active bands of the proteins in this spectral region to particular types of modified residues based solely on spectral positions.

Hence, definitive assignments of all wavelength bands to particular residues awaits further characterization of the modified proteins and examination of systems in which unique modification of particular groups can be performed unambiguously. The location of the azoamino acid derivatives in the primary sequence of some of these proteins and the effects of these modifications on enzymatic function constitutes a continuing study in this laboratory. It would appear that such optically active azo protein derivatives should prove valuable in future studies of the overall and local conformations of proteins.

Acknowledgment

We thank Drs. James F. Riordan and David D. Ulmer for their valuable advice and interest.

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Myosin Structure as Revealed by Simultaneous Electrophoresis of Heavy and Light Subunits*

B. Paterson and R. C. Strohman†

ABSTRACT: A method for the simultaneous resolution of the sulfonyl derivatives of the large and small subunits of myosin on sodium dodecyl sulfate acrylamide gels is presented. DEAE-Sephadex chromatographed myosin (myosin-II) isolated from chicken or rabbit muscle contains only two low molecular weight components when extracted at low pH (6.2) and high ionic strength (μ = 0.5) in the absence of magnesium ions. Myosin-I, extracted by high pH (9.5) and low ionic strength (μ = 0.10), containing magnesium, shows three major and one minor low molecular weight components after chromatography on DEAE-Sephadex. Of the two additional components extracted at high pH, one is tentatively identified as actin. The low molecular weight components released from myosin by sodium dodecyl sulfate treatment are identical with those released by exposure to a pH greater than 10.5. Molecular weight estimates for the two low molecular weight components of myosin-II are made from their electrophoretic mobilities on acrylamide gels calibrated for molecular size. Preliminary molecular weight estimates for the two low molecular weight components are 18,500–19,500 and 32,100–33,000, respectively.

Recent work on the structure of the myosin molecule suggests that the molecule is composed of at least two low molecular weight protein chains (LMP)[1] of average molecular weight 20,000–30,000 (Gershman *et al.*, 1966; Frederiksen and Holtzer, 1968) in addition to the two heavy or fibrous (f) subunits of molecular weight 200,000 (Kielly and Harrington, 1960; Gazith *et al.*, 1970; Frederiksen and Holtzer, 1968). The LMP components are released from the parent molecule by a number of different procedures: treatment with urea or guanidine hydrochloride, exposure to alkaline solutions above pH 10.5, chemical modifications involving

* From the Department of Zoology, University of California, Berkeley, California. *Received March 6, 1970.* One of us (R. C. S.) was supported by U. S. Public Health Service Grant 13882.

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[1] LMP = low molecular weight protein.

succinylation or acetylation, exposure to specific salts, heat treatment, and, as reported in this paper, treatment with the anionic detergent, sodium dodecyl sulfate (Gershman *et al.*, 1968). There exists, however, some controversy concerning the number of LMP components associated with myosin and whether they are tightly bound artifacts or functional parts of the parent molecule.

The work of Gaetjens *et al.* (1968) indicates three major and one minor component among the alkali-dissociated LMP subunits released from twice-chromatographed, highly purified myosin. Locker and Hagyard (1968) obtain 2–5 LMP components for acetylated, DEAE-cellulose chromatographed rabbit cardiac, red, and white muscle myosins. Gershman *et al.* (1966), Gershman and Dreizen (1970), and Dreizen and Gershman (1970) have demonstrated four to six bands by cellulose acetate electrophoresis of isolated alkali-dissociated LMP components released from unchromatographed rabbit myosin. The isolation of at least one LMP component from highly purified subfragment-1 of myosin suggests that each f-subunit has associated with it a single LMP component (Trotta *et al.*, 1968; Lowey *et al.*, 1969).

The evidence bearing on the functional significance of the LMP components is equally mixed. Thus Weeds (1969), and Gazith *et al.* (1970), have reported that DTNB (5,5-dithiobis-(2-nitrobenzoic acid)) treatment of rabbit myosin will preferentially release one of the LMP components without substantially impairing the ATPase activity of the treated myosin. Gaetjens *et al.* (1968) have concluded that functional myosin, as judged by ATPase activity and actin combining ability, cannot be recovered from solutions of myosin that have been exposed to conditions which fully dissociate the LMP components. But Frederiksen and Holtzer (1968), Stracher (1969), and Dreizen and Gershman (1970) have reported that ATPase activity and/or actin combining ability can be recovered in solutions of fully dissociated myosin under the appropriate reassociating conditions. In the latter two cases mixing and reassociation was allowed to proceed after separation of the f-subunit from the LMP components.

A number of these differences and ambiguities are, in all probability, generated by the variety of methods used to extract and purify myosin, and the limited sensitivities of the methods used to determine the extent of the dissociation of LMP components. Work in this laboratory on the kinetics of synthesis of myosin subunits by cultured muscle cells has had to meet most of these problems. It became apparent that many of the difficulties encountered in attempts to clearly identify the myosin subunits could be avoided if one could electrophorese rapidly and simultaneously in the same system both the f-subunit and the LMP components.

There are, however, formidable problems attending the electrophoresis of the f-subunit. Myosin or the f-subunit is only soluble in solutions of high ionic strength. Such solutions are particularly unfavorable for electrophoresis due to the temperature and convection effects generated by solvents of such high conductivity. Since the LMP components are soluble in low ionic strength solutions it is usually necessary to isolate them from either the parent molecule or f-subunit prior to electrophoresis.

There have been several attempts to resolve the f-subunit of myosin on polyacrylamide gels. Thus, Small *et al.* (1961) developed a urea–acrylamide system which depends on high concentrations of urea (12 M) and elevated temperatures (45–50°) to retain the f-subunits in solution at low ionic strengths. We have found this system very difficult to work with. As Small *et al.* (1961) have made clear, run times are long (15–24 hr), resolution of components is poor, and a large proportion of the protein remains aggregated at the site of sample application never entering the body of the gel. More recently, Florini and Brivio (1969), using Small's method, have demonstrated apparent heterogenity of chicken myosin in different concentrations of urea. In addition, Stark *et al.* (1960) have demonstrated that cyanate ion, a natural equilibrium product of urea, is easily formed in concentrated urea solutions of high temperature and pH, the conditions which characterize the electrophoresis system described by Small. At equilibrium, under the mild conditions of room temperature and neutral pH, Stark reports that 8 M urea solutions may be 0.02 M in cyanate ion. The presence of cyanate ion in protein solutions results in the carbamylation of free amino groups, ϵ amino groups, and sulfhydryl groups (Stark *et al.*, 1960) which, in turn, alters the native charge and electrophoretic behavior of the protein. However, this system has been used, with apparent success, as an assay system for various muscle proteins synthesized in a cell-free system (Heywood *et al.*, 1967; Heywood and Rich, 1968; Heywood and Nwagwu, 1969).

The work reported here, using sodium dodecyl sulfate to dissociate and solubilize the subunits of myosin, describes a rapid, highly reproducible, and extremely sensitive electrophoresis method for the simultaneous resolution of the sulfonyl derivatives of the myosin subunits. Some of the earlier experiments dealing with the number and degree of dissociation of LMP components of myosin are reexamined and many of the ambiguities are apparently resolved.

Materials and Methods

Myosin Isolation Procedures. Leg muscle from 5- to 14-day posthatch, white leghorn chickens was finely minced with two passes through a prechilled Universal-400 meat grinder and the mince was extracted in one of two different ways. In procedure I myosin was extracted according to Baril (Baril *et al.*, 1966) except that sodium salts were used in all instances. The extraction solution contained 0.02 M pyrophosphate and 1 mM $MgCl_2$ at pH 9.5. Myosin was precipitated from the crude extract by a tenfold dilution with ice-cold, glass-distilled water rather than with ammonium sulfate. This preparation is known as myosin-I. In procedure II the mince was extracted with three volumes (w/v) of Hasselbach–Schneider buffer (1951) containing 0.47 M KCl–0.01 M KH_2PO_4–0.01 M sodium pyrophosphate at pH 6.2, for 30–60 min while stirring slowly in the cold. The extract was separated from the residue by centrifugation at 8000*g* for 10 min and was then diluted tenfold with ice-cold, glass-distilled water to precipitate the myosin. This preparation is known as myosin-II. In both procedures subsequent steps were identical. The precipitates of crude myosin were redissolved with enough 2 M NaCl–0.1 M L-histidine, pH 6.8 (NaCl–histidine buffer), to bring the final concentration of NaCl to 0.5 M. This solution was then further diluted to 0.3 M NaCl and spun at 35,000 rpm in the Spinco Model L ultracentrifuge (40 rotor) for 1 hr to pellet any actomyosin.

