SEPARATION AND PURIFICATION OF MYOSIN SUBUNITS

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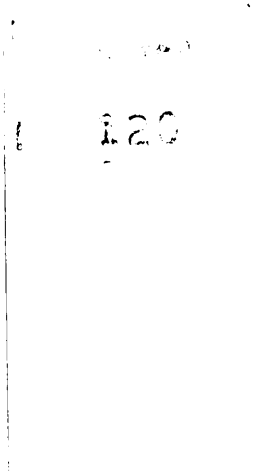

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ABSTRACT

SEPARATION AND PURIFICATION OF

MYOSIN SUBUNITS

by Peter John Bechtel

Myosin is involved in ATPase activity, actin binding and as a contractile component of muscle. The myosin molecule is currently believed to consist of two large subunits with an approximate molecular weight of 210,000 and two or three small subunits having a molecular weight in the range of 20,000-30,000. The nature of the small subunits has been extensively investigated due to the ease of extraction and the small molecular size. Few studies have dealt with the large subunits because of extensive aggregation and other problems involved in handling large molecular weight proteins.

The present study was undertaken to investigate the heterogeneity of myosin subunits with electrophoretic techniques. Myosin small subunits exhibited three bands on a 7% acrylamide disc gel electrophoretic system. The three bands were common to all preparations and were not altered by alkylation with different agents. Myosin large subunits did not migrate in this electrophoretic system.

Electrophoresis of myosin small subunits was observed on a 3.5% acrylamide matrix with sodium dodecyl sulfate (SDS) buffer. The small subunits migrated as three bands, which indicated different molecular weights for the three components. In this electrophoretic system, the large subunits of myosin migrated as one band. Thus, results indicate that the two large subunits have similar molecular weights.

Electrophoresis of myosin large subunits on a 3.5% acrylamide gel and in a 12 M urea buffer indicated that the large subunits migrated as one zone. However, this system yielded poor gels. Since heterogeneity was not observed, it was concluded the two large subunits had similar charges.

The second phase of this study involved the isoelectric focusing of myosin and its subunits on 3.5% acrylamide gel in 12 M urea solvent. The large subunits focused as one or two bands, however, the composition of the bands was not determined. The large subunits had isoelectric points between pH 6.4 and 7.2. Small subunits focused as three or more bands having isoelectric points between pH 4.9 and 5.5. When whole myosin was isoelectrofocused, it dissociated into both large and small subunits, which focused at their respective isoelectric points.

In the third phase of this study, the amino acid composition and end group analysis of myosin and its subunits were investigated. The amino acid composition of myosin from rabbit back muscle was similar to that of myosin from other muscles of the rabbit. The large subunits had an amino acid composition similar to that of whole myosin. The amino acid composition of myosin small subunits was different from that of either whole myosin or the large subunits, having a high phenylalanine to tyrosine ratio (3:1) and a large proline content.

Hydrazinolysis, C-terminal analysis of myosin and its large subunits resulted in the release of submolar quantities of several amino acids. Although several free amino acids were detected, lack of duplication precluded any conclusions on the composition of the terminal end group of

myosin. C-terminal analysis of myosin large subunits using carboxypeptidase A and B did not consistantly yield one or two common end groups. However, carboxypeptidase A and B analysis of whole myosin did yield two moles of isoleucine per mole of myosin. Since isoleucine is the carboxy terminus of myosin small subunits, it was concluded that myosin contains two small subunits per molecule.

The final phase of this study dealt with purification of myosin subunits. Large subunits separated by column chromatography on Sephadex G-100 utilizing either an 8 M urea or 5 M LiCl buffer did not yield a totally pure large subunit preparation. However, chromatography on a Sephadex G-100 column using a 5 M guanidine-HCl solvent resulted in purified large subunits free from small subunit contamination.

Myosin small subunits were column chromatographed on Sephadex DEAE A-25 with a KCl gradient. Two peaks resulted, one containing two subunits and the second containing a third subunit. The two subunits found in the first peak appeared to be tightly bound to the large subunits upon pH fractionation of large and small subunits.

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By

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INTRODUCTION

The contractile proteins are involved in muscle movement and structure. Since they also compose a major portion of muscle, therefore, they make a significant contribution to the protein content of meat. This group of proteins is of interest to the food scientist for its nutritional value and as a component of many composite food products.

Myosin is the major constituent of the contractile protein complex, being involved in the contractile process and structural unity of the individual myofibril. Myosin has been known to exist for many years, but due to preparation contamination, protein aggregation and lack of methodology for handling high molecular weight proteins, its physical parameters have not been well delineated. Recent methodology advances in protein separatory techniques have provided new techniques for studying large proteins such as myosin.

The current model of myosin consists of two large subunits and two or three small subunits (Dreizen and Gershman, 1970). Both large and small subunits are required for enzymatic activity; however exact stoichiometric relationships are not totally defined. The number of small subunits is thought to be two, however conflicting reports exist in regard to their amino acid composition and molecular weight. Myosin large subunits have not been separated from each other.

Most studies have dealt with the enzymatically active peptide fragment of the large subunit.

The present investigation was undertaken to isolate, identify and partially characterize large and small subunits from myosin. Also, this investigation examined the heterogeneity of the large subunits of myosin.

REVIEW OF LITERATURE

Structure of Myosin in Muscle

Striated muscle is composed of many small bundles of myofibrils. Individual myofibrils consist of thick and thin filaments surrounded by sarcoplasmic fluid (Huxley, 1960). Thick filaments are composed of myosin, and the thin filaments of actin, tropomyosin and the troponins (Ebashi et al., 1969).

Thick and thin filaments, and their overlap result in the I, A and H bands and the M line of the muscle sarcomere. Huxley (1960) showed the I band consists of thin filaments and the H band consists of thick filaments. The A band is composed of interdigitated thick and thin filaments. Thin filaments are hexagonally arranged around the thick filaments, thus forming a geometric periodicity.

According to Huxley (1960) thick filaments consist only of myosin, with the possible exception of a protein located at the M line (Pepe, 1967a). They are approximately 1.6 μm long and 12.0 nm wide. Along the length of the myosin filament two cross bridges rotate every 120 degrees with respect to one another, thus, forming a 6/2 helix, which makes one turn every 42.9 nm along the thick filaments (Huxley and Brown, 1967).

The thick filaments are divided into equal halves by the M line; each half containing myosin molecules of similar polarity but the two halves having opposite polarity (Huxley, 1963). Huxley

(1963) has observed the two halves of thick filaments are joined together by interaction of the tails of myosin molecules, resulting in the H band. The H band is bisected by the M line, which is the location of "M substance" (Masaki, 1968; Maruyama and Ebashi, 1970; Eaton and Pepe, 1971). Although the role of "M substance" is not completely known, Pepe (1967a) believes it is involved in thick filament formation and/or in the lateral association of myosin aggregates. Thick filament formation has been observed in vitro using pure myosin and the appropriate ionic and pH conditions (Huxley, 1963; Josephs and Harrington, 1966; Kaminer and Bell, 1966).

Dreizen and Gershman (1970a) using the biochemical analytical procedures of Perry et al. (1965) and certain theoretical considerations have shown the stoichiometric relationships of actin and myosin in muscle. They concluded that each myosin cross bridge consists of one myosin molecule. Bendall (1969) using the data from Perry et al. (1965) calculated that actin and myosin are present in muscle in a ratio of four to one. When he made calculations using known lengths and numbers of molecules in the thick and thin filaments, however, he obtained a ratio of 8.7 actin molecules to one myosin molecule. The 8.7 to 1 ratio of actin to myosin is equivalent to 54% actin in the total content of myofibrillar proteins, which is incongruous with the 50% myosin figure calculated

by Perry et al. (1965) and by Huxley (1960). However, Bendall (1969) noted that if each cross bridge contains not one, but two myosin molecules the dilemma would be resolved. Although this postulation resolves the conflict with the calculations of Bendall (1969), it still does not agree with those presented by Dreizen and Gershman (1970a). Bendall (1969) has pointed out that two myosin molecules per cross link results in a clumsy structure and that the concept should be reinvestigated.

Enzymatic Properties of Myosin

Contraction of muscle involves the conversion of chemical energy to mechanical work. The chemical energy is delivered in the form of ATP, which is enzymatically hydrolyzed by myosin and results in changing the state of the actomyosin complex (Engelhardt and Ljubimova, 1939). At high ionic strengths ATP causes the actomyosin complex to dissociate (Gergely, 1956), but at lower ionic strengths, the complex can either clear or superprecipitate (Spicer, 1952; Maruyama and Gergely, 1962).

Myosin reacts with nucleotide triphosphates according to the following simplified reaction (Bendall, 1969):

$$M + NTP \rightleftharpoons M - NTP \xrightarrow{H_2O} M \begin{matrix} \nearrow NDP \\ \searrow Pi \end{matrix} \rightleftharpoons M + NDP + Pi \quad (1)$$

When:

M = myosin

NTP = nucleotide triphosphate

NDP = nucleotide diphosphate

Pi = inorganic phosphate

Although this reaction is inhibited by its products, Alberty (1968) showed that equilibrium favored hydrolysis.

Stoichiometric studies of ATPase activity per myosin molecule have led to conflicting experimental results. Work by Nanninga and Mommaerts (1960) resulted in slightly more than one mole of ATP bound per mole of myosin. Schliselfeld and Barany (1968) have shown molar quantities of myosin and heavy meromyosin bind 1.7 and 1.6 moles of ATP, respectively. Other studies by Kiely and Martonosi (1969) have demonstrated that ADP binds myosin in a ratio of 1.2-1.6. Nauss et al. (1969) found pyrophosphate was bound at 1.82 moles per mole (5×10^5 g) of myosin, 1.83 moles per mole (3.6×10^5 g) of heavy meromyosin and 0.85 moles per mole (10^5 g) subfragment-1. Natural actomyosin bound 1.0 mole of pyrophosphate per mole (5×10^5 g) of myosin.

Eisenberg and Moos (1970) using a kinetic analysis showed myosin and heavy meromyosin contained approximately two ATP binding sites per molecule, while subfragment-1 contained only one binding site. This study was in general agreement with values reported by

Murphy and Morales (1970), Morita and Shimizu (1969) and Lowey and Luck (1969). Further support for the kinetic analysis of Eisenberg and Moos (1970) was found in the ATP binding ratios of myosin and heavy meromyosin to subfragment-1 by Hayashi and Tonomura (1970).

Myosin ATPase activity is believed to be associated with the head region of the myosin molecule. Frederiksen and Holtzer (1968) first demonstrated the dependence of myosin ATPase on the presence of light- and heavy-myosin subunits, which are known to be located in the myosin head. They separated the subunits by adjusting the pH to 10.5-11.0 and found the separated subunits exhibited no ATPase activity. When the pH was adjusted to 7.0 and the subunits were recombined, 50 percent of the ATPase activity was restored. Gershman et al. (1969) also demonstrated that myosin ATPase activity was dependent on the interaction of light- and heavy-myosin chains. They separated the subunits by titrating myosin to pH 11.0. They then precipitated the large subunits with potassium citrate, which left the light subunits in solution. The purified heavy and light subunits did not have ATPase activity, but the recombination of subunits resulted in restoration of about 15 percent of the initial activity.

