Further Purification and Characterization of Slime Mold Myosin and Slime Mold Actin*

M. R. Adelman† and Edwin W. Taylor‡

ABSTRACT: Starting with the portion of partially purified slime mold actomyosin which was voided from Sephadex G-200 at low ionic strength slime mold myosin was further purified by DEAE-cellulose chromatography and salt fractionation with ammonium sulfate. Slime mold myosin had about three times the specific Ca^{2+}–adenosine triphosphatase activity of rabbit myosin assayed under identical conditions. The enzyme was free of nucleic acids and nearly all material present sedimented as a single species with $s^0_{20,w}$ = 6.40 S in 0.50 M KCl at pH 7.4. By gel chromatography on Sepharose 4B the enzyme was found to have a high particle asymmetry with a diffusion coefficient similar to rabbit myosin An approximate molecular weight of 4.6×10^5 was obtained. Slime mold myosin formed an actomyosin complex with rabbit actin and was similar to rabbit myosin in some of its enzymatic properties. The main differences from the rabbit enzyme were its solubility and low degree of aggregation at low ionic strength and the lack of a strong magnesium inhibition of the adenosine triphosphatase activity. Slime mold actin was prepared from the protein peak which was retarded on the G-200 column. The material was first polymerized by the addition of KCl and $MgCl_2$ and separated by high-speed centrifugation. At low ionic strength slime mold actin existed as a low molecular weight protein with $s^0_{20,w}$ = 3.2 S. In the presence of salt the actin formed rapidly sedimenting asymmetric particles: several schlieren boundaries were present. Slime mold actin had adenosine triphosphate binding properties similar to muscle actin, formed an actomyosin complex with rabbit myosin, and activated the Mg^{2+}–adenosine triphosphatase activity of the mammalian enzyme at low ionic strength.

In the preceding paper (Adelman and Taylor, 1969) we reported the isolation of an actomyosin-like protein from the plasmodium of the acellular slime mold, *Physarum polycephalum*. It was shown that the actomyosin could be resolved into actin- and myosin-like components by column chromatography on Sephadex G-200 at low ionic strength. In this paper we describe the further purification and characterization of slime mold myosin and slime mold actin.

Materials and Methods

The culturing and harvesting of slime mold plasmodium was described in the preceding paper (Adelman and Taylor, 1969). An outline of the over-all purification scheme is presented in Figure 1. The steps leading to the fractions designated G-200 actin and G-200 enzyme have been described (Adelman and Taylor, 1969) as have the following: protein and Ca^{2+}–ATPase assays, viscometry, electron microscopy, measurement of radioisotopes, and general aspects of salt fractionation and column chromatography.

Ion-Exchange Resins. Bio-Rad DEAE-cellulose (Calbiochem), DEAE-Sephadex A-25 (Pharmacia), and Whatman DE-32 grade DEAE-cellulose (Reeve Angel) were prepared according to the manufacturers' instructions. Columns were packed under 10–20-cm pressure heads and thoroughly flushed with starting buffer. Low flow rates, both for application of protein solutions and for subsequent elution, were maintained by use of a pump. Linearity of salt gradients used for elution was checked by monitoring effluent fractions refractometrically with a water-jacketed (T = 22°) Zeiss refractometer.

Centrifugation. Preparative centrifugations—always at 3°—are described using the notation (*e.g.*) 30′-27K-#30 (85,000) to denote a 30-min spin at 27,000 rpm in the Spinco No. 30 rotor for which conditions the maximum g force is ~85,000g. Extrapolated s values were determined using a Spinco Model E equipped with schlieren optics, an RTIC unit, and electronic speed control. Double-sector, 12-mm, filled-Epon centerpieces were used. Dialyzed stock protein solutions were diluted volumetrically with their dialysates. Runs were at 20°; standard computational techniques (Schachman, 1959) were used.

Miscellaneous. Rabbit myosin and actin were prepared as described by Finlayson and Taylor (1969) and Finlayson *et al.* (1969). Heavy meromyosin was prepared essentially according to Young *et al.* (1965) except that the tryptic digestion was for 90 sec and the final purification on Sephadex G-200 was omitted. Spectrophotometry, pH measurements, and the sources of most reagents have been described (Adelman and Taylor, 1969). NaATP, -ADP, -AMP, and -ITP were obtained from Nutritional Biochemicals Corp., NaGTP from P & L Biochemicals, Inc., and [^{3}H]ATP from Schwarz BioResearch, Inc. We thank Dr. Jiro Suzuki for [γ-^{32}P]ATP; Dr. Robert Haselkorn provided samples of tobacco mosaic virus and turnip yellow mosaic virus.

* From the Department of Biophysics, University of Chicago, Chicago, Illinois. *Received July 11, 1969.* M. R. A. was supported by a National Science Foundation Graduate Fellowship, The James Franck Dissertation Fellowship, and U. S. Public Health Service Training Grant GM 780. This research was supported by a U. S. Public Health Service Research Grant GM 10992 and the Life Insurance Medical Research Fund.

† Present address: The Rockefeller University, New York, N. Y. 10021.

‡ Address inquiries to the Department of Biophysics, Chicago, Ill. 60637.

Washed plasmodium

↓ Homogenize in one volume 0.05 M $Na_4P_2O_7$–0.01 M Tris-maleate (pH 6.8). Clarify 45-27K-#30 (85,000)

Crude extract

↓ Salt fractionation with $(NH_4)_2SO_4$, 0–45% at pH 6.5, then 0–35% at pH 6.0. Resuspend in 0.01 M Tris (pH 7.8) and clarify 30'-27K-#30 (85,000)

Crude actomyosin

↓ Column chromatography on Sephadex G-25 in TAM buffer

Desalted actomyosin

Column chromatography on Sephadex G-200 in TAM

Voided peak = G-200 enzyme	Retarded peak = G-200 actin
↓ step elution from DEAE-cellulose	↓ Clarify 30'-27K-#30 (85,000), add salt to final conditions: 0.15 M KCl–0.01 M Tris-maleate–0.01 M $MgCl_2$ at pH 6.1–6.2. Store 1 hr in cold. Pellet 2–3 hr [60K-#65 (314,000)]
DEAE–enzyme	
↓ salt fractionation: 45–60% saturation with $(NH_4)_2SO_4$ at pH 6.5	
Slime mold myosin	Slime mold actin

FIGURE 1: Outline of procedure for purification of slime mold myosin and slime mold actin.

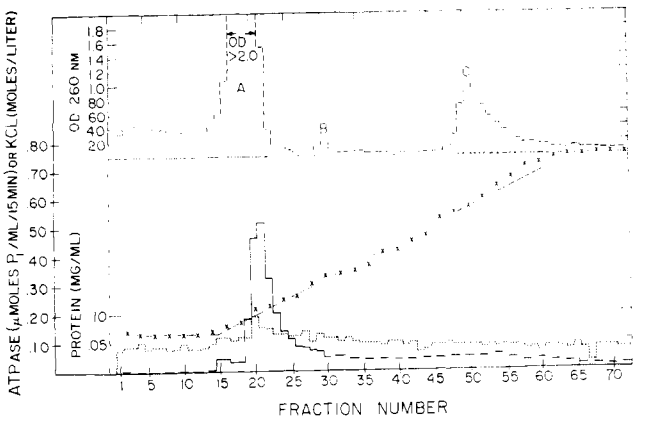

FIGURE 2: Gradient elution of slime mold myosin from DEAE-cellulose. The column (1.5 × 23 cm) contained DEAE-cellulose (Bio-Rad, 0.7 mequiv/g) equilibrated with 1 mM Tris (pH 8). The G-200 enzyme aliquot (18 ml) contained 0.83 mg of protein/ml with a specific ATPase activity of 1.6 μM P_i/mg per 15 min and was applied at a flow rate of ~20 ml/hr. The column was washed with two bed volumes of 0.125 M KCl–0.01 M Tris (pH 7.8) at ~20 ml/hr and then eluted with a 200-ml salt gradient [0.125 M KCl–1.0 M KCl, both in 0.01 M Tris (pH 7.8)] at ~9 ml/hr. Fractions (2.5 ml) were assayed for ultraviolet absorption at 260 nm (– – – –), protein content (· · · ·), ATPase activity (——), and refractive index (X—X) plotted as equivalent KCl concentration. The protein concentration in the fractions was quite low and since the Tris buffer made a relatively large contribution (~0.037 mg/ml) to the assayed protein this background was not subtracted from the data plotted here. Ultraviolet spectra of zones A, B, and C gave OD_{260nm}/OD_{280nm} ratios of 6.5, 2.5, and 2.0, respectively. Zones A and C were assumed to be ATP (present in the applied G-200 enzyme) and nucleic acids, respectively; zone B was reproducible but was not identified. The input enzyme for this experiment came from a preparation in which the desalted actomyosin (Figure 1) was clarified 60K-#65 (314,000) prior to G-200 chromatography. This high-speed centrifugation was used in a few experiments but was eliminated from the standard procedure because it resulted in a variable but often large loss of ATPase activity. The loss was apparently due to slow aggregation of the enzyme as the buffer ATP was hydrolyzed; addition of excess ATP prevented the loss but caused some actin to repolymerize.

