Nagai, Y., Lapiere, C. M., and Gross, J. (1966), *Biochemistry 5*, 3123.
Nishihara, T., and Doty, P. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 411.
Piez, K. A. (1965), *Biochemistry 4*, 2590.
Piez, K. A., and Gross, J. (1960), *J. Biol. Chem. 235*, 995.
Piez, K. A., Martin, G. R., Kang, A. H., and Bornstein, P. (1966), *Biochemistry 5*, 3813.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Simha, R. (1940), *J. Phys. Chem. 44*, 25.
Yang, J. T. (1961), *Advan. Protein Chem. 16*, 323.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Chromatography of Myosin on Diethylaminoethyl-Sephadex A-50*

E. G. Richards, C.-S. Chung, D. B. Menzel, and H. S. Olcott

ABSTRACT: A chromatographic system using DEAE-Sephadex A-50 with 0.04 M pyrophosphate buffer, pH 7.5, and a linear KCl gradient to 0.50 M KCl separates monomeric myosin from aggregated myosin, other unidentified proteins, and ribonucleic acid (RNA). The procedure has been applied to myosin preparations from skeletal muscle of rabbit, chicken, and four species of fish.

With good preparations, adenosine triphosphatase activity across the peak varied about 20% and was greater than the starting material. As determined by sedimentation equilibrium, the molecular weight of chicken myosin purified by this procedure was found to be 5.0×10^5 g mole^{-1}.

Several investigators have employed column chromatography for the purification of myosin. Brahms (1959) and Perry (1960) used DEAE-cellulose with 0.2 M KCl buffered with Tris to pH 7.4, and obtained some degree of fractionation, but the bulk of the material passed directly through the column without retention. Perry (1960) also employed a buffer system (0.16 M KCl–0.02 M Tris, pH 8.2) with a KCl gradient that resulted in a separation of myosin from ribonucleoprotein and other proteins that passed through unretarded. However, the adenosine triphosphatase (ATPase)[1] activity varied considerably across the myosin peak, and dimers not present in the starting material were seen in the myosin purified by this procedure. Based on the work of Brahms and Brezner (1961), who showed that myosin was soluble in polyphosphates at low ionic strength, Asai (1963) used DEAE-cellulose columns, ATP in the solvent, and a KCl gradient to obtain chromatograms that were similar to those obtained with the pH 8.2 system of Perry (1960). The ATPase activity was more nearly constant across the peak, but myosin aggregates were present in the trailing part of the peak. Asai (1963) also mentioned that similar results were obtained with DEAE-cellulose and pyrophosphate as the solvent. Smoller and Fineberg (1964) observed an enhancement in ATPase activity when they purified mouse myosin by gel filtration with Sephadex G-200. Baril *et al.* (1964, 1966) used DEAE-cellulose and eluted stepwise with pyrophosphate–KCl buffers at pH 8.5 for the purification of chick myosin. They achieved separation of myosin from RNA and also from myosin–RNA complexes. The purified myosin appeared to be monomeric, but its ATPase activity was reduced.

We have developed a chromatographic procedure, with DEAE-Sephadex A-50 and KCl gradient in pyrophosphate or phosphate buffer, that separates monomeric myosin from aggregated myosins, other unidentified proteins, and RNA. This system is suitable both as an analytical tool to examine the purity of myosin preparations and as a final purification step. It should be of general utility, for similar chromatographic profiles were obtained for myosin preparations from fresh skeletal white muscle of rabbit, chicken, striped bass, skipjack, and bluefin tuna, and frozen skeletal muscle of yellowfin tuna. The ATPase activity of the peak fractions was greater than that of the starting material. The 5′-adenylic acid deaminase activity present in the original myosin was considerably re-

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[1] Abbreviation used: ATPase, adenosine triphosphatase; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates.

duced upon purification. The molecular weight of purified chicken myosin, as measured by sedimentation equilibrium, was found to be about 5.0×10^5 g mole^{-1}.

Experimental Section

Materials. Sephadex G-25 and DEAE-Sephadex A-50, coarse and beaded, and A-25, coarse, were obtained from Pharmacia; DEAE-cellulose (Cellex D, 0.98 mequiv/g) and phosphonic acid cellulose (Cellex-P, 0.72 mequiv/g) from Bio-Rad; and histidine–HCl, AMP, ADP, and ATP from Sigma. Other reagents were analytical grade.

Analytical Methods. Spectrophotometric measurements were performed with either the Zeiss spectrophotometer Model M4QIII or the Cary recording spectrophotometer Model 15. The Radiometer titragraph (type SBR2C) was used for titration experiments.

Protein concentrations were estimated by micro-Kjeldahl, assuming 16% nitrogen. As tests for the presence of RNA and DNA in chromatographic fractions, the orcinol and diphenylamine methods, respectively, were used. The KCl gradient was followed with a conductivity meter (Radiometer, type CDM2c). To test for the presence of actomyosin in myosin preparations, the effect of ATP on turbidity was examined (Rice *et al.*, 1963).

The ATPase activity of myosin solutions was measured in 0.1 M Tris–maleate, 0.01 M $CaCl_2$, 4 mM ATP, pH 6.5, at 30°; the myosin concentration was about 0.1 mg/ml, or lower (J. Marshall, 1963, personal communication). Two-milliliter samples were incubated for 5 min, then the reaction was stopped by the addition of 1 ml of cold 15% trichloroacetic acid. After centrifugation to remove precipitated protein, the liberated inorganic phosphate present in the supernatant was determined by the Fiske–Subbarow method (Leloir and Cardini, 1957). The values were corrected from measurements made on solutions with trichloroacetic acid added before the beginning of the reaction. The specific activity was expressed as μmoles of P_i/5 min per mg of protein.

Fractions from chromatography experiments were examined for 5′-adenylic acid deaminase activity according to a method modified from that of Nikiforuk and Colowick (1955). The reaction mixture contained 2.5 ml of 0.5 M KCl–0.05 M citrate–4.1×10^{-2} mM AMP, pH 6.5, and 0.1 ml of protein solution. The reaction was followed by measuring the decrease in absorbance at 265 mμ. The specific activity was defined in terms of the initial slope, a change in absorbance of 0.001 unit/min per mg of protein.

Examination for myokinase activity was made according to a modified method of Colowick (1955). Myosin at about 1.5 mg/ml was dialyzed against 0.5 M KCl–0.1 M Tris, pH 7.5. The reaction mixture contained 0.5 ml of 0.1 M $MgCl_2$ for each 8 ml of myosin solution. The reaction, carried out at 30°, was started by the addition of a suitable amount of concentrated ADP or ATP. Aliquots of 2 ml were removed at intervals (starting with zero time) and analyzed for inorganic phosphate (liberated by myosin ATPase from the ATP formed by any myokinase present) in the same manner as for the ATPase determination. A blank determination was made in which all components were present, except for the myosin. Specific activity was defined in terms of micromoles of P_i/minute per milligram of protein.

Preparation of Myosin. Myosin was extracted from the muscle of New Zealand white rabbits (backs only), New Hampshire chickens (breast), striped bass (*Roccus saxatilis*), and three species of tuna, yellowfin (*Thunnus albacares*), bluefin (*Thunnus thynnus*), and skipjack (*Katsuwonis pelamis*). White muscle only was used from the fish. All operations were carried out in a 4° cold room; centrifugations were performed near 0° with a Servall centrifuge RC-2 or a Spinco preparative ultracentrifuge Model L-2.