Dreizen and Gershman (1970a) criticized the work of Frederiksen and Holtzer (1968) for the high recombined subunit ATPase activity and for failing to protect ATPase during pH adjustment.

Gaetjens et al. (1968), using an unprotected ATPase system with pH 11.2 dissociating conditions, concluded that the loss of small subunits resulted in termination of ATPase activity and of actin binding. However, reconstitution of small and large myosin subunits did not restore ATPase activity.

Dreizen and Gershman (1970b) found 1.6 ADP binding sites per myosin molecule. They also observed that the number of ADP binding sites decreased directly with the ratio of light to heavy chains. In recombination experiments, they also noted that Ca^{++} --ATPase was progressively lost as the ratio of light- to heavy-chains decreased. From these experiments, they postulated that ATPase activity and ADP binding involve interactions with both light- and heavy-chains, and that bound ADP may bridge light- and heavy-chains within each myosin protomer.

There are two general classes of thiol groups in myosin, whose blocking affects ATPase activity. Kielley and Bradley (1956) found addition of sulfhydryl agents, such as N-ethylmaleimide and p-mercuribenzoate, to the S_1 thiol groups stimulated Ca^{++} induced ATPase and inhibited K^+ EDTA induced ATPase. When the S_2 thiol groups were reacted, all ATPase activity was lost. Trotta et al. (1968) reported peptide fragments containing large portions of S_1 and S_2 thiol groups were released during tryptic digestion of heavy meromyosin and its subfragment-1. ATPase activity was not affected by the loss of these fragments.

Seidel et al. (1970a) labeled myosin thiol groups with nitroxide free radicals for electron spin resonance studies. After tryptic digestion of subfragment-1, the labeled thiol groups were not released. These results plus others (Seidel et al., 1971) did not support the conclusions of Trotta et al. (1968). Seidel et al. (1970a) then postulated that myosin had two equivalent heavy chains, each containing one active ATP site and one S_1 group. Later Seidel et al. (1970b) concluded that myosin contains two S_2 thiol groups per molecule and that the reactivity differs for S_1 and S_2 thiol groups.

The exact sequence of events whereby myosin hydrolyzes ATP and binds to actin is not fully understood. Taylor et al. (1970) utilizing a kinetic analysis of myosin products concluded that the rate limiting step of ATP hydrolysis is product dissociation. Also, their analysis implied the ATPase sites on each protomer were interacting in some manner. Tonomura et al. (1969) performed a group of experiments to determine the sequence of events for the myosin, ATP and actin interactions. Results were similar to those of Taylor et al. (1970), but a more complex mechanism was used to explain the results.

Kominz (1970), using a pH-stat with acetyl phosphate and acetokinase as an ATP-regenerating system, found evidence for an ATP hydrolytic site and an ATP clearing site in both actomyosin

and myofibrils. He proposed the presence of an ATPase cycle, which would couple ATPase activity and actin binding by an interaction and exchange of ATP bound at the clearing site and the ATP split at the hydrolytic site.

Interaction of Myosin with Actin

Basic features of the sliding-filament contraction model of Huxley (1960) have been almost universally accepted. Huxley's (1969) review of the evidence for direct interaction of thick and thin filaments has reinforced the view that the sliding force is a direct consequence of intimate contact between actin and myosin. Although neither the thick nor thin filaments change length during the process, the actin-myosin interaction results in muscle contraction and relaxation. This fact plus other evidence, including the work of Pepe (1967b), led to a contraction model in which the thin actin filaments are bound to the flexible heads of myosin molecules.

The myosin molecule is the site of hydrolysis, the site of actin binding, and also the location of the force translating step of contraction (Dreizen and Gershman (1970a). Pepe (1967b) using antibody staining experiments proposed a myosin molecule containing both a flexible neck region and a second flexible section associated with the area connecting the heavy- and light-meromyosins.

Using x-ray data, Huxley (1968) concluded that the myosin head is connected to the unflexible thick filament by two joints. The joints are separated by a portion of the myosin molecule, but are not bound to the thick filament.

Lowey et al. (1969) elucidated the structure of myosin with matrix bound papain. They found that a water soluble nonaggregating peptide connected the myosin head to the tail region. Huxley (1969) suggested this peptide was of the proper size and had the necessary physical characteristics to connect the two proposed myosin joints without binding to the thick filament.

The interaction of myosin with actin results in the head region of the molecule advancing. After reviewing three models that could account for this observation, Dreizen and Gershman (1970a) favored a torsional model over the conventional linear or screw models. They indicated that the torsional model is in better agreement with electron microscopic and low angle x-ray diffraction data. This model involves a rotational or spiral thrust of crossbridges, interaction of myosin with actin followed by a longitudinal advance of the myosin head. Huxley and Brown (1967) reported evidence for crossbridges capable of swinging freely around the thick filaments, and thus bringing the crossbridges and actin filaments into direct contact. However, they did not indicate the manner of crossbridge movement.

Several investigators (Gratzer and Lowey, 1968; Cheung and Morales, 1969) have probed the conformational changes occurring in myosin. Using optical rotatory dispersion and far ultraviolet absorption techniques, Gratzer and Lowey (1968) concluded that α-helix conformational changes did not take place when either ATP or pyrophosphate interacted with myosin. This experiment precluded α-helix conformational changes, but recognized the possibility of small induced conformational changes.

Imamura et al. (1970) using H^+ ion product detection procedures found evidence that the purine rings of ATP and ITP cause slight conformational changes on the active sites of myosin. Minor conformational changes in the myosin head region were observed by Cheung and Morales (1969). Their study involved the attachment of a fluorescing dye 8-anilino-1-naphthalenesulfonate to previously modified myosin and native myosin.

Quinlivan et al. (1969) attached nitroxide spin labels to myosin SH groups and then modified myosin ATPase activity with p-mercuribenzoate. They concluded that the ATPase thiol groups were heterogeneous. They further concluded that minor conformational changes could occur. Current evidence for myosin binding and enzymatic activity does not favor α-helix conformational changes, but does imply minor conformational changes in the head of the myosin molecule.

Physical Properties of Myosin

Godfrey and Harrington (1970a, b) investigated myosin monomer-dimer relationships and found them dependent on pH and ionic strength. Using high speed sedimentation equilibrium studies they concluded that the monomeric molecular weight for myosin was 458,000. Gershman et al. (1969) performed a series of high speed sedimentation equilibrium experiments and concluded that myosin had a molecular weight of 468,000 (± 10,000). Using high speed sedimentation equilibrium experiments, Rossomando and Piez (1970) reported the molecular weight of myosin to be 465,000. In this case, myosin was chromatographed on an agarose column to obtain monomeric molecules. Using osmometry the number-average molecular weight of myosin was reported as 470,000 by Tonomura et al. (1966).

The length of the myosin molecule was estimated to be approximately 140-160 nm by Lowey et al. (1969) and Cohen et al. (1970). Electron micrographs presented by Slayter and Lowey (1967) showed one end of the myosin rod to have two globular head regions. The myosin molecule contained about 57 percent α-helix, which formed the rod section of the molecule. The globular ends of the myosin molecule had a diameter of 7.0 nm and a low α-helix content.

Tsao (1953) found that myosin was composed of light and heavy protein constituents. Kominz et al. (1959) separated the light

and heavy subunits by pH adjustment and performed amino acid analysis, end group analysis and molecular weight studies on the small molecular weight proteins. This study established the fact that small proteins were associated with the large subunits, but failed to discern the biological function of these entities. However, small protein components were thought to represent either contaminants or proteolytic breakdown products until Dreizen et al. (1967) showed that myosin dissociated into two types of myosin subunits in 5 M guanidine-HCl.

Frederiksen and Holtzer (1968) and Gershman et al. (1969) separated small and large myosin subunits and demonstrated that both large and small subunits were required for ATPase activity. Numerous methods exist for the separation of large from small subunits, including succinylation (Huriaux et al., 1967), acetylation (Locker and Hagyard, 1967a), carboxymethylation (Locker and Hagyard, 1967a), heat treatment (Locker and Hagyard, 1967b), adjustment of pH (Frederiksen and Holtzer, 1968), urea treatment (Gazith et al., 1970) and varying the concentration of salts (Gershman and Dreizen 1970).

Determination of the structure of myosin has been greatly aided by the use of proteolytic enzymes. Using limited tryptic hydrolysis of myosin, Gergely (1953) produced two fragments, heavy and light meromyosin. Lowey et al. (1969) demonstrated that limited tryptic

degradation of heavy meromyosin results in formation of two subfragments--the enzymatically active S-1 subfragment and the S-2 subfragment with a high α-helix content.

Kominz et al. (1965) found papain cleaved myosin into the meromyosins, simultaneously hydrolyzing the bonds attaching the globular portion to the rod portion. Lowey et al. (1969) also employed insoluble papain to treat insoluble myosin and obtained myosin rods and subfragment S-1. The major limitation to proteolytic enzyme analysis has been uneven cleavage at specific sites and fragmentation of some peptides (Lowey et al., 1969).

The amino acid content of whole myosin has been well documented by Kominz et al. (1954). Gröschel-Stewart (1971) has shown minor differences in the Arg, Pro, Ile and Leu contents of smooth and striated human muscle. Minor amino acid differences in myosin from different species of domestic animals were observed by Bodwell (1971).

Amino acid analysis has shown myosin contains small quantities of 3-methylhistidine, ε-N-monomethyllysine and ε-N-trimethyllysine. Johnson et al. (1967) using adult rabbit muscle found approximately 2 residues of 3-methylhistidine per mole of myosin. Tryptic digests of myosin yielded approximately 1 residue of 3-methylhistidine per mole of subfragment-1. They concluded that this unusual amino acid composed part of the primary structure of myosin.

Kuehl and Adelstein (1970) found 3-methylhistidine to be absent from red muscle fibers and cardiac myosin. Mixed muscle, which is composed of both red and white fibers, contained 1.3-1.7 residues of 3-methylhistidine per 500,000 g of myosin. This study also showed that the 3-methylhistidine residue was common to muscle from different animal species.

ε -N-monomethyllysine and ε -N-trimethyllysine were reported to be present in myosin by Hardy et al. (1970), Huszar and Elzinga (1969) and Kuehl and Adelstein (1969). Hardy et al. (1970) isolated subfragment-1 and found 0.59 residues of ε-N-methyllysine and 1.75 residues of ε-N-trimethyllysine per 100,000 g. Kuehl and Adelstein (1969) also isolated subfragment-1 and found 0.29 residues of ε-N-monomethyllysine and 1.5 residues of ε-N-trimethyllysine per 100,000 g of protein.

Hardy et al. (1970) stated that methylation of both histidine and lysine occurs after peptide bond synthesis. Huszar and Elzinga (1971a) were unable to determine whether methylation of histidine occurred before or after folding of the polypeptide chains. Although specific residues are methylated enzymatically, they indicated that enzyme recognition of the methylation site is not understood.