Results

A. Slime Mold Myosin. Several attempts were made to purify the G-200 enzyme by gradient elution on columns of DEAE-cellulose or DEAE-Sephadex; the results of one such experiment are shown in Figure 2. In all cases, even when the salt gradient was begun at zero salt concentration, no activity was detected in the eluate until the concentration reached approximately 0.15 M KCl. The enzyme profile then rose sharply, peaked at about 0.20 M KCl, and trailed off in a shoulder which extended out to high salt concentrations. [Similar profiles have been obtained in the chromatography of rabbit myosin on DEAE-cellulose (Asai, 1963)]. Although this "tailing" was minimized by low elution rates and was less pronounced on DEAE-Sephadex than on DEAE-cellulose, a significant fraction (*ca.* 25%) of the recovered activity was spread out in this shoulder. Usually only 30–50% of the input Ca^{2+}–ATPase was recovered. Because of difficulties in accurately assaying small amounts of protein in some of the effluent fractions (see Figure 2) the estimates of purification obtained by the column elution were subject to large error. However, the peak fractions generally had a specific ATPase activity about three to five times that of the input. Despite this considerable purification it was not feasible routinely to purify the G-200 enzyme by gradient elution because the recoveries were so low and because even the peak effluent fractions contained protein at such a low concentration (C = <0.05 mg/ml).

A more satisfactory routine purification of the G-200 enzyme was obtained using DEAE-cellulose under conditions approximating batch procedures; Table I gives the details of a representative experiment. As a precaution against possible aggregation excess ATP (1 mM) was added to the G-200 enzyme which was then applied to a small column of resin. Under these conditions of high sample volume–bed volume ratio (normally ~3:1) the columns were loaded to about three-fourths of their capacity as judged by the yellow coloration due to binding of the pigment in the input. As expected, the salt concentrations appropriate for step elution of the enzyme were found to be slightly different from those suggested by Figure 2. The columns were first washed with 0.05 M KCl–0.01 M Tris (pH 7.8), and the enzyme was then eluted, at a low flow rate, with 0.175 M KCl–0.01 M Tris (pH 7.8). Normally more than 80% of the input Ca^{2+}–ATPase was recovered; the elution profile was sufficiently sharp that a few fractions corresponded with 70–80% of the input and these were pooled for further use. The DEAE–enzyme thus obtained was two to three times as concentrated (in terms of enzyme units per milliliter) as the input and had nearly twice as high a specific ATPase activity. Nucleic acid contaminants (zone C in Figure 2) were removed by this batch treatment with DEAE-cellulose.

The final step in the purification of slime mold myosin was a refractionation by salt precipitation with ammonium sulfate. On the basis of the two experiments indicated in Table II, the use of 45–60% saturation limits was adopted as routine (Figure 1). Normally about 60% of the DEAE-cellulose-purified enzyme used for the final salt fractionation was recovered, with 45% (75% of the recovery) in the 45–60% fraction. The final preparations, designated slime mold myosin, were always centrifuged at high speed [30'-60K-#65 (314,000)], to assure removal of any aggregates, and recoveries refer to material thus clarified. Slime mold myosin had a specific activity nearly twice that of the input DEAE–enzyme.

TABLE I: Purification of G-200 Enzyme by Step Elution from DEAE-cellulose.[a]

Step	Effluent Contents: Total Protein (mg)	Effluent Contents: Total ATPase (μM P_i/15 min)	% Applied ATPase
Application of G-200 enzyme	~1	Negligible	
Elution with 0.05 M KCl–0.01 M Tris (pH 7.8)	9	<2	
Elution with 0.175 M KCl–0.01 M Tris (pH 7.8)	51	400	79

[a] The applied sample consisted of 135 ml of G-200 enzyme (0.86 mg/ml; Ca^{2+}–ATPase activity = 4.4 μM P_i/mg per 15 min) plus 1.3 ml (130 μM) ATP. The column was a 3.5 cm diameter × 5 cm high bed of Whatman, DE-32 DEAE-cellulose equilibrated with 0.01 M Tris (pH 7.8). The enzyme was applied at a flow rate of ~120 ml/hr and the column was then eluted with 100 ml of 0.05 M KCl–0.01 M Tris (pH 7.8) at a flow rate of ~60 ml/hr. The enzyme was eluted with 100 ml of 0.175 M KCl–0.01 M Tris (pH 7.8), at a flow rate of ~30 ml/hr. Fractions (22 ml for first two stages, 10.8 ml for enzyme elution) were collected and assayed for protein content and ATPase activity. Since there was still measurable enzyme activity in the final fraction, the 79% recovery is a slight underestimate. Fractions pooled as DEAE–enzyme constituted a total volume of ~43 ml containing ~1.09 mg of protein/ml with a specific ATPase activity of ~7.87 μM P_i/mg per 15 min.

As a summary Table III is presented here: it gives the protein and Ca^{2+}–ATPase figures assayed at each stage of a large-scale purification. With the exception of the spurious high recovery of ATPase in salt fraction I, the data in Table III are fairly representative. Normally 15–20% of the ATPase in the plasmodial extract was recovered as slime mold myosin with an approximately 25-fold increase in specific activity; about 10 mg of enzyme was obtained from 100 g of washed plasmodium. All final preparations of slime mold myosin had specific Ca^{2+}–ATPase activities between 10 and 15 μM P_i per mg per 15 min; the slime mold enzyme was about three times as active as rabbit myosin (4.2–4.8 μM P_i/mg per 15 min for various preparations) assayed under identical conditions.

Solutions of slime mold myosin were colorless and water clear. The ultraviolet spectrum (Figure 3) was that of a fairly typical protein: there was a maximum at 278 nm, a minimum at 250 nm, and a shoulder at 290 nm. The OD_{280nm}/OD_{260nm} ratios observed (1.6–1.7) suggested little nucleic

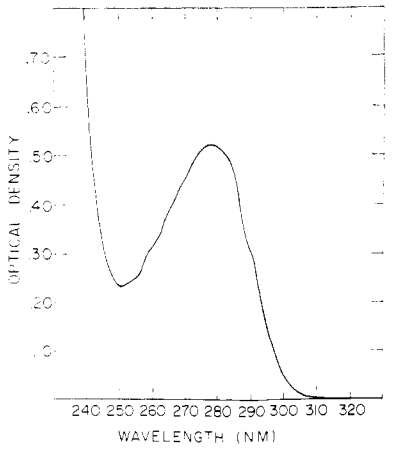

FIGURE 3: Ultraviolet absorption spectrum of slime mold myosin. Protein in 0.50 M KCl–0.01 M Tris (pH 7.4) at a concentration of 1.0 mg/ml. An OD_{320nm} of 0.035 relative to dialysate was subtracted as a turbidity correction; OD_{280nm}/OD_{260nm} = 1.66.

TABLE II: Salt Fractionation of DEAE–Enzyme.[a]

	Fractionation Limits (% Saturation)	Protein (mg)	Ca^{2+}–ATPase Activity (μM P_i/mg per 15 min)	Recovery of Input ATPase (%)
Expt 1	0–40	8.85	2.38	6.3
	40–50	5.92	7.58	13.5
	50–66	9.80	13.9	41.0
Expt 2	0–45	10.9	3.78	12.7
	45–60[b]	10.0	15.1	45.9

[a] In each case fractionation was carried out with saturated $(NH_4)_2SO_4$ at pH 6.5. The appropriate volume of salt solution was added, the mixture was left standing for ~30 min, and the precipitate was collected by centrifugation: 30′-27K-#30 (85,000g). The supernatant was then submitted to further fractionation in a similar manner. Pellets were resuspended in 0.50 M KCl–0.01 M Tris (pH 7.4), dialyzed overnight *vs.* the same buffer, and clarified 30′-60K-#65 (314,000g). For expt 1 the input consisted of 39 ml of DEAE-enzyme containing 42.5 mg of protein with a specific ATPase activity of 7.9 μM P_i/mg per 15 min. For expt 2 the input consisted of 41 ml of DEAE–enzyme containing 41 mg of protein with a specific ATPase activity of 7.9 μM P_i/mg per 15 min. [b] After this fraction was pelleted the supernatant was brought to 75% saturation but no further precipitate was formed.

TABLE III: Recoveries of Protein and ATPase During Large-Scale Purification of Slime Mold Myosin.[a]

Stage	Volume (ml)	Protein (mg/ml)	Ca^{2+}–ATPase (μM P_i/mg per 15 min)	Total Protein (mg/100 g of washed plasmodium)	Total ATPase (μM P_i/15 min per 100 g of washed plasmodium)
Crude extract	175	12.3	0.42	1845	775
Salt fraction I	43.5 (42.5)	25.5	0.88	951	837
Salt fraction II (crude actomyosin)	64 (62.5)	9.6	1.06	539	571
Desalted actomyosin	101 (91)	5.25	1.04	476	495
G-200 Enzyme	146 (140)	0.75	3.75	110	413
DEAE-enzyme	42.8 (41)	1.01	7.92	44.7	354
45–60% Fraction (slime mold myosin)	4	2.50	15.1	10.9	165

[a] The figures given are for a typical large-scale purification. Normally 130–140 g of plasmodium was used and of the resulting crude extract 175 ml was submitted to further fractionation. Volumes indicated are those measured (before dialyses) for entire fraction; volume figures in parentheses indicate amounts actually used in the next step in the procedure. Protein content and ATPase activities of desalted actomyosin, G-200 enzyme, and DEAE-enzyme were determined on pooled column effluents without dialyses. Other figures were determined on samples dialyzed overnight *vs.* 0.50 M KCl–0.01 M Tris (pH 7.8) (pH 7.4 for final enzyme fraction). The total protein and total ATPase figures are normalized to a hypothetical input of 100 g of mold assuming 1.5 ml of crude extract/g of washed plasmodium and taking account of the small aliquots removed at each step for analyses.

acid contamination. Examination in the analytical ultracentrifuge (Figure 4) showed that most material sedimented as a single boundary with $s_{20,w}$ of 5–6. There was always some more slowly sedimenting material but, in the best preparations, this impurity was estimated as considerably less than 25%. Figure 5 presents the variation, with protein concentration, of $s_{20,w}$ for slime mold myosin in 0.50 M KCl–0.01 M Tris (pH 7.4). The extrapolated value of 6.38, coupled with values of 6.39, and 6.45 obtained with different enzyme preparations under similar conditions, indicates as $s^0_{20,w}$ of 6.40 for slime mold myosin.[1] For purposes of comparison we dialyzed rabbit myosin *vs.* the KCl–Tris solvent, centrifuged the preparation 30′-60K-#65 (314,000) and, under the conditions of Figure 5, found $s^0_{20,w} = 6.0$.