Myosin was prepared by two methods. The first was modified from a procedure used by M. Morales and described by Asai (1963). The muscle was ground twice with an Oster electric grinder, Model 516, exposed surfaces of which were coated with silicone grease. The minced muscle was extracted for 10 min with three times its weight of buffer containing 0.37 M KCl–0.05 M histidine–0.4 mM ATP, pH 6.8. The separation of the extract from the residue was accomplished by centrifugation at 9000*g* for 10 min. The myosin was precipitated by dilution with nine volumes of water and collected by centrifugation at 15,000 rpm in the Servall centrifuge equipped with the continuous flow apparatus set at a flow rate of about 250 ml/min. The precipitate was gathered in plastic tubes placed inside the stainless steel tubes, and dissolved either by addition of concentrated buffer to bring the final concentration in the resulting solution to 0.6 M KCl–0.025 M histidine at pH 6.8, or by addition of solid KCl to a concentration of 0.6 M. After the myosin solution had been carefully diluted with water to 0.3 M KCl, the solution was centrifuged 2 hr in the Spinco L-2 at 21,000 rpm in the no. 21 rotor or at 19,000 rpm in the no. 19 rotor to remove actomyosin and aggregated myosin. Precipitation (with six to seven volumes of water), collection of precipitate (with the continuous flow apparatus for volumes greater than 3 l.), and resolution of precipitate, dilution to 0.3 M KCl, and centrifugation in the Spinco L-2 (40,000 rpm in the 40 rotor) were repeated. (A third precipitation cycle could be used but was found to be unnecessary for myosin that was to be purified by chromatography.) Myosin to be used without delay was then dialyzed overnight against the desired buffer, or, if not, it was stored in 50% glycerine–0.6 M KCl–0.025 M histidine (or 0.01 M phosphate), pH 6.8, at −20°. More recently we have employed with this method an extraction buffer of lower ionic strength (0.3 M KCl–0.05 M histidine–0.2 mM ATP, pH 6.80) (Tonomura *et al.*, 1966).

For the second method, used only with frozen muscle from fish, myosin was prepared according to a modified procedure of Connell (1960). It gave very high yields

(50% of the available myosin) as compared to the first method, but only with frozen muscle. About 300 g of muscle mince was extracted for 10 min with three volumes of 0.05 μM phosphate buffer, pH 7.5 (3.38 mM KH_2PO_4–15.5 mM $K_2HPO_4 \cdot 3H_2O$), and the supernatant was removed by centrifugation for 10 min at 9000*g* in the Servall centrifuge. The extraction and centrifugation were repeated two or three more times. At this point the residue was nearly white, indicating that most of the myoglobin had been removed. The residue was extracted immediately with the buffer used by Connell (1960), but to avoid the formation of gelatinous lumps, the residue was first mixed with two volumes (based on original weight of mince) of water, and then with one volume of three times concentrated buffer. This solution contained, in the order of mixing, 30 ml of 33.8 mM KH_2PO_4–Na_2HPO_4, 54 ml of 2.5 M KCl, 1.5 ml of 1 M $MgCl_2$, and 15 ml of 0.2 M $Na_4P_2O_7$ (to give a volume of about 100 ml) with pH adjusted to 7.17 to give 7.5 upon dilution. The buffer was prepared shortly before use to avoid the formation of a precipitate. The extraction, with occasional stirring, was allowed to proceed 15–30 min. Then the procedure described above was followed for one or two cycles. Samples of myosin prepared by the above methods were shown to be free of actomyosin by the light-scattering test of Rice *et al.* (1963).

Chromatography Procedure. The myosin solution was dialyzed overnight against 0.04 M sodium pyrophosphate, pH 7.50 ($Na_4P_2O_7 \cdot 10H_2O$, adjusted with HCl), or 0.15 M potassium phosphate, pH 7.50. To avoid precipitation of myosin in concentrated solutions during dialysis, sufficient neutralized pyrophosphate was added to bring the initial concentration inside the sac to 0.04 M. The myosin was added to the washed DEAE-Sephadex A-50 column at a concentration lower than 1.5% to prevent plugging the column (Perry, 1960). (For analytical purposes 15–150 mg of myosin was added to 2 × 30 or 1.5 × 30 cm columns; for preparative purposes, 0.5–1.4 g to 4 × 50 or 2.5 × 100 cm columns.) The myosin was eluted with a linear gradient, using equal volumes (200 ml for small columns and 1000 ml for large columns) of 0.04 M pyrophosphate and 0.5 M KCl–0.04 M pyrophosphate, or 0.15 M potassium phosphate and 0.5 M KCl–0.15 M potassium phosphate, pH 7.5, for all solutions. Then 0.5 M KOH was added until the pH of the eluate was 13. The absorbance of the fractions was read at 280 mμ. The DEAE-Sephadex A-50 could be reused after several washings with 0.5 M NaOH and neutralization with HCl.

Concentration of Myosin Solutions. The myosin eluate was usually too dilute to permit precipitation by dilution with water, hence other methods of concentration were explored. Ultrafiltration resulted in the formation of a gel on the inside surface of the sac. Dry Sephadex G-25 added directly to the myosin solution resulted in the loss of about one-half of the protein, probably due to the formation of concentrated gel around the Sephadex particles. Drying powders sprinkled on the outside of a dialysis sac containing the myosin solution (Baril *et al.*, 1966) were not used because of the possibility of contamination with unknown impurities. Dialysis against buffers at the isoelectric point or at a low ionic strength precipitated myosin from dilute solutions, but this method was avoided for myosin to be used for physical studies because of the possibility of aggregation during prolonged dialysis. The procedure finally adopted was as follows. The myosin solution was first dialyzed against 1 M KCl–0.025 M histidine, pH 6.8, then against an equal volume of glycerol at 0–4°. The glycerol was stirred with a stream of nitrogen gas. The resulting solution of myosin in 50% glycerol, now reduced to one-fourth its volume, was stored at −20° for future use. When needed, it was precipitated by the addition of nine volumes of water or dialyzed against buffer.

Analytical Ultracentrifugation. The experiments were performed with a Spinco Model E ultracentrifuge equipped with temperature control system, Rayleigh interferometer, including the adjustable optical components (Gropper, 1964), and the ultraviolet absorption optical system. The optical system was aligned according to the procedure of E. G. Richards, R. H. Haschemeyer, D. E. Teller, and H. K. Schachman (in preparation). The patterns obtained from the ultracentrifuge were read with a Nikon shadowgraph Model 6C equipped with a 50 power objective lens. All runs were performed at 4° or slightly lower.

The sedimentation equilibrium experiments were performed according to the procedure of E. G. Richards, D. E. Teller, and H. K. Schachman (in preparation). A brief description of some of the most pertinent steps is given here.

An aliquot from the chromatographic experiments was diluted to the maximum desired concentration, then dialyzed against buffer for 1–2 days. The necessary dilutions for the other concentrations were made volumetrically by slow addition of the buffer to the myosin solution, stirring carefully with the pipet. A sedimentation velocity experiment at 59,780 rpm was first performed on the solution of maximum concentration, using both Rayleigh interference and schlieren optics. If the photographs indicated the presence of aggregates, the equilibrium experiment was not performed. If the solution was free of dimers, the myosin concentration, in fringes, was determined from a synthetic boundary experiment; a base-line pattern was obtained after mixing the cell contents without disturbing the orientation of the cell in the rotor. The sedimentation equilibrium experiment was performed simultaneously on four solutions of about 2.2 mm in length, using the six-cell rotor (AN-G) and masked upper window holders with ordinary interference cells (Teller, 1965). Fluorocarbon oil FC-43 was added to bring the solution column into view in the openings in the window holders. As an aid for the location of the hinge point (the position in the cell where the equilibrium concentration is equal to the initial concentration), the white-light fringe was brought into view in the diffraction envelope by the addition of the appropriate amount of 1,3-butanediol to the

reference liquids (Richards and Schachman, 1959). The equilibrium velocity was chosen so that the cell containing the highest concentration would still have resolvable fringes at the bottom of the cell (about 150 fringes/cm), in this instance, 4327 rpm; the rotor did not precess at this speed. Two base-line patterns, one with the filter in place and the other with white light, were taken at the beginning of the centrifugation. A preliminary period of sedimentation at a higher velocity was employed to reach the equilibrium distribution more rapidly. Pictures were taken at approximately 12-hr intervals for 3.5 days, long after the equilibrium distribution was achieved. Final equilibrium patterns were taken with the filter in place and also with white light. The cell contents were redistributed without disturbing the orientation of the cells and another pair of base-line patterns was obtained. After the completion of the equilibrium experiment, sedimentation velocity experiments with schlieren optics were performed with the three remaining myosin solutions.

