ELECTROPHORETIC STUDIES ON MYOSIN

by 6408

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INTRODUCTION

The structure of the myosin molecule has long been the concern of many muscle and protein biochemists. Most attempts to clarify the inconsistencies in the molecular weight and subunit structure have just contributed to the existent confusion and frustration. Many of the difficulties in pursuing these objectives have been attributed to the large size of the myosin molecule, its unique and somewhat undesirable solubility properties, and the ease by which it readily forms high molecular weight aggregates.

The understanding of the mechanism of muscle contraction has also been of major concern to many physiologists and biochemists. Most proposed theories have been developed on the basis of morphological observations and from the results of physical measurements. While the importance of these observations and measurements has been recognized for a wide-angle view of the inter-relationships of muscle fibers and of their contractile proteins, the need for a detailed understanding of the structure of these contractile proteins at the molecular level is of equal or greater importance in the elucidation of the molecular mechanism of muscle contraction.

One persistent uncertainty at the molecular level has been the number and uniqueness of the subunits comprising the major portion of the myosin molecule. While the number and nature of

the low molecular weight proteins associated with the "head" region of myosin is now well understood, and recent evidence for the number of subunits comprising the "head" and "tail" regions of myosin is quite convincing, little is yet known about the uniqueness of these subunits. The research presented in this thesis deals with the determination of both the number and uniqueness of the major myosin subunits.

LITERATURE REVIEW

Muscle, Structural Considerations

The contractile unit of the muscle cell has been defined as the sarcomere, composed of Z-lines (lateral limits), A-band, I-band, H-zone and M-line (Fig. 1). Within the sarcomere, parallel arrays of myosin filaments (thick filaments) are surrounded by parallel filaments of actin (thin filaments) in a hexagonal lattice (Fig. 1). As illustrated by Huxley (1969), the thick myosin filaments, by a reversal in polarity at the center of the sarcomere (M-line), extend out toward the Z-lines. Similarly, from each Z-line the thin filaments extend into the adjacent sarcomeres. The interaction between the thick and thin filaments always occurs in a portion of the region known as the A-band, which is defined by the length of the myosin filaments. Interaction never occurs in the H-zone, located between the actin filaments of each sarcomere and consisting only of myosin filaments.

The thick myosin filaments are composed of parallel arrays

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Fig. 1. Diagram of the structural inter-relationships of skeletal muscle (Bloom and Fawcett, 1968).

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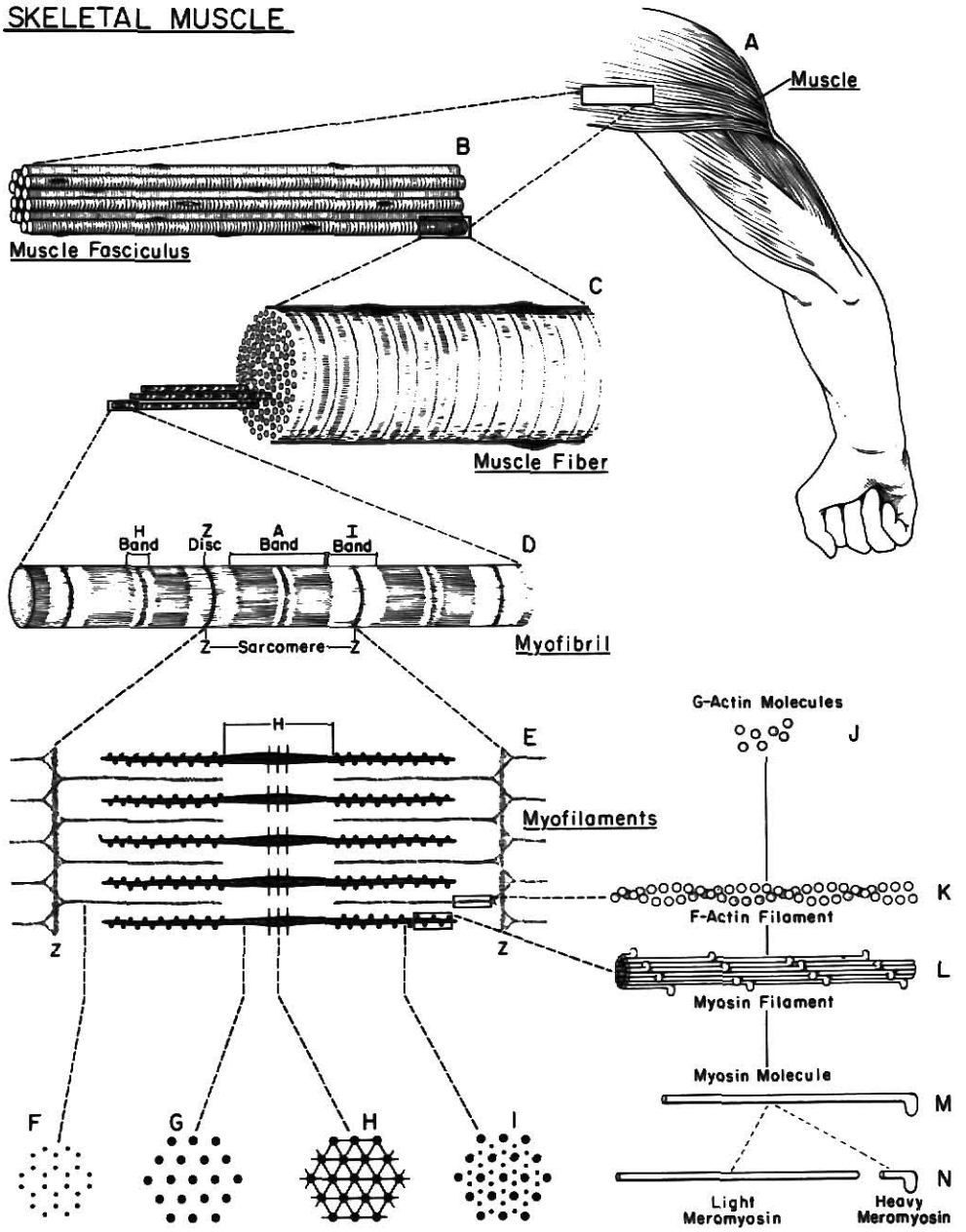

SKELETAL MUSCLE
A
Muscle
B
Muscle Fasciculus
C
Muscle Fiber
H Band
Z Disc
A Band
I Band
D
Z—Sarcomere—Z
Myofibril
H
E
Myofilaments
Z
Z
G-Actin Molecules
J
K
F-Actin Filament
L
Myosin Filament
Myosin Molecule
M
N
Light Meromyosin
Heavy Meromyosin
F
G
H
I

of myosin molecules. Extending out from each myosin molecule at regular intervals along the thick filaments are globular appendages, referred to as "heads", which interact with the actin molecules of the thin filaments. The "tails" of the myosin molecules are highly helical polypeptide chains which form the backbone of the thick filament. The appendages or cross-bridges appear along the filament as two helices, each with a pitch of 429 A^{o} and a cross-bridge spacing of 143 A^{o} (Huxley and Brown, 1967). Each cross-bridge occurs directly opposite another, located at the same level on the opposite side of the filament. These sets of cross-bridges are rotated 120^{o} from each adjacent set. A view at the end of and in the direction of the axis shows the arrangement of the cross-bridges in an apparent hexagonal lattice (Fig. 1) consisting of a three-fold axis (Huxley and Brown, 1967).

The thin filaments are composed of two strands of globular actin monomers. These strands have been shown to be coiled around each other in a helix with a pitch of 740 A^{o} and approximately 14 actin monomers per turn (Millman et al., 1967). Several other proteins are known to be associated with the thin filaments. In addition to the 740 A^{o} pitch, a pitch of approximately 400 A^{o} has been observed to occur in the helical filaments of actin (Huxley and Brown, 1967; Hanson and Lowy, 1963; Worthington, 1959). Tropomyosin, composed of tropomyosin B and troponin (Ebashi and Kodama, 1966), is a very helical protein of approximately 400 A^{o} in length. It has been proposed to be located adjacent to the thin filaments, probably within the

additional helical pitch (Ebashi et al., 1967), and to be involved in the binding of divalent calcium or magnesium ions (Cohen and Longley, 1966). Troponin, spaced at 400 A^{o} intervals along the thin filament, has been proposed by Ebashi to be a globular protein which provides the receptor site of calcium or magnesium binding. It is composed of two functionally distinct components, troponin A and B. Troponin A is thought to be associated with calcium sensitivity. Troponin B, when complexed with tropomyosin B, has been shown to be an inhibitor of actomyosin ATPase activity (Hartshorne and Mueller, 1968; Hartshorne et al., 1969). Both tropomyosin B and troponin have been shown to be necessary in the binding of divalent cations but, on the basis of a strong binding constant, troponin has been suggested to be primarily responsible for calcium binding during the activation of contraction (Yasui et al., 1968). Alpha-actinin, first isolated by Ebashi and Ebashi (1965), is another protein known to strongly bind to actin filaments (Briskey et al., 1967). It is known to be composed of a 10S and a 6S component (Nonomura, 1967). Although the function of the 10S component is not yet known, the 6S component which is located in the Z-band has been demonstrated to be functional in the lateral association of F-actin particles (Kawamura et al., 1969). Beta-actinin has been shown to regulate the length of F-actin filaments (Maruyama and Kawamura, 1968).

Recent improvements in the design of x-ray diffraction equipment have provided a detailed picture of the inter-relationships between thick and thin filaments. During contraction,

little, if any, change has been shown to occur in the helices of actin (Elliott et al., 1967) or in the lengths of the filaments of either actin or myosin (Huxley and Brown, 1967). However a considerable change has been observed in the pattern of cross-bridges along the filaments of myosin (Huxley et al., 1965). This change has been suggested to be a random angular displacement of the cross-bridges, the purpose of which seems to be the search for binding sites on the thin filaments (Huxley and Brown, 1967). Although the separation between thick and thin filaments is known to increase during contraction, the force generated by each cross-bridge has been observed to remain constant at all times (Gordon et al., 1966).

There have been many reviews published on the mechanism of muscle contraction (Perry, 1968; Szent-Gyorgyi, 1968; Huxley, 1969; Young, 1969; Dreizen and Gershman, 1970b). Most of the evidence has supported a sliding filament model in which the actin and myosin filaments interact by some mechanism which allows them to move past each other during contraction and extension (Huxley and Hanson, 1954; Huxley and Brown, 1967). It has been generally assumed that this movement is linear in relationship to the attachment of any single longitudinal row of cross-bridges with an actin filament. However little conclusive proof has been offered for this type of model.

A rotational movement of the thick filaments by several possible mechanisms has also been proposed as a model for contracting muscle (Dreizen and Gershman, 1970b). This concept

involves three possible mechanisms for the movement of the thick and thin filaments past each other. The first is a linear movement of the filaments in which there occurs a possible radial movement of the cross-bridges in search of binding sites on stationary actin filaments (Huxley and Brown, 1967). The second is a screw-type mechanism in which the myosin filaments move along stationary actin filaments by means of a screw motion. The third is a torsional mechanism in which the movement of the thick filaments past the thin filaments is propagated by an outward rotational movement of the cross-bridges and subsequent interaction with actin on the thin filament. Most observations have seemed to partially support the last proposed mechanism although many ambiguities and a lack of agreement remain (Lowey and Cohen, 1962; Davies, 1963; Huxley and Brown, 1967; Pepe, 1967). Regardless of the results obtained at the macromolecular level, the eventual elucidation of the structure of the myosin molecule seems ultimately necessary in the final understanding of the actual mechanism of muscle contraction.

Myosin, Structural Considerations

The precise structure of the myosin molecule remains as one of the great mysteries of muscle research. Within the last ten to fifteen years, a great deal of research has been performed on myosin resulting in few unambiguous structural models. Although still somewhat vague, progress has been made in the elucidation of the structure. The entire molecule is known

to consist of a head and a tail region. The head region is thought to contain an extensive amount of folding in a globular structure, whereas the tail region is thought to exist primarily as a highly coiled alpha-helix which is known to associate with other myosin tails to form the backbone of the thick filaments in the sarcomere. Studies have resulted, with some uncertainty, in a molecular weight of 400,000 to 600,000.

The structure of the myosin molecule was early examined with the use of the proteolytic enzymes, trypsin, chymotrypsin, and papain. Trypsin has been shown to cleave the molecule into heavy meromyosin (HMM) and light meromyosin (LMM) fragments (Perry, 1951; Gergely, 1953; Szent-Gyorgyi, 1953). LMM, consisting of a large portion of the tail region, has a molecular weight of approximately 130,000 (Lowey and Holtzer, 1959; Szent-Gyorgyi et al., 1960; Lowey and Cohen, 1962). HMM, consisting of the entire head region and a small portion of the tail, has a molecular weight of 340,000 (Lowey and Holtzer, 1959; Lowey and Cohen, 1961). LMM has been shown to have the same solubility properties as myosin, comprise most of the backbone of the thick filament, have no ATPase activity or actin-binding properties (Huxley, 1963), and have a high helical content (Lowey and Cohen, 1962).

Heavy meromyosin has been shown to be soluble at low ionic strength, comprise part of the thick filamentous backbone, form the cross-bridges between the thick and thin filaments, and contain the ATPase activity and actin-binding properties of

myosin (Huxley, 1963). Prolonged treatment of HMM with trypsin or treatment of myosin with papain produces a smaller fragment, subfragment 1 (S-1) (Mueller and Perry, 1962; Lowey et al., 1969). It has been shown to have a molecular weight of approximately 120,000 (Weeds and Baker, 1968), contain the ATPase and actin-binding properties of myosin (Mueller and Perry, 1962), and exist as the larger of the two fragments obtained by trypsin digestion of HMM (Weeds and Baker, 1968). Since equal rotational relaxation times from polarization of fluorescence studies on HMM and S-1 were obtained, it was suggested that three S-1 particles comprise heavy meromyosin (Young et al., 1965). An extensive amount of work, reported by Lowey and co-workers in 1969, presents electron micrographs of and summarizes the relationships between the various dimensions of myosin fragments produced by proteolytic enzymes.

There has been a great deal of interest in the low molecular weight protein (LMP) associated to the myosin molecule. Early conclusions about the nature of this material generally regarded it as a contaminant or a product of proteolytic fragmentation. Later observations demonstrated its dissociation from myosin at an alkaline pH (Connell and Olcott, 1961), in urea and guanidine solutions (Connell and Olcott, 1961; Small et al., 1961; Young et al., 1961), and at high temperatures (Yasui et al., 1960). In 1966, LMP was demonstrated to be an integral part of the myosin molecule (Dreizen et al., 1966).

Early measurements established that the average molecular

weight of the LMP was approximately 20,000 (Gershman et al., 1966; Locker and Hagyard, 1967b). Later experiments indicated the presence of a larger component of approximately 32,000 (Frederiksen and Holtzer, 1968). Most recently the LMP has been well characterized as two distinct species, one of molecular weight 18,500 - 19,500, and the other of 32,100 - 33,000 (Paterson and Strohman, 1970).