As shown by the upper trace of Figure 4B there was little sign of large-scale aggregation when slime mold myosin was dialyzed *vs.* 0.15 M KCl at pH 6.8, although the slight increase in s value might indicate dimerization. In another experiment enzyme previously dialyzed *vs.* 0.50 M KCl–0.01 M Tris (pH 7.8) was dialyzed *vs.* 0.075 M KCl at pH 7.8: again there was no large-scale aggregation but a slight increase in s value. In a third experiment samples of slime mold myosin were dialyzed *vs.* 0.01 M Tris (pH 7.8) containing, respectively, 0.00, 0.05, and 0.50 M KCl. The three samples had identical ATPase activities. The solution at high ionic strength showed only a "monomer" boundary, but the low ionic strength samples had, in addition, a second boundary with $s_{20,w} \sim 9$–10. This faster species accounted for about half the material in the sample at 0.00 M KCl but there was no sign of larger aggregates.

As a possibility for further purification, we examined the elution of the enzyme from a column of Sepharose 4B. The results are presented in Figure 6, which also indicates the elution positions of several other macromolecules. The data in Figure 6 were treated according to the theory of Ackers (1964) so as to obtain an estimate of the Stokes radius, "a," and therefrom the diffusion coefficient of slime mold myosin. The elution positions of tobacco mosaic virus and inorganic phosphate were assumed to be at $K_D = 0$ and $K_D = 1$, respectively. The measured values of K_D and the known values of "a" for catalase and turnip yellow mosaic virus were used to compute independent estimates of r, the column calibration parameter in Ackers' (1964) representation. For catalase $K_D = 0.718$, $a = 52$ Å (Siegel and Monty, 1966), and $r = 688$ Å. For TYMV $K_D = 0.325$, $a = 143$ Å [computed from data in DeRosier (1965) and DeRosier and Haselkorn (1966)], $r = 627$ Å. For the following computations the mean value, $r = 658$ Å, was used.

Rabbit myosin eluted from the Sepharose column at $K_D = 0.219$ from which a Stokes radius of 192 Å was derived. The equivalent diffusion coefficient, $D_{20,w} = 1.12 \times 10^{-7}$ cm²/sec, was in good agreement with the value of 1.05×10^{-7} cm²/sec reported by Parrish and Mommaerts (1954). Rabbit heavy meromyosin eluted at $K_D = 0.402$ from which $a = 125$ Å and $D_{20,w} = 1.72 \times 10^{-7}$ cm²/sec were computed. The accuracy of the latter may be appreciated by substituting it, along with an assumed $\bar{v} = 0.73$, and $s^0_{20,w} = 7.0$–7.2 (Szent-Györgyi, 1953; Lowey and Holtzer, 1959) into the Svedberg equation: the molecular weight of 367,000–377,000 thus obtained is in reasonable agreement with the currently accepted range of figures (Gergely, 1966).

[1] The preliminary value of $s^0_{20,w} = 7.0$ obtained in an early experiment (Adelman *et al.*, 1968) was not reproduced in any of the later preparations and may have been due to faulty temperature regulation of the model E.

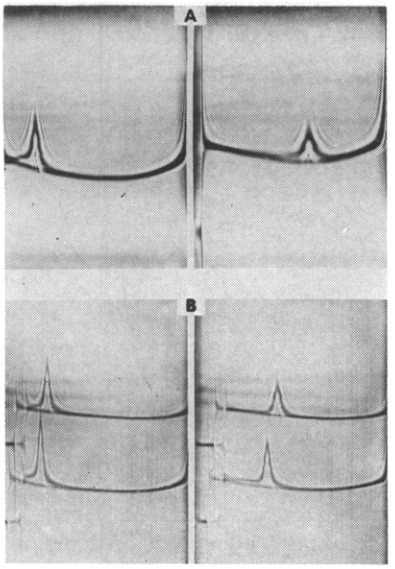

FIGURE 4: Boundary centrifugation of slime mold myosin. (A) Enzyme at 2.0 mg/ml in 0.50 M KCl–0.01 M Tris (pH 7.4). Sedimentation at 52,000 rpm, 20°. Pictures were taken ~42 and 122 min after maximum speed was reached, at phase-plate angles of 45 and 40°, respectively; $s_{20,w}$ = 5.99. (B) Both traces represent enzyme from the same preparation (different from that shown in A). Upper trace: protein at 2.0 mg/ml in 0.15 M KCl–0.01 M Tris-maleate (pH 6.8); $s_{20,w}$ = 6.37. Lower trace: protein at 2.5 mg/ml in 0.50 M KCl–0.01 M Tris-maleate (pH 6.8); $s_{20,w}$ = 5.6. Sedimentation at 52,000 rpm, 20°. Pictures were taken ~26 and 58 min after maximum speed was reached, at a phase-plate angle of 60°. The apparently greater amount of slowly sedimenting material in the lower trace (*cf.* upper) may be due to the somewhat higher protein concentration and the greater mismatch of solvent and solution menisci.

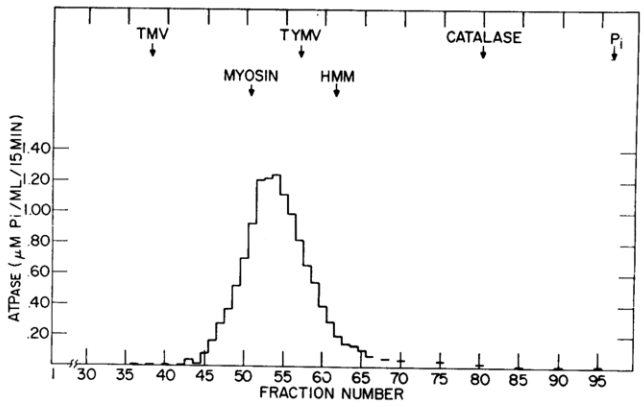

FIGURE 6: Gel filtration of slime mold myosin on Sepharose 4B. The column (2.5 × 39.5 cm) was equilibrated and eluted with 0.50 M KCl–0.01 M Tris–5 mM β-mercaptoethanol (pH 7.4). The applied zone (2.1 ml) contained 3.6 mg of enzyme, with a specific activity of 10.6 μM P_i/mg per 15 min, in 0.50 M KCl–0.01 M Tris (pH 7.4) plus 2% (w/v) sucrose. Fractions of 2.1 ml were eluted at a flow rate of 12 ml/hr and aliquots were assayed for Ca^{2+}–ATPase. Recovery of enzymatic activity was 68%. The protein content was too low and buffer background too high to allow a meaningful determination of protein distribution. The arrows indicate the elution positions of tobacco mosaic virus (TMV), turnip yellow mosaic virus (TYMV), catalase, P_i, rabbit myosin, and rabbit heavy meromyosin (HMM). For all marker runs conditions were identical with those above except that β-mercaptoethanol was not included in the buffer. TMV and TYMV were located by OD_{260nm}; catalase was assayed according to Martin and Ames (1961); myosin (64% activity recovered) and heavy meromyosin (33% activity recovered) were located by ATPase assay under standard conditions. When chromatographed in the absence of β-mercaptoethanol the slime mold myosin was recovered at ~12% efficiency in a broad peak centered between fractions 55 and 56.

When slime mold myosin was chromatographed, in 0.50 M KCl–0.01 M Tris (pH 7.4), the activity recovery was very low (Figure 6). Addition of β-mercaptoethanol to the column buffer resulted in much more satisfactory recovery. Slime mold myosin eluted at K_D = 0.268 giving values of a = 171 Å and $D_{20,w} = 1.26 \times 10^{-7}$ cm²/sec. Use of this value for D, along with $s^0_{20,w}$ = 6.40 and $\bar{v}$ = 0.73, in the Svedberg equation indicates the molecular weight of the slime mold enzyme to be 458,000.

Slime mold myosin was fairly stable: samples stored in ice at C = 1–3 mg/ml lost only 10–20% of their Ca^{2+}–ATPase activity in the first 2 or 3 days. The samples, referred to above, in 0.00, 0.05, and 0.50 M KCl at pH 7.8 showed no differences in their slow loss of activity. In one experiment a sample stored at 0° in 0.50 M KCl at pH 7.8 retained 75% of its activity after 5 days; when examined in the model E after a further 4-day storage the preparation gave a major boundary

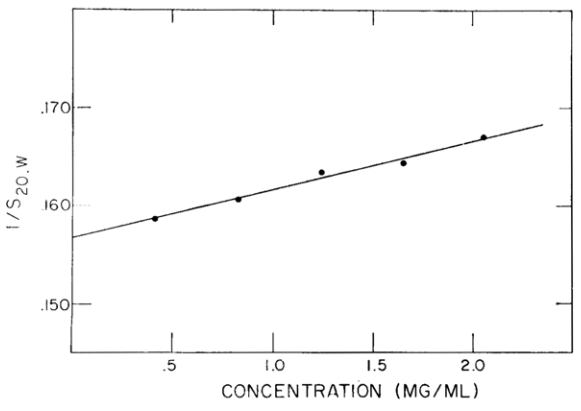

FIGURE 5: Concentration dependence of $s_{20,w}$ of slime mold myosin. The protein was in 0.50 M KCl–0.01 M Tris (pH 7.4). Centrifugation was at 20°; 52,000 rpm; $s^0_{20,w}$ = 6.38.