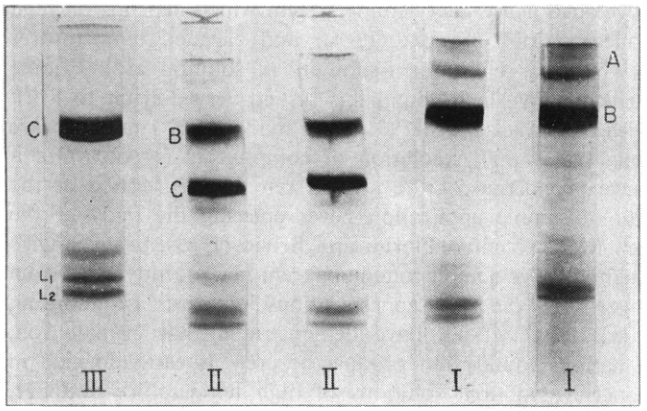

FIGURE 1: Effects of sulfonation on the electrophoretic mobility of the f-subunit of myosin. Preparations are of unchromatographed chick myosin-I. Resolution in the LMP region of preparations I and II is obscured for these samples from earlier phases of this work: (I) no sulfonation, electrophoresed for 2 hr; (II) 50 mM sodium sulfite, electrophoresed for 2 hr; (III) 100 mM sodium sulfite, electrophoresed for 90 min.

The supernatant solution was subjected to two more reprecipitation cycles and was finally resuspended in 0.5 M NaCl–histidine buffer and 50% glycerol for storage at −20°. Procedures I and II produced myosins with 276 mμ/254 mμ absorbancy ratios of 1.4 and 1.55, respectively. These are the unchromatographed preparations of chicken leg muscle myosins-I and -II.

The rabbit myosin preparations used in these experiments were the generous gifts of Dr. E. Glen Richards and were extracted according to procedure II and were chromatographed on Sephadex-DEAE A-50 in 0.04 M sodium pyrophosphate buffer, pH 7.5, according to Richards *et al.* (1967).

Chromatography of the Myosin Preparations. The glycerol–myosin preparations were dialyzed directly into column buffer (0.04 M sodium pyrophosphate, pH 7.5) and were loaded onto columns of either Sephadex-DEAE A-50 or Whatman microgranular DE-52 at a protein concentration of less than 1.0%. Myosin was eluted from the columns with a linear gradient of increasing NaCl concentration from zero to 0.5 M NaCl made up in two to four column volumes of buffer. Fractions were monitored for protein at 278 mμ and peak fractions from the column were pooled for dialysis against low ionic strength buffer (0.04 M NaCl–2 mM L-histidine, pH 6.8) to precipitate the myosin. Column chromatographed myosins were resuspended in 0.5 M NaCl–histidine buffer and 50% glycerol for storage at −20°. The 276 mμ/254 mμ absorbancy ratio for chromatographed myosins was 2.0–2.5 and was essentially free of RNA.

Preparation of Rabbit Actin. Column-purified rabbit actin was a generous gift from Dr. Eloise Clark. This actin was completely free of tropomyosin as judged by acrylamide gel electrophoresis.

Preparation of LMP Components. DEAE-chromatographed chicken myosins-I and -II, in 0.5 M NaCl–0.025 M L-histidine, pH 6.8 at 0°, were quickly mixed with enough 1.0 M dimethylamine-HCl, pH 11.0, to bring the pH of the myosin solution quickly to 11. This mixture was allowed to stand for 1 hr in the cold, and was then diluted 10- to 15-fold with ice-cold, glass-distilled water to precipitate the myosin. Myosin was centrifuged off at 10,000 rpm for 10 min and the supernatant was lyphilized. The resulting dried protein was extracted for 1 hr with 0.04 M NaCl–histidine buffer, clarified at 10,000 rpm in the Sorvall centrifuge, and stored at 0° until used. This solution contained the LMP components.

Sulfonation of Myosin. One volume of sulfonating reagent containing 0.2 M sodium sulfite–0.2 M Tris, pH 8.5, 1.0% (w/v) sodium dodecyl sulfate, and 25% (v/v) glycerol was mixed with one volume of protein solution at a concentration of 2–5 mg/ml. The protein solutions were first dialyzed against a potassium-free solution of 0.3 M NaCl–histidine buffer, pH 6.8, containing 0.1% mercaptoethanol. The reaction mixture was incubated in a wide-mouth, open vessel protected from dust at 27° for about 2 hr. A reaction vessel was chosen to give the reaction mixture a surface to volume ratio of one, for the reaction is driven by the oxidation of free sulfhydryl groups to disulfide groups which are then split in the sulfonation reaction (Cole, 1967). The high-temperature conditions were used to keep the sodium dodecyl sulfate in solution and to accelerate the reaction rate. The sulfonation reaction mixture always contained sodium sulfite in a 14-fold excess to β-mercaptoethanol.

Lithium Chloride Treatment of Myosin. Precipitated pellets of unchromatographed myosins were resuspended in a small volume of LiCl solvent to a final concentration of 4 M LiCl–25 mM Tris, pH 7.5, and 2.5 mM Mg^{2+}. This material was then layered onto a 1.25 × 20 cm Sephadex, G-200 column equilibrated with the same solvent, and run at the maximal flow rates suggested in the Sephadex Manual (Pharmacia Fine Chemicals, Inc., 1967). Fractions (2 ml) were collected and the absorbance at 278 mμ was used to indicate protein content of the fractions. The entire column step took a minimum of 6 hr. We were unable to attain the flow rates reported by Stracher (1969) for a 1.5 × 20 cm column of Sephadex G-200.

DTNB Treatment of Myosin. DTNB [5,5-dithiobis-(2-nitrobenzoic acid)], myosin, and DTNB-released LMP components were prepared from unchromatographed myosins according to Weeds (1969) and Gazith *et al.* (1970). Pellets of myosin were solubilized with the addition of 2 M NaCl–0.1 M L-histidine, pH 6.8, to a final concentration of 0.5 M NaCl. This solution of myosin was mixed with an equal volume of DTNB solution containing 0.02 M DTNB–0.5 M NaCl–0.05 M Tris, pH 8.5. The reaction was stopped after 15 min by diluting the mixture tenfold with ice-cold, glass-distilled water to precipitate the DTNB-myosin from the DTNB-released LMP components. The supernatant solution containing the released LMP components was freeze-dried, redissolved in 0.04 M NaCl–histidine buffer, dialyzed against the same buffer containing 0.1% β-mercaptoethanol, and stored at 0° until further use. The DTNB-myosin was solubilized with the addition of 2.0 M NaCl–histidine buffer, pH 6.8, to 0.5 M NaCl, and was dialyzed against the same buffer with 0.1% β-mercaptoethanol to remove the DTNB reagent. DTNB-myosin was occasionally chromatographed on a 1.25 × 20 cm Sephadex G-200 column equilibrated with 0.5 M NaCl–0.05 M Tris, pH 8.5. The column flow rate was 8 ml/cm² hr and 2-ml fractions were collected and monitored for protein at 278 mμ.