The fringe positions of the equilibrium patterns were corrected for base-line curvature, using the patterns taken at the beginning and the end of the run. The hinge point was located by comparison of the white-light base line and equilibrium patterns. The initial concentration for the most concentrated solution was determined from the synthetic boundary run, with correction made for curvature of the base line. The initial concentrations for the other solutions were calculated using the appropriate dilution factor.

A computer program was used to calculate the apparent molecular weights $M_w^{app} = (2RT/(1 - \bar{V}\rho)\omega^2) \cdot ((c_b - c_m)/c_0(r_b^2 - r_m^2))$ and $M_z^{app} = (M_{w,b}^{app}c_b - M_{w,m}^{app}c_m)/(c_b - c_m)$, where R is the gas constant; T, the absolute temperature; $\bar{V}$, the partial specific volume; ρ, the density of the solvent; ω, the angular velocity; c, the concentration; and r, the radius measured from the center of rotation. The subscripts m and b refer to the meniscus and the bottom of the cell, and c_0 is the initial concentration. The values for $M_{w,b}^{app}$ and $M_{w,m}^{app}$ were obtained from the slope of the ln c *vs.* r^2 plot at r_b^2 and r_m^2. As a check to see that all the material was accounted for, molecular weight data were obtained from both the conservation of mass treatment and from hinge-point labeling (Richards and Schachman, 1959).

The values for M_w^{app} and M_z^{app} were extrapolated to zero concentration according to the equations of Fujita (1962), $1/M_w^{app} = 1/M_w + B_1^*((c_m + c_b)/2) + \ldots\ldots +$ and $1/M_z^{app} = 1/M_z + B_1^*(c_m + c_b) + \ldots\ldots +$, where M_w and M_z are the weight-average and z-average molecular weights. For polydisperse systems, B_1^* represents a complicated average of activity coefficients and partial specific volumes. For a two-component system, $B_1^* = B_1 + \bar{V}/M + \ldots\ldots +$, where B_1 is the first term in the expansion of the activity coefficient y as a power series in concentration, $\ln y = MB_1c + \ldots\ldots +$.

To convert concentrations from units of fringes to conventional units, 41.33 fringes corresponded to a solution at 1 g/100 ml (Woods *et al.*, 1963). The value used for $\bar{V}$ at 5° was 0.720 (Kay, 1960).

The plot of concentration *vs.* position in the cell for the velocity experiment with interference optics was obtained by combination of data from a pattern taken after the boundary had traversed one-half the cell and a base-line pattern taken immediately after reaching the velocity of the experiment. The two plots of fringe position were superimposed near the meniscus. The measured sedimentation coefficients were corrected to standard conditions in the usual manner.

Results

Pyrophosphate–Sephadex-A-50 System. Typical elution patterns from DEAE-Sephadex A-50 columns with pyrophosphate as the solvent and a KCl gradient (followed by a KOH wash) are shown in Figures 1–3. In general, the patterns from all such experiments were similar, resembling those obtained on DEAE-cellulose by Perry (1960) with KCl–Tris at pH 8.2 and by Asai (1963) with ATP. Three or four small peaks of varying shape appeared before the main peak. Turbidity in the starting material, if any, appeared in the first peak, representing the void volume of the column. In sedimentation properties the protein responsible resembled the aggregated myosin (synthetic thick filaments) of Huxley (1963). Its ATPase activity, when present, was low. That it was not actomyosin was shown by two facts. (1) Actomyosin was absent by the turbidity test of Rice *et al.* (1963), and (2) preparations of known actomyosin when chromatographed under the same conditions gave very broad elution patterns. The other small intermediate peaks were not identified. The main peak appeared at about 0.13 M KCl, and was always broader than expected for a homogeneous protein. Its identification as myosin followed from its sedimentation and enzymatic properties. With some preparations a component appeared as a shoulder on the leading edge of the myosin peak. Sedimentation velocity experiments performed on this material revealed several faster moving peaks, suggestive of the myosin dimers and trimers reported by other workers (Johnson and Rowe, 1961). Similar examinations of fractions taken from the trailing edge did not show the presence of aggregated myosin. The number of components eluted *after* myosin varied from one to three. The first and second appeared during the gradient, and the third, after the pH change following the addition of alkali. They were identified as RNA with trace amounts of protein by their absorption maxima at 260 mμ and by a positive orcinol reaction. These components exhibited sedimentation properties suggestive of molecular weights greater than 10^5; no further attempt was made to characterize them. Their amount could be reduced by increasing the number of reprecipitation cycles.

The results obtained from the chromatography of rabbit myosin are shown in Figure 1. This myosin peak was sharper than that obtained from any other experiment. The peaks seen at the void volume of the column and those representing RNA components also

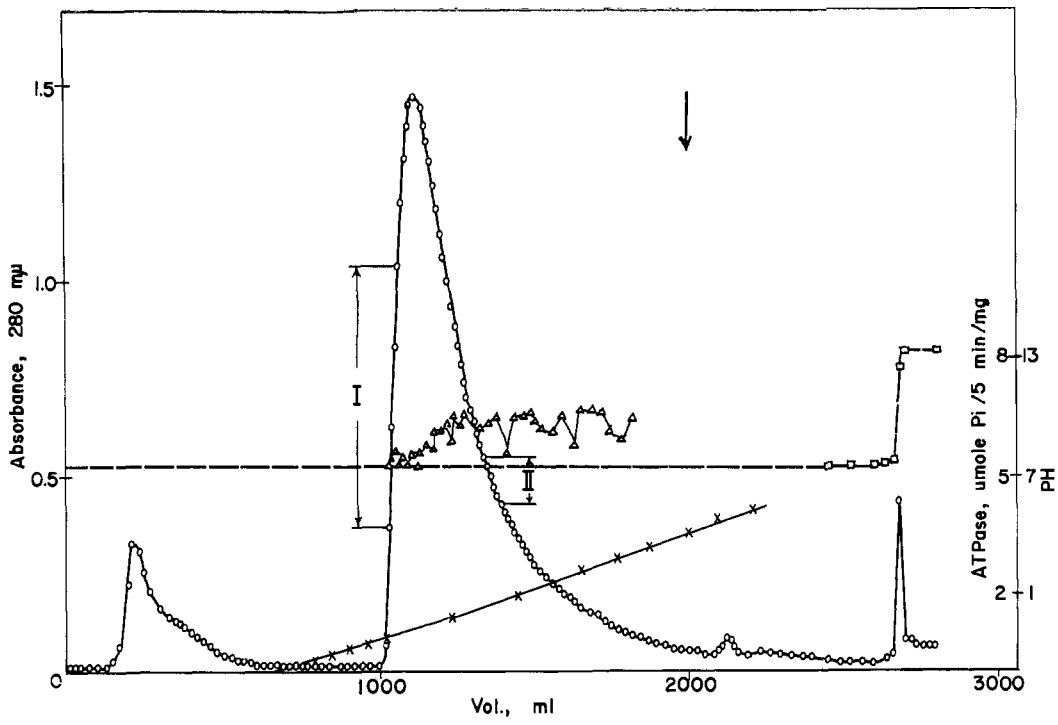

FIGURE 1: Chromatography of rabbit myosin on DEAE-Sephadex A-50. Myosin (95 ml of 1.2%) (prepared by method 1, second extraction buffer) in 0.04 M sodium pyrophosphate, pH 7.5, was applied to a 2.5 × 95 cm column equilibrated with the same buffer. A linear gradient (1 l. of 0.04 M sodium pyrophosphate, 1 l. of 0.5 M KCl–0.04 M sodium pyrophosphate, both at pH 7.5) was applied at 20 ml/hr followed by 0.05 M KOH at arrow until the pH of the eluate reached 13. Fractions of 21 ml were collected to a total volume of 530 ml, then 10.7-ml fractions were collected; recovery, 97%. Open circles, absorption at 280 mμ; triangles, specific ATPase activity; crosses, KCl concentration in eluate; squares, pH of eluate.