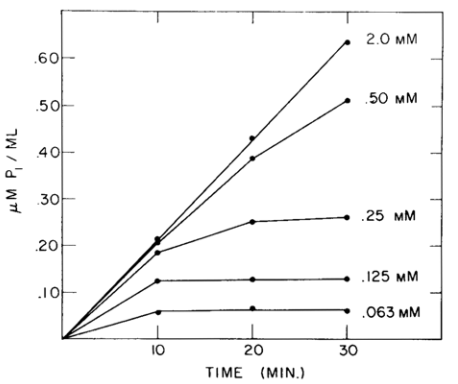

FIGURE 7: ATPase activity of slime mold myosin. The time course of P_i liberation is shown for a series of ATP concentrations indicated to the right of the respective curves. Assays were carried out under standard conditions of Ca^{2+} activation with slime mold myosin at a concentration of 0.026 mg/ml.

TABLE IV: Substrate Specificity of Slime Mold Myosin.[a]

Substrate	Activity (μM P_i/mg per 15 min)
ATP	10.3
ITP	6.89
GTP	3.11
ADP	(1.46)[b]
AMP	<0.073
PP_i	<0.024[c]

[a] Assays were at room temperature in 0.50 M KCl–0.05 M Tris–0.01 M $CaCl_2$ (pH 8.0). All substrates were present at a final concentration of 2 mM. [b] P_i liberation was nonlinear with time and plateaued at a level corresponding with 0.05 μmole of phosphate/ml. This was assumed to be due to *ca.* 2–3% contamination of the ADP with ATP. [c] A precipitate (possibly calcium pyrophosphate) formed during the assay.

with $s_{20,w}$ = 5.7 and an increased amount of slowly sedimenting material but showed no signs of formation of rapidly migrating boundaries.

The data of Figure 7 demonstrate that, in the presence of Ca^{2+} at pH 8, slime mold myosin hydrolyzed only the terminal phosphate of ATP. The enzyme appeared to be substrate saturated at such low levels of ATP that it was not possible, using the chemical assay for P_i, to estimate an apparent K_M. Measurements using the actomyosin (data not shown) indicated the ATPase activity was constant over a range of ATP concentration from below 1 mM to at least 10 mM. Other nucleoside triphosphates were hydrolyzed by slime mold myosin, although at lower rates than was ATP (Table IV). ADP, AMP, and PP_i were not hydrolyzed. An Arrhenius plot of the temperature dependence of the Ca^{2+}–ATPase (Figure 8) gave an energy of activation of about 12 kcal/mole; Barany (1967) reported a similar value for cat myosin determined under comparable conditions.

TABLE V: Dependence of ATPase Activity of Slime Mold and Rabbit Myosins on Divalent Ions.[a]

Addition	ATPase Activity (μM P_i/mg per 15 min)		
	Slime Mold Myosin		Rabbit Myosin
	Expt 1	Expt 2	
No added divalent ion	0.79	0.79	3.14
Ca^{2+}	13.0	10.8	4.16
Mg^{2+}	0.49	0.49	0.029
EDTA	0.49	0.47	26.6

[a] Assays were at room temperature in 0.50 M KCl–0.05 M Tris (pH 8.0). ATP was present at 2 mM final concentration. Additions, as indicated, were: Ca^{2+}, 10 mM $CaCl_2$; Mg^{2+}, 10 mM $MgCl_2$; and EDTA, 1 mM Na_2EDTA.

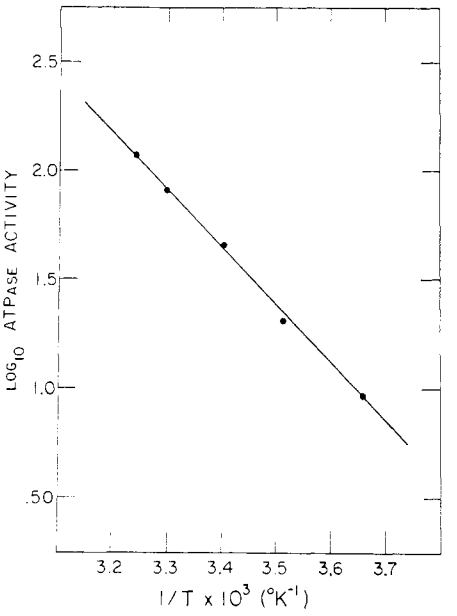

FIGURE 8: Arrhenius plot of the temperature dependence of slime mold myosin Ca^{2+}–ATPase activity. Log activity values are in arbitrary units: the specific activity at room temperature (~21°) was 10.3 μM P_i/mg per 15 min. The slope of the line indicates an energy of activation of ~12.1 kcal/mole; ΔH^* at 20° is therefore about 11.5 kcal/mole.

A comparison of the pH dependence of slime mold and rabbit myosins assayed under similar conditions is presented in Figure 9. While the slime mold enzyme showed a profile somewhat like the rabbit enzyme, its acid optimum was less pronounced and the trough was at pH ~6–6.5 as compared with the well-known minimum at ~pH 7.5 for rabbit myosin.

The requirements of the enzyme for divalent ions are given in Table V. The slime mold myosin was strongly activated by Ca^{2+} and showed a Ca:Mg activity ratio of about 25. The enzyme was not activated by EDTA, although under identical assay conditions, EDTA activation of rabbit myosin was evident.

As judged by analytical ultracentrifugation slime mold myosin formed an ATP-dissociable actomyosin-like complex with

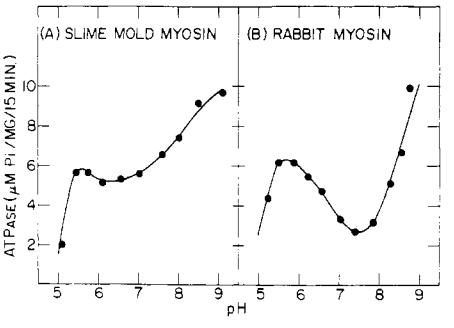

FIGURE 9: Comparison of the pH dependence of the ATPase activity of slime mold and rabbit myosins. (A) Slime mold myosin. Assays at 23° (water bath) in 0.50 M KCl, 10 mM $CaCl_2$, 2 mM ATP, and 50 mM Tris-maleate buffer at all pH values. The activity of this preparation was ~9.6 μM/mg per 15 min under standard conditions. (B) Rabbit myosin. Assays at room temperature in 0.50 M KCl, 10 mM $CaCl_2$, 2 mM ATP, and 25 mM Tris-maleate buffer at all pH values.

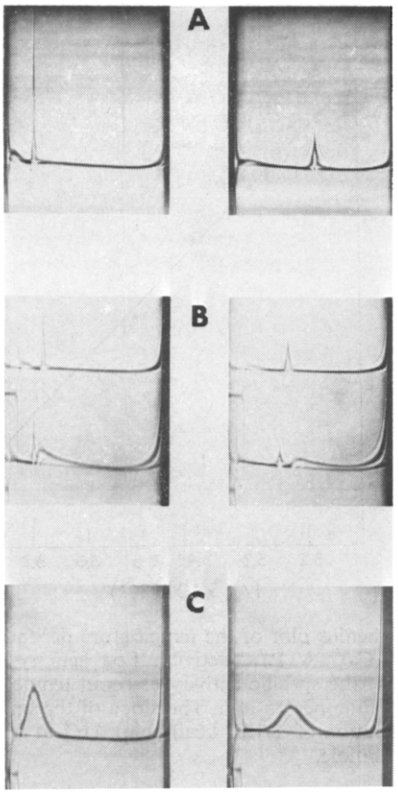

FIGURE 10: Boundary centrifugation of slime mold actin under various conditions. (A) F-actin in 0.15 M KCl–0.01 M Tris-maleate (pH 7.0) at a concentration of 1.7 mg/ml. Pictures were taken, at a phase-plate angle of 55°, 8 and 32 min after the maximum speed of 40,000 rpm was reached; $T = 20°$, $s_{20,w} = 28.5$. (B) Both samples derive from the same preparation of G-200 actin (see text). Upper trace: a standard preparation of F-actin at a concentration of 1.2 mg/ml, $s_{20,w} = 30.2$. Lower trace: a preparation of low-speed actin (polymerized at pH 7) at a concentration of 3.6 mg/ml. $s_{20,w}$ values are 32.4 and ~46. Both actin preparations were in 0.15 M KCl–0.01 M Tris-maleate (pH 7.0). Pictures were taken, at a phase angle of 50°, 8 and 16 min after the maximum speed of 40,000 rpm was reached, $T = 20°$. (C) A preparation of actin in the globular form. Protein was at a concentration of 5 mg/ml in 1 mM Tris–1 mM ATP–5 mM β-mercaptoethanol (pH 7.8). Pictures were taken, at a phase-plate angle of 50°, 52 and 148 min after the maximum speed of 52,000 rpm was reached; $T = 20°$ $s_{20,w} = 2.7$.