Electrophoresis Conditions for Myosin. Electrophoresis was performed in a continuous buffer system using a 0.025

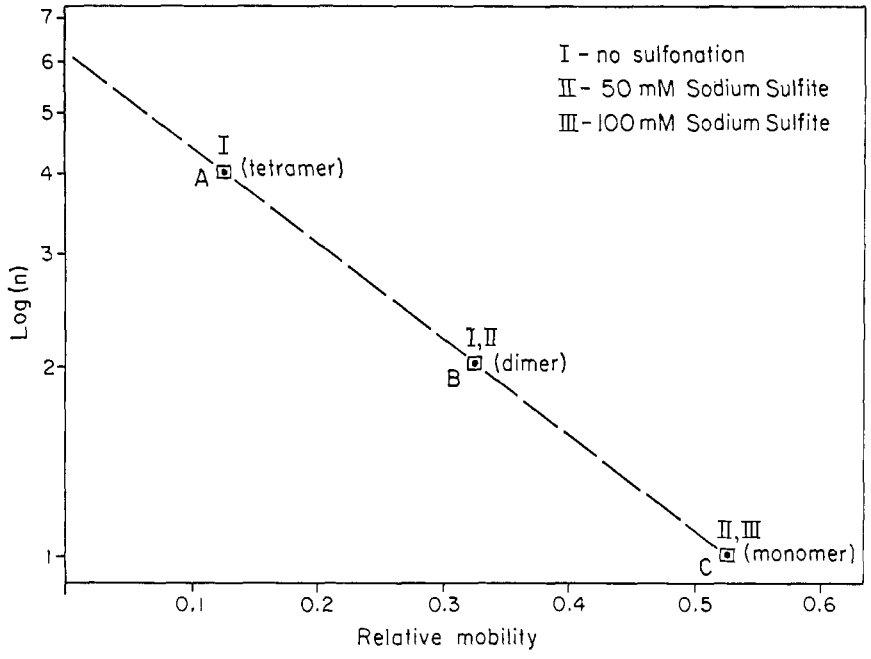

FIGURE 2: Plot of relative mobility of aggregates of myosin f-subunits *vs.* the log of the assigned aggregate numbers. The relative mobilities of bands A, B, and C (relative to band L_1 in Figure 1) were assigned n values of 4, 2, and 1, respectively, corresponding to the tetramer, dimer, and monomer states of the myosin f-subunit.

Tris–glycine buffer, pH 8.8, 0.05% sodium dodecyl sulfate (w/v), and 10% glycerol (v/v) on 3.36% acrylamide gels. For a 25-ml gel solution the following procedure was used. Stock solutions of 2% bisacrylamide and 40% acrylamide, filtered through Whatman No. 1, were maintained. A portion (2.1 ml) of each stock was mixed with 11.8 ml of water, 6.25 ml of stock buffer (116 g of glycine and 24 g of Tris, final pH 8.8), 2.5 ml of glycerol, 20 μl of TEMED,[2] and 0.1 ml of 12.5% sodium dodecyl sulfate. The stock buffer was diluted fourfold and made 0.05% in sodium dodecyl sulfate and 10% in glycerol for chamber buffer. The gel solution was degassed and then polymerized with the addition of 0.1 ml of fresh 10% amonium persulfate (w/v). The system was prerun for 15 min at 100 V (constant voltage) prior to sample application in order to avoid problems introduced by residual amonium persulfate. Electrophoresis runs performed without sodium dodecyl sulfate in the buffer used a 0.05 M Tris–glycine buffer, pH 8.8, throughout and were run at a constant 125 V for 1 hr. Subsequent procedures were identical. Up to 50-μl aliquots of the sulfonated protein solution at 2–5 mg/ml were layered directly on top of the running gel and electrophoresis was performed at a constant 50 V (approximately 1 mA/tube) for 1.5–2 hr. Gels were stained for 20 min–1 hr in 0.1% Amido Black (w/v), 20% acetic acid (v/v) at 90–95°, allowed to cool for 10 min, and destained electrophoretically. Gel scanning was performed with a Gilford linear transport on a Beckman DU at 650 mμ using the medium slit (0.10 × 2.36 mm) with a scanning rate of 2 cm/sec.

Acrylamide gels (10%) were prepared according to Weber and Osborn (1969) except that 0.025 M Tris–glycine buffer was used in place of phosphate buffer. Gels were run at 160 V and 3 mA/tube for 1.5–2 hr and were stained with coomassie blue.

[2] Abbreviation used is: TEMED, *N,N,N*^{1},*N*^{1}-tetramethylethylenediamine, purchased from Eastman Chemicals.

Results

Effects of Sulfonation on the Electrophoretic Mobility of Myosin Subunits. In the presence of sodium dodecyl sulfate myosin is completely soluble at low ionic strength but its native structural and enzymatic properties are irreversibly lost (Hartshorne and Stracher, 1966). As shown below, sodium dodecyl sulfate will also dissociate the LMP components which are then resolvable from one another and from the f-subunit on polyacrylamide gels run with sodium dodecyl sulfate in the buffer system. In early phases of this work it was found that once myosin was solubilized in sodium dodecyl sulfate some reactive sulfhydryl groups were apparently exposed to oxidation, resulting in the formation of aggregates through disulfide bond formation. Sodium sulfite was found to reverse aggregate formation most effectively when compared to acetylation or treatment with β-mercaptoethanol or dithiothreitol. In the case of freshly prepared myosin solutions, sulfonation entirely prevents aggregation. The effects of sulfonation on the electrophoretic mobility of subunits obtained from chick myosin-I are given in Figure 1. Bands A and B, near the top of the gels have been identified as tetramer and dimer aggregates, respectively, of the monomeric unit in band C. It is apparent that the extent of aggregation is inversely proportional to the concentration of the sulfonating reagent, sodium sulfite. With 100 mM sodium sulfite the high molecular weight aggregates in bands A and B are eliminated and all of the protein now moves in band C.

The degree of aggregation (monomer, dimer, and tetramer)

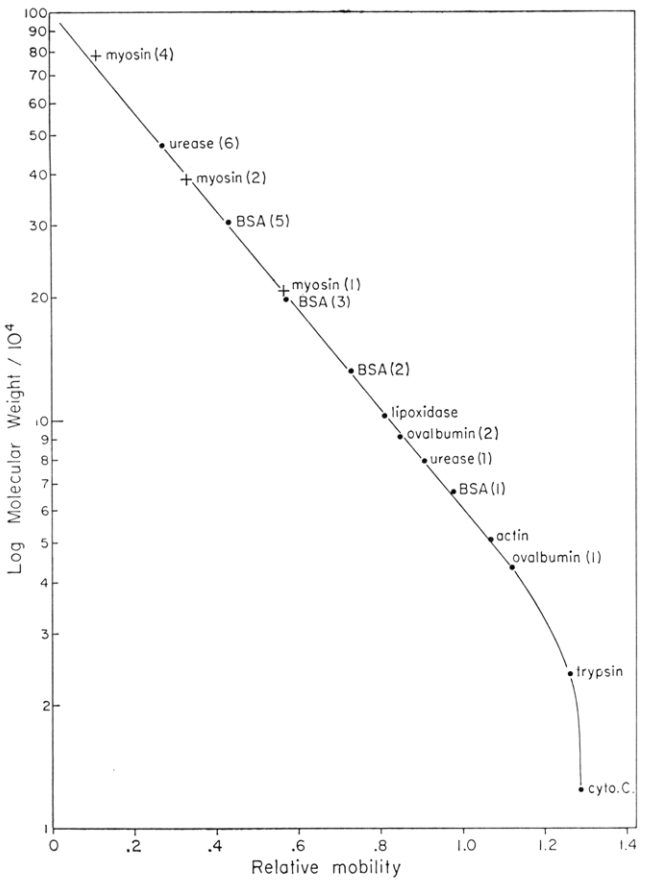

FIGURE 3: Molecular weight calibration curve for 3.36% acrylamide gels in 0.05% sodium dodecyl sulfate. Molecular weights were taken from Dunker and Rueckerts (1969), and from Klotz and Darnall (1969). Chemicals were purchased from the following: bovine serum albumin from Pentex Inc.; cytochrome *c* from Calbiochem; lipoxodase and ovalbumin from Nutritional Biochemical Corp.; trypsin from Armour Pharmaceutical Co; urease from Sigma. The actin was a generous gift from Dr. Eloise Clark. The mobilities of all proteins were normalized to the mobility of the dye marker band (R_F 1). The numbers in parentheses after some proteins indicate the number of subunits in that particular species.