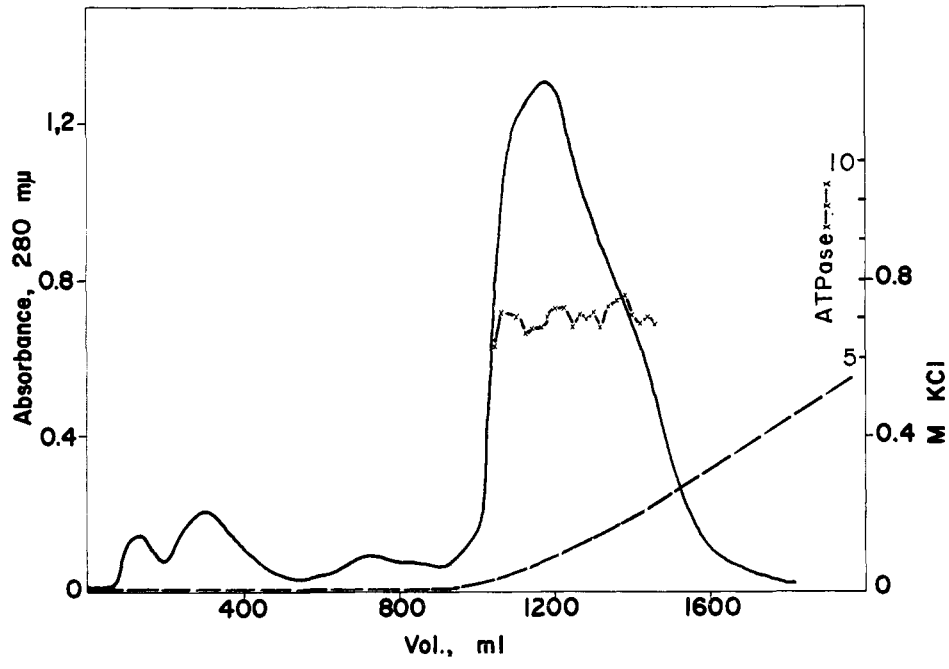

FIGURE 2: Chromatography of chicken myosin on DEAE-Sephadex A-50. Myosin (88 ml of 1.4%) (prepared by method 1, first extraction buffer) was applied to a 4.0 × 50 cm column. A linear gradient (1 l. of 0.04 M sodium pyrophosphate, 1.1 of 1 M KCl–0.04 M sodium pyrophosphate) was applied at 42 ml/hr and 20-ml fractions were collected. Solid line, absorbance at 280 mμ; dashed line, KCl concentration of eluate; crosses, specific ATPase activity.

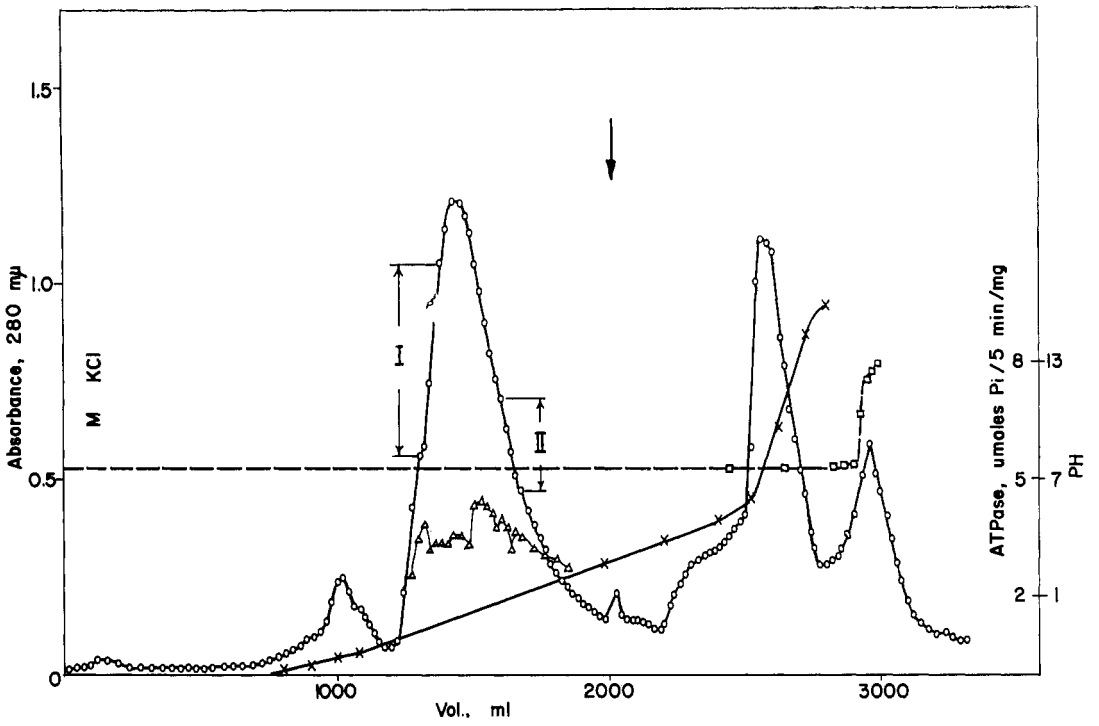

FIGURE 3: Chromatography on DEAE-Sephadex A-50 of myosin prepared from frozen yellowfin tuna. Myosin (100 ml of 2%) (prepared by method 2 and stored in glycerol) was applied to a 4 × 50 cm column. Solvent and gradient were the same as Figure 1, except that at arrow, 1 l. of 1 M KCl–0.5 M NaOH was added. Flow rate, 30 ml/hr; 20 ml/tube were collected. Symbols are the same as in Figure 1.

were much smaller than those seen with other preparations.

With chicken myosin prepared from fresh breast muscle, there were three minor components that appeared before myosin (Figure 2). The suggestion of a shoulder on the trailing edge of the myosin peak is a frequent occurrence.

A profile of a myosin prepared from frozen yellowfin tuna is shown in Figure 3. Only one minor component appeared before the myosin peak, which was broader than those shown in Figure 1 and 2. Three RNA components were present in rather large amounts, presumably arising from the cellular breakdown in the frozen tissues. Similar elution patterns were obtained with myosin prepared from fresh striped bass and bluefin and skipjack tuna.

ATPase Activity. The specific ATPase activity of the rabbit myosin (Figure 1) rose from about 5.3 units at the leading edge of the peak to 6.4 units halfway along the trailing edge and remained nearly constant. Fluctuations in activity from tube to tube were ignored. For myosin prepared from chicken muscle (Figure 2), the specific activity was essentially constant. However, the activity of the myosin from frozen yellowfin tuna (Figure 3) was much lower. Moreover, it rose from 2.5 units at the leading edge to 4.3 at the peak, and gradually dropped to 2.5 for the trailing fractions.

Rechromatography. When pooled fractions of the myosin peak were rechromatographed, the elution diagram usually resembled that of the original, with only traces of the other components. Pooled fractions eluted from both sides of the myosin peak (at 0.10 and 0.18 M KCl) from the experiment with rabbit myosin (Figure 1) were dialyzed against the starting buffer and rechromatographed on smaller columns (Figure 4). The samples were too dilute to see any unabsorbed material originally present in the pooled fractions I and II. Except for a trace of dimer in fraction I, appearing before the main peak, the two absorbance profiles were the same within experimental error. However, in both cases the specific activities rose, then fell. The specific activity profile for fraction I was generally lower than that for fraction II, as were the activity levels before rechromatography. It is difficult to explain how two fractions originally eluted at different KCl concentrations and having different specific activities can be eluted at the same KCl concentration, yet still retain the original specific activities.

For the experiment with frozen yellowfin tuna shown in Figure 3, five fractions on both sides of the peak were combined, concentrated with glycerine, precipitated, redissolved, dialyzed into the starting buffer, and rechromatographed on smaller columns. The elution diagrams are shown in Figure 5. Again the myosin was eluted at the same KCl concentration. A considerable amount of aggregate was present in the leading edge of the peak for fraction I.

