspondingly larger. In a typical experiment the pooled G-200 enzyme contained 0.75–1.0 mg of protein/ml with a specific activity three to four times that of the desalted actoymosin and accounted for ~70 to 80% of the input Ca^{2+}–ATPase activity.

As indicated in Figure 8 the protein profile showed, in addition to the large voided peak, a major zone of retarded material. This second peak, which accounted for 30–40% of the total protein, eluted somewhat behind bovine plasma albumin. When crude slime mold actomyosin was chromatographed on Sephadex G-200 at high ionic strength in the presence of ATP, the retarded protein peak was not seen; instead all protein eluted as an asymmetric peak which emerged at the void volume of the column but trailed considerably into the region corresponding to lower molecular weight material. The fact that a large percentage of the protein in the crude slime mold actomyosin chromatographed as a discrete low molecular weight species at low, but not at high, ionic strength suggested that much of the material in the retarded peak was the presumptive actin-like subunit. The bar labeled "A" in Figure 8 indicates the fractions pooled and designated G-200 actin. In most experiments the G-200 actin fraction contained ~0.5–1.0 mg of protein/ml and represented ~20–30% of the protein in the original aliquot of desalted actomyosin.

Discussion

The data presented here, as well as in the following paper, provide strong evidence for the presence, in the slime mold, of an actomyosin-like complex resolvable into actin- and myosin-like components. Several of our techniques and findings deserve further comment, especially since our preparations of slime mold actomyosin differ from those of Ts'o *et al.* (1956a,-b, 1957a,b), Nakajima (1960, 1964), and Hatano and Tazawa (1968); in addition, slime mold actomyosin does not seem to be identical with striated muscle actomyosin.

In its ability to show a sharp and reversible viscosity response to ATP (Figure 3) slime mold actomyosin satisfies the test most often used to identify muscle-like proteins. Our preparations, however, show complex responses to other adenosine nucleotides and have relatively low ATP sensitivity (= A.S. = 30–50% compared with values of 100–200% for striated muscle actomyosin; Portzehl *et al.*, 1950). Some of these differences are probably due to the large amount of impurities in our preparations; it should be noted, however, that the equation defining A.S. (Portzehl *et al.*, 1950) makes this number formally insensitive to the degree of contamination if the impurities are relatively nonviscous and do not interact with the actomyosin-like protein. While Hatano and Tazawa (1968) have reported values of A.S. = 100–130% for plasmodial myosin B, the preparations of Nakajima (1960, 1964) gave A.S. = 40–60% and myxomyosin had a comparably low ATP response (A.S. = 25% computed from Figure 9 of Ts'o *et al.*, 1957b). As Nakajima pointed out, low A.S. values have been reported for some smooth muscle actomyosins (Needham and Cawkwell, 1956; Laszt and Hamoir, 1961; Mallin, 1965a,b) but not for all (Huys, 1960; Laszt and Hamoir, 1961; Needham and Williams, 1963; Filo *et al.*, 1963; Ruegg *et al.*, 1965; Barany *et al.*, 1966). Since Weber and von Kerekjarto (1952) found the ATP sensitivity of reconstituted striated muscle actomyosin to vary with myosin:actin ratio, it is possible that the differences in A. S. values for the various actomyosins reflect differences in the *in vivo* myosin:actin ratios and/or in the recoveries after purification of the two components, rather than distinct properties of the actin- or myosin-like species. As discussed below, we suggest that slime mold actomyosin has a low myosin:actin ratio when compared with rabbit striated muscle actomyosin.

The idea (Nakajima, 1960) that slime mold actomyosin is more similar to smooth than to striated muscle actomyosin is consistent with the ATPase data presented here. Potassium activation of the Ca^{2+}–ATPase (*cf.* Table III) has been reported for several smooth muscle actomyosins (Needham and Cawkwell, 1956; Needham and Williams, 1963; Filo *et al.*, 1963; Ruegg *et al.*, 1965; Schirmer, 1965), whereas striated muscle actomyosin is a K^+-inhibited Ca^{2+}–ATPase (Mommaerts and Seraidarian, 1947). Also, the slime mold protein, in analogy with most of the smooth muscle actomyosins, did not show the low ionic strength Mg^{2+} activation characteristic of striated muscle actomyosins. Since the Mg^{2+}–ATPase of the striated muscle protein is observable only when the complex is insoluble and superprecipitates (Maruyama and Gergely, 1962), our findings may merely reflect the solubility properties of slime mold actomyosin.