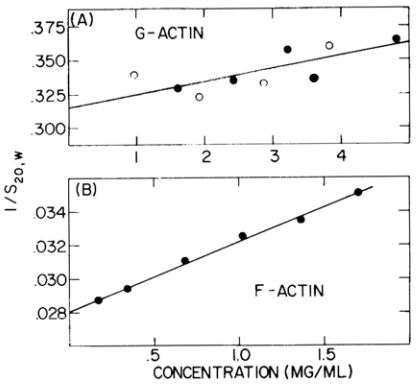

FIGURE 11: Concentration dependence of slime mold actin sedimentation coefficients. (A) Globular slime mold actin in 1 mM Tris–1 mM ATP–5 mM β-mercaptoethanol (pH 7.8). Sedimentation at 52,000 rpm, 20°; (○) Standard preparation, (●) Material pelleted at low speed. The regression line gives a value for $s^0_{20,w} = 3.17$. Regression fits to data (○) and (●) separately give $s^0_{20,w} = 3.13$ and 3.22, respectively. (B) Fibrous slime mold actin in 0.15 M KCl–0.01 M Tris-maleate (pH 7.0). Sedimentation at 40,000 rpm, 20°; $s^0_{20,w} = 35.6$.

slime mold actin and with actin prepared from rabbit skeletal muscle. These complexes sedimented very rapidly, however, and the experiments were thus somewhat less satisfying than those (see below) demonstrating interaction between slime mold actin and rabbit myosin.

B. Slime Mold Actin. The procedure most commonly used to prepare actin from the pooled fractions designated G-200 actin is indicated in Figure 1. The G-200 actin was first centrifuged briefly to remove aggregated material; since little protein was pelleted by this step it was sometimes omitted. The solution was then diluted by about 10% with a concentrated salt solution (1.5 M KCl–0.10 M $MgCl_2$–0.10 M Tris-maleate, pH 6.2) and the pH was adjusted, if necessary, to pH 6.1–6.2. A fairly concentrated buffer was employed because the addition of Mg^{2+} to poorly buffered solutions of protein plus ATP resulted in a large drop in pH which, in some cases, was sufficient to cause precipitation. The solution was allowed to stand for about 1 hr in the cold room; longer storage times or storage at room temperature did not markedly increase the yield of polymerized actin, as judged by the amount of protein pelleted by high-speed centrifugation (2 or 3 hr 60K-#65 [314,000]). The resulting pellets varied from nearly translucent to whitish in appearance and accounted for 30–50% of the protein originally present in the G-200 actin.

Slime mold actin in the polymerized, or F form was routinely obtained by gently homogenizing the pellets in 0.15 M KCl–0.01 M Tris-maleate (pH 7.0). In some early experiments 1 mM ATP was included in the resuspension buffer, but the nucleotide was eliminated when its presence was found to have no significant effect. After a brief centrifugation [*e.g.*, 15 min 35K-#40 (110,000)] to remove undissolved material the clear and colorless F-actin solutions contained roughly two-thirds of the protein originally pelleted; the yield at this point was about 25–40 mg of protein/100 g of washed plasmodium.

As indicated in Figure 10A, examination of such F-actin preparations in the analytical ultracentrifuge revealed a small variable amount of slowly sedimenting material and a major high s species which gave a sharp symmetrical boundary having $s^0_{20,w} \simeq 36$ (Figure 11B). Similar schlieren patterns were also obtained when F-actin solutions were dialyzed briefly (3–6 hr) against 0.15 M KCl–0.01 M Tris-maleate (pH 7.0). However, overnight dialysis caused the appearance of poorly defined more rapidly sedimenting material ($s_{20,w} > 40$) as well as an increase in the low s boundary; this apparent breakdown was enhanced when the dialysis was against 0.50 M KCl at pH 7.

Only under the above conditions (Figure 1) was it possible to obtain preparations of slime mold actin with a single symmetrical high s boundary. Changes in the procedure led to preparations which contained variable amounts of an additional higher s species. This material formed an symmetric boundary, apparently hypersharp on the low s side, which spread very rapidly. The s value of this boundary was difficult to measure precisely but several estimates of $s_{20,w}$ near or greater

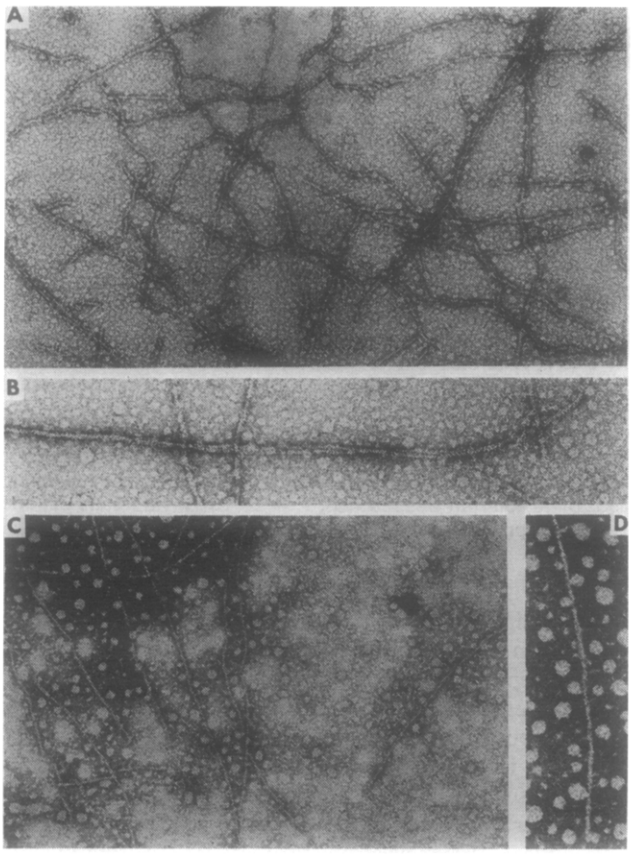

FIGURE 12: Electron micrographs of slime mold actin in the polymerized form. A and B show material stained with 1% uranyl acetate; C and D show material stained with 1% phosphotungstic acid. A and C were taken at a direct magnification of 21,000 and are printed at a total magnification of 62,000. B and D were taken at a direct magnification of 30,000 and are printed at a total magnification of 88,500.

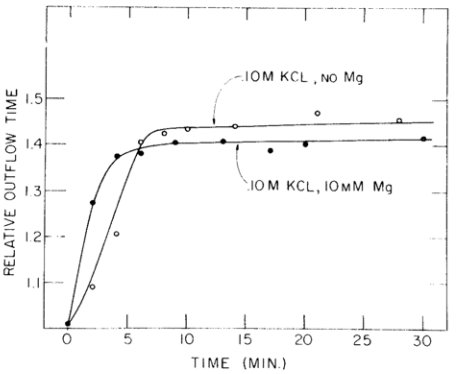

FIGURE 13: Increase of solution viscosity during polymerization of slime mold actin. Each viscometer contained 0.9 ml of actin in 1 mM Tris–1 mM ATP (pH 8). At zero minutes each viscometer received 0.1 ml of a concentrated buffer solution giving final conditions of 0.10 M KCl–0.01 M Tris-maleate (pH 7) (○) or 0.10 M KCl–0.01 M $MgCl_2$–0.01 M Tris-maleate (●). The final protein concentration was 1.25 mg/ml.

than 50 were obtained. For simplicity we refer to the rapidly spreading asymmetric boundary as "50 S" and the sharp symmetric boundary as "30 S." Figure 10B, lower trace, provides one example of an actin preparation with both "30S" and "50-S" species. In some preparations, which showed both 30S and 50S species, the addition of 10 mM Mg^{2+} resulted in a shift of material from the 50S to the 30S boundary. Although this Mg^{2+} effect was explored briefly (Adelman, 1969), its significance remains obscure.

Formation of the 50S species was not due, *per se*, to the G-200 chromatography and subsequent manipulations, since a similar boundary appeared upon addition of salt to desalted actomyosin (Adelman and Taylor, 1969) which had been prepared in 1 mM Tris–1 mM ATP (pH 8) and stored overnight. Inclusion of a mild reducing agent in the elution buffer for both Sephadex columns (Figure 1) greatly minimized the amount of 50S material in the final actin preparations: 5 mM cysteine was used in many experiments but the best results were obtained using TAM buffer.[2] It was also found desirable to minimize the time involved in the G-200 chromatography (by using the short wide column described previously (Adelman and Taylor, 1969) and to polymerize the G-200 actin at pH ~6.1–6.2.

Recent experiments indicate that even with the above precautions much 50S material was formed but was partially eliminated from the final F-actin preparations by the high-speed pelleting and resuspension steps. When polymerized G-200 actin solutions were centrifuged at lower speed, namely, 3 hr-27K–#30 (85,000) the resulting protein pellets were actually larger and dissolved more completely than those obtained by the standard high-speed centrifugation. Such "low-speed" F-actins represented a larger percentage of the G-200 actin and contained considerably more "50S" material than did the usual F-actin. (Compare Figure 10B upper and lower traces, both of which are derived from the same G-200 actin solution.)

Examination of F-actin in the electron microscope (Figure 12) revealed fibers with diameters of 50–80 Å and lengths up to 1 μ or more. The diameter of the strands often varied in a manner suggesting the twisting of subfibers about one another; in some places a two-strand structure was detectable (Figure 12D). There was in all preparations a variable but large amount of globular material with diameters ranging from below 200 Å to above 400 Å. The relative amounts of fibers and globules bore no obvious relation to the amounts of 30S and 50S species.

Slime mold actin in the depolymerized or G form was obtained by gently homogenizing the pellets of polymerized actin, usually in TAM buffer. Ultracentrifugal analyses indicated depolymerization was rapid when pellets were dissolved at concentrations of 1–2 mg/ml. Normally, however, the G-actin solutions were dialyzed for several hours (overnight for extrapolated s value measurements) before high-speed clarification (30′-60K-#65 [314,000]). About 75% or more of the protein pelleted at high speed from repolymerized G-200 actin was recovered in these G-actin preparations; yields of up to 50 mg of protein/100 g of washed plasmodium were thus obtained.