Results of recent studies widely disagree on the number of unique LMP species. The number varies from as few as one (Gaetjens et al., 1968) to as many as four to six (Dreizen and Gershman, 1970a; Gershman and Dreizen, 1970) depending on the methods used to obtain the data. It has been widely accepted that the LMP participates in the ATPase activity of myosin but cannot be removed without irreversible loss of activity (Gaetjens et al., 1968). However, some investigations have demonstrated at least a partial restoration of ATPase activity after complete removal of LMP by using appropriate reassociating conditions (Frederiksen and Holtzer, 1968; Stracher, 1969; Dreizen and Gershman, 1970a). Several studies have shown that the LMP components are bound to the heavy meromyosin (HMM) region at the head of the myosin molecule (Gershman et al., 1966; Bárány and Oppenheimer, 1967; Huriaux et al., 1967; Locker and Hagyard, 1967b).

Early molecular weight values of approximately 600,000 (Kielley and Harrington, 1960; Woods et al., 1963; Mueller, 1964; Young et al., 1964) were obtained on myosin prepared from

ammonium sulfate fractionation and largely were thought to reflect the presence of myosin dimers (Dreizen et al., 1967) as well as LMP. Several ultracentrifugal methods, including high-speed sedimentation-equilibrium, the Archibald approach-to-equilibrium, and low-speed sedimentation-equilibrium, were used to obtain a molecular weight of a better quality myosin (Stracher and Dreizen, 1966). The high-speed sedimentation-equilibrium method, used to resolve monomeric myosin from any multimers, resulted in a molecular weight of 468,000 (Gershman et al., 1969). The Archibald technique resulted in an early molecular weight of 530,000 which decreased to 470,000 after several hours (Gershman et al., 1969). These values seemed to indicate the presence of multimers, even from the refined methods used to isolate myosin. Low-speed sedimentation-equilibrium resulted in a weight-average molecular weight of 520,000 after 45 hours of centrifugation and 800,000 after 160 hours. Extrapolation to zero time resulted in a molecular weight of approximately 460,000 (Gershman et al., 1969).

Most of the early structural work on the myosin molecule favored a model containing three equivalent polypeptide chains (Small et al., 1961; Young et al., 1961). It was determined largely on the basis of molecular weight studies of the undissociated myosin molecule in conjunction with those of the dissociated molecule in urea or guanidine. Additional support was gained from quantitative measurements on S-1 (Young et al., 1965), kinetic studies of proteolytic fragmentation (Segal et

al., 1967), results on radioactively labeled sulfhydryls (Kimura and Kielley, 1966), and the number of ADP binding sites of heavy meromyosin (Young, 1967). Once molecular weight data from ultracentrifuge studies were corrected for the presence of LMP, and aggregated myosin and the second virial coefficient (Gershman et al., 1969) from the molecular shape of the molecule were considered, the emergence of a two-stranded model replaced that for a three-stranded structure. Results of electron microscopy, in addition to revised molecular weight data, strongly supported this model (Frederiksen and Holtzer, 1968; Lowey et al., 1969; Gazith et al., 1970).

Electrophoresis of Myosin

Electrophoresis, the migration of charged molecules through a medium in an electrical field, has been extensively used in the separation and purification of proteins. The use of zone electrophoresis, the migration of charged molecules through a liquid medium supported in a porous material, has resulted in greater resolutions from the sieving effect on the molecule due to its size and shape as it passes through the pores of the support medium (Smithies, 1955). Starch gels, first developed by Smithies (1955), have been widely used in the separation of molecules on the basis of net charge and molecular size. Polyacrylamide has more recently been used as a porous gel-support medium, and has generally replaced starch gels in electrophoretic systems (Raymond and Weinstraub, 1959; Raymond and Nakamichi, 1962).

Reproducible gel matrices, complete insolubility, gel stability under a wide range of conditions, increased gel strength, and relative ease in handling have all contributed to the gradual transition from the use of starch to polyacrylamide in zone electrophoresis.

The most recent development in the separation of charged molecules is disc electrophoresis (Ornstein, 1964; Davis, 1964). By utilizing Kohlrausch's "regulating function" (Kohlrausch, 1897) in the establishment of pH discontinuities in vertical columns of polyacrylamide gels, Ornstein and Davis were able to increase the resolution as a result of the stacking of different molecular species and hence demonstrate separations which were previously unobtainable by conventional electrophoretic techniques. By this method charged molecules are concentrated into thin starting zones from which they are then separated according to net charge and molecular size. Davis (1964) has presented a procedure applicable only to a limited number of pH values. However, Williams and Reisfeld (1964) have presented the ground rules by which the method can be extended to a wide range of pH values.

Considerable electrophoretic work has been performed on the myosin molecule. Recent studies have employed gels of low concentrations (Florini and Brivio, 1969; Paterson and Strohman, 1970), although a great deal of difficulty in handling these gels has been reported. Difficulties with the solubility of myosin in the low ionic strength buffers required by electrophoretic

systems have also been reported. This problem has been overcome by the use of urea-acrylamide systems in maintaining the solubility of myosin during electrophoresis (Small et al., 1961). Small and co-workers observed several polydisperse bands in urea concentrations of 4 to 10 M. In 12 M urea and temperatures of 45 to 50°C, a single band was observed. However, long electrophoresis times were necessitated by the relatively high gel concentrations employed. Much of the myosin sample appeared to be located at the surface of the gel, never having entered the gel. More recently, in addition to the observed LMP components, Florini and Brivio (1969) demonstrated an apparent heterogeneity of succinylated and acetylated myosin.

Most of the previous electrophoretic work on the determination of the subunit structure of myosin seemed only to add to the uncertainty that already existed. Since most results were obtained using myosin of questionable purity and since the utilization of highly advantageous electrophoretic systems possessing undesirable technical difficulties was usually avoided, the stage was thus set for the research presented in this thesis.

MATERIALS AND METHODS

Isolation and Purification of Myosin

Isolation and Purification

Myosin was prepared and purified by the procedure of Harris and Suelter (1967). A medium-sized young male rabbit was fasted for 24 hours and sacrificed. Both adductor magnus muscles were excised, trimmed of fat, washed, and immediately cooled on ice. All subsequent isolation steps and buffer pH adjustments were conducted at 3°C. Muscles were minced through 1.5 mm holes with a Howard Tissue Press (Howard Apparatus Co., Millis, Mass.) adapted for use in a Carver Press (Macalester Bicknell Co., Millville, N.J.). Twenty-five grams of the combined minced muscle was homogenized for 15 seconds in a Waring Blender with 100 ml of buffer containing 0.3 M KCl, 0.09 M KH_2PO_4 (pH 6.5; μ = 0.55). The suspension was stirred for 15 minutes. The ionic strength (μ) was then reduced from 0.55 to 0.3 with the addition of 83 ml of cold, deionized, distilled water. The suspension was briefly stirred, allowed to stand for 15 minutes, and centrifuged at 13,000 X g for five hours. Myosin was precipitated by dialysis of the supernatant against 10 X the volume of water overnight. Before use, dialysis tubing was prepared by boiling in 10 mM acetic acid for 30 minutes, washing thoroughly with deionized, distilled water, and equilibrating

at 3°C with cold, deionized, distilled water for at least 30 minutes. Myosin was further purified by two additional cycles through the 0.55μ dissolution, 0.3μ adjustment, and precipitation by dialysis overnight. The final precipitate was dissolved in 60 ml of 0.4 M KCl, 0.04 M Tris-HCl (pH 7.8). The resulting solution was then clarified by centrifugation at 25,000 X g for 1 hour.

Cellulose phosphate was purchased from Sigma Chemical Co. (St. Louis, Mo.) as Cellulose Phosphate Cation Exchanger (0.85 m equivalents per gram). The cellulose phosphate was washed successively with 0.5 M KOH, 0.5 M HCl, 0.5 M KOH, and 5 mM EDTA solution, as well as exhaustive washings with deionized water between each washing. After the final wash, the resin was suspended in 0.4 M KCl, 0.04 M Tris-HCl (pH 7.8), degassed under vacuum, and packed in a 25 X 400 mm Kontes (Kontes, Vineland, N.J.) column, for a final column height of approximately 16 cm. A volume of 50 ml of myosin (approximately 250 mg protein) in 0.4 M KCl, 0.04 M Tris-HCl (pH 7.8) was carefully layered on top of the cellulose phosphate and eluted at a rate of approximately 32 ml per hour. The flow rate was controlled by regulating and finally maintaining a constant head of pressure with a Mariotte bottle (Moyer and Kingdon, 1969). The column eluant was constantly monitored (A_{254nm}) using an ISCO Model UA Ultraviolet Analyzer (Instrumentation Specialties Co., Lincoln, Neb.) while collecting 5 ml fractions. Accurate absorbance values at 260 nm were subsequently obtained (Gilford 240 Spectrophotometer,

Gilford Instrument Laboratories, Inc., Oberlin, Ohio) for each fraction and converted to protein concentrations by calculating the absorptivity from a Biuret protein assay of the fraction containing the highest A_{260nm} value.

DEAE cellulose was purchased from Sigma Chemical Co. (St. Louis, Mo.) as DEAE Cellulose (diethylaminoethyl cellulose) Anion Exchanger (1.0 m equivalents per gram). It was prepared and packed into a column as previously described for cellulose phosphate. A 50 ml volume of myosin (approximately 150 mg of protein) from the cellulose phosphate column was layered on the DEAE cellulose column and eluted as described for the cellulose phosphate column. The column eluant was monitored, collected in fractions, and assayed for protein concentration as previously described. Fractions with a protein concentration equal to or greater than approximately 0.5 mg per ml were combined into one volume, dialyzed against three changes of 10 X the volume of 6 M urea, and stored at $3^{o}C$ for later use.

ATPase Activity of Myosin

Myosin ATPase activities were determined with the use of adenosine-5'-triphosphate-γ-^{32}P (ATP-γ-^{32}P) purchased from New England Nuclear (Boston, Mass.). ATPase activities were assayed ($30^{o}C$) in 20 mM Tris (pH 7.0 at $30^{o}C$), 10 mM KCl, 5 mM $CaCl_2$ and 4 mM ATP (0.26 uC of ATP-γ-^{32}P) in a final volume of 0.5 ml (Harris and Suelter, 1967; Keleman and Mühlrad, 1969). Myosin in a volume of 0.1 ml (50 µg of protein) was added to start the reaction. Exactly 5 minutes later the reaction was

stopped by adding 0.5 ml of cold 20% (w/v) trichloroacetic acid (TCA) containing 50 mM NaH_2PO_4. Unreacted ATP and residual ADP were removed from solution by centrifugation after 0.5 ml of Norit A suspension was added to and thoroughly mixed with each reaction. Norit A was prepared by washing 15 grams with 100 ml of 1 N HCl for 3 minutes, centrifuging, pouring off the supernatant, washing with H_2O until the pH of the supernatant was equal to that of the distilled water, and suspending in H_2O to a final volume of 100 ml. One hundred lambda aliquots of the supernatant (containing 10^3-10^4 dpm of cleaved ^{32}P) were added to liquid scintillation counting vials containing 10 ml of scintillation solvent (two parts toluene containing 0.3% (w/v) 2,5-diphenyloxazole (PPO) and one part Triton X-100). Each vial was thoroughly mixed to insure complete dissolution of the aliquots and was counted for a minimum of two minutes in a Beckman Liquid Scintillation Counting System (Model LS-200B). Appropriate blanks were similarly prepared. Activities of each fraction were obtained in duplicate and reported as ATPase specific activity (μ moles ATP cleaved $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$).

AMP Deaminase Activity

The procedure used by Harris and Suelter (1967) was directly followed. Aliquots of protein solutions after DEAE cellulose chromatography as well as before and after cellulose phosphate chromatography were assayed simultaneously for AMP deaminase activity at 265 nm on a Gilford Model 240 Spectrophotometer equipped with an automatic sample handling system.

Absorbance Ratios: 280 nm:260 nm

Absorbance values at 260 nm and 280 nm were obtained at 3°C on aliquots of combined protein fractions after DEAE cellulose chromatography, before cellulose phosphate chromatography, and after cellulose phosphate chromatography. All values were measured on a Gilford Model 240 Spectrophotometer, pre-equilibrated at 3°C and purged with nitrogen.

Isoelectric Focusing of Myosin

Isoelectric focusing of myosin was performed using an LKB 8101 electrofocusing column (LKB Instruments, Inc., Rockville, Md.) and a Buchler Voltage and Current Regulated D. C. Power Supply (Buchler Instruments Division of Nuclear-Chicago Corp., Fort Lee, N.J.). All solutions were prepared as described in the LKB 8100 Ampholine Electrofocusing Equipment Instruction Manual. A sucrose density gradient containing 3 M urea and LKB (pH 3-10) Ampholine Ampholytes was prepared and layered into the column manually as described in the instruction manual. Fifty ml of the myosin preparation (approximately 100 mg of protein) was equally distributed between the light and dense sucrose gradient solutions, resulting initially in an equal distribution of protein throughout the column. The anode was connected to the electrode at the top of the column and the cathode to that at the bottom. A starting voltage of 400 volts was gradually increased to 1000 volts and maintained for the 72 hour run time. The column

contents were eluted (1.64 ml/min), monitored (A_{260nm}) at 4°C, and fractions were collected. The pH of each fraction was measured at 4°C using a Beckman single electrode (Model No. 39030) connected to a Beckman Century SS pH meter (Beckman Instruments Inc., Fullerton, Calif.).