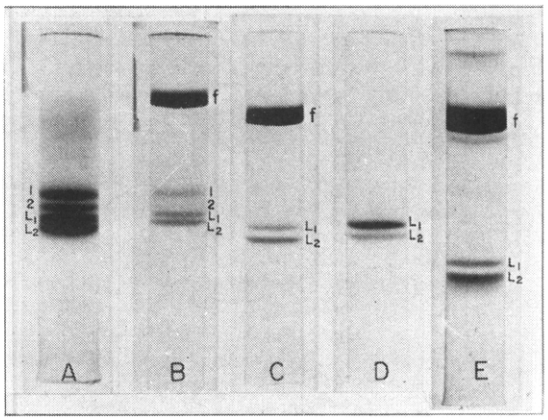

FIGURE 4: Identification of the light components (LMP) of myosin. All preparations were made with chromatographed myosins: (A) alkali-extracted LMP components of chick myosin-I; (B) chick myosin-I; (C) chick myosin-II; (D) alkali-extracted LMP components of chick myosin-II; (E) rabbit myosin-II. Samples A and B were electrophoresed 90 min; samples C and D were electrophoresed 110 mins; sample E was electrophoresed for 2 hr and 10 min.

was determined on the basis of the relative mobilities of each band with band L_1 as the internal marker. The fast-moving bands, 1, 2, L_1, and L_2, will be discussed in detail below. Sulfonation has no effect on the electrophoretic mobilities of bands L_1 and L_2. It was assumed that the molecular weight of the species in each of the bands A and B was some integral multiple (*n*) of a minimal molecular weight represented by the species in band C. If this model is correct then, according to Shapiro *et al.* (1967), a plot of log *n vs.* the relative mobility of each band A, B, and C should yield a straight line when *n* values of 4, 2, and 1 are assigned, respectively. This is seen to be the case in the log plot given in Figure 2. Further evidence for the identity of the material in band C as the f-subunit of myosin is obtained from Figure 3. In this electrophoresis system, calibrated for molecular weight, the material in band C falls into the molecular weight range of 200,000, the expected value for the f-subunit of myosin (Gazith *et al.*, 1970). It should be pointed out here that even though this gel system uses a different buffer system and contains half the sodium dodecyl sulfate concentration used by Shapiro *et al.* (1967) and by Shapiro and Maizel (1969), separation is still according to molecular size. Under our conditions the useful separation range appears to run from approximately 40,000 to 800,000 molecular weight.

The fact that the aggregate plot in Figure 2 is a straight line also indicates complete dissociation of LMP from f-components in sodium dodecyl sulfate. If dissociation was incomplete one would expect some skewing of the curve to higher molecular weight values proceeding from monomers to tetramers.

Identification of the LMP Components of Myosin. Because of the differences in number of LMP components reported by various workers (discussed above) we made an effort to see if variations in the myosin extraction procedure would yield variations in number or heterogeniety of these components. These data for chick myosin are summarized in Figures 4–6. Both myosins-I and -II were chromatographed on DEAE columns and a typical elution profile is given in Figure 5. Fractions obtained across the peak in all cases had 276 mμ/254 mμ absorbancy ratios greater than 2.0. Samples of each of the chromatographed myosins were sulfonated, electrophoresed, and compared for LMP component distribution. This material is also compared to the LMP components isolated from chromatographed myosins following treatment of the two myosins at pH 11.0. A representative comparison is shown in Figure 4. Myosin-I yields four LMP components (bands 1, 2, L_1, L_2) which migrate exactly with the four components released by alkali treatment. Myosin-II, however, yields only two LMP components (bands L_1, L_2) and again these migrate with the two components released by alkali treatment of myosin-II. When the gels of myosin-I and myosin-II in Figure 4 are scanned the result is the tracing given in Figure 6. Such scans permit an approximate estimation of the relative proportions of light and heavy components as judged from areas under the peaks. This was done for chromatographed and unchromatographed myosin. For unchromatographed myosin-I (not shown), bands 1, 2, L_1, and L_2 comprise roughly 20% of the total protein dis-

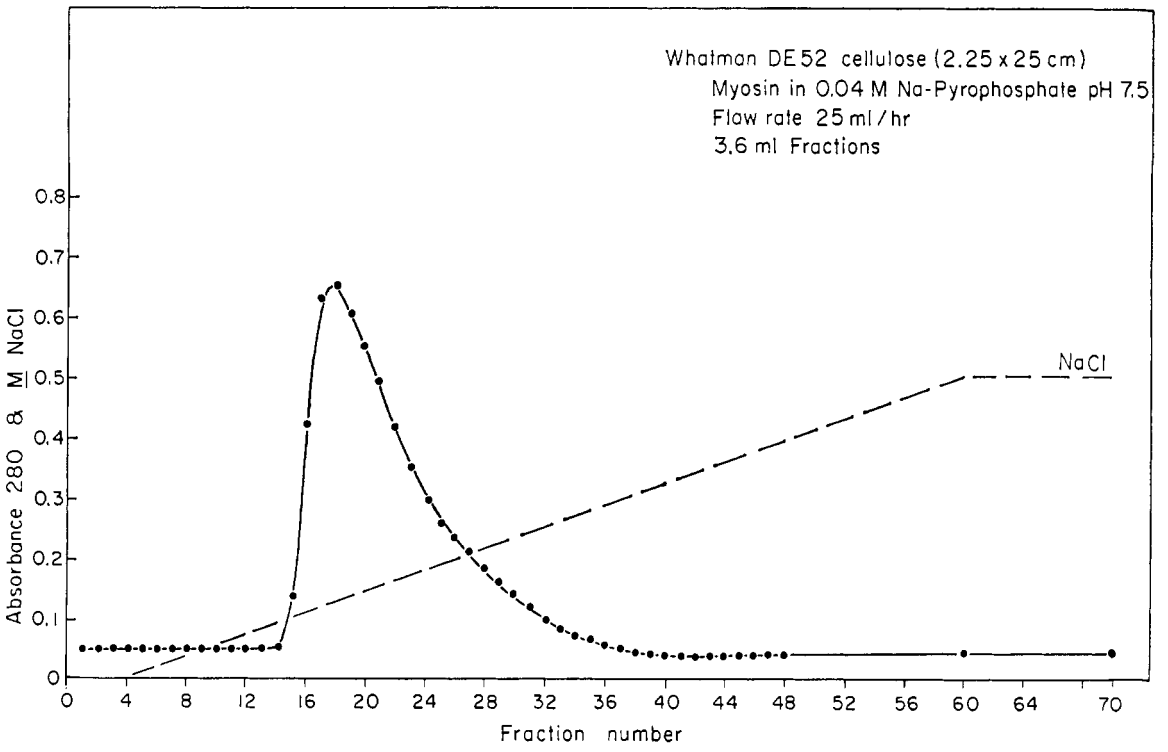

FIGURE 5: DEAE chromatography of chick myosin-II. The elution gradient was applied after washing the column with two column volumes of column buffer. Fractions were collected with the addition of the gradient. Myosin from this column had a 276 mμ/254 mμ absorbancy ratio of 2.50 and was considered free of RNA.