Sequential amino acid analysis of myosin has not progressed beyond the preliminary stages. Huszar and Elzinga (1971a), using

subtractive Edman degradation and carboxypeptidase digestion of peptides from tryptic digestion of cyanogen bromide S-β-carboxy-amido-methyl subfragment-1, elucidated the amino acid sequence around the 3-methylhistidine residue of myosin. Huszar and Elzinga (1971b) using similar procedures have also located and determined the sequence of a decapeptide containing ε-N-trimethyllysine. The other ε-N-trimethyllysine residue was located in a peptide possessing a different solubility, and therefore, probably has a different amino acid sequence. Yamashita et al. (1964, 1965) have isolated and sequenced two small thiol peptides believed to be involved in myosin ATPase activity.

Two major studies on sequencing myosin thiol groups have been completed. Weeds and Hartley (1968) using a disulphid-ethiol interchange reaction followed by enzymatic digestion sequenced 16 unique myosin groups. Using a different labelling and purification technique Kimura and Kielley (1966) sequenced 15 unique thiol groups. When the data from both studies were tabulated, myosin appeared to contain 22 or more unique Cys-peptide groups (Weeds and Hartley, 1968).

The carboxyl terminus of myosin was extensively investigated by Locker (1954; 1956). Using carboxypeptidase, Locker (1954) obtained one end group of isoleucine per 300,000 g, one alanine per 500,000 g, one valine per 600,000 g and one leucine per 800,000 g of myosin. Using hydrazinolysis, he also found small amounts of

glutamic acid, serine, alanine, isoleucine and traces of glycine. Using carboxypeptidase, Locker (1956) later concluded that isoleucine was the major amino acid released from the carboxyl terminus.

Sarno et al. (1965) using carboxypeptidase A concluded that isoleucine was the C-terminal amino acid of myosin. They then calculated myosin had a minimum molecular weight of 133,000 and heavy meromyosin had a minimum molecular weight of 80,000. No calculations were made using valine, alanine, threonine or serine, which were released at approximately one third of the rate of isoleucine. Kominz et al. (1959) using carboxypeptidase obtained 1.4 isoleucine residues per 420,000g of myosin. They also found 1.0 isoleucine residue was released per 29,000g of carbonate subunits. This was the first indication that isoleucine was associated with the small subunits of myosin. Trotta et al. (1968) found one mole of isoleucine was released per mole of subfragment-I.

N-terminal end group analysis of myosin was first attempted by Bailey (1951). He used the FDNB method for end group detection, but results were negative. Therefore, he concluded that myosin may have a cyclic or looped conformation. Gaetjens et al. (1964) using a carbamylation procedure found no free NH_2-terminal residues in L-myosin. Offer (1965) obtained evidence of N-acetyl serine as the N-terminal amino acid of myosin. The N-terminal sequence

consisted of N-Acetyl-Ser-Ser-Asp-Ala-Asp, with a yield of two sequences per 600,000g. It is not known whether or not this sequence exists in the small or large subunits.

Electrophoretic and Chromatographic Properties of Myosin

Electrophoresis of myosin has been limited by the large molecular weight of the myosin molecule, aggregation of the molecules and preparative impurities. Free boundry electrophoresis was employed by Oppenheimer et al. (1967). They found one peak with moving boundry electrophoresis. When the single peak was subjected to polyacrylamide gel electrophoresis it was shown to be heterogenous.

Electrophoresis of myosin in gel matrix systems has been limited due to its high molecular weight. However, it has been successfully electrophoresed by the addition of dissociating agents, which reduce the molecule to its subunits. Urea, a nonionic dissociating agent, has been used in polyacrylamide gel by Rampton (1969), Florini and Brivio (1969), Heywood et al. (1967) and Small et al. (1961). Small et al. (1961) used 12 M urea to solubilize myosin on a polyacrylamide flat bed matrix and obtained a multibanded pattern. Heywood et al. (1967) found myosin migrated as one band in a 12 M urea disc gel system.

Rampton (1969) used 7 M urea in a polyacrylamide disc gel matrix for electrophoresing different myosin preparations. His results indicated that preparation purity differed, and that large subunits of myosin migrated in two or more bands. However, the cause of multibanding was not investigated. Florini and Brivio (1969) used 9 M urea and 2.2% acrylamide for electrophoresing different chemical modifications of myosin. Results were difficult to interpret, since large quantities of protein did not migrate and a number of proteins appeared to form aggregated complexes.

Urea was used with starch gel in the studies of Parsons et al. (1969) and Gröschel-Stewart (1971). In order to compare myosin from various muscles, Parsons et al. (1969) used 8 M urea in a 16% starch matrix. Results indicated that myosins from different muscles have unique densitometric tracings. Gröschel-Stewart electrophoresied myosin in an acid-urea-starch system previously developed by Smithies et al. (1962) and found pectorial and uterine myosin behaved differently.

Sodium dodecyl sulfate, an anionic dissociating agent, has been used in electrophoresing whole myosin by Weber and Osborn (1969) and Paterson and Strohman (1970). Sodium dodecyl sulfate complexes with proteins and peptides, resulting in an electrophoretic migration proportional to the molecular weight of the

protein (Dunker and Rueckert, 1969; Weber and Osborn, 1969; Fish et al., 1970; Reynolds and Tanford, 1970). A molecular weight close to 200,000 was obtained by Weber and Osborn (1969) for large subunits of myosin, and a molecular weight of 16,000-23,000 for three minor components of myosin.

Paterson and Strohman (1970) performed a complete electrophoretic study on the large and small subunits of myosin. The large subunits moved as one band on 3.36% acrylamide gels in a sodium dodecyl sulfate system, and had a monomeric molecular weight of approximately 200,000. They found two small subunits with corresponding molecular weights of 18,500-19,500 and 32,100-33,000. Results using sodium dodecyl sulfate electrophoretic techniques indicated that the dissociated myosin complex could be effectively separated on a gel matrix.

Column chromatography of myosin has become an almost universally accepted step in most purification procedures. Richards et al. (1967), Rossomando and Piez (1970) and Harris and Suelter (1967) developed purification systems to remove nucleic acids or other proteins and aggregates from whole myosin.

Column chromatography has been used by Stracher (1969) in separation of large and small subunits. He separated the large and small subunits of myosin, which had previously been dissociated in 5 M LiCl, using a Sephadex G-200 column equilibrated with 5 M

LiCl. Using the same solvent system and matrix, Paterson and Strohman (1970) found the large subunit fraction contained some small subunits. They were unable to obtain the rapid flow rate reported by Stracher (1969).

Using 5 M guanidine-HCl as the dissociating agent and column buffer, Gazith et al. (1970) and Kuehl and Adelstein (1970) separated previously dissociated myosin subunits on Sephadex G-150 or G-100 columns. Gazith et al. (1970) separated myosin subunits on Sephadex G-150 that had been equilibrated with 3 M urea. They found that low molarity urea solutions were ideal for dissociating and collecting the low molecular weight subunits, but the purity of the large subunits was questionable.

Locker and Hagyard (1967a) dissociated myosin by an acetylation procedure and separated the dissociated subunits with DEAE cellulose using a KCl gradient as the eluant. However, they found that the usefulness of the method was limited by the difficulty in obtaining pure components from the various peaks.

In all cases, separation of myosin subunits by column chromatography first required subunit dissociation followed by the separatory procedure.

Properties of Myosin Large Subunits

Molecular weight determination of large subunits from myosin requires dissociation of the complexed subunits. Small et al. (1961) observed that total dissociation of the myosin complex occurred in urea concentrations above 10 M. Their sedimentation studies indicated a molecular weight for myosin small subunits of 180,000g per mole. Using 5 M guanidine-HCl as the dissociating agent, Kielley and Harrington (1960), Gershman et al. (1969) and Gazith et al. (1970) determined the molecular weight for the large subunits. Molecular weights of 197,000 were reported by Kielley and Harrington (1960), while values of 212,000 (± 5,000) and 194,000g per mole were reported by Gershman et al. (1969) and by Gazith et al. (1970), respectively.

Sodium dodecyl sulfate was employed by Weber and Osborn (1969) and Paterson and Strohman (1970). Using the disc gel technique, they determined the molecular weight of large myosin subunits. Results indicated a molecular weight of approximately 200,000g per mole. However, dissociation of the large subunits did not occur in high molarity salt solutions (Gershman et al., 1970).

The amino acid composition of purified heavy subunits is believed to be similar to that of whole myosin. Perry et al. (1970) showed that 3-methylhistidine, ε-N-monomethyllysine and ε-N-trimethyllysine were absent in the light subunits, and appear to be

located in the subfragment-1 portion of the myosin molecule. Although C-terminal analysis of purified heavy subunits was attempted by Gershman et al. (1969) using carboxypeptidase-A, they did not report any significant yield of C-terminal amino acid residues. The N-terminal amino acid of purified heavy subunits has not been investigated.

Myosin large subunits appear to migrate as a single band in sodium dodecyl sulfate electrophoretic systems (Paterson and Strohman, 1970; and Weber and Osborn, 1969). Using urea to dissociate and solubilize myosin, Rampton (1969), Florini and Brivio (1969) and Small et al. (1961) produced multiple band formations on polyacrylamide gel. However, none of these workers unequivocally demonstrated that the multiple bands represented purified monomeric myosin large subunits.

Gershman et al. (1969) separated and purified myosin heavy subunits by repeated alkaline fractionation or by repeated low molarity guanidine fractionation. Column chromatography with a 3 M urea solvent and a Sephadex matrix was used to purify heavy subunits by Gazith et al. (1970). Kuehl and Adelstein (1970) isolated and purified heavy subunits with a Sephadex G-150 column and 5 M guanadine-HCl solvent. Gaetjens et al. (1968) purified heavy subunits by an extensive washing procedure after the initial pH adjustment and precipitation. The original pH adjustment and

ammonium sulfate precipitation procedure of Kominz et al. (1959) was used by Frederiksen and Holtzer (1968), after recycling and other modifications.

Properties of Myosin Light Subunits

The molecular weight of small subunits from myosin has been found to range from 17,100 to 33,000 (Lowey, 1971; Paterson and Strohman, 1970). Kominz et al. (1959) and Frederiksen and Holtzer (1968) estimated the molecular weight of small subunits from myosin to be approximately 30,000g per mole. A molecular weight of approximately 20,000 was reported by Gershman et al. (1969). Locker and Hagyard (1967a) obtained molecular weight values ranging from 17,000 to 20,000g per mole. These values were later verified by Weeds (1970). Using a sodium dodecyl sulfate electrophoretic system, Paterson and Strohman (1970) obtained molecular weights of 18,500-19,500 and 32,100-33,000 for the two small subunits of myosin. Lowey (1971) using a similar system obtained values of 17,000 and 25,000 for the two rapidly moving electrophoretic bands.

Amino acid analysis of myosin small subunits was accomplished by Kominz et al. (1959), Oppenheimer et al. (1967) and Gazith et al. (1970). Although the amino acid analyses were different for various small subunits, all had high phenylalanine to tyrosine ratios. Locker and Hagyard (1967a) and Gaetjens et al. (1968)

analyzed small individual myosin components. Results indicated that the small components had high phenylalanine to tyrosine ratios, and that one component exhibited a different proline content. Kuehl and Adelstein (1970) did not find any methylated histidine or methylated lysine in the light myosin components.