Boundary centrifugation of slime mold G-actin at low ionic strength revealed no rapidly sedimenting material:

[2] TAM buffer is composed of 1 mM Tris–1 mM ATP–5 mM β-mercaptoethanol (pH 7.8).

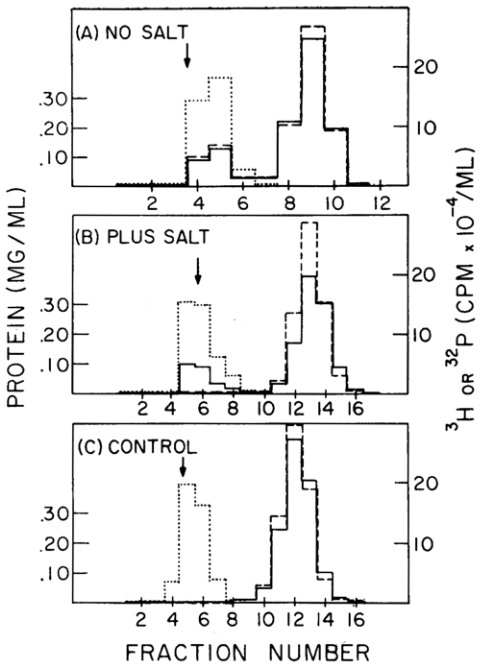

FIGURE 14: Nucleotide binding to slime mold actin. The pelleted actin was resuspended at a concentration of 1.2 mg/ml in 1 mM Tris (pH 8) with no added ATP. Three 1-ml aliquots received the following treatments. To samples A and B were added 25-μl aliquots of a solution containing [^{3}H]ATP and [γ-^{32}P]ATP at a total concentration of 4 μmoles of ATP/ml. The isotope contents of this stock had been adjusted to give 1.12×10^7 ^{32}P cpm/μmole of ATP and 1.06×10^7 ^{3}H cpm/μmole of ATP. Sample C received 25-μl of radioisotope-free ATP at 4 μM/ml. All three samples were stored 1 hr on ice and then brought to room temperature. Samples B and C were then polymerized by addition of 0.1 ml of concentrated buffer to give the approximate final conditions of 0.10 M KCl–0.01 M $MgCl_2$–0.01 M Tris-maleate (pH 6.2). After 1 hr 25 μl of ^{3}H, [^{32}P]ATP solution was added to sample C. (Chromatography on A and B was begun at this point.) and it was allowed to stand ~1 hr more at room temperature. All three samples were chromatographed on small (1 × 15 cm) Sephadex G-100 columns equilibrated and eluted with the following: sample A—1 mM Tris (pH 8); samples B and C—0.10 M KCl–0.01 M $MgCl_2$–0.01 M Tris-maleate (pH 6.8). For each column fifteen 20-drop fractions were collected; mean volumes per fraction were A = 1.7 ml, B = 1.35 ml, and C = 1.40 ml. Fractions were assayed for protein (· · · ·), ^{3}H cpm (——), and ^{32}P cpm (– – – –). The fraction number axis for A is slightly expanded to facilitate comparison with B and C. The void volume of each column was determined using Blue Dextran and is indicated by an arrow above each profile. Quenching of both isotopes due to protein or buffer was assumed small because the recovery of ^{3}H and ^{32}P counts was 85 to 95% of that expected on the basis of the counts per minute of a 1:100 dilution of the stock label (in water). The recovery of ^{32}P cpm was slightly higher than that for ^{3}H.

a single low s boundary was present which showed a very slight asymmetry on the high s side (Figure 10C). Globular slime mold actin had $s^0_{20,w}$ = 3.2 S (Figure 11A); neither the boundary sedimentation profiles nor the extrapolated s values (compare filled and open circles in Figure 11A) were significantly different for G-actins derived from preparations which, in the polymerized form, showed large differences in the amount of 50S material.

The addition of KCl or KCl plus Mg^{2+} to solutions of depolymerized slime mold actin resulted in a rapid rise in solution viscosity (Figure 13) and the reappearance of

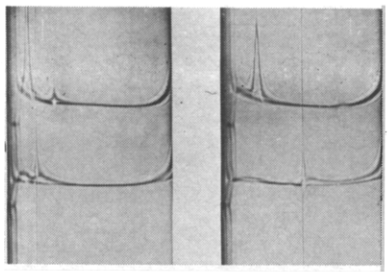

FIGURE 15: Interaction of slime mold actin with rabbit myosin. The myosin was in 0.50 M KCl–0.01 M Tris (pH 7.4). The actin was in 0.15 M KCl–0.01 M Tris-maleate (pH 7.0). Equal aliquots of actin and 0.85 M KCl–0.01 M Tris (pH 7.8) were added to the myosin to give final conditions of ~0.50 M KCl (pH 7.4). The lower trace represents 2.4 mg/ml of myosin and 0.77 mg/ml of actin (final concentrations). The upper trace shows the same solution after 10% dilution to give a final concentration of 10 mM ATP. Pictures were taken, at a phase angle of 55°, 16 and 56 min after the maximum speed of 40,000 rpm was reached, T = 20°. $s_{20,w}$ values are 5.4 and 23.9 in the upper trace and 16.1 in the lower trace.

rapidly sedimenting material. Actins thus polymerized from the G form had relatively more 50S material than did the F-actin prepared directly from the same high-speed pellets. The samples used for the measurements in Figure 13 were removed from the viscometers and examined in the ultracentrifuge: in both samples most material sedimented in the form of "30S" and "50S" boundaries but the relative amount of the latter was higher in the sample to which only KCl had been added. While electron micrographs of solutions of G-actin showed no fibrous material and very few 200–400-Å globules, images similar to those in Figure 12 were detectable after the addition of salt.

The results of an experiment to test the adenosine nucleotide binding properties of slime mold actin are shown in Figure 14. When G-actin was incubated with a mixture of [^{3}H]ATP and [γ-^{32}P]ATP and then chromatographed on Sephadex G-100 in the absence of salt, a nearly voided protein peak separated from the excess of nucleotide and carried with it ^{3}H and ^{32}P counts in the same ratio as was present in the input ATP (Figure 14A). When the G-actin was similarly incubated with doubly labeled ATP, then polymerized by the addition of salt, and chromatographed in the presence of salt, the voided protein peak carried with it the same amount of ^{3}H but very little ^{32}P: the ratio of ^{3}H:^{32}P cpm increased from ~1:1 (Figure 14A) to greater than 50:1 (Figure 14B). Thus, while the bound nucleotide was retained, the addition of salt caused the release of (at least) the terminal phosphate. When the actin was polymerized prior to incubation with ATP (Figure 14C), column chromatography revealed no ^{32}P and very little ^{3}H in the protein peak. Allowing for dilution of the labeled ATP with the unlabeled nucleotide initially added (see Figure 14), the binding of labeled nucleotide to the F-actin in Figure 14C was less than 15% of that in Figure 14B.

The ^{3}H cpm/mg of protein in fractions 4 and 5 of Figure 14A and fractions 5 and 6 of Figure 14B indicated a binding site molecular weight of 68,000 ± 5,000 (mean and range), whereas the ^{32}P cpm/mg of protein in fractions 4 and 5 of Figure 14A gave mol wt 63,000 ± 4,000. The difference in estimated molecular weights probably reflected a slightly larger quenching for ^{3}H. These estimates for the molecular

TABLE VI: Slime Mold Actin Activation of Rabbit Myosin ATPase.[a]

		Total K^+ (moles/l.)	ATPase (μM P_i/mg of myosin per 15 min	Activation Factor
Expt 1	Myosin	0.031	0.17	4.1
	Myosin plus actin	0.039	0.69	
Expt 2	Myosin	0.016	0.23	12
	Myosin plus actin	0.024	2.77	

[a] Assays were carried out at room temperature in the presence of 1 mM $MgCl_2$–0.1 mM $CaCl_2$–1 mM ATP at pH 7.0. The buffer for expt 1 was 0.025 M KCl–0.025 M Tris-maleate. The buffer for expt 2 was 0.01 M KCl–0.01 M Tris-maleate. For each experiment rabbit myosin (dialyzed *vs.* 0.50 M KCl–0.01 M Tris, pH 7.4) was present at a final concentration of 0.165 mg/ml. The slime mold actin (a preparation pelleted at low speed and resuspended in 0.15 M KCl–0.01 M Tris-maleate, pH 7) was present at a final concentration of 0.14 mg/ml. The K^+ concentrations indicated include the salt introduced by the protein aliquots. For each experiment the actin, with no myosin present, was tested and had no detectable ATPase activity.

weight of the actin monomer assumed that (a) the protein was pure and undenatured, (b) nucleotide exchanged rapidly and was firmly bound during column chromatography, and (c) the protein reacted in the Lowry assay (Lowry *et al.*, 1951) identically with bovine serum albumin. The error in c could not be determined but probable errors in a and b would tend to give an overestimate for the molecular weight of the binding site.

Slime mold actin was shown to interact with rabbit myosin by two methods. When the two proteins were mixed and examined in the analytical ultracentrifuge (Figure 15, lower trace) only a major rapidly sedimenting species was detected. The addition of ATP resulted in the reappearance of distinct myosin and actin boundaries (Figure 15, upper trace) indicating the ATP-induced dissociation of an actomyosin complex. The data of Table VI show that slime mold actin was capable of activating the low ionic strength Mg^{2+}–ATPase of rabbit myosin: thus the slime mold protein could substitute for rabbit actin in this enzymatic assay for a functional actomyosin complex.