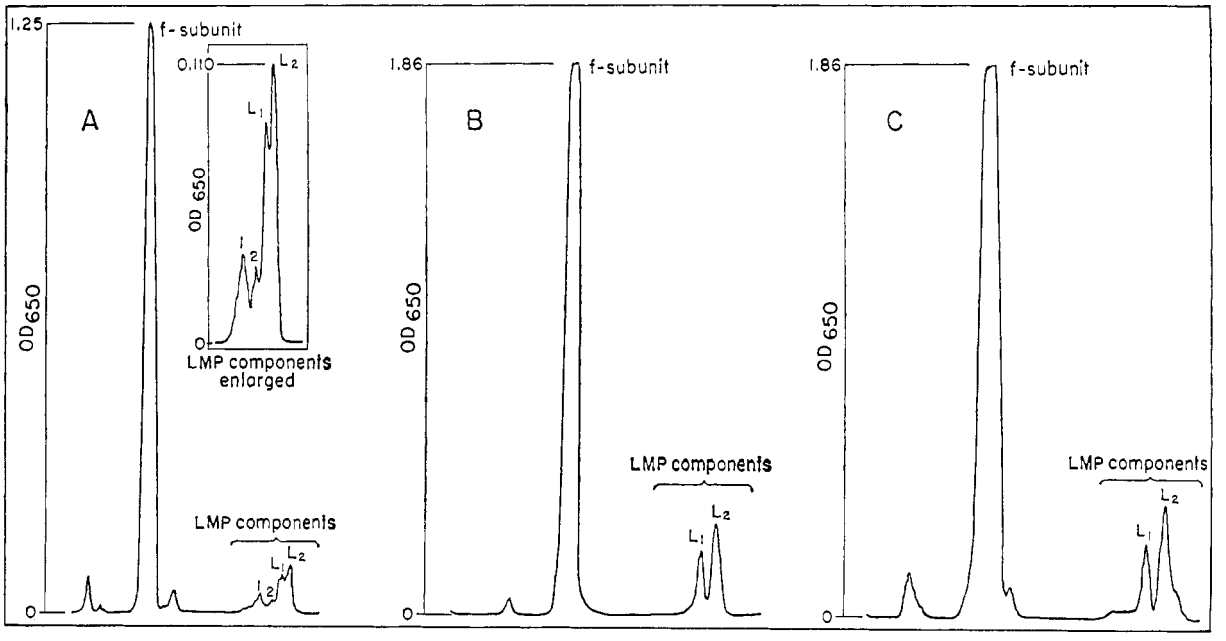

FIGURE 6: Scans of the DEAE-chromatographed myosins. Gels were scanned at 650 mμ using a scan rate of 2 cm/sec: (A) chick myosin-I; (B) chick myosin-II; and (C) rabbit myosin-II. Pictures of these same gels are given in Figure 4. Migration is from left (−) to right (+).

tributed between the f-subunit and the LMP components. In chromatographed myosin-I (Figure 6) these bands represent 16.5% of the total protein. For chromatographed (Figure 6) and unchromatographed (not shown) chicken or rabbit myosin-II, the LMP components represent approximately 13.5% of the total protein distributed between the f-subunit and the LMP components. These estimates must be viewed as approximate only. The scans were of stained bands on the gels and the amount of dye bound is greatly influenced by the presence of residual sodium dodecyl sulfate.

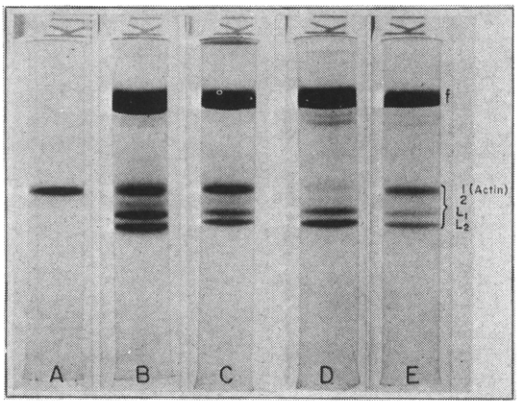

FIGURE 7: Identification of actin in the LMP components of chick myosin-I. All preparations are of unchromatographed chick myosins-I and -II: (A) rabbit actin; (B) chick myosin-I; (C) chick myosin-I and actin; (D) chick myosin-II; (E) chick myosin-II and actin. All samples were electrophoresed for 90 min.

The amount of dye bound per unit amount of protein on acrylamide gels has not been shown to be linear over a broad protein concentration range or to be constant for different proteins. On the other hand, the estimates gain reliability from the work of Kihara and Kuno (1968) and Bramhall *et al.* (1969) which shows that Amido Black binds linearly in proportion to protein concentration over a 5- to 200-μg range for serum albumin precipitated on filter papers or nitrocellulose membranes.

The differences exhibited by myosin-I and -II strongly suggest that the pyrophosphate extraction at high pH in the presence of Mg ion has extracted protein components other than myosin which are nevertheless tenaciously bound to myosin. They are clearly not separated from myosin-I by chromatography over DEAE-Sephadex, but are released by high pH and by sodium dodecyl sulfate. These additional

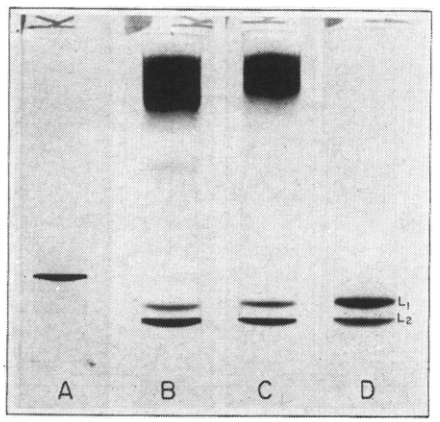

FIGURE 8: Electrophoresis of actin, chick myosin-II, and chick myosin-II LMP components in the absence of sodium dodecyl sulfate. All proteins were sulfonated in the presence of sodium dodecyl sulfate. Aliquots of (25 μl) of each sample were layered on the gels and contained approximately 0.125 mg of sodium dodecyl sulfate each: (A) actin; (B) unchromatographed chick myosin-II; (C) chromatographed chick myosin-II; (D) chromatographed chick myosin-II LMP components. Samples were electrophoresed for 60 min in 0.05 M Tris–glycerin buffer, pH 8.8, without sodium dodecyl sulfate, at 125 V (constant).

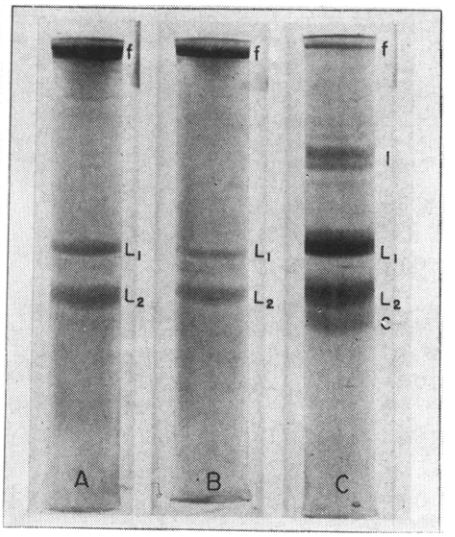

FIGURE 9: Electrophoresis on 10% acrylamide gels in 0.1% sodium dodecyl sulfate: (A) chromatographed chick myosin-II; (B) chromatographed rabbit myosin-II; (C) DTNB-released components from unchromatographed chick myosin-I. Samples were sulfonated for 2 hr then electrophoresed at 160 V (approximately 3 mA/tube) for 2 hr. Gels were stained in coomassie blue according to Weber and Osborn (1969). Gel C is included here to show that in 10%, as well as in 3.36% gels (compare Figure 11), DTNB-released material includes chains identical with L1, L2 of highly purified, chromatographed myosin-II. Of the contaminants appearing from this unchromatographed preparation, band 1 migrates with the same mobility as actin; band C is normally resolved on 10% gels from myosin-I but not from myosin-II and has not been identified.

components which migrate on the gels as bands 1 and 2 are completely missing from myosin-II preparations.

Band 1 in the LMP region of gels of myosin-I has tentatively been identified as actin. As indicated in Figure 7, a highly purified preparation of actin runs as a single band with the same electrophoretic mobility as band 1 from myosin-I. Mixing experiments in Figure 7 show, in addition, that actin added to myosin-I yields no new bands but that actin added to myosin-II gives rise to an additive picture, that is, actin (band 1) plus bands L_1 and L_2 that closely resembles the LMP pattern for myosin-I. All material in Figure 7 is from unchromatographed myosins. There is a slight suggestion of an actin band in unchromatographed myosin-II but this is apparently removed by chromatography on DEAE columns (see Figure 8).