Weeds (1969, 1970) reported that the non-DTNB extractable small subunits contained one thiol sequence. Some 27 residues were common to the two subunits. The difference between the two subunits was the absence of several tryptic peptides from one subunit. However, Weeds (1970) emphasized that over 150 amino acid residues were common to both small subunits.

Using carboxypeptidase or carboxypeptidase A, Kominz et al. (1959), Gershman et al. (1966), and Weeds (1967) determined that the C-terminus of myosin small subunits consisted of an isoleucine residue.

Locker and Hagyard (1967a, 1967b, 1968) have performed extensive electrophoretic studies of the small subunits of myosin for various muscle fiber types, for various species and to ascertain the value of various purification procedures. They found that all species or muscle types did not always yield the three expected electrophoretic bands. Also, they used preparative gel electrophoresis to isolate the various small subunits for molecular weight determinations and amino acid analysis.

Gaetjens et al. (1968), Weeds (1969) and Gazith et al. (1970) have used conventional gel procedures to study myosin small subunits. Gaetjens et al. (1968) found four bands associated with myosin small subunits. Weeds (1969) electrophoretically demonstrated the purity of the DTNB extractable small subunit, and still found two bands remained after DTNB extraction. Gershman and Dreizen (1970) used a cellulose acetate electrophoretic system and obtained 4 small subunit bands. Perrie and Perry (1970) examined myosin small subunits using polyacrylamide-gel electrophoresis in 8 M urea. Four small subunit bands were evident from freshly prepared material, while the number of small subunits increased with storage.

Electrophoretic systems incorporating sodium dodecyl sulfate have been used by Lowey (1970) and by Paterson and Strohman (1970) to determine the molecular weights and electrophoretic heterogeneity of small subunits. Results of these studies indicate that whole myosin contained two small subunits with different molecular weights.

Frederiksen and Holtzer (1968) calculated that two small subunits plus two large subunits comprise the myosin molecule. Gershman et al. (1969) postulated that there are 2.7 (± 0.3) light subunits per myosin molecule. These two studies involved accurate molecular weight estimations for the whole myosin molecule and for its large and small subunits. However, both groups reported different values for the molecular weights.

Gazith et al. (1970) and Weeds (1969) removed a portion of the myosin small subunits with DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) extraction without significantly affecting the myosin ATPase activity. Later Weeds (1970) produced subfragment-1 and found a single light subunit associated with the cleaved heavy subunit segment. Since the DTNB extractable small unit was not found to be associated with subfragment-1 and was absent in cardiac muscle, Weeds (1969, 1970) postulated that the DTNB protein may be a contaminant. Although the selectivity of DTNB extraction of the small subunits has been questioned by Paterson and Strohman (1970), they still concluded that myosin had two small subunits.

MATERIALS AND METHODS

General Procedures

Protein determinations were made using the biuret method or spectrophotometrically using an extinction coefficient of 350 cm^2 per g for light subunits (Stracher, 1969) and an extinction coefficient of 500 cm^2 per g for heavy subunits and whole myosin (Gazith et al., 1970). Deionized distilled water was used in all experiments. Urea was purified by running 10 M solutions through Rexyn I-300 resin 4 times. Purity was ascertained from conductivity readings.

Protein Preparation

Myosin was prepared from the back muscle of 4-6 pound female rabbits using the procedure of Kessler and Spicer (1952) as modified by Kominz et al. (1959). The animals were stunned by a blow on the head and killed by bleeding. The muscle was removed immediately and placed in crushed ice. After cooling, the muscle was weighed (approximately 250 g/rabbit) and ground in a Waring blendor with 750 ml of pH 6.5 Guba-Straub buffer at 4°C for 15 sec. This solution was stirred for 15-20 min at 4°C and diluted with 2700 ml of 4°C water, followed by filtration through two layers of

cheese cloth. Then 6,000 ml of 4°C water was added to the filtrate, and the myosin was allowed to settle to the bottom of the container.

After the myosin had settled out, all excess liquid was siphoned off and the precipitate was collected by centrifugation at 13,000xg for 15-25 min at 4°C. The KCl concentration of the precipitated myosin was brought to 0.5 M by adding the necessary amount of 3 M KCl. The volume was adjusted to 300 ml with 4°C neutral 0.5 M KCl, and stirred overnight at 4°C.

A solution of 500 mg of ATP, 5 ml of H_2O and 1.4 ml N NaOH was added to 0.36 ml of 1 M $MgCl_2$, 5.6 ml of H_2O and 0.4 ml of 3 M KCl. The resultant pH 6.5 solution was added to 300 ml of the previously prepared myosin. The resultant myosin-ATP solution was centrifuged at 78,000xg for 180 min at 4°C in a Beckman model-L ultracentrifuge. The supernatant was poured off and diluted with ten volumes of 4°C water.

The precipitated myosin was then collected by centrifugation at 13,000xg for 15-25 min at 4°C. The myosin was adjusted to 0.5 M KCl, and the volume was brought to 300 ml with 4°C neutral 0.5 M KCl. It was then stirred at 4°C over night. The above ultra-centrifugation and precipitation procedure was repeated twice in order to purify the myosin. Immediately after purification, a biuret protein determination was made and the supernatant volume was noted. The myosin was then precipitated by dilution with 10

volumes of water. The precipitate was dissolved in 0.5 M KCl. If the myosin was not used immediately, it was mixed with cold glycerol (v/v) and stored at -20°C.

Large myosin subunits were prepared from both fresh and stored myosin. A total of 1600 mg of myosin in 250 ml of neutral 0.5 M KCl and 0.1% β-mercaptoethanol were stirred together, and the pH was adjusted to 11.0-11.3 by addition of 1 M ethylenediamine or 1 M KOH at 4°C. The mixture was stored at 4°C for 60 min. The pH was then lowered to 6.0-7.0 with 1 M HCl, after which it was diluted with 10 volumes of cold water. The resultant precipitated protein was collected by centrifugation at 13,000xg for 10-15 min, and was subsequently redissolved in neutral 0.5 M KCl. The cycle of pH adjustment, dilution, centrifugation and redissolving of the precipitated protein was repeated up to five times and resulted in purified large subunits. After the cyclic dissociation and removal of myosin small subunits, the large subunits were washed twice with neutral 0.05 M KCl. The remaining large subunits were then stirred overnight in neutral 0.5 M KCl and used immediately, or else were stored in 50% glycerol at -20°C.

Myosin small subunits were prepared from both freshly prepared and stored myosin. The supernatant solutions from preparation of the large subunits were treated by two different methods in order to purify the small myosin subunits. One purification

method involved dialysis of the supernatant against water followed by lyophilization. The lyophilized sample was dialyzed against a neutral 0.05 M KCl solution and centrifuged at 13,000xg for 20 min. The final supernatant was lyophilized. The purification and lyophilization cycle was repeated twice. The other purification method involved concentrating the supernatant using an Amicon cell with either an Amicon PM-10 or a Millipore membrane. The concentrated supernatant was dialyzed and lyophilized as before. The purified small subunits were then lyophilized and stored at -20°C until used.

Reduction, Alkylation and Succinylation

Reduction with β-mercaptoethanol and alkylation with 4-vinyl pyridine was accomplished by a modification of the method of Friedman et al. (1970). A total of 200 mg of protein in 10 ml of the appropriate buffer, 10 ml of Cavins buffer (16.11 g tris, 7.12 g HNO_3, 0.75 g KCl and 1 mg EDTA in 200 ml of water) and 80 ml of 10 M deionized urea were stirred under a nitrogen atmosphere while 0.2 ml of β-mercaptoethanol was added. The solution was then stoppered and held overnight at 4°C. The reduced solution was then brought to 23°C and stirred for 90 min. Afterwards, 0.33 ml of 4-vinyl pyridine was added, and the solution was dialyzed against the appropriate buffer.

Other alkylation procedures using ethylenimine and iodoacetamide followed the above method, except that the appropriate reagent was substituted for 4-vinyl pyridine. Alkylation with iodoacetic acid was similar to the above procedure, except that the reaction was chemically halted by addition of β-mercaptoethanol prior to dialysis.

Sulfonated myosin and large subunits were produced by the method of Paterson and Strohman (1970). This method involved incubation of myosin in 0.2 M sodium sulfite, 0.2 M tris, 1.0% sodium dodecyl sulfate and 0.05% β-mercaptoethanol at pH 8.5 for 12.0 min at 27°C with an air oxidation system.

Succinylation of whole myosin and of large subunits was accomplished utilizing a modification of the method of Oppenheimer et al. (1967). Reduced and alkylated protein in 0.5 M KCl, pH 7.0, was reacted with 1.4 mg of succinic anhydride per mg of protein. The succinic anhydride was powdered and added over a 60 min time span. The temperature was kept below 5°C, and pH 7.0 was maintained with 1 M NaOH. After 60 min, the product was dialyzed against neutral 0.05 M KCl.

An alternate succinylation procedure involved reaction of myosin in 6 M urea with 1 mg of succinic anhydride per mg of protein. The pH was maintained at 8.2, and the temperature at 23°C. The solution was then dialyzed against a neutral 0.05 M KCl buffer as outlined above.

Isoelectric Focusing

Solutions of reduced and alkylated protein were made 12 M with ultra pure urea (Mann Chem. Co.) and incubated at 43°C for 60 min or more prior to the addition of the ampholines and gel solution. Gel solutions were made using warm (43°C) 12 M urea solvent and contained 0.12% bisacrylamide, 0.036% temed (N,N,N',N' Tetramethylethyenediamine), 0.00036% riboflavin, and either 3.5 or 5.0% acrylamide. Two ml of gel solution, 0.1 ml of sample solution (0.05-0.5 mg protein) and 0.1 ml of pH 3 to 10 ampholines (40% stock, LKB Produkter A. B.) were mixed, and aliquots were put into 13 cm gel tubes having an inner diameter of 0.45 cm. The gels were photopolymerized at 43°C for 1 hr and placed in a BioRad electrophoresis apparatus with the lower, jacketed buffer chamber maintained at 43°C. Pre-heated 1.5% phosphoric acid was placed in the positive electrode chamber and 1.5% ethylenediamine in the negative chamber.

For electrofocusing, an LKB power supply was used to maintain a constant potential of 80 volts for variable times. Gels to be stained were removed and fixed in several changes of 5% trichloro-acetic acid -5% sulfosalicylic acid. The gels were stained in 4% trichloracetic acid -4% sulfosalicylic acid, 16% methanol, 0.00125% coomassie blue, and destained with several changes of 5% trichlor-acetic acid -5% sulfosalicylic acid. For determining isoelectric

focused gel pH gradients, unstained reference gels were cut into 1 cm sections. The sections were left in 3 ml of H_2O for 4 hr prior to measurement of pH. All pH gradient measurements, and sample runs for determining isoelectric points of stained bands, were made on 5% gels due to their better handling properties. The isoelectric point of the protein bands in the stained gels was determined by comparing the band location with the pH at the same location in the reference gel. In all cases, each reference gel contained the same identical protein as the corresponding stained sample gel.