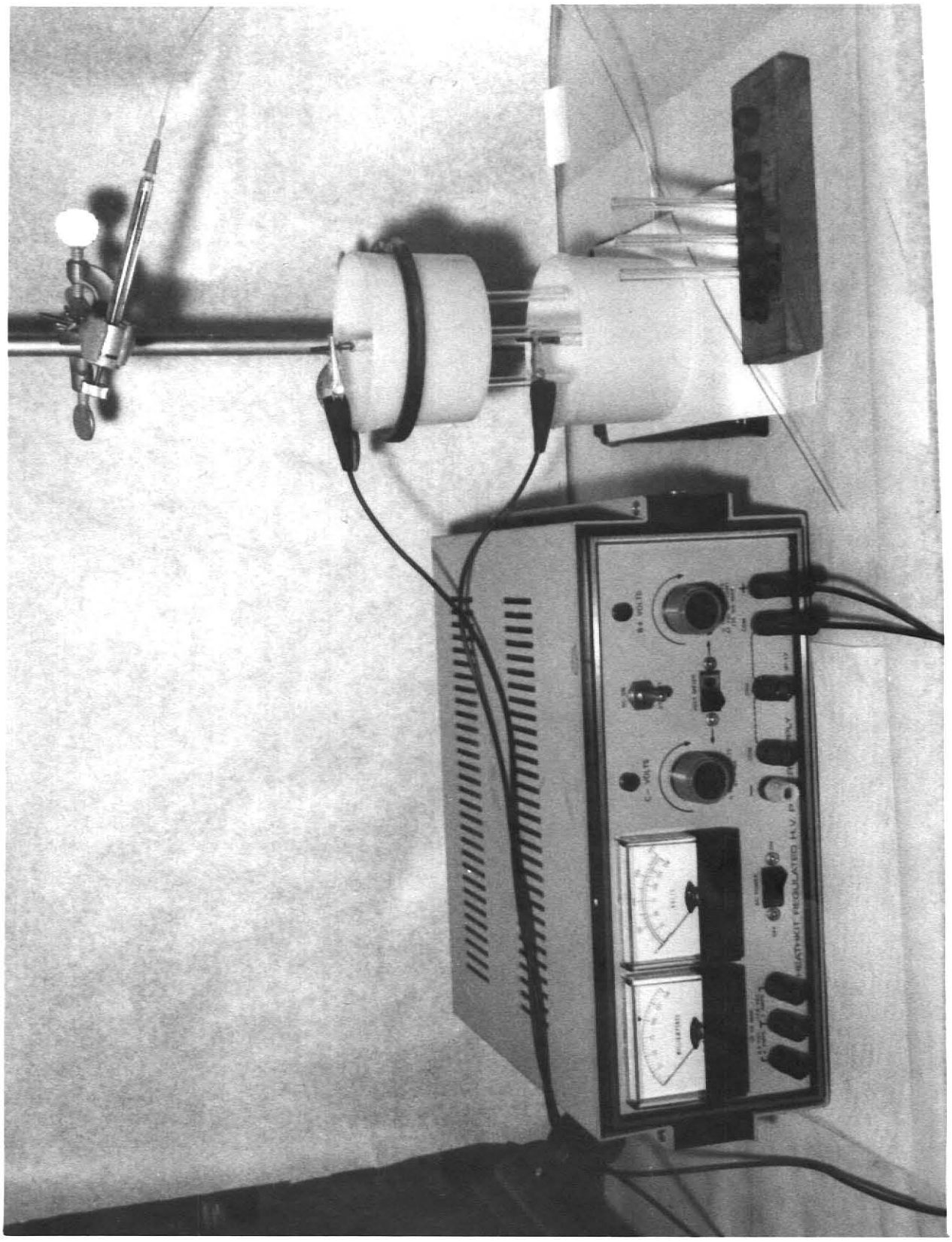
Polyacrylamide Disc Gel Electrophoresis of Myosin

Equipment

Tray buffer reservoirs and gel tubes were similar to those described by Davis (1964) and are shown in Figure 2. Electrodes consisted of platinum wire spirally wound (6-7 turns) on a plexiglass rod (7 mm diameter, 63 mm long) and soldered (silver solder) to a copper terminal. A Heathkit Model IP-17 High Voltage Power Supply (Heath Co., Benton Harbor, Mich.) was employed for all electrophoresis experiments.

Reagents. Acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylethylenediamine (TEMED), and Kodak Photoflow 200 were obtained from Eastman Kodak Co., (Rochester, N.Y.); ammonium persulfate and bromophenol blue from Fisher Scientific (Pittsburgh, Pa.); glycine, 7S gamma globulin, and bovine serum albumin (fatty acid poor) from Schwarz-Mann (Orangeburg, N.Y.); urea and sulfosalicylic acid from Mallinckrodt (St. Louis, Mo.); sodium lauryl sulfate (SDS), dithiothreitol (Cleland's Reagent), Coomassie brilliant blue, Sephadex G-25, and 2-mercaptoethanol (Type 1) from Sigma Chemical Co. (St. Louis, Mo.). Component-I, a phosphoprotein of 190,000 MW, was obtained from Dr. R. E. Clegg.

Fig. 2. Disc electrophoresis apparatus.



All reagents were reagent grade and were used without further purification.

Stock Solutions. The stock solutions were generally those used by Davis (1964) and are summarized in Table 1. It should be noted that, in general, a stock acrylamide solution of 16% (w/v), comonomer being 5% by weight of monomer, was maintained and diluted accordingly to give the desired final acrylamide concentration, usually 4% (w/v) or less.

The polymerization catalyst was ammonium persulfate. For gel polymerization at room temperature, the stock catalyst concentration was 0.14%. For gel polymerization at 40°C in 11 M urea, the initial catalyst concentration was 0.56%. In both systems the total number of moles of catalyst present during gel polymerization was the same.

Stock myosin solutions were prepared from myosin stored in 6 M urea at 3°C. When myosin in high concentrations of urea was desired, enough solid urea was dissolved in myosin stored in 6 M urea to give the final desired urea concentration.

Working Solutions. The working solutions were based upon those employed by Davis (1964). Several variations were used to facilitate the handling of the various component solutions within the system as indicated in Table 2. The small-pore running gel at room temperature, with or without urea, was prepared in exactly the same way as described by Davis (1964). Due to the requirements of a larger volume for the dissolution of the urea, polymerization of gels in 11 M urea at 40°C was accomplished with

Table 1. Stock Solutions for Disc Electrophoresis

Buffer Sol'n A (running gel)

1 N HCl	48 ml
Tris (Base)	36.6 gms
TEMED	0.23 ml
Water	to 100 ml

(pH 8.9)

Buffer Sol'n B (stacking & sample)

1 N HCl	48 ml
Tris (Base)	5.98 gms
Water	to 100 ml

(pH 6.7)

Acrylamide Stock Sol'n

Acrylamide	16 gms
Bis	0.80 gms
Water	to 100 ml

Stock Tray Buffer

Tris (Base)	6.0 gms
Glycine	28.8 gms
Water	to 1000 ml

(pH 8.3)

Stock 40% Sucrose

Sucrose	40 gms
Water	to 100 ml

Catalyst A

Ammonium persulfate	0.14 gms
Water	to 100 ml

Catalyst B

Ammonium persulfate	0.56 gms
Water	to 100 ml

Sulfosalicylic Acid Fixer

Sulfosalicylic acid	20 gms
Water	to 100 ml

Coomassie Stain

Coomassie brilliant blue	0.25 gms
Water	to 100 ml

Photo-flow Solution

Kodak Photo-flow 200	1 ml
Water	to 200 ml

the use of a catalyst four times more concentrated.

The stacking and sample layers were prepared without the use of a large pore gel. The stacking layer was initially prepared as a pipetable slurry of a suspension of Sephadex G-25 in sucrose and the appropriate buffer. However, when using low percent gels (large pore size), it became necessary to use

Table 2. Working Solutions for Disc Electrophoresis

	System	1	2	3	4	5	6	7	8	9
	Urea Conc (M)	6	6	6	11	11	11	11	11	11
	Temperature (°C)	25	25	25	40	40	40	40	40	40
Running Gel	Urea (gms)	4	4	4	10.58	10.58	10.58	10.58	10.58	10.58
	Buffer A (ml)	1	1	1	1	1	1	1	1	1
	Acryl (%)	10.4	11.04	11.04	20	16	16	16	16	16
	Acryl (ml)	2	2	2	2	2	2.5	3	4	5
	H_2O (ml)	1	1	1	4	4	3.5	3	2	1
	Persulfate (%)	.14	.14	.14	.56	.56	.56	.56	.56	.56
	Persulfate (ml)	4	4	4	1	1	1	1	1	1
Stacking Solution	Sephadex (Yes or No)	Y	Y	Y	N	N	N	N	N	N
	Urea (gms)	6.2	6.2	6.2	16.5	16.5	16.5	16.5	16.5	16.5
	Buffer B (ml)	2	2	2	4	4	4	4	4	4
	40% Sucrose (ml)	6	6	6	8.5	8.5	8.5	8.5	8.5	8.5
	H_2O (ml)	4.5	4.5	4.5	-	-	-	-	-	-
Sample Solution	Urea (gms)	.8	.8	.7	.96	1.06	1.06	1.06	1.06	1.06
	Stock Myosin (ml)	.24	.24	.24	.8	.8	.8	.8	.8	.8
	Urea Conc in Stock Myosin (M)	0	0	6	11	11	11	11	11	11
	Buffer B (ml)	.2	.2	.2	.2	.2	.2	.2	.2	.2
	40% Sucrose (ml)	.6	.6	.6	.6	.6	.6	.6	.6	.6
	H_2O (ml)	.56	.56	.56	-	-	-	-	-	-
Tray Buffer	Urea (gms)	180	180	180	540	540	540	540	540	540
	Urea (Conc) (M)	3	3	3	9	9	9	9	9	9
	10 X Tray Buffer (ml)	100	100	100	100	100	100	100	100	100
	Total volume (liters)	1	1	1	1	1	1	1	1	1

higher concentrations of sucrose in place of Sephadex as a stabilizing and anti-convection medium. The sample layer was similarly prepared in the appropriate buffer and sucrose. However, the sucrose concentration was less than that in the stacking layer. This established a large enough difference in density between the two solutions that the sample could easily be layered on top of the stacking layer.

The tray buffers were prepared with urea concentrations slightly less than those in the running gel, stacking layer, and sample layer to decrease the rate of diffusion across the interface of the sample layer and the upper tray buffer.

Molecular weight studies were conducted by polyacrylamide gel zone electrophoresis instead of disc gel electrophoresis because of the highly charged nature of the protein-SDS complex. Working solutions for molecular weight studies were modifications of the above solutions and are described in Table 3. The stacking phase was omitted and Tris-glycine buffer (pH 8.3) was used throughout the system. The sample solution contained protein in Tris-glycine buffer, 0.1% (w/v) SDS, and 5% (w/v) sucrose.

Methods

Electrophoresis Procedure. Six hard glass gel tubes were washed with detergent and rinsed thoroughly with distilled water. Each tube was then dipped several times into, and allowed to soak for one minute in a 1:200 dilution of Kodak Photo-flow 200. The tubes were then ventilated in an upright position and allowed to air dry for one hour. One end of each tube was then sealed with

Table 3. Working Solutions for Molecular Weight Determinations by Electrophoresis.

System		A	B
Gel Conc (%)		2.5	3.0
Temperature (°C)		24	24
Running Gel	16% Acrylamide (ml)	3.90	4.69
	10 X Tris-glycine Buffer (ml)	2.50	2.50
	1% (w/v) SDS	2.50	2.50
	TEMED (ml)	0.02	0.02
	H_2O (ml)	14.33	13.54
	0.56% (w/v) Persulfate (ml)	1.75	1.75
Sample Sol'n	Protein in Tris-glycine Buffer, 0.1% SDS, 5% Sucrose (ml)	0.05/gel	0.10/gel
Tray Buffer	1% (w/v) SDS (ml)	100	100
	10 X Tris-glycine Buffer (ml)	100	100
	Total volume (liters)	1	1

a B-D Vacutainer stopper and secured in a vertical position by means of a wood support.

The gel solution was prepared by dissolving the appropriate amount of solid urea in a specified volume of Buffer A, acrylamide solution, and distilled water (Table 2). The ammonium persulfate solution, the non-polymerized gel solution, and the gel tubes were then temperature equilibrated for at least 30 minutes. A specified volume of the catalyst was added, with stirring, to the gel solution. Using a Pasteur pipette, the gel tubes were then filled approximately 4/5 full with the catalyzed gel solution. A small volume of water was then carefully introduced into

the gel tubes with a 500 μ liter screw-plunger Hamilton (Hamilton Co., Whittier, Cal.) Gastight syringe #1750 (connected to a short length of 0.075" o.d. polyethylene tubing) and layered over the gel solutions. The water layer was replaced by 6 M urea in the systems containing 11 M urea at 40°C. The solutions were then allowed to polymerize undisturbed for a period of one hour.

The stacking solution was prepared by dissolving the appropriate amount of solid urea in a specified volume of Buffer B, 40% sucrose, and distilled water (Table 2). When Sephadex was used as an anti-convection medium in the systems containing 6 M urea, 2 grams of Sephadex G-25 was suspended in the specified volume (Table 2) of stacking solution and allowed to equilibrate with constant stirring for at least 6 hours prior to use. The stacking solution was then allowed to temperature equilibrate for at least 30 minutes before being layered into the gel tubes.

The sample solution was similarly prepared by dissolving the appropriate amount of solid urea in a specified volume of stock myosin solution, Buffer B, 40% sucrose, and distilled water. It was also temperature equilibrated for at least 30 minutes prior to use.

The tray buffer was prepared by dissolving solid urea in one liter of solution containing 100 ml of stock Tris-glycine buffer (pH 8.3). The tray buffer was then divided into equal volumes designated as upper and lower tray buffer. When a marker dye was used during electrophoresis, 1 ml of 0.001%

(w/v) bromophenol blue was mixed with the upper tray buffer. Both upper and lower tray buffers were temperature equilibrated before use.

After gel polymerization (about 1 hour), the layer of water or 6 M urea was removed from the top of the gel with a syringe. A 1 ml syringe was then used to layer enough spacer solution over the gel that the total thickness of the spacer layer was approximately 0.5 cm. A clean 1 ml syringe with 0.01 ml graduations was used to carefully layer exactly 0.1 ml of sample over the stacking layer by resting the needle tip of the syringe against the inner wall of the gel tube and allowing the sample to slowly run down the wall of the tube and layer on top of the spacer layer. A clean 1 ml syringe was then used to carefully introduce and layer the upper tray buffer over the sample layer until the tube was filled. Each tube was removed from the B-D Vacutainer stopper by carefully pinching the stopper and fastened into the upper tray reservoir by securing it into the grommets in the reservoir. The tops of the tubes were positioned approximately 1 mm over the upper edge of the grommet in which they were fastened. The upper reservoir was then secured in a ring stand, positioned over the lower reservoir, and lowered to a point over the lower reservoir, which had previously been filled 2/3 full with lower tray buffer, where the bottoms of the gel tubes were immersed approximately 1 cm into the lower tray buffer. The gel tubes were adjusted to a vertical position in such a way that their bottom tips were equidistant from adjacent tubes and from the electrode positioned

in the center of the lower reservoir. Trapped air bubbles at the bottom of the gels were removed with a syringe.

In order to prevent or minimize loss of sample into the upper tray buffer and to allow more efficient stacking, a reduction in the initial current, usually 2 ma per tube, was used for approximately 30 minutes, followed by a final current of 4 ma per tube. During electrophoresis the current was periodically checked and adjusted accordingly. Electrophoresis was terminated either after a pre-determined amount of time, at timed intervals during a time-variable run, or after the band from a marker dye had reached the bottom of the gel. Termination of electrophoresis in one or more, but not all, gel tubes was accomplished by briefly turning off the power and removing the specific tubes by simultaneously inserting solid glass rods into the grommets.