The method of electrophoresis used here separates protein components on the basis of molecular size. We have considered the possibility that the two LMP components obtained from myosin-II might actually be comprised of additional proteins of similar size but different charge. However, when sulfonated myosin-II, or actin are electrophoresed in a buffer system containing no sodium dodecyl sulfate no further resolution of LMP components or actin is obtained. This result is seen in Figure 8. It is apparent that the sodium dodecyl sulfate originally used to dissociate the myosin has been electrophoresed away from the protein and has left the gel. The f-subunit no longer remains in solution. The result of sodium dodecyl sulfate removal therefore is a smearing out of the f-subunits at the top of the gels. The LMP components continue to be resolved, now on the

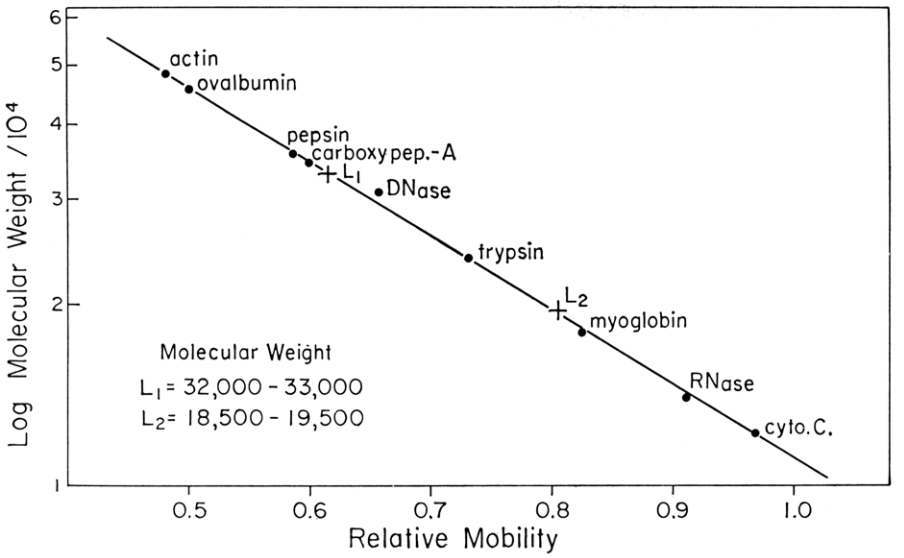

FIGURE 10: Molecular weight calibration curve for 10% acrylamide gels run in 0.1% sodium dodecyl sulfate. Molecular weights were taken from Weber and Osborn (1969), Dunker and Rueckerts (1969), and from Klotz and Darnall (1969). Chemicals were purchased or obtained from the following sources: the rabbit actin was a generous gift from Dr. Eloise Clark; ovalbumin and cytochrome *c* (horse) from Calbiochem; pepsin, carboxypeptidase A, myoglobin, from Mann Biochemicals; DNase, RNase, and trypsin from Worthington. The mobilities of the proteins were normalized to the mobility of the dye marker band (R_F 1).

basis of size and charge, as two distinct bands, L_1 and L_2. The actin also continues to be resolved as a single band with a different mobility than the two LMP components, L_1 and L_2. Putnam and Neurath (1945) have shown that if sodium dodecyl sulfate–protein complexes are electrophoresed in a system containing no sodium dodecyl sulfate, the detergent moves rapidly away from the slower moving protein components. Shapiro and Maizel (1969) have presented evidence indicating that the sodium dodecyl sulfate–protein complex is a weak one and under conditions of electrophoresis there is a continuous rapid exchange of detergent molecules between those in the complex and those in the electrophoresis buffer system.

Molecular Weight Estimates for LMP Components L_1 and L_2. The reported values for the molecular weights of the LMP components of myosin range from 17,000 to 32,000 (Gershman and Dreizen, 1970; Locker and Hagyard, 1967a,b; Frederiksen and Holtzer, 1968; Weber and Osborn, 1969; Gaetjens *et al.*, 1968) with an average around 20,000 for each component. DEAE-chromatographed myosin-II from chicken and rabbit were sulfonated for 2 hr and electrophoresed in the 10% acrylamide sodium dodecyl sulfate

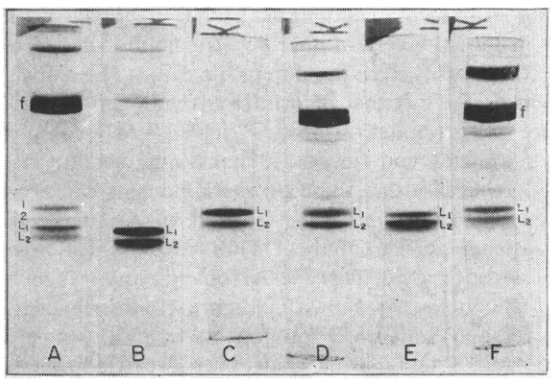

FIGURE 11: Electrophoresis of DTNB-LMP components and DTNB-myosins. All preparations are from unchromatographed myosins: (A) DTNB chick myosin-I; (B) DTNB-LMB components from chick myosin-I; (C) DTNB-LMP components form chick myosin-II; (D) DTNB chick myosin-II; (E) DTNB-LMP components from rabbit myosin-II; (F) DTNB rabbit myosin II. Samples were electrophoresed for about 90 min. The slight aggregation of the f-subunit after DTNB treatment is a highly reproducible phenomenon and has not been explained at this time.

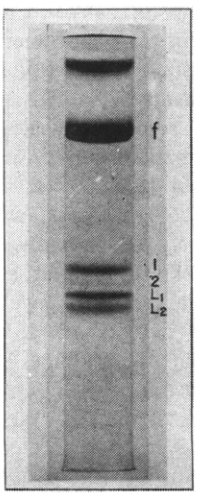

FIGURE 12: DTNB chick myosin-I after chromatography on Sephadex G-200. The myosin used here was not chromatographed on DEAE prior to treatment with DTNB. The sample was electrophoresed for 90 min.

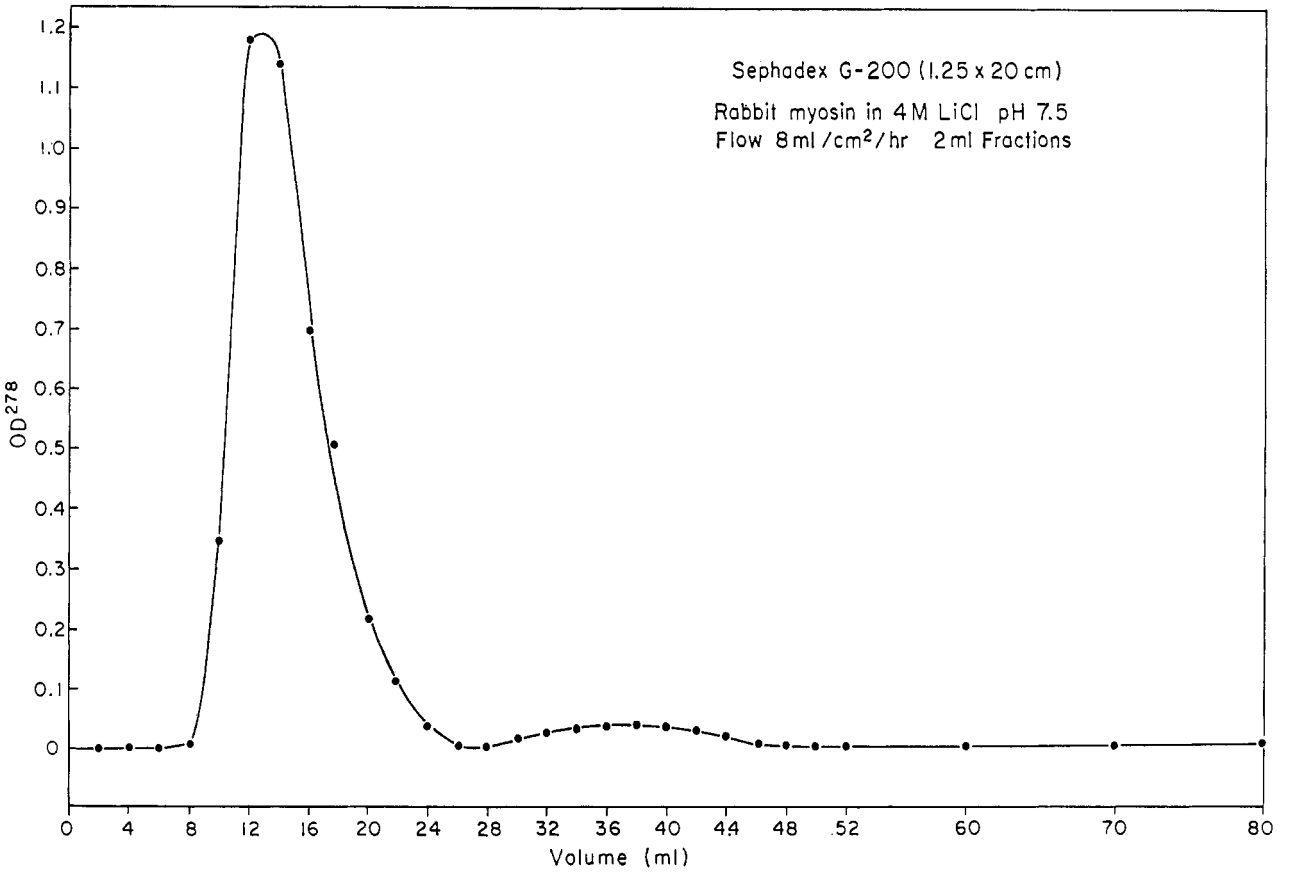

FIGURE 13: Chromatography of rabbit myosin-II in 4 M lithium chloride on Sephadex G-200. Unchromatographed rabbit myosin-II was exposed to 4 M lithium chloride as in Methods. The column procedure required a minimum of 6 hr.