Electrophoresis

Disc gel electrophoresis was carried out utilizing the same instrument as previously described for isoelectric focusing. Three gel systems were used. The first system consisted of a 7% separation gel, a spacer gel, and a pH 8.2 tris-glycine buffer system. This system for electrophoresis was essentially that of Canal Industries, and utilized their stock solutions and procedures. The proteins used in this system had been reduced and alkylated with 4-vinyl pyridine and dialyzed against 0.05 M tris-glycine and 0.4 M KCl, pH 8.2, buffer for 24 hr.

The second system of electrophoresis consisted of a 3.5% gel composed of equal weights of the four following solutions: (1)

3.5 g acrylamide, 0.16 g bis and 21.4 g water; (2) 0.035 ml temed, 1.21 g tris, 0.75 g glycine and 23 g water-pH 8.5; (3) 3 g urea, 0.1 g SDS (sodium dodecyl sulfate), 0.001 g EDTA and 21.9 g water; (4) 0.07 g APS (ammonium persulfate) and 25 g H_2O. After mixing, the solution was placed in tubes and several drops of water were added to assure a flat gel interface. The gel was then allowed to polymerize for 60 min in the absence of light. The chamber buffer, pH 8.2, consisted of 0.05 M tris-glycine, 0.5 M urea, 0.1% SDS and 0.01% EDTA, and on occasions, 0.1% β-mercaptoethanol.

The protein was reduced and alkylated and was then dialyzed for 48 hr against a 0.4 M NaCl solution or the electrophoresis buffer. After dialysis, the protein was incubated in a 1% SDS solution at 43°C for 60 min. The amount of protein applied to each gel was 0.1 to 0.025 mg, although occasionally larger amounts were applied. Staining of the gels was accomplished with 20% acetic acid and 0.05% coomassie brilliant blue, and then the gel was destained with 7% acetic acid. When it was necessary to examine the bands immediately, the method of Chrambach et al. (1967) was used. A constant current power supply was maintained at 2.5 mA per tube for 120 minutes.

The third electrophoretic system employed a 12 M urea solvent in a 3.5% acrylamide matrix. The gel formulation was as follows: 1.75 g acrylamide, 0.2 mg riboflavin, 0.06 g bis, 0.02 ml temed,

0.4 g tris, 0.3 g glycine and 48 ml of 12 M urea made to pH 8.5 with HCl when necessary. The gel was poured in tubes and photopolymerized. The protein solution in 12 M urea was next layered on top of the photopolymerized gel, followed by a small amount of gel solution to keep the protein from diffusing into the buffer chamber. The buffer consisted of 0.05 M tris-glycine at pH 8.2 and 12 M urea. The electrophoresis apparatus was maintained in a 43°C in an isothermal oven. A constant current power supply was maintained at 1.5 to 2.5 mA per tube for 3 to 5 hr. Staining was carried out as described above for 3.5% SDS electrophoresis.

Carboxypeptidase A and B End Group Analysis

The basic method of Ambler (1967) was used with modifications for carboxypeptidase A and B end group analysis. Carboxypeptidase A and B treated with diisopropyl phosphorofluoridate, were purchased (Sigma Chem. Co. or Worthington Biochemical Co.) and stored at the appropriate temperature. Carboxypeptidase A was solubilized by washing with water, and the protein was then suspended in 0.1 ml of a 1% sodium bicarbonate solution at 4°C. The crystals were dissolved with the drop wise addition of cold 0.1 M NaOH, and the pH was adjusted to 8-10. Carboxypeptidase B was washed once with water and solubilized with 1 M NaCl at 4°C.

Myosin and myosin heavy subunits were reduced and alkylated followed by succinylation, and finally were dialyzed against pH 8.5, 0.2 M N-ethylmorpholine acetate buffer. Digestion was initiated by combining approximately 0.8 μM of protein with carboxypeptidase A (25:1, w/w) or with carboxypeptidase B (100:1, w/w) and a known amount of norleucine, which was used as an internal standard.

The protein buffer and enzyme blanks were run simultaneously with shaking. Enzymatic cleavage occurred at 37°C. Aliquots were removed at 15, 30, 60 and 300 min, and cleavage was halted by lowering the pH to approximately 3 by adding Dowex 50W-8X resin. The resin was washed three times with water, and the amino acids were eluted with two 5 M NH_4OH washes. The samples were evaporated to dryness and redissolved in amino acid analyzer buffer, filtered and frozen at -20°C. Samples were later analyzed for amino acid composition.

Hydrazinolysis End Group Analysis

End group analysis by hydrozinolysis was carried out using a modification of the procedure of Fraenkel-Conrat and Tsung (1967). Myosin and myosin large subunits were reduced and alkylated. They were then dialyzed against water at 4°C for three days, lyophilized

and dried overnight above P_2O_5. The reaction was initiated by addition of 2 ml of 97% purity hydrazine to 0.2 μM of protein. Incubation of the various sample fractions was at 100°C for 8, 16 and 24 hr or at 110°C for 8 hr. Samples were removed, placed in a vacuum desiccator and dried over P_2O_5.

Amberlite IRC-50 columns were used to separate the free amino acids from the hydrazides. The dried samples were dissolved in water and a known amount of norleucine was added as an internal standard. This solution was put on the column and was washed extensively with water and a 0.1 M ammonium acetate solution. The washings were combined, evaporated and redissolved in amino acid buffer. Free amino acids were identified using an amino acid analyzer.

Samples prepared by hydrazinolysis were run on thin layer chromatography plates (Eastman cellulose-6064). The developing solvent consisted of butanol, ammonium acetate and water (80:20:20). Identification of the free amino acids was made using a cadmium chloride-ninhydrin spray (CdCl-100 mg, water-10 ml, ammonium acetate -5 ml, acetone-100 ml, ninhydrin-1 g).

Column Chromatography

Whole myosin in 5 M LiCl was chromatographed on Sephadex G-200, G-150 or G-100 columns. A 2.5 cm diameter Sephadex column was

used at a temperature of either 4 or 23°C. Flow rates, column buffers and samples preparations and amounts were varied to optimize the separatory properties of the columns. The columns were monitored with a Beckman DB spectrophotometer, and samples collected in a refrigerated fraction collector.

Several 8 M urea columns were employed in attempts to separate large from small subunits. The columns were 2.5 cm in diameter by 100 cm in length, and contained Sephadex G-100 equilibrated with urea buffer (8 M urea, 0.025 M tris, 0.1% β-mercaptoethanol -pH 8.1). The proteins were dialyzed against 8 M urea buffer, applied to the column, and developed at approximately 8 ml per hr. Analysis and collection were carried out as described earlier herein.

Columns equilibrated with 5 M guanidine-HCl were employed to separate the small from the large subunits. The solvent was 5 M guanidine-HCl (Mann ultra pure), 0.1% mercaptoethanol, 0.005 M EDTA and 0.05 M KCl at pH 7.2, on a Sephadex G-150 matrix. A 2.5 x 100 cm column was connected to a fraction collector. The rate of development was approximately 12 ml per hr and each fraction was analyzed by the method of Davison (1968). The proteins were dialyzed against the column buffer and introduced on the column by adding sucrose to the sample and layering the sample under the column buffer.

Ion exchange chromatography was used to separate the small myosin subunits. A 2.5 x 33 cm column with a Sephadex DEAE A-25 matrix was used with a linear gradient consisting of 1,500 ml of 0.05 M KCl, 0.05 M tris (pH 8.5) and 1,500 ml of 0.75 M KCl, 0.05 M tris (pH 8.5). The column rate was 40 ml per hr and 10 ml fractions were collected. The column was monitored at a wave length of 280 nm. Individual tubes were monitored at 230 nm. Individual tubes were also assayed for protein using a modification of the Moore and Stein (1954) quantitative ninhydrin procedure.

Amino Acid Analysis

Samples for amino acid analysis were dialyzed for 3 days against water and then lyophilized. The lyophilized samples were weighed, and put in digestion flasks with 1 ml of 5.55 N constant boiling HCl per 2.5 mg of protein.

Digestion was conducted for 22 hr in a 110°C isothermal oven. The digests were removed from the oven and dried over NaOH in a vacuum desiccator. The residue was dissolved in amino acid buffer and stored at -20°C until analyzed.

A Hitachi Perkin-Elmer model KLA-3B amino acid analyzer with an automatic sample loader was utilized. Samples were analyzed separately for acidic-neutral and basic amino acids. Amino acid standards (Beckman Co.) were run approximately every four runs.

Samples from end group analysis were analyzed in 1 ml of buffer, which contained 0.02-0.04 μM of amino acid. In order to obtain accuracy in calculations, a minimum of 0.02 μM of each amino acid was required.

RESULTS AND DISCUSSION

Isoelectric Focusing

Initial isoelectric focusing experiments were performed in a conventional liquid matrix isoelectric focusing cell containing 6 M urea as a solubilizing and dissociating agent. A 440 ml LKB isoelectric focusing column was used with a pH 3-10 ampholine. Reduced and alkylated whole myosin was dialyzed against 6 M urea prior to initiation of the pH gradient. The column was started with a current of 16 mA, and 48 hr later after the current had fallen to 2.7 mA the column was unloaded. The sample of myosin appeared to be soluble prior to beginning isoelectric focusing. After 24 hr, however, the protein had precipitated into three sharply focused bands. Two of these bands contained approximately equal amounts of protein and were located within 1 cm of one another. After 48 hr, these two bands had drifted together and some protein had gravitated to the bottom of the column. Visual inspection indicated that the third band contained less protein and was located at a lower pH than the two previously described bands.

Figure 1 shows the pH gradient as reconstructed from the individual column fractions. The location of the bands previously described are shown. The pH range of the faint band was approximately 5.0-5.6, while the pH range of both heavy bands was approximately

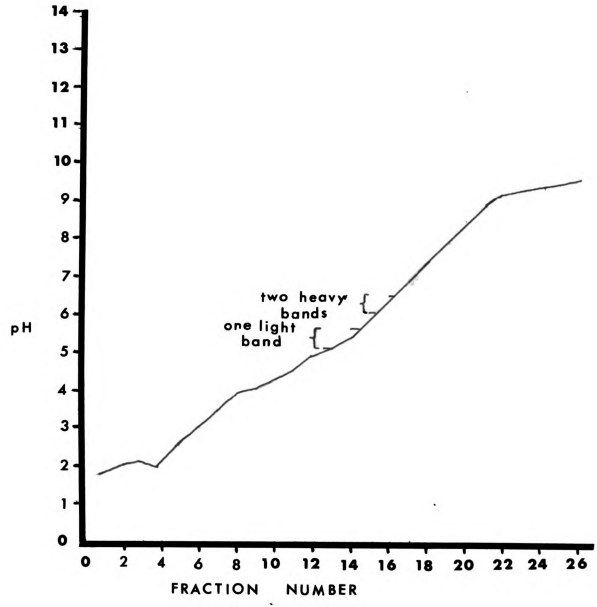


Figure 1. Isoelectric focusing of myosin using a conventional column with an aqueous 6 M urea matrix. Locations of bands are indicated.