Upon completion of electrophoresis, the gels were transferred from the gel tubes to test tubes containing 20% (w/v) sulfosalicylic acid (pre-equilibrated to the temperature of electrophoresis) for fixation (precipitation) of the protein-containing-bands within the gel. Removal of the gels from the gel tubes was accomplished by inserting a section of 23 gauge stainless steel tubing, the tip of which had been filed smooth, between the gel and the inner wall of the tube, rimming the gel in a spiral motion, and pushing the gel out of the tube using the same spiral motion after the tip of the tubing had emerged from the bottom of the tube. After fixing for 20 to 30 minutes at the same temperature as electrophoresis, in a position of

approximately 30° from a horizontal position (necessitated by the extreme flexibility of the low percent gels), the sulfosalicylic acid was carefully poured from the test tubes and replaced by an equal volume of 0.25% Coomassie brilliant blue to stain for protein (St. Groth et al., 1963; Fish et al., 1969). The test tubes were then positioned as before and occasionally rotated for uniform staining. Staining time varied from 30-60 minutes at 40°C to 3-4 hours for gels at room temperature. Destaining was performed by carefully pouring off the stain and subjecting the gels to enough changes of distilled water until the background stain was sufficiently low for gel scanning and photography. All gels were finally stored in stoppered test tubes containing 7% (w/v) acetic acid.

Stained gels were scanned on a Coleman Autoset Spectrophotometer (modified for gel scanning) at either 500 nm or 625 nm. After destaining, gels were transferred to a Gilford 2412 gel cuvette and positioned on a carriage driven by a stepper motor connected to a Heath multi-speed chart drive. Gel scans were recorded on a Heath Servo-Recorder Model EUW-20A. Velocities of 200 seconds per inch for the carriage and 50 seconds per inch for the recorder were usually employed.

Cyanate Assay. Cyanate produced from high concentrations of urea at elevated temperatures was determined by a modification of E. A. Werner's procedure (1923).

The reagents used for this analysis were pyridine, chloroform, 1% (w/v) copper sulfate (anhydrous), and standard (50 mM)

potassium cyanate. The standard, containing 0 to 50 μ moles of potassium cyanate, and sample to be analyzed were added to a series of conical glass stoppered centrifuge tubes. An 0.5 ml volume of copper sulfate solution was added and mixed. This was followed by the addition of 0.5 ml of pyridine and sufficient deionized, distilled water to increase the volume to 10 ml. All tubes were vigorously mixed and allowed to stand at room temperature. After 30 minutes, 2 ml of chloroform was added and each tube was vigorously mixed twice for 15 seconds with 10 to 15 minute intervals between each mixing. The chloroform layer was then removed and the absorbance measured at 705 nm.

Preparation of Myosin for Prevention of Carbamylation. Three grams of solid urea was dissolved in 5 ml of myosin stored in 6 M urea to give a final volume of 7.27 ml of myosin in 11 M urea. The pH was immediately adjusted to approximately 2 at 40°C with 6 N HCl. The solution was then stored at 40°C for 48 hours, after which a 2 ml aliquot was dialyzed overnight against 500 ml of 11 M urea. Fresh dialysate was prepared and dialysis was repeated for an additional four hours. The myosin in 11 M urea was then removed from the dialysis bag and used as stock myosin which was subjected to electrophoresis within 24 hours.

Molecular Weight Studies with Sodium Dodecyl Sulfate. Component-I and 7S gamma globulin were separately dissolved in enough 6 M urea for a final concentration of approximately 5 mg per ml. Enough solid urea for a final concentration of 11 M was added separately to equal volumes of each of the reference protein solutions, as well as myosin (in 6 M urea), and dissolved. Each

protein solution in 11 M urea was then stored at 40°C for approximately 48 hours, after which enough SDS was added to each sample to give a 1% (w/v) SDS concentration in each. Each protein solution was then stored at 40°C for 5 hours. Subsequently, a 2 ml volume of each protein was separately and simultaneously dialyzed against 500 ml of dialysate containing 0.1% (w/v) SDS and full-strength Tris-glycine tray buffer (pH 8.3) for 2 hours at 40°C. Dialysis was repeated for 2 hours using fresh dialysate. Each sample was finally dialyzed overnight at room temperature against 1 liter of Tris-glycine buffer (pH 8.3) containing 0.1% (w/v) SDS and 5% (w/v) sucrose. Samples were removed from the dialysis tubing and separately stored for electrophoresis.

Solutions were carefully poured into gel tubes to prevent foaming, covered with a small volume of water, and allowed to polymerize for 2 hours. The following proteins and protein mixtures were run simultaneously after being covered with tray buffer containing 0.1% SDS: myosin, 7S gamma globulin, Component-I, myosin + 7S gamma globulin, myosin + Component-I, and an equal mixture of all three. All tubes were secured in the upper reservoir and electrophoresis was performed with a current of 2 ma per tube for fifteen minutes. Gels were rimmed, fixed, stained, and destained as previously described (pp. 31-32).

RESULTS AND DISCUSSION

Isolation and Purification of Myosin

General Observations. The method employed by Harris and Suelter (1967) was selected for the isolation and purification of myosin because of the relatively short time required to obtain myosin and the high degree of purity in which it could be obtained. Other methods (Kielley and Harrington, 1960; Perry, 1960; Takahashi et al., 1962; Asai, 1963) require longer isolation procedures, more elaborate purification techniques, and result in a less pure preparation of myosin.

The adductor magnus muscle (a predominantly white muscle) of the rabbit was chosen as the source of myosin since it is the largest easily-accessible muscle in the hind leg of the rabbit and voluminous literature already exists on rabbit myosin. The use of a single muscle guarded against large differences in myosin molecules that may occur between muscles but did not avoid those differences in myosin that may be present between red and white muscle fibers in the adductor magnus.

Initial attempts at extraction and isolation of crude myosin from non-fasted rabbits resulted repeatedly in pre-chromatographic solutions of a very opaque appearance. All attempts to clarify these solutions were unsuccessful. Since it was conceivable that this opaqueness could have been the result of glycogen binding

to myosin, rabbits were fasted for 24 hours prior to sacrifice. Isolation of crude myosin from fasted rabbits resulted in the disappearance of the opaqueness. Hence all subsequent animals were fasted overnight prior to sacrifice.

Protein Concentration and ATPase Activity Profiles. Protein concentration profiles from cellulose phosphate columns consistently demonstrated a small shoulder on the trailing side of the curve (Figure 3). Since this shoulder exhibited an increase in ATPase activity, it seemed that the trailing shoulder was probably due to a high molecular weight (aggregated) form of myosin or a small amount of contaminating actomyosin. The magnitude of the shoulder and the accompanying increase in ATPase activity was greatly reduced in the profile from the DEAE cellulose column (Figure 4).

ATPase specific activity profiles were similar to those reported by Harris and Suelter (1967) but the magnitude of the values was about two-fold higher. Since the only variation in isolation procedures was the greater specificity in the source of myosin, this increase in specific activity could have been the result of such a variation. All attempts to assay fractions for ATPase activity with a protein concentration less than 0.5 mg per ml were unsuccessful.

AMP Deaminase Activities: $A_{280nm}:A_{260nm}$ Ratios. AMP deaminase activities were reduced to nearly zero after chromatography on a cellulose phosphate column (Table 4). Chromatography on DEAE cellulose further purified myosin from AMP deaminase contamination.

Fig. 3. Cellulose phosphate chromatography of myosin. The column (2.5 cm X 16 cm) was eluted at 32 ml per hour and 5 ml fractions collected. Protein concentration profile (—— · ——). ATPase specific activity profile (—— ▲ ——).

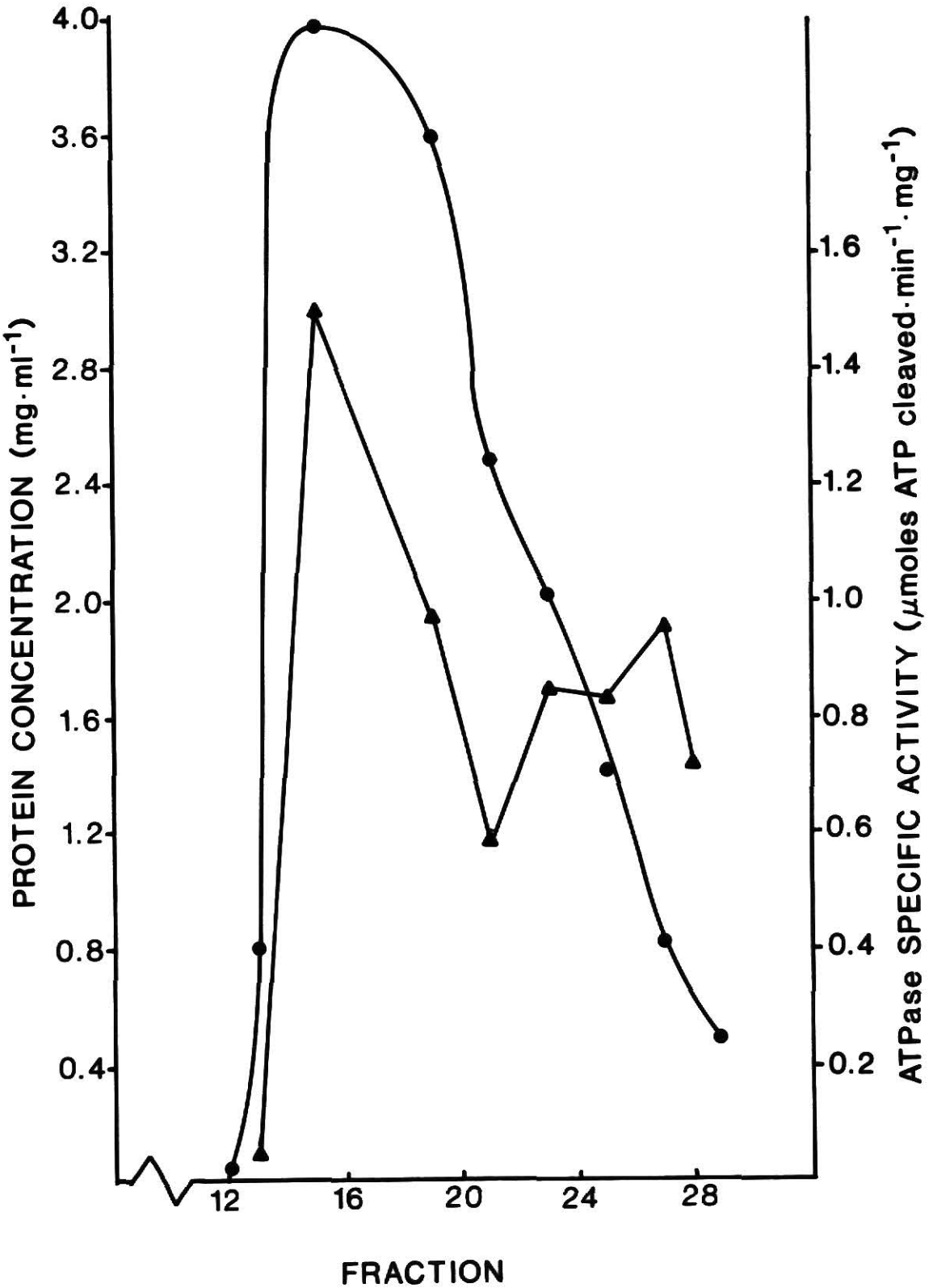

4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
PROTEIN CONCENTRATION (mg·ml⁻¹)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ATPase SPECIFIC ACTIVITY (μmoles ATP cleaved·min⁻¹·mg⁻¹)
12
16
20
24
28
FRACTION

Fig. 4. DEAE (diethylaminoethyl) cellulose chromatography of myosin. The column (2.5 cm X 16 cm) was eluted at 32 ml per hour and 5 ml fractions collected. Protein concentration profile (—— · ——). ATPase specific activity profile (—— ▲ ——).

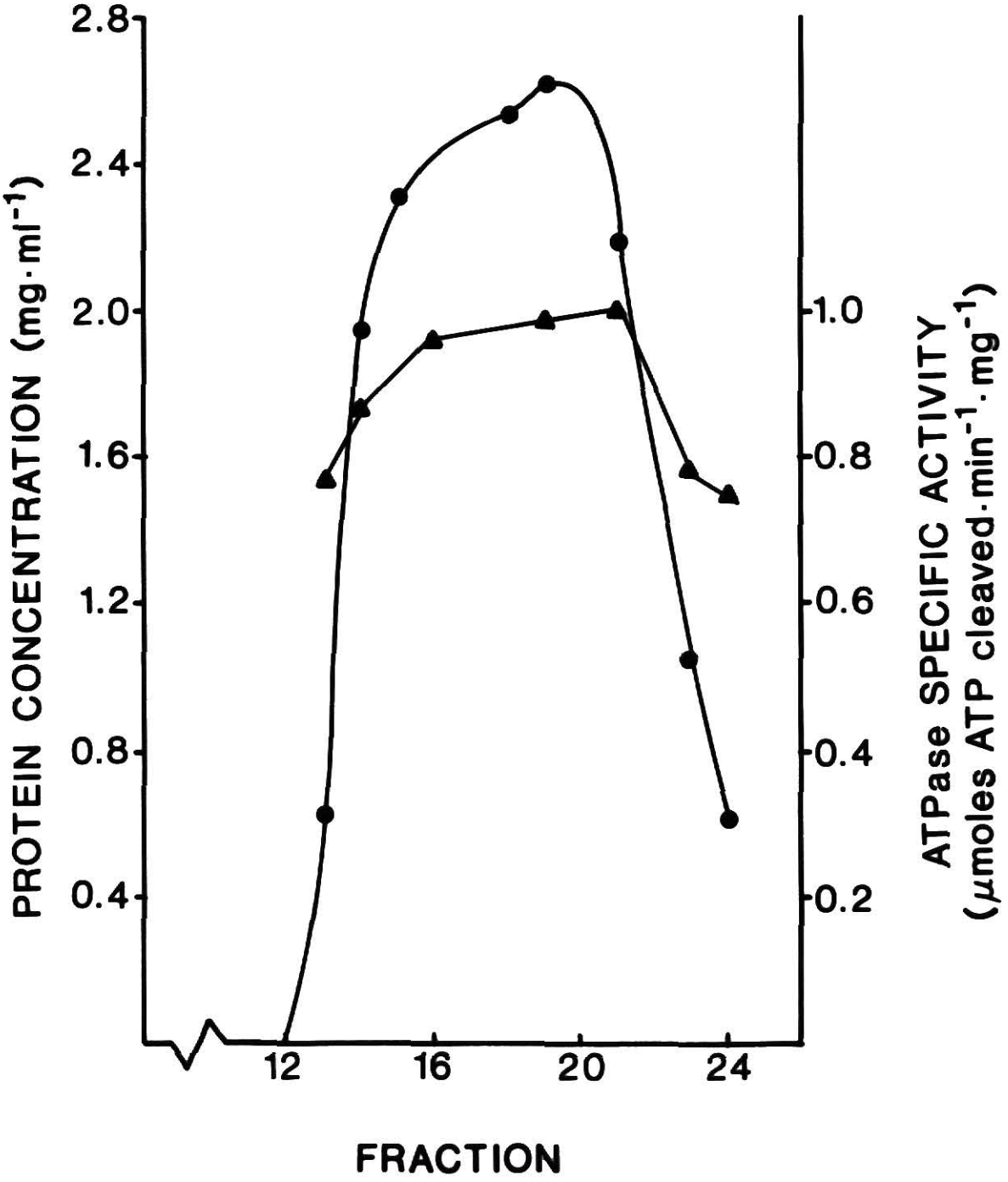

PROTEIN CONCENTRATION (mg·ml⁻¹)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
ATPase SPECIFIC ACTIVITY
(μmoles ATP cleaved·min⁻¹·mg⁻¹)
1.0
0.8
0.6
0.4
0.2
12
16
20
24
FRACTION

Table 4. AMP Deaminase Activity and A_{280nm}:A_{260nm} Ratios

	AMP Deaminase Specific Activity (μ moles $\cdot$ min^{-1} $\cdot$ mg^{-1})	A_{280nm}:A_{260nm}
Before Cellulose Phosphate Chromatography	0.035	1.16
After Cellulose Phosphate Chromatography	0.0005	1.25
After DEAE Cellulose Chromatography	0.0004	1.70

The A_{280nm}:A_{260nm} ratios in Table 4 are similar to those previously reported (Harris and Suelter, 1967) and indicate that nucleic acid contamination was greatly reduced by chromatography on DEAE cellulose. A small degree of purification was detected by cellulose phosphate chromatography (Table 4).