We have used zone sedimentation in sucrose gradients (Figure 5) to verify that the effect of ATP upon the viscosity (Figure 3) and boundary centrifugation properties (Figure 4) of the slime mold protein is to produce dissociation into actin- and myosin-like components. This sucrose gradient dissociation test should be of general utility in actomyosin studies,[7] especially for work on primitive motile systems. The test is essentially an enzymatic assay; consequently only small amounts of material are required and the test can be applied to rather impure protein fractions. The sucrose gradient assay is a more direct demonstration of dissociation than is the measurement of a change in relative viscosity, especially when electron microscopy can also be used (Figures 2 and 5) to show that fibrous material is present in the "plus ATP" gradient but is clearly resolved from the Ca^{2+}–ATPase activity. However, in view of the relative instability of the actin-like high s material under conditions of zone sedimentation (Figure 5B), it may prove difficult to demonstrate the actin-like component of other primitive actomyosins by the above methods alone. Although the conditions for sedimentation were not chosen to give an accurate measure of the sedimentation constant, the value of $s = 7 \pm 1$ obtained for slime mold myosin is in the range found for various myosins (Holtzer and Lowey, 1959; Johnson and Rowe, 1960; Barany *et al.*, 1966).

Careful examination of Figures 4 and 5 indicates distinct differences between our preparations of slime mold actomyosin and rabbit striated muscle actomyosin. Allowing for

[7] To demonstrate the generality of the test we subjected rabbit striated muscle actomyosin to centrifugation under the conditions of Figure 5A, but for 1 hr only. In the absence of ATP all Ca^{2+}–ATPase sedimented more rapidly and heterogeneously than was the case with slime mold actomyosin. When ATP was present in the applied zone, all Ca^{2+}–ATPase sedimented as the expected sharp low s peak.

the slowly sedimenting impurities (lower traces of Figure 4) it is clear that much less mass is transferred to the low *s* region upon addition of ATP (upper traces of Figure 4) than would be expected if the remaining high *s* peak represents the myosin-free actin from an actomyosin containing, on a mass basis, approximately three parts myosin and one part actin (Szent-Györgyi, 1947; Tonomura *et al.*, 1962). The experiments carried out on the desalted actomyosin (Figures 7 and 8 and Results) indicate that most of the high *s* material is reversibly converted into a low molecular weight species at low ionic strength. Data presented in the following paper further verify the actin-like nature of this material and allow us to estimate that the myosin:actin ratio (mass basis) of crude slime mold actomyosin is between 1:1 and 1:3. In preliminary experiments we have demonstrated by boundary centrifugation that rabbit myosin will form an ATP-dissociable complex with the slime mold actomyosin, again suggesting that the latter is myosin-poor actomyosin. Since our studies on crude extracts indicate we have maximized extraction of both actin- and myosin-like components and because a very conservative salt fractionation was carried out (Table II and Results), we suggest that the slime mold contains an excess of actin over myosin, as compared with rabbit striated muscle. A similar suggestion was made by Hatano and Tazawa (1968).

Slime mold actomyosin prepared by our procedure is soluble at low ionic strength, and in this respect our results agree with those of Ts'o *et al.* (1956a,b, 1957a,b) on myxomyosin. Since Nakajima's (1960, 1964) preparations of plasmodial myosin B were insoluble at low ionic strength, we prepared extracts according to his procedure. Attempts to duplicate his dilution precipitation were unsuccessful but by dialyzing the extracts to low ionic strength (pH $\leq$6.5) we obtained precipitates which contained the bulk of the Ca^{2+}–ATPase in the crude extract. This material was very difficult to resuspend and even after thorough homogenization at high ionic strength it was present in the form of large aggregates. Hatano and Tazawa (1968) reported similar difficulties but found that by including EDTA in the extraction medium it was possible to prepare a plasmodial myosin B which was insoluble at low ionic strength and dissolved readily at higher salt concentrations. Low ionic strength insolubility is, of course, not a property specific to actomyosin:DNA–protein complexes are also precipitated at lowered salt concentrations (Allfrey, 1959) and, since prolonged extraction with alkaline salt solutions tends to solubilize nuclear contents (Allfrey, 1959), the solubility properties of impure actomyosins prepared using such extraction conditions should be viewed with caution.