6.0-6.5. These figures are in general agreement with the values presented later herein for isoelectric focused myosin on a polyacrylamide matrix.

A SDS disc gel system was used to electrophorese the faint and heavy bands. The two heavy bands from isoelectric focusing formed a single diffuse band upon SDS electrophoresis. Results indicated that the two large heavy bands obtained upon isoelectric focusing myosin may have contained heavy myosin subunits. However, the subunit mobility was greater than expected and the band focused over a wider area. One possible cause of variation could have been the interaction of ampholites with the large subunits. The small band was electrophoresed and found to contain small myosin subunits as well as other low mobility fractions. These fractions could have been either aggregated small subunits, large subunits or aggregated ampholite-protein products.

The conventional isoelectric focusing apparatus used in this experiment was a cylindrical glass container designed for stabilization of liquid solutions. A liquid matrix was employed consisting of an aqueous suspension of ampholites (aliphatic polyaminopolycarboxyclic acids) and 6 M urea, which was used as a nonionic solubilizing agent. Focusing occurred as the solubilized myosin subunits migrated to their respective isoelectric points.

The liquid matrix system is not applicable to all proteins, thus, limiting the usefulness of the technique. Protein precipitation

was a major problem. Three possible causes of this phenomena were observed. First, a large group of proteins are not soluble in low ionic strength solutions. Myosin is one such insoluble protein. Secondly, some proteins react with ampholites, resulting in precipitated products. Succinylated myosin reacts this way, but could be resolublized by the addition of urea. Thirdly, many proteins tend to precipitate as they approach their isoelectric point. Other problems were also encountered with the liquid matrix system, including gradient stabilization and removal of the focused protein bands.

Protein precipitation results in aggregation and contamination of other non-precipitated protein bands. In the matrix used, precipitated protein drifted through the matrix, possibly contaminating other focused bands. It appears doubtful whether or not a more concentrated sucrose gradient would keep the precipitated proteins from drifting to the bottom of the column. To avoid the precipitation problems, either the protein must be prevented from drifting through the matrix, or the protein must be solubilized over the entire pH range.

Proteins can be solubilized with urea concentrations up to 6 M. Even in the presence of high urea concentrations some proteins, such as myosin, precipitate near their isoelectric point. The feasibility of incorporating larger amounts of urea in the aqueous matrix is

limited, since additional urea decreases the density gradient and lends little support to the pH gradient.

Due to the problems encountered in using the liquid matrix, a modified method for the isoelectric focusing of proteins in high urea concentrations was developed. The polyacrylamide isoelectric focusing procedures described by Dale and Latner (1968), Awden (1968), Wrigley (1968) and Catsimpoolas (1969) were modified for use with large molecular weight proteins. The myosin molecule with a molecular weight of approximately 460,000 g per mole resulted in low mobility on polyacrylamide gel. To isoelectric focus myosin on polyacrylamide gel, therefore, it was necessary to dissociate the molecule into its subunits, and focus the subunits in the dissociating solution. Since Small et al. (1961) had previously dissociated and electrophoresed myosin in 12 M urea, this approach was adopted. The temperature was maintained at 43°C to keep the urea in solution.

Whole myosin, large subunits and small subunits were isoelectrically focused using a 12 M urea solvent and 3.5% acrylamide matrix at 43°C as shown in figure 2. The large subunits (A-I, B-I, C-I, D-I) were shown to have different isoelectric points than the small subunits (B-II, D-II). This is clearly shown by comparing the positions of the large and small subunits on the gels. Although the large subunits (C-I) focused as a single band in gel C, two bands are apparent in gels A, B and D (figure 2). Purified large subunits

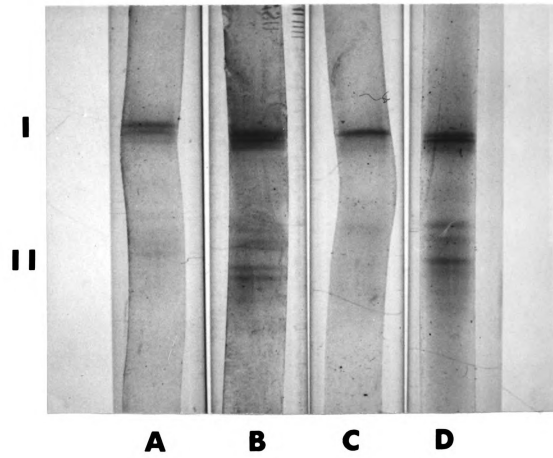


Figure 2. Isoelectric focusing of native, reduced and alkylated myosin on 3.5% acrylamide gel with 12 M urea as the solvent at 43°C. A = whole reduced and alkylated myosin (low concentration); B = native myosin; C = reduced and alkylated large subunits; D = whole myosin reduced and alkylated. Band area I contains the large subunits and area II contains small subunits.

migrated as one band in gel C, but other isoelectric focused gels of the same protein preparation yielded two bands in the region occupied by the large subunits. Both native myosin and reduced - alkylated whole myosin gels were obtained with one band or one band plus several minor bands in the large subunit area (figure 2, area I).

The small subunits in figure 2 (B-II and D-II) separated into multibanded patterns. Gel B-II is native myosin (no thiol protection) and has a complex small subunit band pattern. However, the same protein (D-II) when reduced, alkylated and focused under identical conditions yielded three distinct bands. Reduction and alkylation of the proteins used in the electrophoretic system resulted in simplified banding patterns similar to those of gel D-II. Gazith et al. (1970) have shown electrophoretically that the number of bands from myosin small subunits were reduced by alkylation of the protein. Thus, it appears that the small subunits may aggregate resulting in bands with unique isoelectric properties.

The large myosin subunits are composed of two bands of relatively equal intensity in gels A-I, B-I and D-I (figure 2). Lowey et al. (1969) concluded that myosin has but two large subunits in a partial α-helix conformation. Interaction between the large subunits is probably limited to noncovalent bonding as shown by the dissociation of the subunits in systems where reducing agents have

not been employed. Therefore, if the two large subunits of myosin are not similar, they could have primary amino acid sequences differing enough to create isoelectric pH differences. If the myosin large subunits have different isoelectric pHs, isoelectric focusing could be used to separate the subunits.

Two bands were observed in the region where large subunits focus, but the composition of these bands is unknown. Possible causes for a two banded structure could be separation into two separate and distinct large subunits of myosin. Other possible causes include aggregated large subunits, undissociated myosin or subunits, products of ampholine-protein interactions, denaturation products and broken pH gradients. Frater (1970) developed a staining method for detection of ampholites and found banding in several acid pH regions. They also found low conductance in the pH 4-5 region, which could have some bearing on protein mobility. The possible formation of artifacts within an acrylamide matrix needs further investigation.

Proof for myosin large subunit heterogeneity would require isolation and characterization of the different components. Several attempts were made to form preparative isoelectric gels on a LKB Unifor column. Results were inconclusive due to mechanical problems.

The 3.5% acrylamide gel was difficult to handle, therefore, a 5% acrylamide matrix was used for isoelectric point determination

for small and large subunits of myosin. Figure 3 depicts three 5% acrylamide gels used for isoelectric point determination. Reduced and alkylated myosin large and small subunits exhibited the characteristic patterns obtained on 3.5% acrylamide gels, however, protein bands were not as well resolved. Distinct bands were observed in gels B and C, but gel A was not as well focused within the given time period. Whole myosin (figure 3-gel C) appears to have two bands in the area associated with the large subunits and three bands in the small subunit area. The band patterns of this gel were in general agreement with the bands of gel D figure 2. Both of these gels contained myosin from a common purification-reduction-alkylation sequence and isoelectric focusing conditions. Gel B (figure 3) contained purified small subunits, but did not appear to contain appreciable quantities of large subunits, which is one criterion of preparation purity.

The pH gradient within the gel matrix of two 5% acrylamide isoelectric focused gels are graphically depicted in figure 4. Table 1 lists the isoelectric points of all detectable bands from six different stained 5% acrylamide gels. The reference gels (figure 4) were run simultaneously under the same conditions using common proteins. Duplicate gels to those shown in figure 3 were sliced perpendicularly. The pH of each sliced segment was determined, and the values are shown graphically in figure 4. The distance the protein had focused

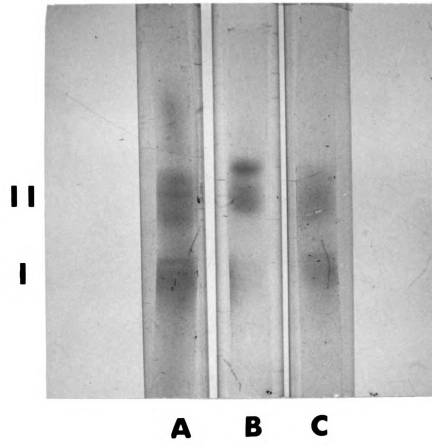


Figure 3. Isoelectric focusing of reduced and alkylated whole myosin, large subunits and small subunits on 5% acrylamide gel with 12 M urea as solvent at 43°C. A = myosin large subunits; B = myosin small subunits; C = whole myosin. Band area I contains large subunits and band area II contains small subunits.