Test for Purity. The dependability of this method in producing myosin of a high degree of purity was demonstrated by subjecting the myosin solutions at each step of the purification process to disc gel electrophoresis. The studies, performed without urea, employed bromophenol blue as a marker dye in the upper tray buffer. In each case electrophoresis was allowed to continue until the marker bands closely approached the bottom of the tubes. Results of these studies demonstrated the removal of one very prominent band after cellulose phosphate chromatography. Treatment with DEAE cellulose columns resulted in the removal of

one additional very weak band. Electrophoresis of the final preparation resulted in several strong bands, all of which were tentatively identified as polymeric forms of myosin. These were preceded by three weak bands, thought to be either three species of low molecular weight protein (LMP) or two species of LMP and one of actin.

Isoelectric Focusing of Myosin

A total of approximately 100-125 mg of protein was uniformly distributed throughout the column. Due to the distribution of the ampholytes to form a pH gradient and to the subsequent migration of myosin to the region of its pI, the current was observed to steadily decrease to a constant value of approximately 2 ma. Within 24 hours after the initial application of voltage, a white fibrous band formed close to the middle of the column and after 72 hours the band appeared to have concentrated into a very thin zone. While the column was being emptied, the band of precipitated material remained attached to the walls. An absorbance scan for protein of the column failed to reveal any protein. The pH gradient in the column was linear from pH 11.3 to 3.1. The approximate pH in the column to which the white band migrated was 5.3 which corresponds very closely to the reported pI of myosin (Florkin and Stotz, Eds., 1963). It was therefore concluded that the white fibrous precipitate consisted of the entire quantity of myosin initially distributed throughout the column.

The absence of any absorbing material was also considered to be additional evidence as to the high degree of purity of myosin obtained from this procedure (Harris and Suelter, 1967).

Electrophoresis of Myosin

Maintenance of High Ionic Strength. Initial experiments were performed by increasing the ionic strength of each phase of the disc electrophoresis system to that of the myosin solution in 0.4 M KCl, 0.04 M Tris buffer (pH 8.3). These experiments were performed with the use of Sephadex G-25 as an anti-convection medium in the stacking region.

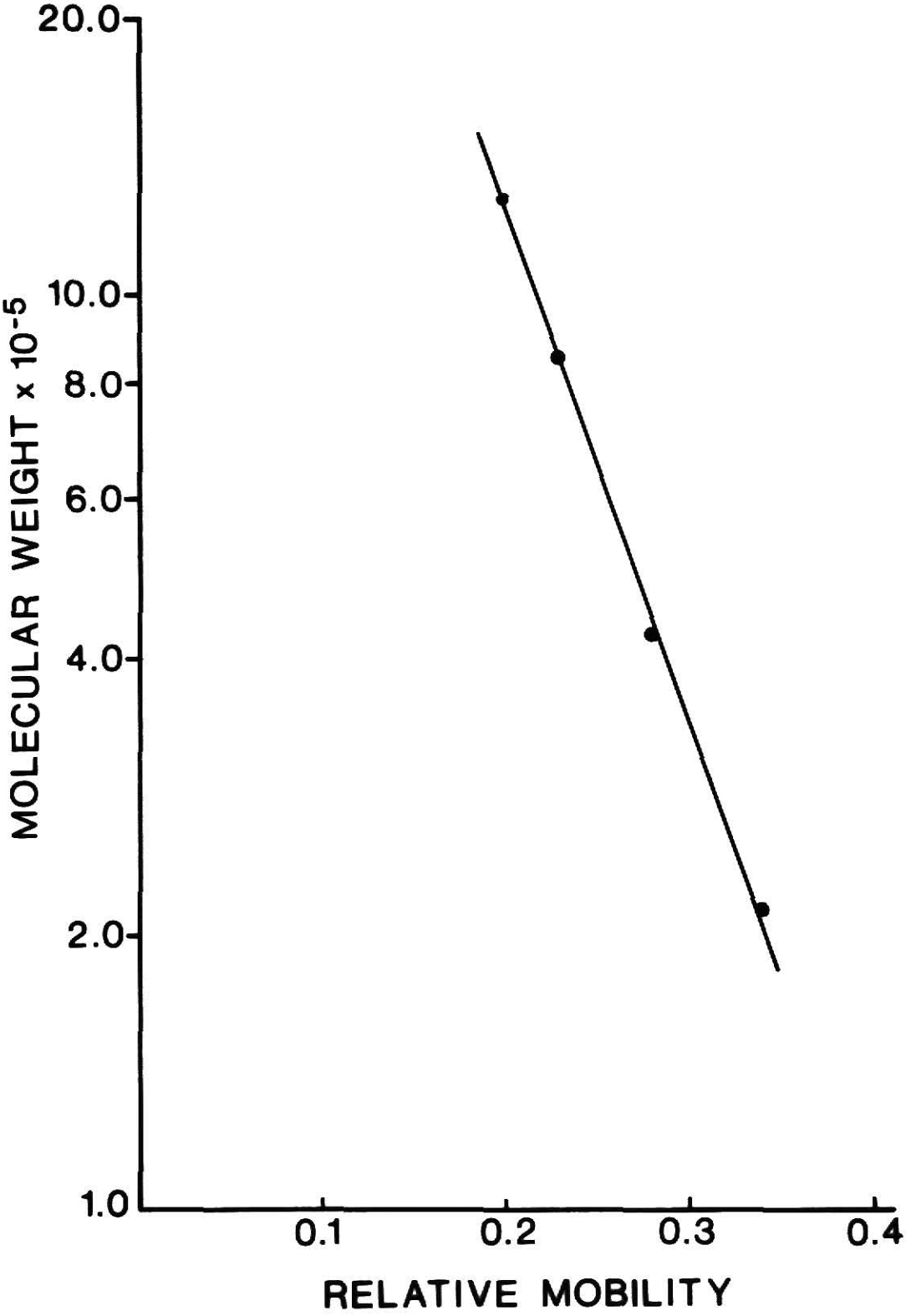
Just after the onset of electrophoresis, a thin, white fibrous band formed at the interface of the sample layer and the upper tray buffer. When bromophenol blue was used as a marker, it appeared to form in front of the white band and subsequently migrate well ahead of it. Also observed was a very large final voltage accompanied by very high temperatures within the gel tubes. This was most likely due to the presence of high salt concentrations throughout the entire system.

When the gels were rimmed and transferred into sulfosalicylic acid fixer, a small amount of white solid material remained attached to and slightly embedded in the top of the gels. At first, this material appeared to be protein since it retained the stain during the destaining process. However, control tubes without protein still resulted in the retention of a small amount of stain at the top of the gel. Since large pore running gels

were being used, it is quite conceivable that the retention of stain could be due to a small amount of Sephadex becoming lodged in the top of the gel during electrophoresis. Sephadex was therefore removed from the stacking region in subsequent experiments and replaced by increased concentrations of sucrose (Table 2).

Electrophoresis in 6 M Urea. Due to the previously described unfavorable conditions encountered during electrophoresis and the observation that myosin was soluble in 6 M urea, salt was completely removed and replaced by urea in the system. Electrophoresis in 6 M urea resulted in lower final voltages and little or no heat production in the gel tubes. The same pattern of bands, as those seen without urea, was observed. Paterson and Strohman (1970) and others (Dunker and Rueckerts, 1969; Shapiro et al., 1967; Shapiro and Maizel, 1969; Weber and Osborn, 1969) have demonstrated the linear relationship between the logarithm of the molecular weight, when using SDS, or the subunit number of multimers and the relative mobility of proteins during polyacrylamide gel electrophoresis. Such a plot of the multiple bands visible in the upper one-third of the gels resulted in a linear relationship of their mobility to a marker band and the logarithm of their subunit number (Figure 5). The fastest migrating of the multiple bands was arbitrarily chosen as the monomer. Each slower migrating band was identified as dimer, tetramer, and hexamer, respectively. It therefore was apparent that the series of multiple bands in the upper one-third of the gel represented aggregated species of myosin molecules. Since the marker band

Fig. 5. Plot of log of subunit number vs. relative mobility for aggregated species of myosin.


20.0
10.0
8.0
6.0
4.0
2.0
1.0
MOLECULAR WEIGHT x 10-5
0.1
0.2
0.3
0.4
RELATIVE MOBILITY

represented the fastest migrating species, the two or three bands between the marker and the multiple bands were assigned as LMP and possibly actin (Paterson and Strohman, 1970).

Further evidence that the series of multiple bands represented aggregated myosin and not myosin molecules polymerized through the formation of disulfide bonds by oxidation was obtained with the use of dithiothreitol (Cleland's Reagent) as a reducing agent. Attempts at eliminating or reducing the number of bands with dithiothreitol (4000 X molar excess to myosin sulfhydryls) were unsuccessful. Instead of a reduction in the number of bands, an increased number, probably due to the partial modification (with insufficient time for a total reaction) of cysteine residues and the resulting variation in the charge of all species at the pH of the running gel (pH 8.9), was observed. It was concluded that the occurrence of multiple arrays of bands was probably a function of low urea concentration and not disulfide bond formation by oxidation.

Electrophoresis in High Concentrations of Urea. Initial experiments were designed to accommodate 12 M urea in the running gel, stacking solution, and sample solution. The tray buffer contained 9 M urea to minimize the diffusion of urea from the sample layer and running gel. Difficulty was encountered with crystallization of urea in the running gel during polymerization and in keeping the gels secured inside the gel tubes during electrophoresis at 45°C. The use of a humid atmosphere had little effect on decreasing or stopping the crystallization of the

solutions containing 12 M urea. Elimination of Photo-flow pre-treatment was also unsuccessful in the prevention of loss of gels. In addition, gels of low concentrations of acrylamide at these high temperatures were quite unmanageable after removal from the glass tubes due to their extreme elasticity.

Attempts at electrophoresis using 11 M urea concentrations in the running gel, stacking solution, and sample solution, with a 5°C reduction in temperature (40°C) were much more successful. The use of a humid atmosphere seemed to be very beneficial in maintaining the dissolution of urea. It was observed that temperature equilibration of the gel solution and catalyst at 40°C before mixing and polymerization resulted in quite reproducible gels in texture and pliability during rimming. In practice, urea (6 M) containing the myosin preparation was always increased to 11 M urea by the dissolution of solid urea and either immediately subjected to electrophoresis or stored at 40°C for various times. The optimal time for treatment of gel tubes with dilute Photo-flow was 30-60 seconds, followed by air drying for 1 hour when 2-3% gels in 11 M urea were used. Total electrophoresis times in the 11 M urea systems varied between 75 and 125 minutes, depending on the gel concentration (usually 2-3%). All attempts at using bromophenol blue as a marker were unsuccessful. Since the pH of each phase of the system was carefully checked and adjusted prior to electrophoresis, it is believed that this failure was due to a high rate of diffusion as a result of the large pore sizes of the gel and the high temperature (40°C).

All electrophoresis experiments at 11 M urea concentrations resulted in the formation of a thin white band at the interface of the sample layer and upper tray buffer approximately 30 seconds after the onset of electrophoresis. This band slowly dispersed when migrating through the sample layer and into the stacking region. The band again became visible when it settled on top of the gel. The accumulation of this material at the top of the gel seemed to be independent of the concentration of the gel or the sample. It is quite likely that the initial formation of this material in a visible band was due to the immediate stacking effect at the top of the sample layer just as the glycine entered from the tray buffer. The initial stacking effect would have concentrated the protein into a thin zone, resulting in a small amount of highly aggregated myosin. These heterogeneous, highly aggregated species of myosin were probably of sufficiently large molecular weight that passage through a gel with even as large a pore size as that of a 2% acrylamide gel would have been virtually impossible. All other forms of myosin within the sample layer were not subjected to the initial concentration and aggregation during the stacking process and consequently could enter the gel.

After the gels were destained at elevated temperatures, a series of bands, similar to those observed in lower concentrations of urea, were observed. However, the bands appeared to be considerably more diffuse. The weak bands previously identified with LMP and actin were absent. It was assumed these had migrated out of the gel during the 75 to 120 minutes required for electrophoresis.

While performing these initial experiments in 11 M urea, it was discovered that the myosin preparation in 11 M urea could be reused for subsequent experiments by storing it at 40°C. The first time myosin (stored for several days in 11 M urea) was prepared for electrophoresis, a very subtle separation in the lead band was observed. Simultaneously, a reduction in the intensity of staining of trailing bands occurred. Electrophoresis of the same myosin stock solution in 11 M urea two days after the first observation resulted in the same subtle separation of the leading band with an apparent complete disappearance of the trailing bands. On the basis of these observations, it became obvious that a series of time studies to determine the time required to resolve the leading band into two distinct bands was necessary.

Time Studies in 11 M Urea. A fresh preparation of myosin in 11 M urea was prepared and electrophoresis was performed at intervals of 0, 24, 48, 72, 96, and 168 hours from the initial time of dissolution in 11 M urea. The result of each experiment is shown in a composite photograph in Figure 6. Scans of a representative stained gel from each experiment are shown in Figure 7.

The 0 hour experiment resulted in a series of bands of which the lead band had migrated approximately one-third the length of the gel. A vaguely stained, very diffuse region was observed in the lower third of the gel. The 24 hour study resulted in a reduction in the intensity of the multiple bands compared to the 0 hour study and an overall increase in the migration of all bands, probably due to a slight variation in the pore size of the gel.

Fig 6. Disc electrophoresis of myosin after 11 M urea treatment for various times. Gel A, 0 hours; Gel b, 24 hours; Gel C, 48 hours; Gel D, 72 hours; Gel E, 96 hours; Gel F, 168 hours. Gels A through D had a total electrophoresis time of 75 minutes, Gel E 95 minutes, and Gel F 105 minutes.