gel system described by Weber and Osborn (1969). Figures 9 and 10 give the results of this experiment. The molecular weight range for the LMP components L_1 and L_2 in both myosins is 18,500–19,500 and 32,000–33,000, respectively. Assuming the molecular weight of the total LMP component is around 50,000 per mole of myosin one can estimate that roughly 10–12% of the total myosin protein is LMP component. This value is in close agreement with the 12–13.5% estimate based on the stain distribution between the f-subunit and the LMP components in the gels. Also, the 10% acrylamide gels failed to resolve any new LMP components that might have been masked in the 3.36% gels.

Treatment of Myosin with DTNB. It has recently been reported by Weeds (1969), and by Gazith *et al.* (1970) that a unique portion of the LMP components can be dissociated from myosin with DTNB, without affecting the ATPase activity of the treated myosin. While we have not investigated the residual ATPase activity of DTNB-treated myosin, we have examined such preparations for extent of dissociation. Chick myosins-I and -II, and rabbit myosin-II were treated with DTNB according to Weeds. The dissociated LMP components were then separated from the reaction mixture. In the following discussion, "DTNB-myosin" refers to myosin reprecipitated from the DTNB reaction mixture by dilution, and "DTNB-LMP" refers to the low molecular weight components recovered from the supernatant solution following dilution. In most of these experiments the acrylamide gels were slightly overloaded with protein in order to obtain good visualization of the LMP components. Under these circumstances, there is always some aggregation of the f-subunit at the top of the gels. The results are shown in Figure 11. It may be seen that DTNB releases both of the LMP components, L_1 and L_2, of all myosins examined. In addition, DTNB treatment does not result in the complete or specific dissociation of one of the LMP components from the parent molecule. This conclusion is based on the fact that myosin recovered from the reaction mixture by precipitation always yielded LMP components when sulfonated and run in gels containing sodium dodecyl sulfate. To ensure that these LMP components had not been trapped by the precipitation step and were actually bound to myosin, a portion of the DTNB-treated chick myosin-I was passed over a Sephadex G-200 column and was analyzed for LMP content. As shown in Figure 12, this myosin continues to show both LMP components L_1 and L_2 in addition to the bands 1 and 2 which have been discussed previously. We conclude, in agreement with Weeds and Gazith, that DTNB does indeed release the LMP components of myosin. It may be that the release shows a preference for one of these although we were not able to draw any definite conclusions on this latter point.

Treatment of Myosin with Lithium Chloride. Gershman *et al.* (1968), Gershman and Dreizen (1970), and Stracher (1969) have previously reported that the LMP components

of myosin can be dissociated from the parent molecule at neutral pH in the presence of 4 M LiCl. Likewise, Tonomura *et al.* (1962) have reported the complete loss of ATPase activity in 3 M LiCl and a decrease in the reduced viscosity (n_{sp}/c) from 2.3 to 0.5 in 3.3 M LiCl. The decrease in ATPase activity was partially reversible in 3.6 M LiCl but was completely irreversible in 4.8 M LiCl.

Stracher (1969) and Dreizen and Gershman (1970) have reported the complete separation of the LMP components from the rest of the myosin molecule after a short exposure to 4 M LiCl in the presence of ATP and Mg ion at neutral pH, and subsequent filtration on Sephadex G-200 or fractionation by LiCl–citrate. In addition, the separate LMP and f-subunit fractions, free of LiCl, could be remixed to recover about 27% of the ATPase activity of the control in the case of the G-200 procedure and 70% of control activity in the case of LiCl–citrate fractionation.

This dissociation procedure was reinvestigated using chick myosins-I and -II, and rabbit myosin-II in order to check the completeness of dissociation of the LMP components in the presence of 4 M LiCl. The myosins were exposed to 4 M LiCl as described under Methods and were then chromatographed on Sephadex G-200. A typical profile for the various preparations of myosin is given in Figure 13. We were unable to attain the rapid separation reported by Stracher (1969). Using the maximal flow rates prescribed for Sephadex G-200 in the Sephadex manual (Pharmacia Fine Chemicals, Inc., 1967) separation required a minimum of 6 hr, well beyond the limits of the experiment for recoverable ATPase activity. The myosin obtained from these columns was sulfonated and electrophoresed to check for LMP content. Figures 14 and 15 show that even after a 6-hr exposure to 4 M LiCl all preparations of G-200 chromatographed myosin still show the presence of LMP components L_1 and L_2. Exposure to LiCl does result in the dissociation of some protein as evidenced by the secondary peak in the column profile of the Sephadex G-200 separation, and the schleirn pattern for chick myosin-II in the 4 M LiCl column solvent, shown in Figure 16. However, dissociation is not complete. On the basis of data taken from gel scans of myosin before and after exposure to LiCl, Figures 6 and 15, no more than 35% of the LMP components is released during treatment over a 6-hr period. Such estimates are subject to protein dye binding effects as discussed above but clearly a substantial amount of the LMP components, L_1 and L_2, remain bound to the f-subunit.

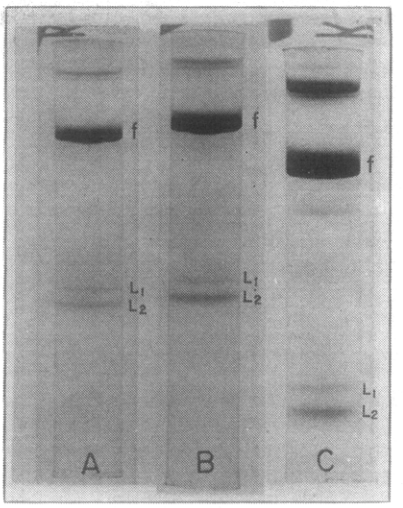

FIGURE 14: Electrophoresis of lithium chloride treated myosins. All preparations are from unchromatographed myosins: (A) chick myosin-I; (B) chick myosin-II; and (C) rabbit myosin-II. The aggregation of the f-subunit is a reproducible phenomenon after exposure to lithium chloride and has not been explained at the present time. Samples A and B were run concurrently for 90 min. Sample C was run for 2 hr.

Discussion

The preparation and electrophoresis of the sulfonyl derivatives of myosin, in the presence of relatively low concentrations of sodium dodecyl sulfate provides a useful analytical method for the assay of myosin subunit structure. Problems inherent in other electrophoresis systems applied to myosin are completely avoided (Small *et al.*, 1961; Florini and Brivio, 1969). The f-subunit migrates as a single species with an estimated molecular weight of 195,000 to 200,000, in agreement with current estimates for the molecular weight of the heavy subunit of myosin (Gazith *et al.*, 1970). With the appropriate extraction procedures the LMP components of myosin are resolved as a class of two distinct components, L_1 and L_2, with approximate molecular weights (estimated from mobilities on acrylamide gels calibrated for molecular size) of 18,500–19,500 and 32,000–33,000, respectively. Such estimates are considered good to an accuracy of at best ±5% (Dunker and Rueckerts, 1969). If one uses these values for the molecular weights of the LMP components and a value of 194,000 for the f-subunit (Gazith *et al.*, 1970) the apparent molecular weight of myosin becomes 440,000, in reasonable agreement with newly published values of 430,000 ± 30,000 (Gazith *et al.*, 1970; Godfrey and Harrington, 1970).