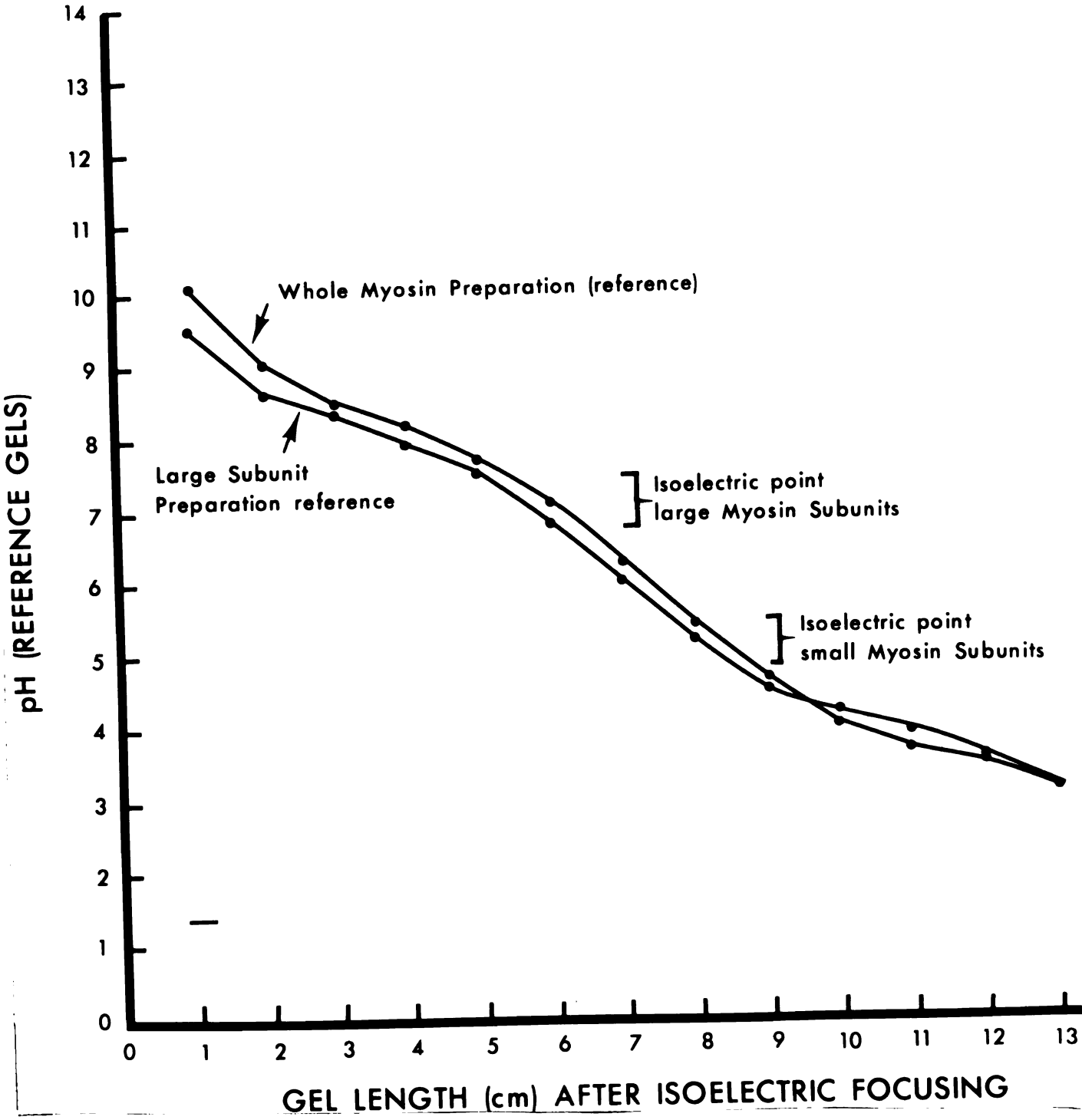


Figure 4. Isoelectric focused gel pH gradients obtained from reference gels showing the location of myosin large and small subunits.

Table 1. Isoelectric Points of Protein Bands of Reduced and Alkylated Myosin on 5% Acrylamide Gels

Preparation:	Whole Myosin		Large Subunits		Small Subunits	
Isoelectric focusing gel number:	1	2	3	4	5	6
Large Subunit	6.7*	6.9-7.2	7.0-7.3	6.7-6.9	6.5-6.7	6.8-7.2**
	6.4	6.7	6.9	6.5	6.4	6.4**
Small Subunit	5.3	5.3	5.5**	5.3**	5.2**	5.0-5.2
	5.0	5.0	5.2**	5.0**	4.9**	4.9
Other	5.9	5.8	5.7**		5.5**	
	4.8					
	4.5					4.5

*Values represent isoelectric points (or ranges of pH) for indicated protein band(s) as determined from reference gels.
**Faint bands.

from the origin could be determined from the stained 5% gel (figure 3) and the corresponding isoelectric points could be obtained from figure 4.

For the large subunits, two isoelectric points ranging between pH 6.4-6.9 and pH 6.5-7.3 were observed. Variations of this magnitude would be expected using these experimental methods. Failure to obtain distinct bands in 5% gels was reflected by the broad pH ranges obtained, possibly this was caused by the lower migration rate of the large subunits due to the higher acrylamide content.

In gel area II (figure 2), three bands are apparent, two of which are thought to represent small subunits. These two bands

focused between pH 4.9 and 5.5 in all cases (Table 1). The bands located at pH values of 5.9, 5.8, 5.7 or 5.5 (Table 1) could also represent small subunits as could the bands assigned an isoelectric point of 4.5 (gels 1 and 6). Assuming two small subunits are present, it is probable that one or more of the faint bands represents an impurity, or possibly a component similar to the DTNB extractable component of Weeds (1969). Another plausible cause of several faint bands could be the aggregation of small subunits. Perrie and Perry (1970) electrophoresed myosin small subunits in 8 M urea and observed that the number of small subunits increased with sample aging. Gershman and Dreizen (1970) also noted the problem of small subunit aggregation.

Bendall (1964) stated that the isoelectric point of myosin in a KCl solution was 5.4, but upon addition of Mg or Ca ions it increased to 9.3. An isoelectric point of 5.4 for the whole myosin molecule is within the range obtained for myosin subunits. However, direct extrapolation of values was impossible due to differing ionic strengths and conformational structures.

Electrophoresis of Myosin Subunits

The Canal Industries electrophoretic procedures and equipment were used to electrophorese myosin small subunits. This technique employed a 7% separation gel and a small spacer gel that were polymerized with ammonium persulfate and riboflavin, respectively. The tank buffers consisted of a tris-glycine pH 8.2 buffer.

The effect of different reduction and alkylation agents on small myosin subunit composition are depicted in figure 5. Gel A was native myosin (unreduced and unalkylated). Although the bands in this gel separated poorly, several faint bands occurred that are not apparent in gels B, C, D and E. The protein preparation used for this gel did not utilize the 8 M urea dissociation step in the reduction-alkylation procedure. Therefore, a portion of the myosin small subunits (gel A) were not free to enter the gel matrix, since they were bound to the protein that accumulated at the gel origin. Gel B shows myosin dissociated in 8 M urea and reduced with β-mercaptoethanol, but unalkylated. This gel contains a split band, which could have been avoided if the reduction step had been followed by alkylation. This can be seen by comparing gel B with gels C, D and E.

Gels C, D and E (figure 5) show examples of small subunits, which have undergone reduction and have been alkylated with different

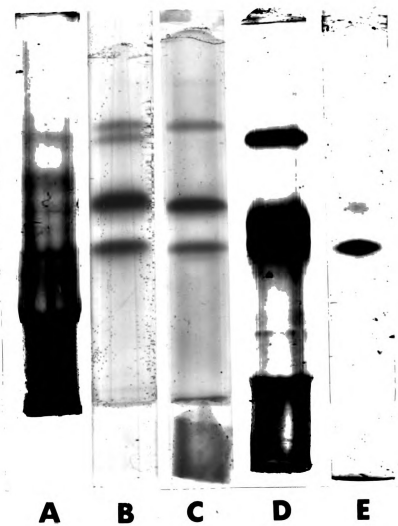


Figure 5. Electrophoretic patterns of whole myosin on 7% acrylamide gel, showing the effects of different reduction and alkylation procedures. A = unreduced and unalkylated whole myosin; B = whole myosin after reduction with β-mercaptoethanol; C = whole myosin after reduction and alkylation with 4-vinyl pyridine; D = whole myosin after reduction and ethylenimine alkylation; E = whole myosin after reduction and iodoacetic acid reaction.

agents. Although results indicate the three alkylation agents were comparable, the mobility of individual components varied on the different gels. Sharp bands resulted upon alkylation with 4-vinyl pyridine and ethyleneimine. The three bands shown on each of these gels represent three different small subunit species, and are characteristic of the myosin preparation used in this study.

Three small subunits were obtained on 7% gel electrophoresis after myosin from rabbit back muscle had been reduced and alkylated (figure 5, gels C, D, E). Weeds (1970) stated that one small subunit was required for each globular head of the myosin molecule in order to retain ATPase activity. Weeds (1969), Gazith et al. (1970) and Paterson and Strohman (1970) have all concluded that whole myosin contains two small subunits. During the course of this study the large and small subunits of myosin were separated by a cyclic pH adjustment sequence. After various cycles, the myosin large subunits were dialyzed against a tris glycine buffer and electrophoresed on the 7% gel system. The whole myosin preparation contained three small subunit bands. After several pH precipitation cycles, however, one band disappeared, leaving the other two small subunits in small quantities.

The selective removal of one small subunit could be construed as evidence for a tighter binding of the two remaining small subunits. However, ATPase activity and actin binding were not investigated due to destruction of the ATPase system during subunit pre-

paration and modification.

The biological function of the third myosin subunit (or impurity) has not been fully investigated. Paterson and Strohman (1970) identified the third small subunit component as actin, and found the presence of extra components dependent on the nature of the myosin preparation. Although actin may be the contaminant in some preparations, further investigation is needed on other light subunit components using different preparation procedures. Also, investigation of the dependence of actin binding on myosin small subunit composition needs additional study. Furthermore, structural implications of a possible third myosin small subunit component have not been fully investigated.

Deaminase and myokinase are possible contaminants in myosin preparations (Gershman and Dreizen, 1970). These proteins were reduced and alkylated in the same manner as the myosin small subunits. After electrophoresis, the resultant patterns from deaminase and myokinase were not well focused and exhibited smaller mobilities than myosin small subunits. Thus, it is concluded that deaminase and myokinase were not responsible for the bands shown on electrophoresis of myosin small subunits.

Attempts to electrophorese myosin have often concentrated on the immobile large subunits, thereby missing the small subunits, which frequently pass completely through the gel and are not observed.

Another common mistake has been the removal of small subunits in procedures involving dissociating conditions followed by precipitation of the large subunits of myosin, and unknowingly discarding the small subunits in the supernatant.

Large subunits and aggregates of myosin were investigated in a 3.5% acrylamide matrix with sodium dodecyl sulfate (SDS) as a dissociating agent. The basic procedure of Dunker and Rueckert (1969) was used with several modifications. This system incorporated SDS (an anionic dissociating agent), urea (a nonionic dissociating agent) and a reduction step to insure monomeric concentration.

Figure 6 contains two SDS disc gels run simultaneously under the same conditions using reduced and alkylated whole myosin. Gel A contains a 3.5% acrylamide matrix and gel B had a 5% acrylamide matrix. Both the large and small myosin subunits in the 3.5% acrylamide matrix (gel A) have migrated further than the comparable subunits in the 5% acrylamide gel. Area I on gels A and B (figure 6) are similar, however, the 5% gel appears to have excluded aggregated myosin large subunits that had previously migrated in gel A. Area II is associated with the myosin small subunits. These two gels illustrate the usefulness of the polyacrylamide SDS electrophoretic system for separation of myosin subunits.

Figure 7 depicts four 3.5% acrylamide disc gels of different myosin preparations. Gels A, C and D show reduced and alkylated

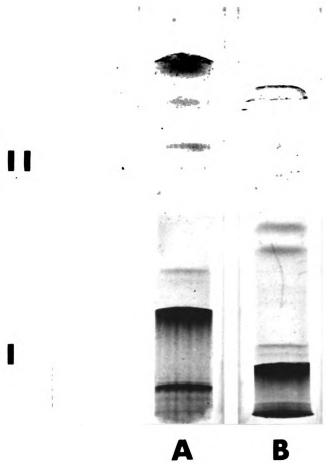


Figure 6. Disc gel electrophoretic patterns of reduced and alkylated whole myosin in SDS buffer. A = whole myosin electrophoresed on a 3.5% acrylamide matrix; B = whole myosin electrophoresed on a 5% acrylamide matrix. Region I shows the area occupied by myosin large subunits, while region II shows the area generally occupied by myosin small subunits.