Discussion

The procedures described in this and in the preceding paper (Adelman and Taylor, 1969) and which are summarized in Figure 1 constitute a reasonably efficient means of preparing fairly pure actin- and myosin-like proteins from *P. polycephalum*. The quantities obtained are sufficient for many types of experiments and the overall preparation time is short: both actin and myosin can be prepared from the same sample of plasmodium in 2 days.

The calcium-activated ATPase we have isolated from slime mold is clearly myosin like by several reasonable criteria. The value of $s^0_{20,w} = 6.4_0$ is in the range of literature values for a variety of myosins, *viz.*, 5.8–6.4 cited by Barany *et al.* (1966). The striking agreement with the commonly cited value for rabbit myosin (Holtzer and Lowey, 1959; Johnson and Rowe, 1960) may be fortuitous: we measured $s^0_{20,w} \sim 6.0$ for the rabbit enzyme and reports of $s^0_{20,w} \sim 6.15$ have appeared (Chung *et al.*, 1967; Gershman *et al.*, 1969). The elution of the slime mold myosin from Sepharose 4B at a position close to that of rabbit myosin demonstrates that both have a similar high particle asymmetry. Although there is as yet no clear theoretical basis for the use of gel chromatography in the measurement of diffusion coefficients, the good agreement between our determinations and literature values for rabbit myosin and heavy meromyosin would appear to justify use of the column-derived value of $D_{20,w} = 1.26 \times 10^{-7}$ cm²/sec for slime mold myosin. With our sedimentation and diffusion data, and an assumed $\bar{v} = 0.73$, we obtain 4.6×10^5 as an estimate of the molecular weight of slime mold myosin; this value is rather close to that of 4.8×10^5 obtained with our data for rabbit myosin and is in fair agreement with most literature values for the mammalian enzyme (Gergely, 1966; Gershman *et al.*, 1969).

While slime mold and rabbit myosin are hydrodynamically similar and while the slime mold enzyme will form an actomyosin complex with rabbit actin, our data do indicate differences between the two enzymes. Slime mold myosin appears to have a much smaller tendency to aggregate at low ionic strength,[3] an observation also reported by Hatano and Tazawa (1968), and does not seem to denature by a process involving large-scale aggregation.

Under our standard assay conditions the Ca^{2+}–ATPase of slime mold myosin is several times that of rabbit myosin. Furthermore, if the previous findings (Adelman and Taylor, 1969) on slime mold actomyosin are relevant here, the slime mold enzyme differs from rabbit myosin and is similar to smooth muscle enzymes in being a K^+-activated Ca^{2+}–ATPase. We were unable to obtain slime mold actin activation of

[3] In view of the work of Josephs and Harrington (1966, 1967, 1968), our observations do not exclude the possibility that slime mold myosin formed larger aggregates (than the *ca.* 10S species) which were broken down by the hydrostatic pressure generated during analytical ultracentrifugation. Since the low ionic strength solutions of the enzyme were not turbid, however, very large aggregates seem definitely excluded.

slime mold myosin Mg^{2+}–ATPase under conditions comparable to those of Table VI; this again suggests an analogy with smooth muscle myosins (Barany *et al.*, 1966).

Although the enzymatic properties of the slime mold enzyme have not as yet been subjected to detailed investigation the following differences from the rabbit enzyme may be relevant to the mechanism of action. The enzyme is not strongly inhibited by Mg^{2+} nor activated by EDTA; the rate of ITP hydrolysis is less than for ATP while the converse is true for the muscle enzyme (Blum, 1960); the minimum in the pH–activity curve is much less pronounced. The lack of versene activation and the shape of the pH–activity curve agree with the results of Nakajima (1960) on plasmodial myosin B.

The slime mold protein we designate actin is fairly similar to the preparations obtained by the totally different procedures of Hatano and coworkers (Hatano and Oosawa, 1966a,b; Hatano *et al.*, 1967) and both proteins are clearly like muscle actin. At low ionic strength slime mold actin exists as a small protein. The extrapolated $s_{20,w}$ of 3.2 may be compared with 3.25 for rabbit actin (Lewis *et al.*, 1963) and 3.7 reported by Hatano and Oosawa (1966b). From the binding of ATP an upper limit of about 6–7 $\times 10^4$ may be estimated as the subunit molecular weight. An alternative estimate may be obtained using a value for the diffusion coefficient derived from the elution position on G-200 Sephadex (Adelman and Taylor, 1969). Again applying Ackers' theory (1964), we use the elution position of bovine serum albumin (K_D = 0.38 in several experiments) to compute a calibration constant, r = 180 Å. (The state of aggregation of bovine serum albumin under the chromatography conditions was checked by dialyzing bovine serum albumin *vs.* the column buffer 1 mM Tris–1 mM ATP–5 mM cysteine (pH 7.8). Boundary centrifugation revealed a single symmetrical peak with $s^0_{20,w}$ = 4.33; thus the column calibration appears to be valid.) The peak fractions normally pooled as G-200 actin were centered at K_D = 0.495 (range of ±0.01 in several experiments) from which a Stokes radius of ~27 Å and a $D_{20,w}$ of $\sim 7.8 \times 10^{-7}$ cm^2/sec may be computed.

Combining this value with s = 3.2 and an assumed $\bar{v}$ = 0.73 gives a molecular weight of 37,000. If a value of $\bar{v}$ = 0.75 (Rees and Young, 1967) is employed the weight becomes 40,000. These estimates are well below the commonly cited figures of 57,000–60,000 for rabbit G-actin (Gergely, 1966) and the value of 57,000 which Hatano and Oosawa (1966b) reported for slime mold actin. Recently, however, Rees and Young (1967) suggested a molecular weight of 46,000–48,000 for chromatographically purified actin, based on measurements with the Yphantis (1964) high-speed sedimentation equilibrium technique and on nucleotide binding. In preliminary experiments, using the Yphantis method, we have obtained a molecluar weight of 44,000 for slime mold G-actin, assuming $\bar{v}$ = 0.73. In addition, work on the 3-methylhistidine content of actin (Johnson *et al.*, 1967; Asatoor and Armstrong, 1967; Gosselin-Rey *et al.*, 1969) and on the C-terminal amino acid content (Johnson and Perry, 1968) strongly suggests that the subunit molecular weight is between 43,000 and 48,000. Hence our rough estimates for the slime mold actin subunit, while low, are clearly not unreasonable.

In the presence of salt, slime mold actin exists as one or more rapidly sedimenting, asymmetric species. The bound adenosine nucleotide, which lacks the terminal (at least) phosphate of the ATP bound to the low salt subunit, is not readily exchanged with free ATP. The actin contains electron microscopically demonstrable fibers which are similar to muscle F-actin (Hanson and Lowy, 1963). Furthermore, the slime mold actin will form an ATP-dissociable complex with rabbit myosin and can activate the low ionic strength Mg^{2+}–ATPase of the mammalian enzyme to a level comparable with that obtained with muscle actin (Barany, 1967). Further comment is required on two aspects of our data on polymerized slime mold actin.

Hatano *et al.* (1967) reported that, in the presence of Mg^{2+}, slime mold actin polymerized to a low viscosity state characterized by the presence of globular material with diameters of 100–600 Å. Since our actin preparations involved exposure to 10 mM $MgCl_2$ (Figure 1) it would at first seem reasonable to assume the ~200–400-Å globules in our preparations of F-actin are similar to the magnesium polymer of Hatano and coworkers. However, our viscosity data indicate no large difference in particle asymmetry between actin polymerized with or without Mg^{2+}, and globules similar to those in Figure 12 are seen in preparations of crude slime mold actomyosin not previously exposed to magnesium (Figure 2A of Adelman and Taylor, 1969). Furthermore globular material as large and heterogeneous as the structures seen electron microscopically would not give the sharp ~30S boundary which we, as well as Hatano *et al.* (1967), detect in the analytical ultracentrifuge. The globules may be an artifact of the electron microscope specimen preparation techniques, but further experiments will be required to clarify this point.

Johnson *et al.* (1963) demonstrated schlieren patterns of muscle actin very similar to the complex ones we have observed with slime mold actin (Figure 10B, lower trace); the boundaries they designated F_1 and F_2 actin appear analogous to our 30S and 50S boundaries, respectively. They concluded that F_2 actin was a side-to-side aggregate of F_1-actin. Normally, muscle actin prepared by the standard acetone powder procedures ("Straub actin") contains primarily rapidly sedimenting material in a boundary resembling our 50S species: there is relatively little material present as a boundary analogous to the "30S" species, which we regard as the more natural form of slime mold actin.

However, several reports have appeared describing natural F-actins, preparations of muscle actin obtained without acetone powder procedures and repetitive cycles of depolymerization and repolymerization (Maruyama *et al.*, 1965; Hama *et al.*, 1965; Maruyama, 1966b; Haga *et al.*, 1966; Hama *et al.*, 1967) and which probably represent virtually intact I-band filaments. Natural F-actins sediment as sharp symmetrical boundaries with extrapolated s values near 40 S and they are markedly prone to breakdown during storage, especially in the absence of excess ATP. The ~30S boundary, ($s^0_{20,w}$ is closer to 40) in our actin preparations is unstable, especially at higher ionic strength; similar observations were reported (Adelman and Taylor, 1969) on the actin-like component of the crude actomyosin and examination of Figure 5 of the preceding paper suggests that Mg–ATP stabilizes the actin. It thus appears that our 30S species is analogous to natural F-actin.