There is no theoretical reason to expect that all actomyosins will be insoluble at low ionic strength. Several reports have appeared describing tonoactomyosins (Laszt and Hamoir, 1961), actomyosins prepared from smooth muscle, which are considerably more soluble at low ionic strength than is rabbit striated muscle actomyosin (Huys, 1961; Filo *et al.*, 1963; Needham and Williams, 1963; Needham and Shoenberg, 1964; Ruegg *et al.*, 1965; Schirmer, 1965; Shoenberg *et al.*, 1966). Since acto heavy meromyosin is soluble at low salt concentration it might be expected that a complex of actin with a soluble myosin would have similar solubility properties. As will be shown in the following paper, slime mold myosin is soluble at low ionic strength.

In view of the well-known solubilizing effect of ATP, PP_i, and other polyanions on muscle myosin and actomyosin (Szent-Györgyi, 1947; Brahms and Brezner, 1961) and the claim (Corsi *et al.*, 1962) that very prolonged dialysis is required to reverse the PP_i solubilization, we considered the possibility that our use of a pyrophosphate-rich extraction medium had increased the low ionic strength solubility of the slime mold actomyosin. We therefore subjected plasmodial extracts prepared without pyrophosphate (extraction buffer no. 1 of Table I) to ammonium sulfate fractionation (Table II). The second salt fraction was dissolved in 0.01 M Tris (pH 7.8) and clarified according to the details of Table II: the resulting solutions contained protein and ATPase at levels similar to those obtained with our standard preparations and were no more turbid than the latter. In one experiment a standard TM–PP_i extract and a high salt extract (Table I, 1) were fractionated according to Table II and aliquots of the resulting actoymosins were dialyzed ~24 hr against high, moderate and low ionic strength solutions (buffers of Figure 4A–C, respectively). All dialyzed aliquots showed little turbidity and had similar ATPase and protein contents. However, when the samples in 0.05 M KCl (pH 6.8) were centrifuged ~20′-27K-No. 30 (85,000), the standard actomyosin was nearly unchanged whereas about 50% of the protein and ATPase pelleted from the other sample. It is possible therefore that our use of a pyrophosphate-rich extraction solution reduces the tendency of the actomyosin to aggregate at low ionic strength. Finally, in an attempt to gain information on the solubility of slime mold actomyosin under "*in vivo*" conditions, we carried out several experiments in which freshly harvested unwashed plasmodium was subject to high-speed centrifugation without prior homogenization. The "cell sap" thus squeezed out was found to contain the RNase-insensitive ~20S peak we consider to be primarily actin-like material. Such "extracts" also contained appreciable Ca^{2+}–ATPase activity the bulk of which obeyed the sucrose gradient dissociation test. On a specific activity basis the enzyme content was fairly low in extracts prepared at very high speed (20′-60K-SW 65 [350,000]) but was considerably higher (~0.16 μM P_i/mg per 15 min) when a lower *g* force was used (30′-27K-No. 30 [85,000]). These experiments therefore also suggest that slime mold actomyosin is soluble under ionic conditions approximating those of the living plasmodium but that the actomyosin may be highly aggregated.

Our use of gel chromatography at low ionic strength (Figure 8) to separate the actin- and myosin-like components of slime mold actomyosin may prove to be of general usefulness in studies of primitive motile systems, especially since differential extraction of the myosin may be impossible when, as is probably the case in the slime mold, the actin is not immobilized in an organized structure like the Z line complex of striated muscle. Our procedure also eliminates the use of an acetone precipitation step in the preparation of actin. Although only relatively small amounts of protein can be handled in a single cycle of the procedure, this is unlikely to be a major drawback in studies of other nonmuscle actomyosins and it is at least partially compensated by the short time required for the separation. It is possible that the procedure will only be successful when used with actomyosins which are soluble at low ionic strength, but it should be pointed out that the myosin component is present at a low concentration in the presence of 1 mM ATP and it is not unreasonable to expect other myosins to be soluble under these conditions (Szent-Györgyi, 1947; Brahms and Brezner, 1961).

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