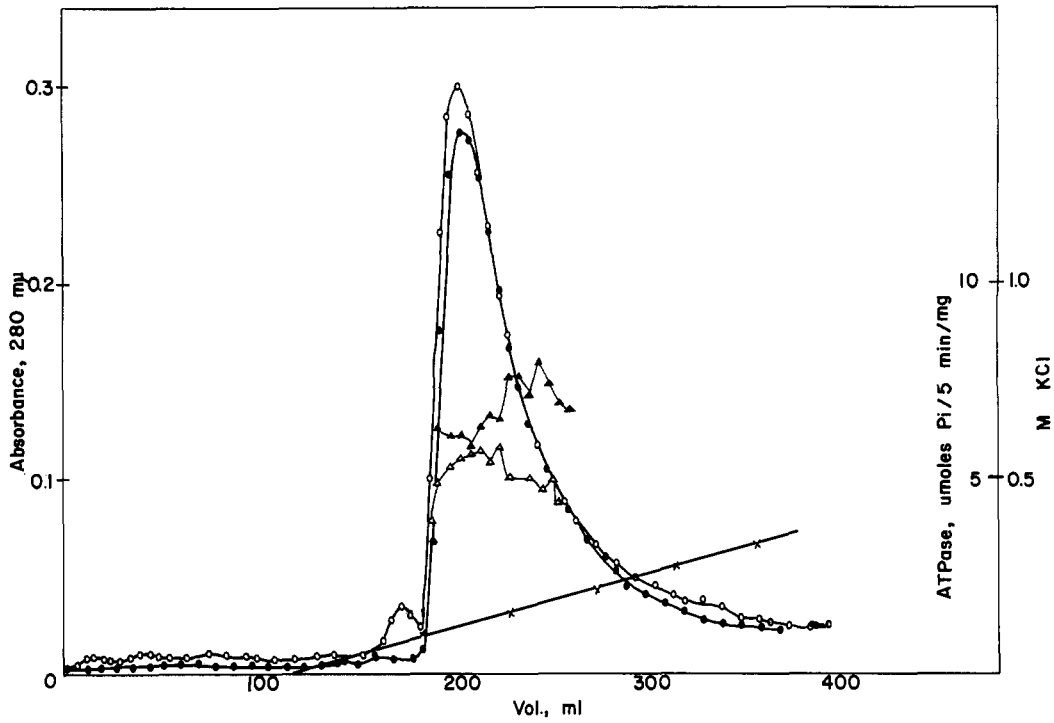

FIGURE 4: Rechromatography of rabbit myosin. Fractions I and II from experiment shown in Figure 1 were dialyzed against starting buffer and applied to separate columns 1.5 × 27 cm. A linear gradient (200 ml of each buffer) was applied at 15 ml/hr and 5-ml fractions were collected. The profiles were superimposed at the KCl concentration (0.12 M) corresponding to the top of the peak. The volume scale corresponds to fraction I. For fraction II, 30 ml should be added. Open and closed symbols refer to fractions I and II, respectively; circles, absorption at 280 mμ; triangles, specific ATPase activity.

Other Elution Systems. Various modifications of the elution systems with the DEAE-Sephadex A-50 column were investigated. The presence of 10^{-4} M $CaCl_2$ did not alter the pattern obtained with the pyrophosphate buffer system. With 0.24 M KCl–0.01 M Tris, pH 7.5, myosin was not retained on the column and the resulting separation into three peaks was not helpful (see Figure 6). This elution pattern was similar to those obtained by Brahms (1959) and by Perry (1960) at pH 7.5.

However, with 0.25 M phosphate buffer at pH 7.5, without chloride ions as a starting buffer, myosin was retained on the column and could not be eluted with a phosphate gradient. Upon the application of a KCl gradient, the resulting elution pattern was similar to that obtained with pyrophosphate. Even with a 0.2 M KCl–0.02 M phosphate starting buffer and a KCl gradient a good resolution was obtained. However, with 0.25 M KCl–0.01 M EDTA, myosin was not retained on the column.

Chromatographic separations were also performed with 0.025 M pyrophosphate at pH 8.7 and 0.01 M pyrophosphate at pH 9.5. The application of a KCl gradient gave elution patterns that were similar to the pyrophosphate system at pH 7.5, but the degree of separation of the various fractions was different. In 0.04 M pyrophosphate at pH 6.5, the myosin solution was more turbid than usual. (The turbidity was reduced upon raising the pH to 7.5.) A similar profile was obtained upon chromatography with this starting buffer except that more material than usual appeared at the void volume. Its turbidity, evident from visual examination of the fractions, suggested that it contained the synthetic thick filaments described by Huxley (1963). The ATP buffer system of Asai (1963) with DEAE-Sephadex A-50 caused extensive shrinkage of the gel with resultant plugging of the column, and was not used.

Other Column Materials. Elution patterns similar to Figure 6 were obtained with DEAE-Sephadex A-25. The failure to retain myosin is not understood.

With phosphonic acid cellulose and the pyrophosphate–KCl gradient system, a single peak followed by a very broad, flat peak was obtained. This system was not investigated further.

Experiments performed with the pyrophosphate buffer system and DEAE-cellulose gave results that were similar to those with DEAE-Sephadex A-50 (Figure 1–3). A detailed comparison of the two materials was not made.

5'-Adenylic Acid Deaminase and Myokinase Activities. To test for 5'-adenylic acid deaminase and myokinase activities, rabbit myosin that had been stored in glycerol at −20° was purified on a 1.5 × 30 cm column of

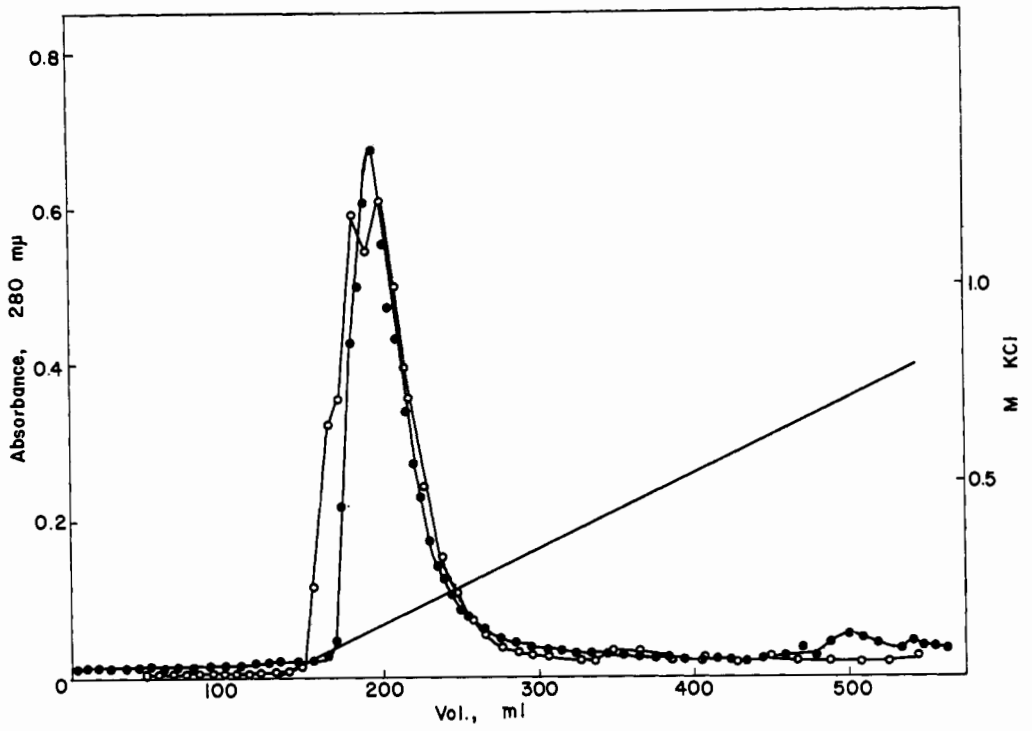

FIGURE 5: Rechromatography of yellowfin tuna myosin. Fractions I and II from experiment shown in Figure 3 were concentrated, precipitated, dissolved, and dialyzed against starting buffer, then applied to separate columns 2 × 30 cm. A linear gradient (250 ml each of 0.04 M sodium pyrophosphate, 1 M KCl–0.04 M sodium pyrophosphate, both at pH 7.5) was applied at 15 ml/hr and 5–10-ml fractions were collected. Symbols are the same as in Figure 4.