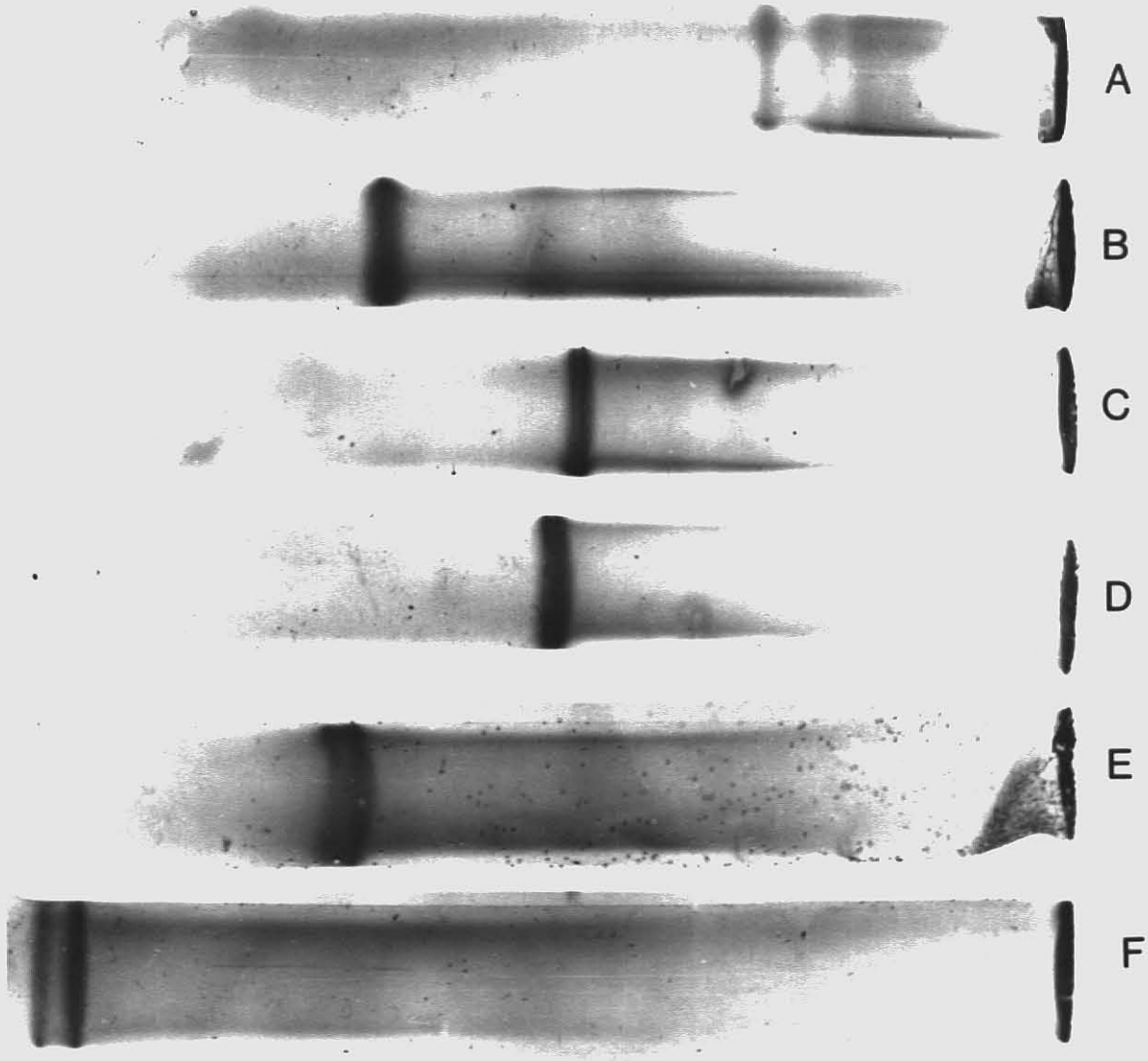
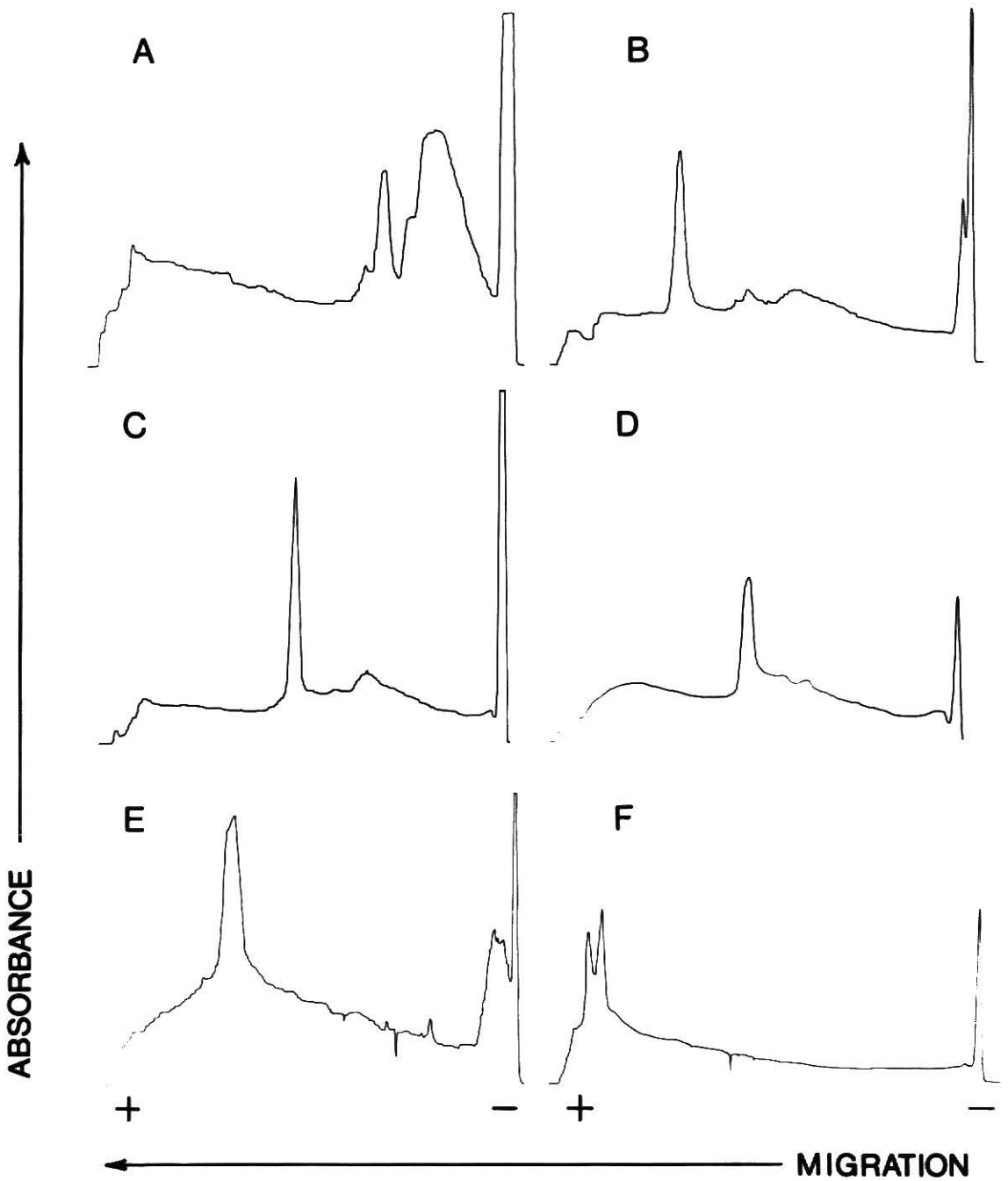

A
B
C
D
E
F

Fig. 7. Densitometric scans of gels from myosin treated with 11 M urea for various times. Scan A, 0 hours (500 nm); Scan B, 24 hours (500 nm); Scan C, 48 hours (500 nm); Scan D, 72 hours (625 nm); Scan E, 96 hours (625 nm); Scan F, 168 hours (625 nm).


A
B
C
D
E
F
ABSORBANCE
+
−
+
−
MIGRATION

The 48 hour study first shows the subtle separation in the lead band with almost complete disappearance of all other trailing bands. The 72 hour study demonstrates the entire disappearance of all trailing bands with a migration of the separated leading band approximately equal to that of the 48 hour study. The longer electrophoresis times used with the 96 and 168 hour treatments gave enhanced separations of the leading band.

The most obvious result of these studies is that the myosin subunits in the form of a dimer of an approximate molecular weight of 468,000 (Gershman et al., 1969) had apparently dissociated into two unresolvable, closely related subunits after 24 hours in 11 M urea. In that case the lead band of the 0 hour study was identified as a subunit dimer having a molecular weight of 468,000. The lack of resolution of the monomeric subunits into two distinct species after 24 hours in 11 M urea was not completely understood. One likely explanation could have been the partial "masking" of the charge differences in the highly globular head regions of the myosin molecule due to insufficient time for complete unfolding. If this were the case, then the results of the 0, 24, and 48 hour studies suggest a possible sequence of events in the unfolding of the myosin molecule into two distinct, but very closely related subunits. It seems quite possible that separation into two subunits first occurs by dissociation in the tail region of the molecule. This is demonstrated by the emergence of a faster migrating band after 24 hours in 11 M urea. Due to incomplete unfolding of the head region of these subunits after

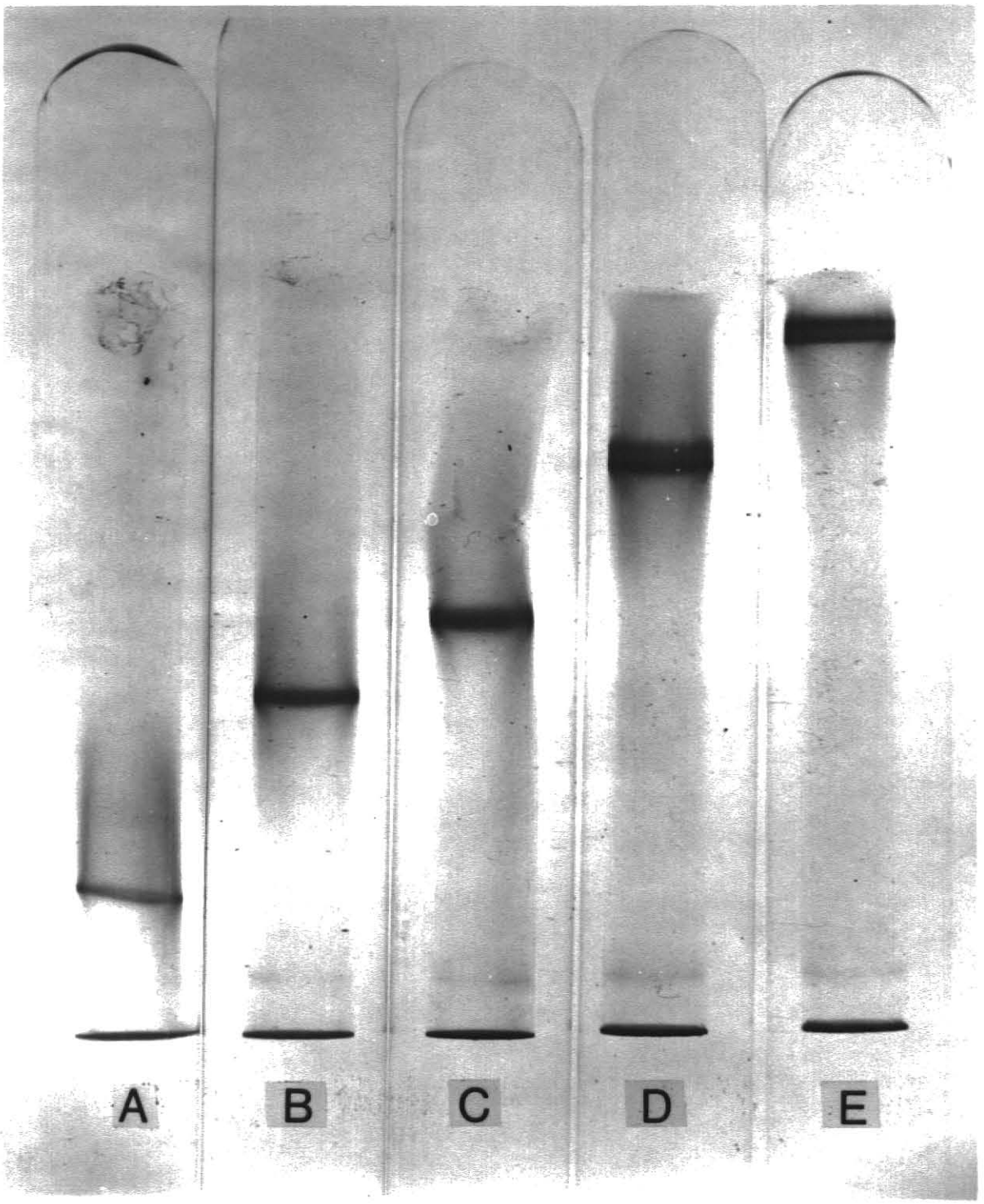
24 hours in 11 M urea, it is quite possible to assume that the net charge on each subunit at this point in the unfolding process is the same. However after continued exposure to 11 M urea, the unfolding of the globular head region of the subunits proceeds to a point where the charges are no longer masked. The subtle difference in charge between the two subunits is then revealed in their electrophoretic separation after 48 hours in 11 M urea.

As demonstrated in Figure 7, the difference in intensity of staining of each band resulted in peaks of unequal sizes. One would expect the peaks to be of equal size, since each myosin molecule dissociates into two subunits. However, if the number of binding sites for the stain differs in each subunit, the size of each peak should similarly differ as demonstrated in Figure 7.

Studies were also performed (myosin stored in 11 M urea for 48 hours) to demonstrate the emergence of the two bands as a function of electrophoresis time during one experiment. The results are shown in Figure 8. Two bands became clearly resolved after a minimum of 90 minutes of electrophoresis time. It is apparent from these results that the difference between the migration rate of each band was very small. Since the molecular weight of the subunits has been established as being identical or very similar (Kielley and Harrington, 1960; Small et al., 1961; Young et al., 1962; Woods et al., 1963), the inherent differences in subunit composition must also be very subtle.

After conditions necessary for the separation of the leading band were established, the need to establish their identity

Fig. 8. Separation of myosin subunits as a function of electrophoresis time. Gel concentration was 2.0% (w/v) in 11 M urea. Electrophoresis times were: Gel A, 45 min.; Gel B, 60 min.; Gel C, 75 min.; Gel D, 90 min.; Gel E, 105 min.