Maruyama (1965a,b, 1966a) has reported the isolation of a protein, β-actinin, which converts Straub actin into natural F-actin; it might be suggested, therefore, that the complex schlieren patterns we have observed for slime mold actin reflect preparations of actin with varying amounts of modifier

protein(s). However, it would be somewhat surprising to find in the slime mold a β-actinin-like protein, if, as Maruyama proposes, this protein functions as a length regulator for the actin filaments in muscle. Clearly further experiments will be required to resolve these complexities.

That slime mold myosin and slime mold actin constitute a considerable proportion of the plasmodial proteins may be demonstrated as follows. Assuming that purified slime mold myosin has a specific Ca^{2+}–ATPase of 15 μM P_i/mg per 15 min and that all the Ca^{2+}–ATPase in the crude actomyosin (usually about 600 μM P_i/15 min per 100 g of plasmodium) is due to the myosin there are about 40 mg of slime mold myosin/100 g of washed plasmodium. The actin content is more difficult to define in view of the complexities discussed above. A minimum estimate of about 40 mg/100 g of washed plasmodium seems reasonable, and the recent results with low-speed pelleting suggest an upper estimate of 100–120 mg/100 g, the mean figure being ~80 mg of actin/100 g of washed plasmodium. If the washed plasmodium is 90–95% water and protein is 50% of the dry mass we estimate 1–2% of the plasmodial protein is slime mold myosin and 2–4% is actin.

It is quite suggestive that the slime mold plasmodium, which manifests unusually vigorous protoplasmic streaming (Kamiya, 1959), contains such large amounts of muscle-like proteins. However, the isolation of actomyosin-, myosin-, and actin-like proteins from a primitive motile system does not prove these proteins cause the observed motility phenomena. Even if the causative role is assumed, it does not follow that the proteins must function in the same way as in muscle. Several reports of 50–70-Å fibers in *Physarum* have appeared (Wohlfarth-Botterman, 1964; Rhea, 1966; Crawley, 1966; Nagai and Kamiya, 1966) and it has been claimed that ATPase activity is detectable around these filaments (Wohlfarth-Botterman, 1964): these are presumably the slime mold actin and slime mold myosin, respectively. There are no signs, however, of thick, myosin-like filaments or of organization of the 50–70-Å filaments into arrangements like the Z-line sarcomere arrays of striated muscle. Also, slime mold myosin does not readily form large aggregates at low ionic strength and slime mold actomyosin can be extracted at fairly low ionic strength or merely by squeezing out the plasmodial "sap" (Adelman and Taylor, 1969). Thus there is no evidence for the type of highly ordered and cross-linked structure on which the sliding filament mechanism is based. It is also questionable whether a model which attributes streaming to the contraction (superprecipitation) of an actomyosin gel is entirely reasonable.

While the available data do indicate that the velocity profile for protoplasmic streaming in slime mold can be produced by a pressure difference (Kamiya, 1959), the required pressure gradients could clearly be generated by mechanisms other than "contractility"; any model predicting overall fluid flow away from the region of energy utilization is potentially satisfactory. Single actin strands with adhering myosin monomers or small aggregates might drive fluid in one direction by a propagated transverse (Taylor, 1965) or helical (Jarosch, 1968) wave, or by an oarlike change in the orientation of adherent myosins. Alternatively, small aggregates (dimers, tetramers) of myosin might bridge actin strands into small bundles which could propagate waves or change intrastrand spacing. Even with a rather random orientation of such force generating "units" in the ectoplasm of the plasmodium the directionality required to guarantee the observed net flow could be accounted for by assuming that some membrane (*i.e.*, cell surface) associated event triggers the mechanochemical event which subsequently propagates undirectionally along the actomyosin fiber or fiber bundle.

Acknowledgments

We thank Dr. William Bloom for access to his electron microscope facilities. Mr. Gerald Grofman assisted with the electron microscopy and production of the illustrations. Mr. Richard Lymn provided samples of the muscle proteins. We also thank Mrs. Chantal Boyd and Mrs. Rose Wheaton for general laboratory assistance.

References

Ackers, G. K. (1964), *Biochemistry 3*, 723.
Adelman, M. R. (1969), Ph.D. Dissertation, University of Chicago, Chicago, Ill.
Adelman, M. R., Borisy, G. G., Shelanski, M. L., Weisenberg, R. C., and Taylor, E. W. (1968), *Fed. Proc. 27*, 1186.
Adelman, M. R., and Taylor, E. W. (1969), *Biochemistry* 8, 0000.
Asai, H. (1963), *Biochemistry 2*, 458.
Asatoor, A. M. and Armstrong, M. D. (1967), *Biochem. Biophys. Res. Commun. 26*, 168.
Barany, M. (1967) *J. Gen. Physiol. 50*, 197.
Barany, M., Barany, K., Gaetjens, E., and Bailin, G. (1966), *Arch. Biochem. Biophys. 113*, 205.
Blum, J. J. (1960), *Arch. Biochem. Biophys. 87*, 104.
Chung, C. S., Richards, E. G., and Olcott, H. S. (1967), *Biochemistry 6*, 3154.
Crawley, J. C. W. (1966) *J. Roy. Microscop. Soc. 85*, 313.
DeRosier, D. J. (1965), Ph.D. Dissertation, University of Chicago, Chicago, Ill.
DeRosier, D. J., and Haselkorn, R. (1966), *J. Mol. Biol. 19*, 52.
Finlayson, B., Lymn, R. W., and Taylor, E. W. (1969), *Biochemistry 8*, 811.
Finlayson, B., and Taylor, E. W. (1969), *Biochemistry 8*, 802.
Gergely, J. (1966), *Ann. Rev. Biochem. 35*, 691.
Gershman, L. C., Stracher, A., and Dreizen, P. (1969), *J. Biol. Chem. 244*, 2726.
Gosselin-Rey, C., Gerday, C., Gaspar-Godfroid, A., and Carsten, M. E. (1969), *Biochim. Biophys. Acta 175*, 165.
Haga, T., Yamamoto, M., Maruyama, K., and Noda, H. (1966), *Biochim. Biophys. Acta 127*, 128.
Hama, H., Maruyama, K., and Noda, H. (1965), *Biochim. Biophys. Acta 102*, 249.
Hama, H., Maruyama, K., and Noda, H. (1967), *Biochim. Biophys. Acta 133*, 251.
Hanson, J., and Lowy, J. (1963), *J. Mol. Biol. 6*, 46.
Hatano, S., and Oosawa, F. (1966a), *J. Cell Physiol. 68*, 197.
Hatano, S., and Oosawa, F. (1966b), *Biochim. Biophys. Acta 127*, 488.
Hatano, S., and Tazawa, M. (1968), *Biochim. Biophys. Acta 154*, 507.
Hatano, S., Totsuka, T., and Oosawa, F. (1967), *Biochim. Biophys. Acta 140*, 109.

Holtzer, A., and Lowey, S. (1959), *J. Am. Chem. Soc. 81*, 1370.
Jarosch, R. (1968), *Z. Naturforsch. 23b*, 17.
Johnson, P., Harris, C. I., and Perry, S. V. (1967), *Biochem. J. 105*, 361.
Johnson, P., Napper, D. H., and Rowe, A. J. (1963), *Biochim. Biophys. Acta* 74, 365.
Johnson, P., and Perry, S. V. (1968), *Biochem. J. 110*, 207.
Johnson, P., and Rowe, A. J. (1960), *Biochem. J. 74*, 432.
Josephs, R., and Harrington, W. F. (1966), *Biochemistry 5*, 3474.
Josephs, R., and Harrington, W. F. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1587.
Josephs, R., and Harrington, W. F. (1968), *Biochemistry 7*, 2834.
Kamiya, N. (1959), *Protoplasmatologia 8*, 1.
Lewis, M. S., Maruyama, K. Carroll, W. R., Kominz, D. R., and Laki, K. (1963), *Biochemistry 2*, 34.
Lowey, S., and Holtzer, A. (1959), *Biochim. Biophys. Acta* 34, 470.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Maruyama, K. (1965a), *Biochim. Biophys. Acta 94*, 208.
Maruyama, K. (1965b), *Biochim. Biophys. Acta 102*, 542.
Maruyama, K. (1966a), *Biochim. Biophys. Acta 126*, 389.
Maruyama, K. (1966b), *Biochem. Z. 345*, 108.
Maruyama, K., Hama, H., Ishikawa, Y. (1965), *Biochim. Biophys. Acta 94*, 200.
Nagai, R., and Kamiya, N. (1966), *Proc. Jap. Acad. 42*, 943.
Nakajima, H. (1960), *Protoplasma 52*, 412.
Parrish, R. G., and Mommaerts, W. F. H. M. (1954), *J. Biol. Chem. 209*, 901.
Rees, M. K., and Young, M. (1967), *J. Biol. Chem. 242*, 4449.
Rhea, R. P. (1966), *J. Ultrastruct. Res. 15*, 349.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.
Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112*, 346.
Szent-Györgyi, A. G. (1953), *Arch. Biochem. Biophys. 42*, 321.
Taylor, E. W. (1965), *in* Symposium on Biorheology: Part IV of the Proceedings of the Fourth International Congress on Rheology, Copley, A. L., Ed., New York, N. Y., Interscience, p 175.
Wohlfarth-Botterman, K. E. (1964) *in* Primitive Motile Systems in Cell Biology, Allen, R. D., and Kamiya, N., Ed, New York, N. Y., Academic, p 79.
Young, D. M., Himmelfarb, S., and Harrington, W. F. (1965), *J. Biol. Chem.* 240, 2428.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.