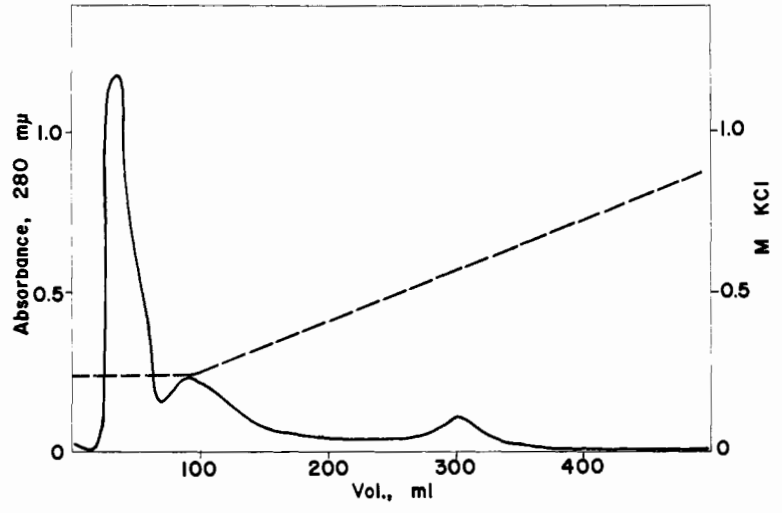

FIGURE 6: Chromatography of striped bass myosin on DEAE-Sephadex A-50 without pyrophosphate. Myosin (10 ml of 1.7%) (prepared by method 3, stored in glycerol) was dialyzed against 0.24 M KCl–0.01 M Tris, pH 7.5, and applied to a 2 × 30 cm column equilibrated with the same buffer. A linear gradient to 1 M KCl was applied at 15 ml/hr. Legend, same as in Figure 2.

DEAE-Sephadex A-50, using 0.094 M phosphate, pH 7.5, and a linear gradient to 0.5 M KCl. (The phosphate system was chosen so that possible effects from pyrophosphate would not have to be considered.) Examination of various fractions revealed that the 5′-adenylic acid deaminase activity was predominantly associated with the fractions appearing at the void volume of the column. Its activity was about 120

units/mg. The activity of the myosin peak was found to be about 10 units/mg, compared to 100 units for the starting material assayed under the same conditions. This reduction of the activity to 10% may be compared to the 33% found by Perry (1960).

Pooled fractions of the myosin peak were dialyzed against 0.04 M KCl–0.01 M histidine, pH 6.3, to precipitate the myosin. After centrifugation the myosin was dissolved in the buffer used for the myokinase assay and dialyzed against the same buffer. With 1.5 mM ADP used as the substrate, the reaction was followed for 1 day. The specific activity of this purified myosin was 1.9×10^{-4} μmole of P_i/min per mg, compared to 4.6×10^{-4} for the starting material. This reduction of about 50% may not be significant, since the assays were not performed at the same time.

The myosin before chromatography was also assayed for myokinase activity with 0.5 mM ATP as the substrate. The initial rate of ATP hydrolysis, owing to ATPase activity, measured for 10 min, was found to be 0.038 μmole of P_i/5 min per mg, about 1% of the value obtained with the usual assay buffer. This decrease was probably primarily due to the presence of magnesium instead of calcium. If it is assumed that all of the ATP was hydrolyzed in the first hour, the liberation of P_i caused by the presence of myokinase during the next 2 hr was 3.2×10^{-4} μmole of P_i/min per mg. This value is in good agreement with that obtained when ADP was used as the substrate.

Effect of Pyrophosphate on ATPase Activity of Myosin. At the time most of these experiments were performed we were aware of the possibility of pyrophosphate inhibition of the ATPase activity of myosin, but not of enhancement. Calculations indicated that the pyrophosphate concentration in the reaction mixture owing to incomplete dialysis of the myosin fractions could be from 0.1 to 0.01 mM, too low to be noticeable as an inhibitor in the presence of 4 mM ATP. Since an aliquot of the starting myosin sample in pyrophosphate was always dialyzed with the chromatographed fractions in the same vessel, the relative activities are valid, even though inhibition or enhancement may have taken place. Moreover, the activity of this starting myosin was within the range found for myosin that had never been exposed to pyrophosphate.

Attempts to evaluate the role of pyrophosphate on the ATPase activity of unchromatographed myosin never exposed to this ion were not reproducible. Three experiments covering a range of pyrophosphate concentrations revealed an enhancement of about 20% at 3.3×10^{-4} M pyrophosphate, while three later experiments performed under the same conditions, except that a different lot of ATP was used, showed no significant effect. Assays performed at the same time with the two different lots of ATP confirmed that their effects on the ATPase assay differed. The pyrophosphate might have influenced the ATPase activity indirectly, possibly by combining with contaminating divalent ions that might have been present in the ATP.

To demonstrate that exposure to pyrophosphate did not permanently alter ATPase activity, an aliquot of myosin was dialyzed against 0.04 M pyrophosphate, pH 7.5, then exhaustively against 0.5 M KCl–5×10^{-4} M borate, pH 7. Another aliquot was dialyzed against another portion of the latter buffer. The activities of the two samples, measured at the same time, were the same within experimental error.

Titration of Pyrophosphate and DEAE-Sephadex A-50. Titration studies were performed to investigate the binding properties of pyrophosphate and DEAE-Sephadex A-50. For 0.04 M pyrophosphate at room temperature it was found that the third and fourth pK values are 6.1 and 8.4. Thus the ionic species predominant at pH 7.5 has three charges, giving an ionic strength of about 0.28 for a solution of 0.04 M $Na_4P_2O_7$ with pH adjusted to 7.5. Titration studies performed in the presence of DEAE-Sephadex failed to reveal any significant difference in the third and fourth pK value of pyrophosphate.

Molecular Weight. The molecular weights of myosins contained in the peak tubes from several chromatography experiments were investigated by sedimentation equilibrium. The results of initial experiments with rabbit myosin and yellowfin tuna myosin were not valid, since aggregation had occurred, as shown by sedimentation velocity studies and by the substantial upward curvature of the ln c *vs.* r^2 plots. This aggregation probably resulted from the method of dilution, in which the protein solution was added to the buffer.

In one experiment with chicken myosin, in which the dilution was performed by addition of the buffer to the protein solution, the myosin did not aggregate; *i.e.*, there were no fast-moving components in sedimentation velocity experiments. This sample was then used for sedimentation velocity and equilibrium experiments at different concentrations. (Preliminary results from this experiment have been reported elsewhere: Richards *et al.*, 1965.)

Four cells containing myosin at concentrations of 3.0, 2.25, 1.5, and 0.75 mg/ml were run simultaneously for 84 hr. That equilibrium had been achieved was demonstrated by the fact that there were no significant differences in fringe positions for patterns obtained at 67 and 84 hr. The plots of ln c *vs.* r^2 displayed downward curvature, indicating that aggregated myosin was not present and that the material exhibited nonideal behavior.