A
B
C
D
E

beyond any reasonable doubt became increasingly important. One source of doubt concerning the identity of the two bands arose from the nature of the LMP associated to the head regions of the myosin molecule. Studies at various laboratories (Locker and Hagyard, 1967a,b; Frederiksen and Holtzer, 1968; Gaetjens et al., 1968; Gershman and Dreizen, 1970) have shown that there are at least two species of LMP, one with a molecular weight of approximately 20,000 and the other of approximately 32,000. It then became increasingly important that the LMP be demonstrated as having migrated out of the gels ahead of the two closely associated bands, the two bands be shown to have not arisen by modification at specific group sites within the protein, and the approximate molecular weight of the two bands be established.

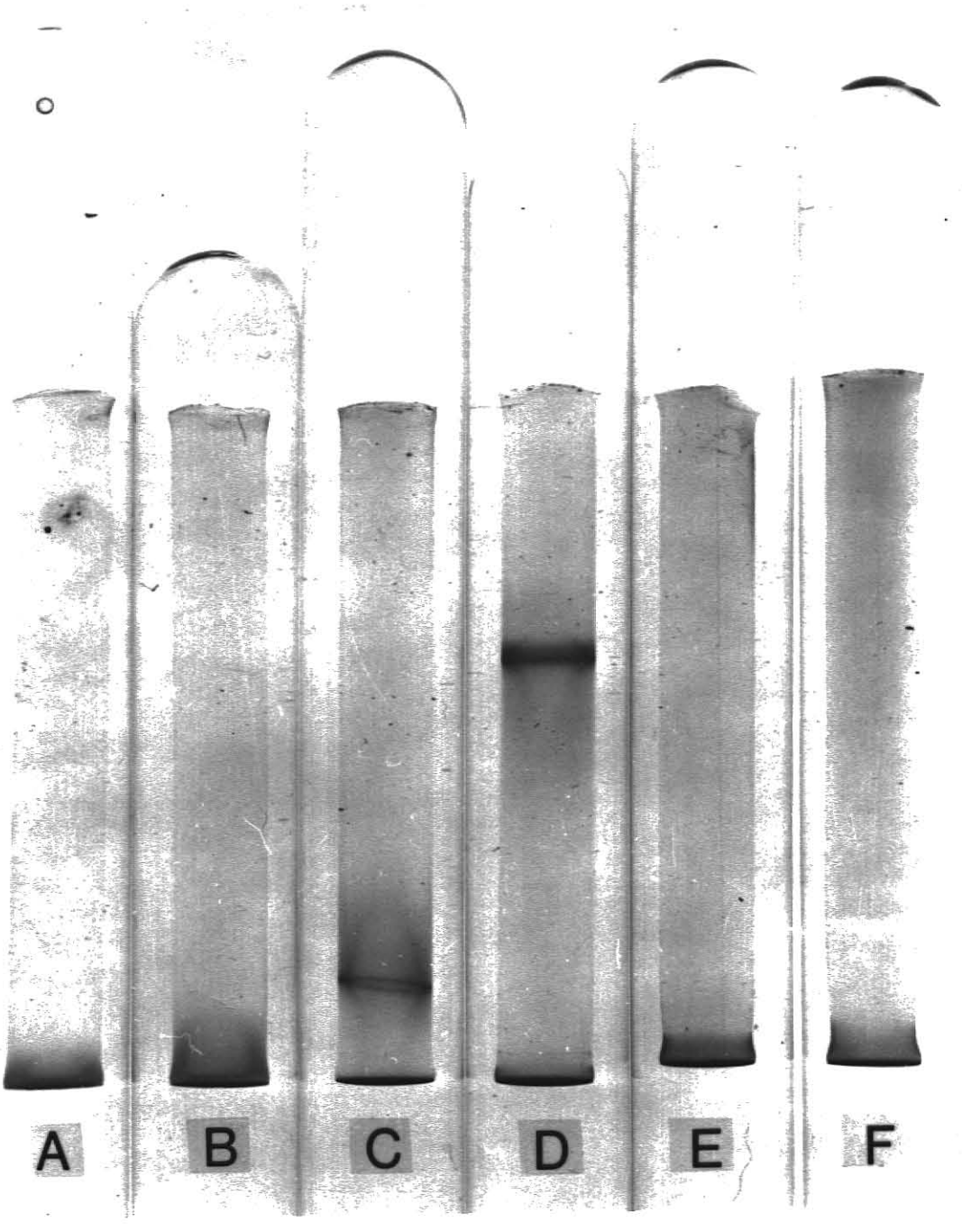
Demonstration of Low Molecular Weight Protein. Several different experiments were performed to demonstrate the presence or absence of LMP. One series of experiments involved the use of higher concentration gels (Table 2). Experiments employing 2.5% gels resulted in the same subtle separation in the leading band. However, experiments employing 3.0 and 4.0% gels resulted in the migration of a heterogeneous single band. The appearance of the band reflected its apparent composition of more than one molecular species. It therefore seemed that separation of the two subunits was only accomplished in pore sizes which did not minimize or lessen the migration differences, apparently only a function of a subtle difference in charge and not molecular size.

The most revealing results were obtained with experiments

employing 4.0% acrylamide gels. In these experiments each gel tube was stopped at timed intervals in an attempt to reveal the actual composition of protein species in the myosin solution. The results of a typical experiment are shown in Figure 9 with electrophoresis times ranging from 15 minutes to 3.5 hours. The 15 minute gel (A) showed a small amount of stained material just beginning to enter the gel. After 30 minutes (B) the stained material still appeared to be very diffuse and had migrated slightly further into the gel. However after 45 minutes (C), a single band appeared and was preceded by the diffuse area of staining. After 90 minutes (D) the band, now appearing as a heterogeneous band, had migrated further toward the bottom of the gel. However the diffuse area of staining had now migrated well ahead of the band and out of the gel. After 150 minutes (E) and 210 minutes (F) of electrophoresis time, no additional material was observed to enter the gel. A small amount of staining was observed at the top of the 150 and 210 minute gels but was believed to be due to the eventual partial collapse of the gels at the top and the subsequent "spilling over" effect of the insoluble stained material at the top of the gel onto the sides of the upper wall of the gel.

It seems apparent that there was some protein material which preceded the only visible band. Since myosin is known to be completely dissociated to its subunits in urea solutions of at least 10 M, the major molecular species in solution must be the monomeric subunit of myosin. The one heterogeneous band apparently represents

Fig. 9. Demonstration of total molecular species present in 11 M urea solutions of myosin. Gel concentrations were 4.0%. Electrophoresis times were: Gel A, 15 min.; Gel B, 30 min.; Gel C, 45 min.; Gel D, 90 min.; Gel E, 150 min.; Gel F, 210 min.


A
B
C
D
E
F

the major molecular species in solution which was preceded by a very diffuse area of protein. It would appear that this band probably constitutes the monomeric subunit of myosin preceded by the only other product of dissociation, the low molecular weight protein. The very diffuse nature of the LMP in the gel is probably representative of the large pore size of the gel (4%) offering little or no resistance to the diffusion of a protein of such small dimensions as compared to a protein approximately ten times larger. The diffusion of the LMP is also probably due to the 45-50°C temperature maintained through the destaining process. The fact that the heterogeneous band represents the only major molecular species is supported by the lack of detection of any other protein from electrophoresis as long as 2½ to 3½ hours.

Modification of Myosin from Carbamylation. It has long been known that ammonium cyanate is a product of urea decomposition and exists in equilibrium with it (Warner, 1942; Dirnhuber and Schütz, 1948; Stark et al., 1960). It has been demonstrated that an 8 M urea solution with a pH greater than 6 has a cyanate concentration of 0.02 M at equilibrium (Stark et al., 1960). Since ammonium cyanate is known to selectively carbamylate amino acid residues containing amino and sulfhydryl groups in alkaline or neutral pH's, it became important to show whether the two bands were a result of partial modification of the myosin molecule.

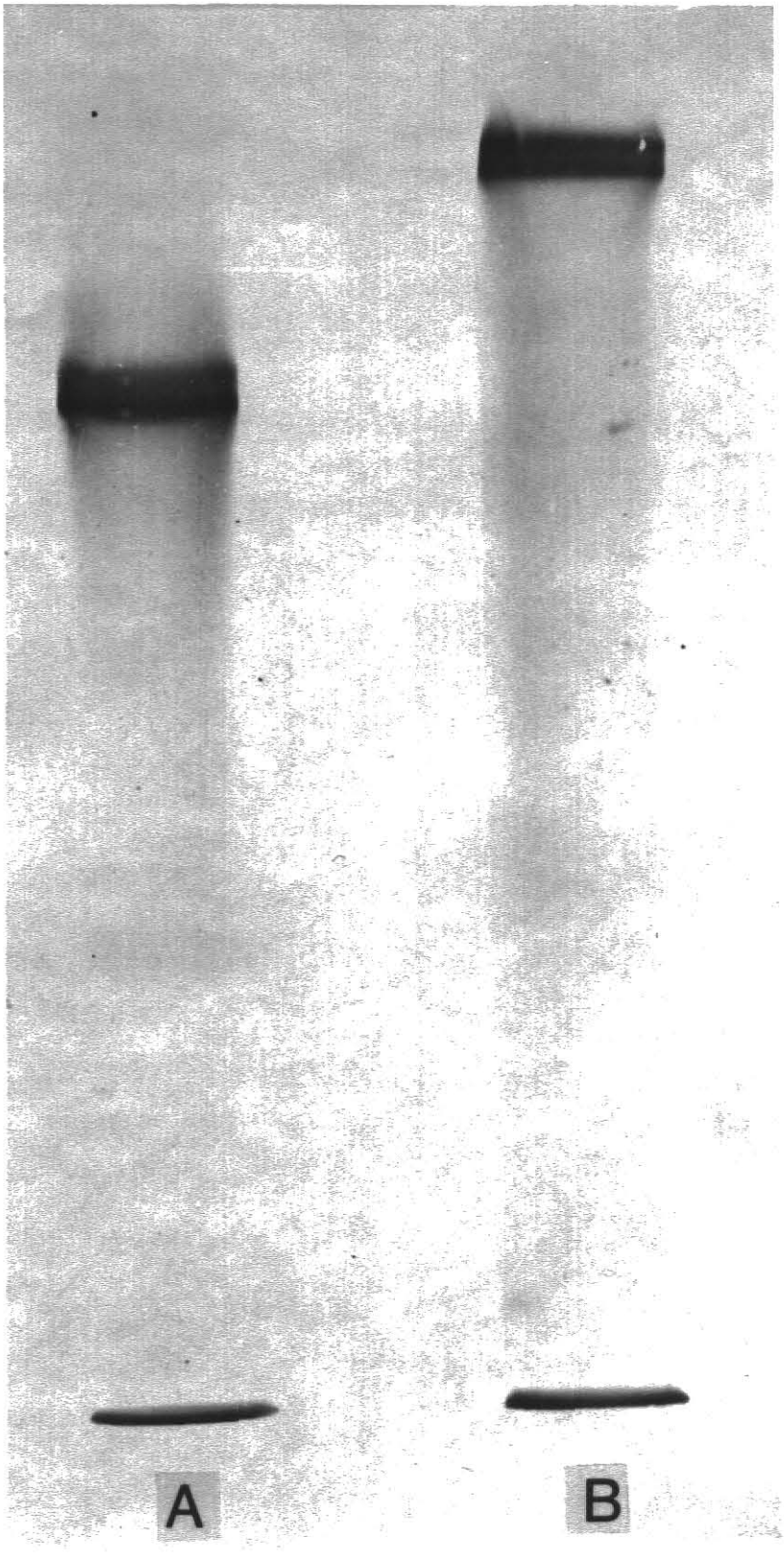
Experiments designed to measure the concentration of cyanate in urea solutions (Werner, 1923) were performed. Results of these experiments showed that the concentration of cyanate

in a myosin - 11 M urea solution at pH 2 after 24 hours of equilibration was less than 1.0 mM. Similar assays showed that the concentrations of cyanate in 11 M urea which had been equilibrated for several days at 40°C and in freshly prepared 11 M urea were 11.6 mM and less than 1.0 mM, respectively.

Since acidification of a urea solution will decompose any cyanate, an experiment involving the equilibration of myosin in 11 M urea at 40°C and pH 2 for at least 48 hours and subsequent electrophoresis was designed. Results of this experiment are shown in Figure 10. It is apparent that the two closely related bands were again visible as the major and only molecular species present. The results presented in Figure 6 indicate that a separation of the subunits does not occur within 24 hours of equilibration in 11 M urea. Therefore if the two bands were due to the modification of myosin at a neutral or alkaline pH, these bands would not have been resolvable within 24 hours after raising the lowered pH by dialysis against 11 M urea at 40°C. Similarly, if the two closely related bands were due to carbamylation of myosin while being stored for several days in 6 M urea, then they probably would have been observed after 24 hours in 11 M urea (the time required for the initial appearance of the monomeric subunit).

If the concentration of cyanate were high enough and the time of its exposure to myosin were long enough, then a complete reaction of cyanate with two identical subunits, assuming equal exposure of all reactive groups, would have resulted in only one band. Similarly, a complete reaction with two non-identical

Fig. 10. Disc electrophoresis of myosin in 11 M urea at pH 2 for 48 hours (40°C). Gel concentrations were 2.0%. Electrophoresis times were: Gel A, 90 min.; Gel B, 105 min.


A
B

subunits would have resulted, in all probability, in two bands. However an incomplete reaction would not necessarily have resulted in only two bands. One would expect that, in this case, many bands indicative of the randomness of incomplete modification would likely have occurred.

Since myosin is known to have several amino and sulfhydryl groups in the helical tail region (Bendall, 1969), the chance of a randomness of modification occurring from an incomplete reaction (resulting in several bands) would seem to greatly outweigh the chance of a partial modification at only one site (resulting in 2 bands). Myosin is also known to contain an abundance of oxidizable groups exposed to its environment in the helical tail region (Bendall, 1969). Modification due to oxidation at 40°C must also be considered as a possible explanation for the emergence of the two bands after 48 hours in 11 M urea. Since studies in 11 M urea did not indicate any change in the staining intensity of each of the two bands with time (Fig. 6), oxidation at only one site (resulting in 2 bands) is highly improbable.

On the weight of the results and the rationale presented in the previous discussion, it would appear that the emergence of 2 bands is due to physical rather than chemical changes.

Molecular Weight Studies in Sodium Dodecyl Sulfate. Further characterization of the two close-running bands was obtained with the establishment of an approximate molecular weight. The usefulness of sodium dodecyl sulfate in molecular weight determinations from acrylamide gel electrophoresis has been repeatedly

demonstrated (Shapiro et al., 1967; Dunker and Rueckerts, 1969; Paterson and Strohman, 1970; Reynolds and Tanford, 1970a). The advantages of employing this method over other methods are the simple and inexpensive equipment needed, the small quantities of sample required, the speed of the method, and the direct relationship of this method with those previously used in myosin subunit studies.