The dissimilar reports on the number of LMP components and their weight percentage of the myosin molecule may be reconciled either on the basis of actin contamination or possibly on the basis of aggregation of the two LMP components (L_1, L_2) reported here. It is possible that dissociation of myosin in sodium dodecyl sulfate prevents aggregation of the LMP components since dissociation can be accomplished at relatively low pH, thus obviating the problems of sulfhydryl exchange and oxidation encountered at higher pH. The presence of the detergent is not, however, sufficient to prevent aggregation of the f-subunits and sulfonation is required to retain these subunits as monomers.

It should be emphasized that in all cases examined by us so far, myosin extracted by the Hasselbach–Schneider (1951) procedure and chromatographed on DEAE columns displays only two LMP components. The details of this comparative myosin study involving several phyla will be published elsewhere. We have considered the possibility that the two LMP components reported here for chicken and rabbit myosin represent isozymic forms of a single LMP component from the red and white muscle contained in our preparations. However, when preparations of pure red and pure white tuna muscle myosin (Ogata and Mori, 1964; Chung *et al.*, 1967) are examined they again each show two LMP components. These preliminary findings on tuna

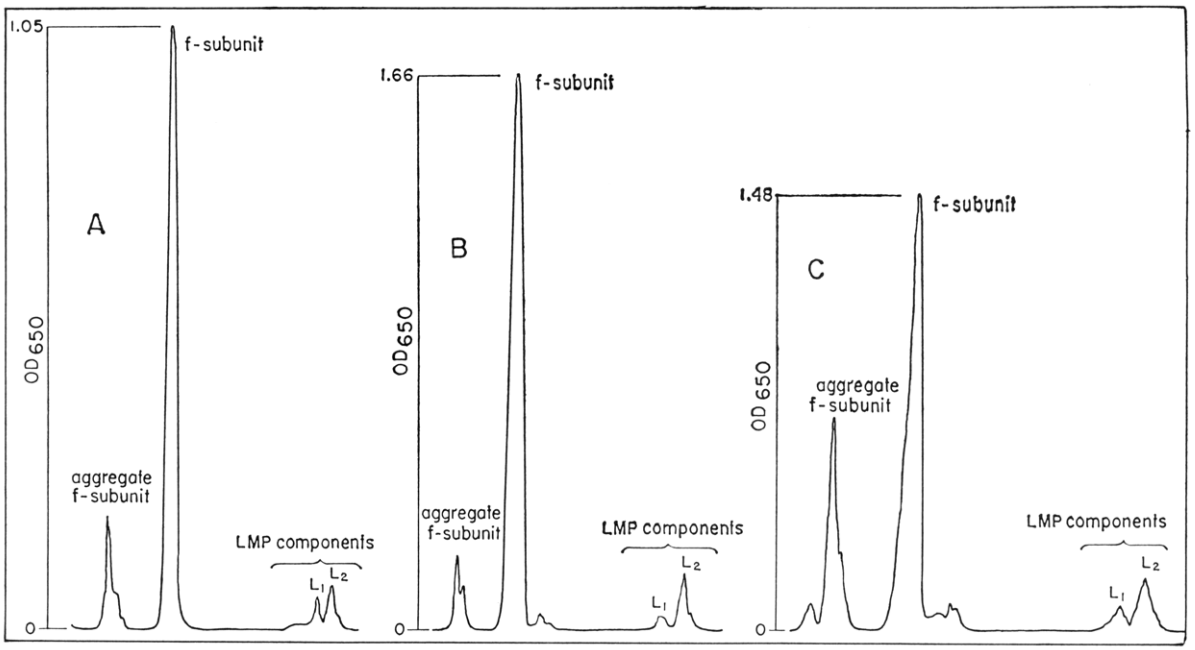

FIGURE 15: Scans of the gels of lithium chloride treated myosins. Gels were scanned at 650 mμ at a rate of 2 cm/sec: (A) chick myosin-I; (B) chick myosin-II; and (C) rabbit myosin-II. Pictures of these same gels are given in Figure 14. Migration is from left (−) to right (+).

muscle (*Euthynnus pelamis*) were carried out in conjunction with Dr. E. G. Richards.

The DTNB treatment of myosin does release LMP components which are identical with those released by sodium dodecyl sulfate or alkali treatments, (Figure 9), but this release is not complete. Substantial amounts of both LMP components, L_1 and L_2, remain with the parent myosin even after filtration over a Sephadex G-200 column. From the evidence presented here one might explain the residual ATPase activity of DTNB-myosins on the basis of undissociated myosin which apparently remains in equilibrium in the DTNB reaction mixture. Thus, even though up to 50% of the LMP components can be removed from myosin with no apparent loss of ATPase activity (Gazith *et al.*, 1970) the remaining undissociated parent molecule might account for the total ATPase activity if some form of activation phenomena were involved. It is well known that EDTA and some sulfhydryl reagents will greatly enhance the ATPase activity of myosin (Sekine and Kielly, 1964; Kielly and Bradley, 1956; Trotta *et al.*, 1968; Stracher and Dreizen, 1968).

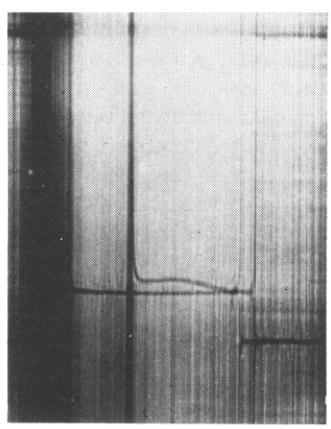

FIGURE 16: Sedimentation pattern for chick myosin-II in the lithium chloride column solvent. The picture was taken after 5 hr at speed: 59,780 rpm, bar angle of 70°, temperature 20°. A standard cell containing just the column solvent was used to give the base line. The preparation was made from unchromatographed chick myosin-II. Sedimentation is from right to left.

Similarly, we were able to demonstrate that treatment of chicken or rabbit myosin with 4 M lithium chloride does not result in the complete dissociation of the LMP components. Lithium chloride treated myosin, when separated from dissociated products on Sephadex G-200 columns could be shown to contain both LMP components, L_1 and L_2. In the light of this evidence the separation and remixing experiments of Stracher (1969) and Dreizen and Gershman (1970) remain open. The recovered ATPase activity reported could result from the activation of incompletely dissociated myosin as discussed above.

The evidence presented here favors the structural model for myosin which includes two identical f-subunits and two globular subunits of similar or dissimilar size (Slayter and Lowey, 1967; Lowey *et al.*, 1969; Godfrey and Harrington, 1970). The data reported here bearing on the globular subunits suggests that the subunits are of dissimilar size and correspond to the LMP components L_1 and L_2. The assymetry in the two LMP components might be explained in one of the following ways. (1) The two different LMP components could be related to a functional duality of the myosin molecule, one component serving to mediate actin binding and the other serving as an ATPase. Lowey *et al.* (1969) have reported that, on a molar basis, actin-activated ATPase of HMM and HMM subfragment 1 of myosin are identical, implying that only one of the two globular heads of myosin is active at a time. In addition, Tokuyama *et al.* (1966)

have shown that the Ca^{2+}-activated ATPase of HMM and HMM subfragment-1 are identical with that of native myosin. This could support the speculation that the globular subunits are dissimilar in size and function. (2) On the other hand, there is evidence to suggest that there are two ATP binding sites per molecule of myosin (Murphy and Morales, 1970; Trotta *et al.*, 1968). If it is assumed that the binding of one ATP molecule requires the presence of an L_1–L_2 subunit pair, then the myosin molecule would have to contain one L_1–L_2 set for every f-chain in order to preserve the binding stoichiometry. The molecular weight of such a molecule, containing two f-subunits, two L_1 subunits, and two L_2 subunits would be about 490,000, currently a high value for the molecular weight of myosin. Such a model, however, has the advantage in that it complies with the notions of molecular symmetry with an L_1–L_2 set on each f-subunit. Clearly further studies will be needed to elucidate the LMP stoichiometry and its relationship to functional myosin activity.

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