The plot of $1/M_z^{app}$ and $1/M_w^{app}$ *vs.* concentration is shown in Figure 7. The equations of the least squares straight lines drawn through the corresponding points are $1/M_w^{app} = 1.98 \times 10^{-6} + 0.17 \times 10^{-6}c$ and $1/M_z^{app} = 1.85 \times 10^{-6} + 0.33 \times 10^{-6}c$, where c is measured in mg/ml. There were no significant differences in results obtained from the conservation of mass treatment or from hinge-point labeling. Even though the uncertainty in M_z^{app} is probably three to five times greater than in M_w^{app} the values of the slopes are consistent in that the value for M_z^{app} is about two times that for M_w^{app}, in agreement with theory (Fujita, 1962). The slope for $1/M_w^{app}$ falls within the range reported by Tonomura *et al.* (1966). From

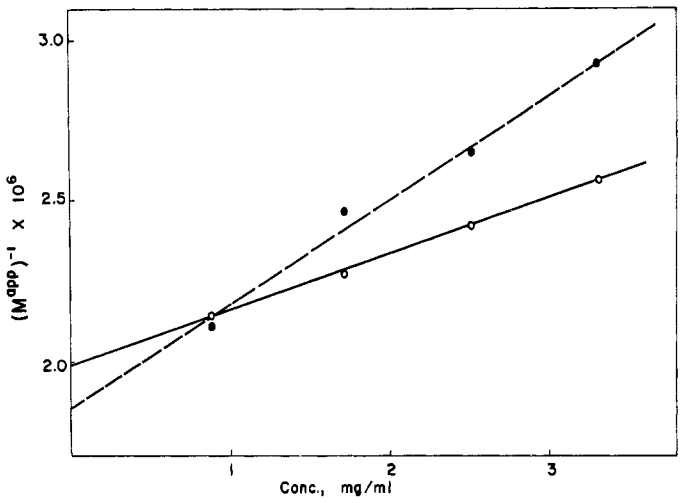

FIGURE 7: Sedimentation equilibrium of chicken myosin. A portion of the peak tube from a chromatography experiment similar to that shown in Figure 2 was dialyzed against 0.6 M KCl–0.01 M Tris, pH 7.0. The dilutions were performed as described in the text. Circles $(M_w^{app})^{-1}$; closed circles $(M_z^{app})^{-1}$.

the intercepts it is found that M_w and M_z are 5.04 and 5.41 $\times$ 10^5 g mole^{-1}. These values are consistent with a mixture containing 90% monomer of molecular weight 4.59 $\times$ 10^5 g mole^{-1} and 10% dimer. However, that dimers were not formed during the prolonged equilibrium centrifugation was demonstrated by subsequent velocity experiments performed with the remaining portions of the solutions stored on ice. We therefore believe that the observed difference between M_z and M_w is insignificant for two reasons: (1) the greater uncertainty in M_z^{app}, and (2) a slightly higher value for M_z^{app} would result from even a small amount of higher aggregates. Thus, we estimate the true molecular weight for the myosin monomer as obtained from chromatography to be 5.0 $\times$ 10^5 g mole^{-1} or slightly lower.

The base lines at the beginning and end of the run were compared to determine whether any myosin precipitated during the prolonged centrifugation. For the cell containing the highest concentration, the two base lines were the same within 0.02 fringe. The second cell leaked upon reacceleration. For the third and fourth cells (the two most dilute solutions), the base lines shifted in a manner that could be explained by a loss in myosin concentration of 0.08 and 0.10 fringe, respectively. Since the error in such measure-

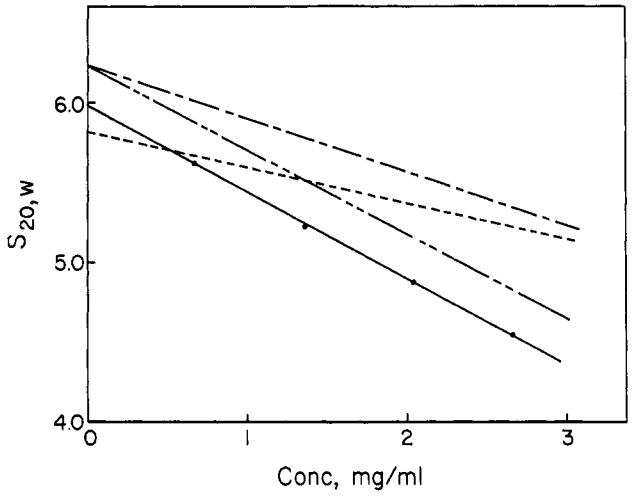

FIGURE 8: Sedimentation velocity of chicken myosin. The same four samples used for the sedimentation equilibrium study shown in Figure 7 were also examined by sedimentation velocity. ●, experimental points for chicken myosin; ——– –, rabbit myosin (von Hippel *et al.*, 1958); – – – –, lobster (Woods *et al.*, 1963); and —— – —, chicken myosin (J. Marshall, 1964, personal communication).

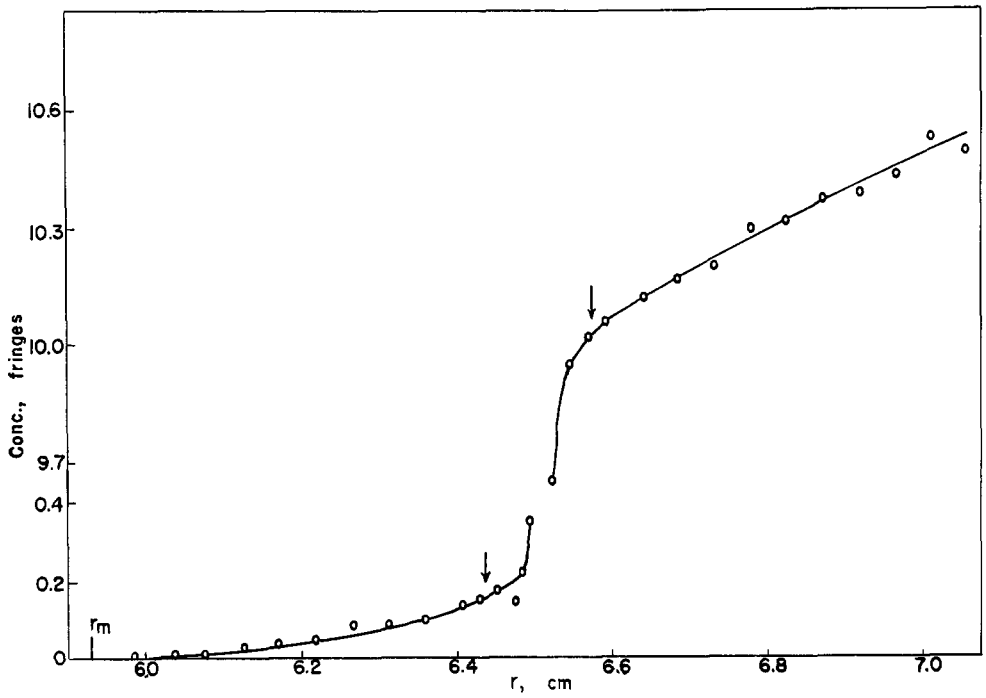

FIGURE 9. Sedimentation velocity of chicken myosin with Rayleigh interference optics. Speed, 59,780 rpm; time, 138 min; temperature 4.6°.

ments is about ±0.05 fringe, the difference is only slightly greater. A lateral shift of the base line can be due to two other things besides precipitation of protein: (1) evaporation of water from either of the two compartments, or (2) distortion of the cell. It cannot be decided which of the three occurred, but if there were precipitation of protein, the value of 0.10 fringe represents only a 3% error in the initial concentration (3.05 fringes) for that cell, and, therefore, a 3% error in M_w.

Sedimentation Velocity. The same four solutions of chromatographed chicken myosin used for the molecular weight determination were also used for the measurement of sedimentation coefficients. The plot of $s_{20,w}$ *vs.* concentration is shown in Figure 8. The least-squares straight line through the four points is governed by the relationship $s_{20,w} = 5.96 - 0.53c$, where c is measured in milligrams per milliliter.

These results are compared with those of other workers in Figure 8. Our value for $s^0_{20,w}$ is within the range reported by these workers, but the slope is greater. Our four points have nearly the same slope as the curve shown by von Hippel *et al.* (1958), but the intercept is not the same. The significance of the higher concentration dependence for our preparation is uncertain because the values represent a single fraction from a single preparation.

Before the equilibrium experiment, the most concentrated solution had been examined by sedimentation velocity using both Rayleigh and schlieren optics. The schlieren pattern showed a single, extremely narrow peak similar to those obtained by other workers; there was no suggestion of faster moving components. However, careful examination of the Rayleigh patterns revealed traces of material of unknown nature sedimenting both slower and faster than the main component (Figure 9). If the material included between the arrows is taken to be myosin, then the slower and faster materials represent about 1 and 5% of the total, respectively.