The nature of the binding of SDS to proteins has been extensively investigated (Pitt-Rivers and Impiombato, 1968; Fish et al., 1970; Reynolds and Tanford, 1970b). It has been shown that all proteins bind 1.4 grams of SDS per gram of protein when the SDS monomer concentration exceeds 8.0×10^{-4} M (Reynolds and Tanford, 1970a). Due to the nature and ratio of SDS binding and the constant charge per unit mass afforded by the highly charged SDS monomer, it has been possible to separate proteins with acrylamide gel electrophoresis on the basis of molecular weight alone.

Initial experiments on myosin alone employed the use of 2-mercaptoethanol as a sulfhydryl reducing agent. Results of these experiments were quite unsatisfactory due to the formation of a series of irregularly spaced multiple bands of unknown origin. Due to the structural nature of one of the reference proteins chosen for use as well as the unsuccessful preliminary SDS experiments, the use of a sulfhydryl reducing agent was discontinued.

Preliminary experiments, to establish the optimal conditions

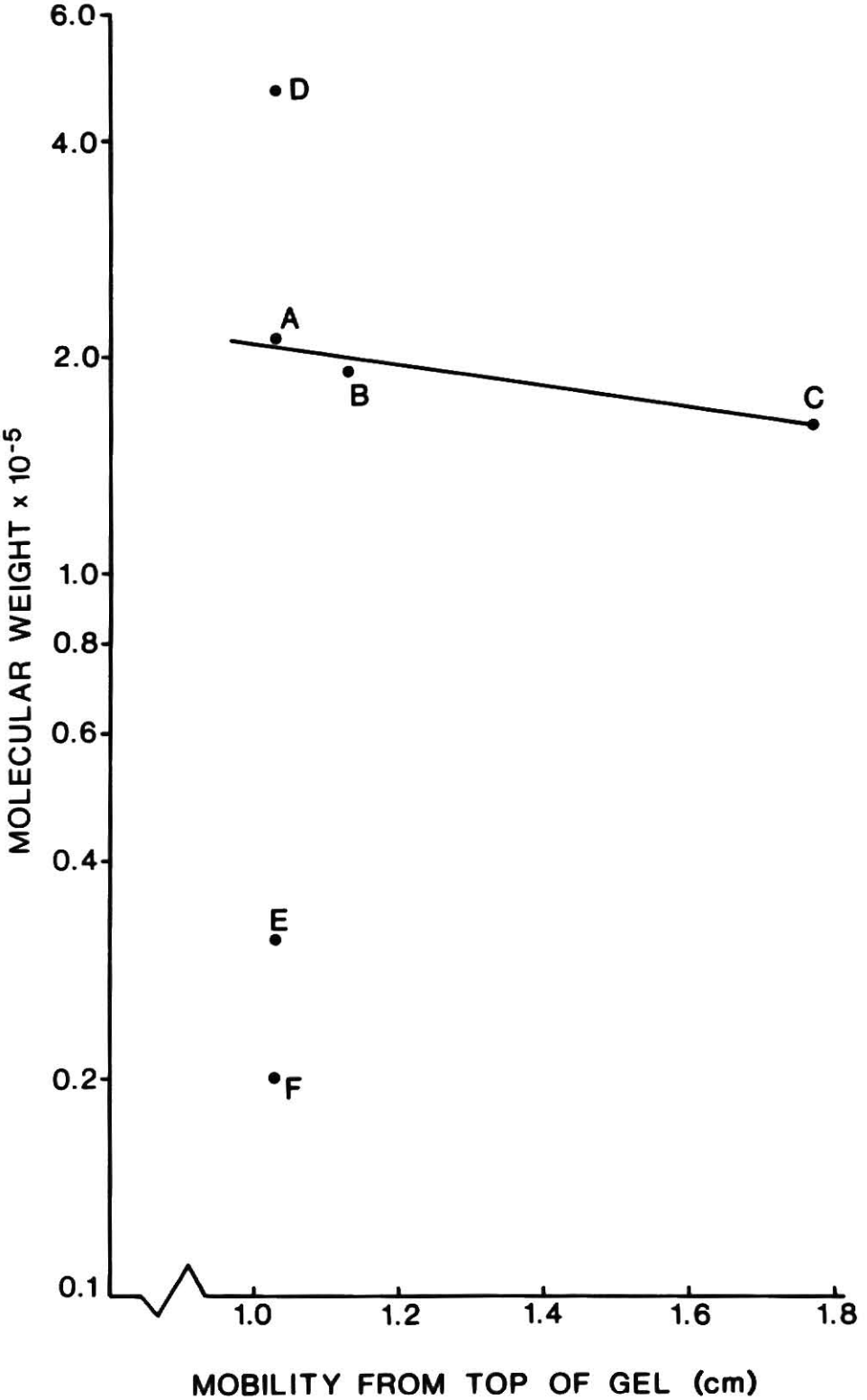
for molecular weight studies, demonstrated that the best resolution was obtained with 3.0% (w/v) acrylamide gels using 100 μ liters of sample and a total electrophoresis time of 15 minutes at 2 ma per gel.

Since very few proteins of an approximate molecular weight of 200,000, suitable for these studies, were available, only two reference proteins were used. One of the reference proteins was 7S gamma globulin of a reported molecular weight of approximately 160,000 (Dunker and Rueckerts, 1969). The other reference protein was Component-I with a reported molecular weight of approximately 190,000 (DeGuzman and Clegg, 1968).

Electrophoresis resulted in the migration of 7S gamma globulin well ahead of Component-I and myosin. Component-I was observed to migrate slightly ahead of myosin. Due to the presence of SDS in the gels during staining, a large amount of background staining occurred and persisted during destaining. Measurements of absolute migrations from the tops of the gels were obtained. The results of a plot of the logarithm of the molecular weight vs. the absolute mobility is presented in Figure 11.

Molecular weight determinations from electrophoresis on acrylamide gels in SDS have been reported to be accurate from at least 10% (Weber and Osborn, 1969) to as low as 2 to 5% (Shapiro and Maizel, 1969) of the actual value. Although it would have been desirable to employ more than two reference proteins for a more accurate standard curve, the results clearly indicate that the molecular weight of the major molecular

Fig. 11. Determination of the molecular weight of the major molecular species of myosin. Experimentally measured molecular weight of myosin monomer, A; migration of reference proteins: Component-I, B; 7S gamma globulin, C; theoretical position of band from myosin preparation if it were undissociated myosin, D; LMP (32,000), E; or LMP (20,000), F.


6.0
4.0
2.0
1.0
0.8
0.6
0.4
0.2
0.1
D
A
B
C
E
F
MOLECULAR WEIGHT x 10-5
1.0
1.2
1.4
1.6
1.8
MOBILITY FROM TOP OF GEL (cm)

species present in the myosin preparation is much closer to the 212,000 molecular weight of the monomeric subunit of myosin than to either the 468,000 value for the entire molecule or to the 32,000 or 20,000 values for LMP. As illustrated in Figure 11, the band representing the major molecular species would have been quite unrelated to those of Component-I or 7S gamma globulin if it were assigned any molecular weight value other than 212,000. The slight deviation from linearity is probably due to either the lack of a greater number of reference proteins available or the absence of a reference marker to establish and standardize relative migrations.

Since the electrophoretic migration of proteins in SDS is a function of their hydrodynamic properties, it would appear that the presence of intramolecular disulfide bonds, altering the effective molecular length and the amount of SDS bound would adversely affect the migration. The intramolecular disulfide bonds in 7S gamma globulin have been described as being numerous and important in maintaining a structure with a molecular weight of approximately 160,000 (Neurath, 1965). Component-I has been reported to contain at least 30 cysteine residues (DeGuzman and Clegg, 1968), all or part of which could probably be easily oxidized to disulfides. Thus Component-I is capable of structural changes due to disulfide bridge formation and resulting alterations in hydrodynamic properties. It would consequently appear necessary to use a reducing agent for molecular weight determinations in SDS.

The reduction of disulfide bonds in 7S gamma globulin would have resulted in the decrease of the average molecular weight from 160,000 to approximately 50,000 (Neurath, 1965). Successful molecular weight determinations of 7S gamma globulin and other proteins from acrylamide gel electrophoresis using SDS has been demonstrated without the use of reducing agents (Dunker and Rueckerts, 1969). Due to the scarcity of suitable reference proteins, the lack of success in using reducing agents in electrophoretic systems as encountered earlier in this research, and the apparent successes of Dunker and Rueckerts, the omission of sulfhydryl reducing agents was felt to be justified.

SUMMARY

The first part of this research employed a method for the isolation of myosin in a highly purified state from the adductor magnus of the rabbit. As demonstrated by high ATPase activities, very low AMP deaminase activities, and high A_{280nm}:A_{260nm} ratios, the method proved to be a very simple and gentle chromatographic procedure for the isolation and purification of myosin. The results of the radioisotopic assay procedure employed for the determination of ATPase activities indicated that this procedure was at least as rapid and probably more convenient and sensitive than the titrimetric (Byrnes and Suelter, 1965) or molybdo-vanado phosphate (Lecocq and Inesi, 1966) methods employed by Harris and Suelter (1967). Protein concentration profiles from cellulose phosphate chromatography consistently demonstrated a trailing shoulder. Since increases in ATPase activities accompanied the shoulder, it appeared the shoulder was probably due to the presence of a small amount of actomyosin or aggregated myosin.

Isoelectric focusing of myosin in 3 M urea resulted in the formation of a fibrous band near the middle of the column. A scan for protein while draining the contents of the column through a flow cell showed the column to be void of soluble protein. Measurement of pH on fractions collected from the column demonstrated that the fibrous band occurred at a volume corresponding to the approximate pI of myosin.

Initial disc electrophoresis experiments on myosin at high salt concentrations or in 6 M urea resulted in the appearance of three bands of low molecular weight material followed by several bands of aggregated myosin. The three bands of low molecular weight material were assigned as two low molecular weight protein (LMP) species and probably a small amount of contaminating actin (Paterson and Strohman, 1970). In all experiments a fibrous band at the interface of the sample layer and upper tray buffer, similar in appearance to that seen during isoelectric focusing, was observed to appear immediately after the onset of electrophoresis. Since this band was observed to migrate to and remain at the top of the gel, it appeared to be a high molecular weight aggregate of myosin.

Electrophoresis of myosin in 12 M urea was unsuccessful whereas electrophoresis immediately after dissolution in 11 M urea (40°C) resulted in the previously described multiple bands without any apparent detection of weaker bands representing LMP. Similar experiments performed on myosin equilibrated in 11 M urea for several days resulted in the appearance of only two closely spaced bands.

Time studies of myosin in 11 M urea resulted in the appearance of the two bands after a minimum of 24 hours in 11 M urea at 40°C. Due to the emergence of a strong band after 24 hours and its resolution into two distinct bands after 48 hours, a mechanism for the dissociation of myosin into two distinct subunits was proposed. It would appear that an initial dissociation occurs

within the tail region of intact myosin and results in two subunits which are initially indistinguishable by electrophoresis. However prolonged exposure to 11 M urea at 40°C results in further unfolding in the head regions of the individual subunits and subsequently a number of previously masked charges are exposed.

Disc electrophoresis of myosin in 4.0% acrylamide gels and 11 M urea after several days demonstrated only one distinct band preceded by a fast migrating, diffuse area of protein. No protein was observed after as long as 3.5 hours of electrophoresis time. It is therefore likely that the single band represented unresolved myosin subunits and the rapidly migrating, diffuse area of protein was LMP.

Assays for ammonium cyanate in 11 M urea solutions (equilibrated at 40°C for several days) showed that 11.6 mM ammonium cyanate was present. However, freshly prepared urea solutions and urea solutions stored at 40°C for 24 hours at pH 2 resulted in a cyanate concentration of less than 1 mM. Electrophoresis of myosin solutions stored in 11 M urea at 40°C for 48 hours at pH 2 resulted in the same two bands. It is therefore highly probable that the two bands are not due to chemical modification by carbamylation.

Experiments to determine the molecular weight of the major molecular species (the two bands) were performed using sodium dodecyl sulfate and polyacrylamide gel electrophoresis. Results established that the molecular weight of the major molecular species was in the vicinity of 200,000 and not 468,000, 32,000,

or 20,000.

The research presented in this thesis has conclusively demonstrated the separation of the large chains of myosin into two electrophoretically distinguishable subunits.

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ELECTROPHORETIC STUDIES ON MYOSIN

by

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ABSTRACT

Myosin was isolated from the adductor magnus muscle of rabbits by the method of Harris and Suelter (Biochim. Biophys. Acta 133, 393 (1967)). The method removed two common contaminants, AMP deaminase and nucleic acids, and yielded preparations of high ATPase activity (myosin ATPase determined with the use of ATP-γ-^{32}P).

Isoelectric focusing of myosin in 3 M urea resulted in a fibrous band that precipitated at pH 5.3, very close to the reported pI of myosin. Monitoring of the column contents indicated the absence of soluble proteins.

Disc electrophoresis of myosin at high ionic strength or in 6 M urea resulted in the formation of a series of bands determined to be the aggregated species of myosin. These bands were preceded by three rapid migrating bands consisting of either two bands of low molecular weight protein (LMP) and one band of contaminating actin or three bands of LMP.

Due to technical difficulties, disc electrophoresis of myosin in 12 M urea (45°C) was unsuccessful. However, disc electrophoresis of myosin and subsequent staining with Coomassie brilliant blue immediately after dissolution in 11 M urea (40°C) resulted in a series of diffuse bands, none of which were attributable to LMP. Myosin equilibrated in 11 M urea (40°C) for several days resulted in only two closely spaced but distinctly

resolved bands.

Time studies of myosin in 11 M urea (40°C) and subsequent disc electrophoresis indicated that the diffuse bands seen initially (0 hr.) transformed into a single prominent band and barely discernible, slower migrating diffuse bands at 24 hours. Treatment of myosin in 11 M urea (40°C) for 48 hours revealed that the single prominent band had dissociated into two close-running bands, with additional time (72, 96 and 168 hours) increasing the resolution of the two bands. On the basis of these results a mechanism was proposed for the dissociation of myosin into electrophoretically unique subunits.

Disc electrophoresis of myosin in 4% acrylamide gels (electrophoresis times ranging from 15 minutes to 3.5 hours) showed the migration of a single sharp band behind which no protein material was observed but preceded by diffuse staining regions during short time runs. These results indicated that the LMP was unable to be observed as sharp bands, probably due to high rates of diffusion, and that the one sharp band was apparently the only major molecular species present.

Urea (11 M) equilibrated at 40°C for several days contained 11.6 mM cyanate which decreased to less than 1 mM cyanate when the pH of the urea (11 M) was initially adjusted to pH 2 and stored for several days at 40°C. Disc electrophoresis of myosin in 11 M urea at pH 2 (40°C) for 48 hours resulted in the appearance of only two close-running bands. These results suggested that the two bands were not a result of chemical modification

during urea equilibration.

Molecular weight estimates of the major molecular species in the myosin preparation was obtained from SDS-polyacrylamide electrophoresis using reference proteins of 160,000 and 190,000 MW.