It should be emphasized that: (1) the changes in the concentration in these regions are so low that they could not be detected with the schlieren optical system, and (2) the material moving ahead of the main component was not the "aggregated" myosin frequently seen as faster moving peaks but material with a broader spectrum of sedimentation properties. This material could be formed from myosin aggregated in different ways or collapsed to different degrees to form molecules with different frictional coefficients. It is also possible that the small concentration changes could be due to convective disturbances, but this is unlikely owing to the stabilizing effect of the density gradient formed by redistributed KCl molecules.

Discussion

The chromatographic system using a DEAE-Sephadex A-50 column with pyrophosphate and a KCl gradient eluate should be of general utility for future studies of myosin, both as an analytical tool to test the purity of myosin and as a preparative method for its further purification. All preparations from the several sources that we have examined gave similar elution patterns.

The amounts of the nonmyosin components varied, but the profile of the myosin peak was remarkably similar, even when the starting material was obtained from frozen muscle and had lower enzymatic activity. Purified myosin from frozen muscle could be used for compositional or physical studies, in spite of this reduced activity.

The procedure removes strongly aggregated myosin appearing at the void volume and RNA. Some myosin dimers may appear at the leading edge of the peak, but the trailing half is monomeric myosin of high purity. Thus, valuable preparations that would ordinarily be discarded because of aggregation could at least be partially salvaged.

It is probable that the purified myosins obtained from DEAE-cellulose with 0.15 M KCl buffer at pH 8.2 (Perry, 1960) or with ATP buffer at pH 7.5 (Asai, 1963) are the same as that obtained with our procedure using DEAE-Sephadex A-50 with pyrophosphate, even though they presented evidence of the presence of myosin aggregates in the purified material, the former by sedimentation velocity and the latter by light scattering. More work with their systems would have to be performed in order to demonstrate the absence of aggregates.

The results obtained from DEAE-Sephadex A-50 with pyrophosphate and with phosphate are similar, suggesting that the basis of separation is the same in both cases. We do not have sufficient data with phosphate to evaluate which is better. For physical studies on myosin, it is recommended that the former system be used, since more work has been done with it. For enzymatic studies the phosphate system would be the one of choice, because of possible effects of pyrophosphate on the ATPase activity.

The fact that myosin is retained on the DEAE-Sephadex A-50 column in the presence of pyrophosphate at a rather high ionic strength (0.28) indicates that its retention and elution are not due merely to changes in ionic strength, but that pyrophosphate ions act as bridges to hold the protein to the charged groups on the column and chloride ions are required to displace it. Phosphate, but not EDTA, acts in the same manner.

The results with DEAE-cellulose and with DEAE-Sephadex A-50 were similar, but the DEAE-Sephadex A-50 was preferred for three reasons. (1) It was more resistant to abrasion; (2) it could be reused; and, most important, (3) the spherical particles gave better flow rates at higher concentrations of myosin.

The peak obtained from the chromatography of myosin on DEAE-Sephadex A-50 with pyrophosphate buffers was broader than that expected for a homogeneous protein. The profiles of leading and trailing fractions upon rechromatography were nearly identical, suggesting that only slight differences exist between them. Preliminary examination of various myosin fractions indicated that there were no significant differences in amino acid composition, molecular weight, sedimentation behavior, and ultraviolet absorption (C. S. Chung and E. G. Richards, 1966, unpublished data).

However, a variation in ATPase activity across the myosin peak was always evident. For good preparations, this variation was only slightly greater than experimental error; Asai (1963) obtained similar results from the chromatography of myosin preparations from rabbit back muscle using DEAE-cellulose with ATP as the solvent. The variation could at least partially be explained by the known differences (Barany *et al.*, 1965; Maddox and Perry, 1966) in the ATPase activities of myosins prepared from red and from white muscle of the same species. Back muscle from rabbit and breast muscle from chicken might contain both kinds of myosin.

In considering the possible heterogeneity of myosin, it is interesting to note that myosin as prepared by most workers represents 30% or less of the total myosin in the muscle. This estimate is based upon a maximum myosin yield of about 2 g/100 g of wet muscle, a myosin content of 34% of the total protein (Hanson and Huxley, 1957), and a protein content for muscle of about 20%. Such myosin is distinguished by certain properties. The 10-min extraction period removes that myosin which is the most easily extractable. Whether this easily extractable myosin comes from myofibrils near the surface of the muscle mince or from partially degraded or newly synthesized thick filaments is not known. Centrifugation in 0.3 M KCl to remove actomyosin and aggregated myosin leaves in the supernatant that myosin which has less tendency to combine with actin or to aggregate into thick filaments. To determine whether myosin is truly a homogeneous protein requires the examination of preparations representing a much larger fraction of the total myosin in a muscle.

A significant difference in ATPase activity across the myosin peak was always observed for preparations from frozen tuna muscle. The material eluted near the center of the peak exhibited higher activity than that at the sides of the peak. These variations could be due to damage suffered by myosin molecules, either during the freezing process or upon storage in the frozen state. It is interesting to note that even though the ATPase activity of the myosin was impaired, it still behaved chromatographically like myosin of high activity.

It should be emphasized that the molecular weight of myosin reported here is only a single value obtained from a single tube of a single myosin preparation. However, the value should have validity because (1) it was demonstrated that the myosin did not aggregate during the prolonged centrifugation, (2) all the protein initially present in the cells at the beginning of the experiment was shown to be still there at the end, and (3) the values for the concentration dependence of M_z^{app} and M_w^{app} are consistent with theory. Further work will be required before the true molecular weight of myosin is established.

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References

Asai, H. (1963), *Biochemistry 2*, 458.

Barany, M., Barany, K., Reckard, T., and Volpe, A. (1965), *Arch. Biochem. Biophys. 109*, 185.

Baril, E. F., Love, D. S., and Herrmann, H. (1964), *Science 146*, 413.

Baril, E. F., Love, D. S., and Herrmann, H. (1966), *J. Biol. Chem. 241*, 822.

Brahms, J. (1959), *J. Am. Chem. Soc. 81*, 4997.

Brahms, J., and Brezner, J. (1961), *Arch. Biochem. Biophys. 95*, 219.

Colowick, S. P. (1955), *Methods Enzymol. 2*, 598.

Connell, J. J. (1960), *Biochem. J. 75*, 530.

Fujita, H. (1962), Mathematical Theory of Sedimentation Analysis, New York, N. Y., Academic.

Gropper, L. (1964), *Anal. Biochem. 7*, 401.

Hanson, J., and Huxley, H. E. (1957), *Biochim. Biophys. Acta 23*, 250.

Huxley, H. E. (1963), *J. Mol. Biol. 7*, 281.

Johnson, P., and Rowe, A. J. (1961), *Biochim. Biophys. Acta 53*, 343.

Kay, C. M. (1960), *Biochim. Biophys. Acta 38*, 420.

Leloir, L. F., and Cardini, C. E. (1957), *Methods Enzymol. 3*, 843.

Maddox, C. E. R., and Perry, S. V. (1966), *Biochem. J. 99*, 8P.

Nikiforuk, G., and Colowick, S. P. (1955), *Methods Enzymol. 2*, 469.

Perry, S. V. (1960), *Biochem. J. 74*, 94.

Rice, R. V., Asai, H., and Morales, M. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 549.

Richards, E. G., Menzel, D. B., Chung, C. S., and Olcott, H. S. (1965), *Federation Proc. 24*, 598.

Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.

Smoller, M., and Fineberg, R. A. (1964), *Biochim. Biophys. Acta 86*, 187.

Teller, D. E. (1965), Ph.D. Dissertation, University of California, Berkeley, Calif.

Tonomura, Y., Appel, P., and Morales, M. F. (1966), *Biochemistry 5*, 515.

von Hippel, P. H., Schachman, H. K., Appel, P., and Morales, M. F. (1958), *Biochim. Biophys. Acta 28*, 504.

Woods, E. F., Himmelfarb, S., and Harrington, W. F (1963), *J. Biol. Chem. 238*, 2374.