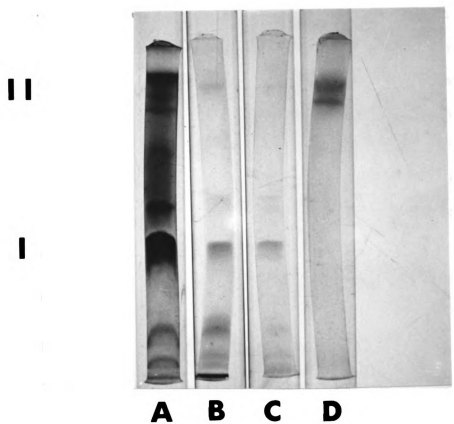


Figure 7. SDS disc gel electrophoretic patterns on 3.5% acrylamide gel of whole myosin, large subunits and small subunits. A = reduced and alkylated whole myosin; B = sulfonated large subunits; C = reduced and alkylated large subunits; and D = reduced and alkylated small subunits. Region I is the location of myosin large subunits and aggregates, while region II is the location of myosin small subunits.

whole myosin, large subunits and small subunits, respectively. Gel A contains bands in both regions I and II as would be expected for whole myosin. The large subunit preparation (gel C) has bands in region I and slight traces of the small subunits located in region II. Gel D appears to be void of bands in region I (large subunits), but has two definite bands located in the small subunit region. Also on gel D, all protein has migrated from the origin, indicating the lack of sample aggregation.

The myosin large subunits separated on gel B (figure 7) were isolated by the procedure described earlier herein. However, the thiol groups were altered by the sulfonation procedure of Paterson and Strohman (1970). A comparison of gel B, which was prepared by the sulfonation procedure of Paterson and Strohman (1970) with gel C, which was prepared by 4-vinyl pyridine alkylation shows the gels were generally similar except for the larger amount of protein at the origin on gel B. The sulfonated large subunits (gel B) migrated the same distance as the reduced and alkylated 4-vinyl pyridine large subunits (gel C). The sulfonation procedure theoretically should have resulted in an increase in the charge on the protein as compared to that on the 4-vinyl pyridine derivative. However, the SDS electrophoretic mobilities were similar. Thus, the SDS protein binding effect apparently overcame the small minor differences in protein charge.

Figure 8 illustrates SDS electrophoresis of myosin and large subunits that had been reduced with β-mercaptoethanol and alkylated with 4-vinyl pyridine. After alkylation gels B, C and D were succinylated. Gel A was a preparation of myosin large subunits that had been reduced and alkylated with 4-vinyl pyridine. The large subunits migrated as one major band with several minor aggregates. When whole reduced and alkylated myosin was electrophoresed, it had a mobility similar to that in gel A. The same protein preparation used on gel A was succinylated and electrophoresed on gels C and D. This modification of the large myosin subunits separated as one band, but the mobility was approximately one half that of unsuccinylated myosin (gel A). The lack of mobility could have been caused by failure of the two large subunits to dissociate in the SDS solvent. However, the change in mobility could be a result of the succinylation procedure, which may have altered the protein charge so that the SDS-protein complex did not overcome the differences in protein charge. Tung and Knight (1971) found that incorporation of different charges into proteins could result in different SDS electrophoretic mobilities.

All SDS 3.5% acrylamide gels separated myosin large subunits into one electrophoretic band. This observation is in agreement with those of Paterson and Strohman (1970) and Weber and Osborn

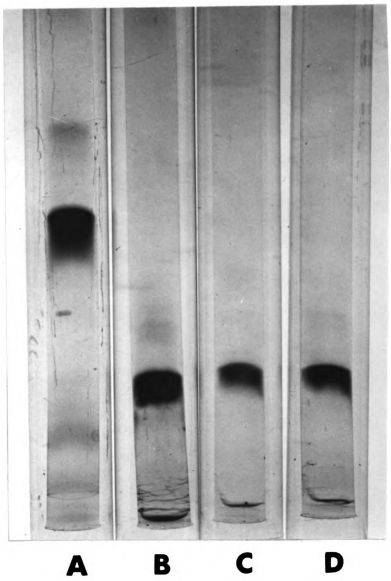


Figure 8. SDS disc gel electrophoretic patterns of whole myosin and large subunits on 3.5% acrylamide gel. A = large subunits reduced and alkylated with 4-vinyl pyridine; B = whole myosin reduced and alkylated with 4-vinyl pyridine and succinylated pH 7.0; C = large subunits reduced and alkylated with 4-vinyl pyridine and succinylated pH 8.2; and D = large subunits reduced and alkylated with 4-vinyl pyridine and succinylated pH 7.0.

(1969). The failure of the two large subunits to migrate as separate bands in a SDS electrophoretic system indicates the two subunits have similar molecular weights.

Disc gel electrophoresis with a 3.5% acrylamide matrix and 12 M urea solvent can be used to detect possible heterogeniety of myosin large subunits. Figure 9 depicts three 12 M urea gels. Gel B consists of reduced and alkylated large subunits that appear to migrate as one band. The origin of this gel shows no aggregation of protein. Gel C shows a small subunit preparation. The resultant migration rate was different than that on either gel A or B.

Figure 10 depicts the results of electrophoresing whole myosin and large subunits in a 3.5% acrylamide matrix with a 12 M urea solvent. Gels A and C were reduced-alkylated and succinylated large subunits and whole myosin, respectively. Gel A was electrophoresed for a longer period of time than gel C. Other conditions being equal this demonstrated that the mobility of the major bands is related to the length of time to which they were subjected to electrophoresis. In gels B and D the unsuccinylated products failed to migrate due to extensive aggregation.

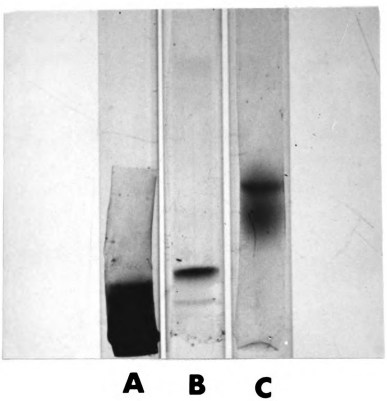


Figure 9. Disc gel electrophoretic patterns of reduced and alkylated myosin subunits on a 3.5% acrylamide matrix with a 12 M urea solvent. A = myosin large subunits (overload); B = myosin large subunits; and C = myosin small subunits.

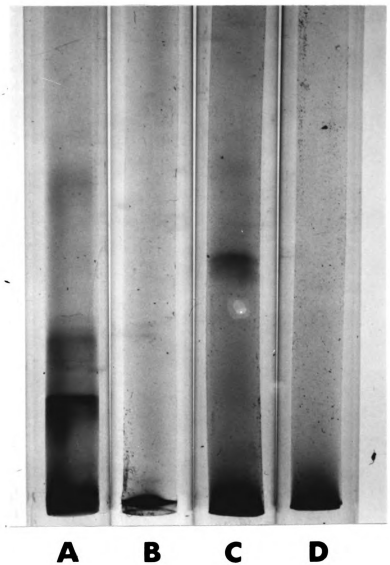


Figure 10. Disc gel electrophoretic patterns of whole myosin and large subunits on a 3.5% acrylamide matrix with a 12 M urea solvent. A = reduced-alkylated and succinylated large subunits; B = reduced and alkylated whole myosin; C = reduced-alkylated and succinylated whole myosin; and D = reduced and alkylated large subunits.

Results from electrophoresing myosin and its large subunits in a 12 M urea buffer were difficult to interpret due to poor band resolution. In all cases the myosin large subunits migrated as one band, which is in agreement with the results of Small et al. (1961) and Heywood et al. (1967). Both studies also utilized 12 M urea on a polyacrylamide matrix. In common with results of the present study long time periods were required to obtain mobility of the protein.

Figure 10 shows that the succinylated myosin products migrated while the comparable unsuccinylated products failed to migrate. This was probably due to the negative charge induced by succinylation on the lysine ε-amino group of the large subunits, which repel each other, thereby, resulting in less aggregation. Therefore, more mobility on acrylamide gel would be achieved. In the succinylated samples, only one major band was associated with the large subunits. Since the subunits migrated as one band in 12 M urea, the two myosin subunits are probably similar in charge, or are undissociated from one another.

Urea was not only used in the reduction-alkylation procedure for myosin, but also in both the isoelectric focusing and the urea electrophoretic systems. The formation of cyanate ion in urea solutions has been well documented by Stark et al. (1960). The cyanate ion is in equilibrium with urea and shifts to ion formation at basic pHs. The cyanate ion is undesirable since it reacts with protein,

resulting in carbamylation of free amino groups and labile functional groups.

Preparation of urea concentrations below 10 M involved recycling over a resin bed until the conductivity was significantly reduced. An example of the results from the recycling procedure for urea is shown in table 2. The data indicate that ions can be removed, but are reformed upon further storage. Several agents were added to urea, but failed to decrease ion formation during storage. A large amount of variability in the initial conductivity of the uncycled urea was observed.

Table 2. Conductivity of 10 M Urea After Cycling Over Resin At Room Temperature.

	μmhos per cm
Uncycled	37.8
One cycle	7.2
Two cycles	6.5
Three cycles	3.9
Four cycles	1.88
Four cycles stored 24-48 hr	258.
Glass distilled water (reference)	1.3

The 12 M urea solutions used for isoelectric focusing and electrophoresis were made from solid urea immediately prior to use. Whenever possible ultrapure urea (Mann-Schwartz Biochemical Co.) was utilized. Since no method was found to completely eliminate cyanate ion, attempts were made to minimize its formation by using fresh urea solutions, using ultrapure urea, recycling low molarity urea over resin and minimizing running times.

Amino Acid Composition of Myosin

The amino acid analysis of myosin is shown in table 3. The values in column A are means for back muscle from two rabbits taken in duplicate. The amino acid composition of myosin from rabbit back muscle (column A) compares favorably with other values for rabbit myosin (columns B, C, D, E). Problems were encountered in the analysis of methionine, resulting in the high standard deviations. Since the samples were reduced and alkylated with 4-vinyl pyridine, cysteine was analyzed as S-β-4 (pyridylethyl)-L-cysteine (PEC). Results of the amino acid analysis of myosin indicated that myosin from rabbit back muscle was similar in composition to other rabbit myosins.

Table 3. Amino Acid Composition of Whole Myosin Expressed Per 1,000 Residues

	A[1]	B[1]	C[2]	D[2]	E[2]
Aspartic Acid	96.36±2.36	98.1±1.8	101.1	99.1	98.5
Glutamic Acid	177.30±6.44	181.1±4.0	184.3	180.8	182.0
Threonine	42.40±4.19	51.0 ±0.3	48.7	47.8	51.0
Serine	51.71±1.28	46.9±0.1	48.7	42.0	45.2
Proline	28.96±2.67	23.1±0.4	26.1	25.7	25.5
Alanine	89.62±1.12	90.9±4.7	92.7	87.5	90.4
Glycine	64.06±4.89	51.3±0.4	46.4	50.1	46.4
Valine	61.51±0.82	50.6±1.1	45.1	54.8	49.8
Methionine	18.90±6.61	23.6±2.1	26.1	26.8	26.7
Isoleucine	50.92±2.01	50.2±2.8	49.9	50.2	48.7
Leucine	95.37±3.44	93.5±6.5	94.0	91.0	93.9
Tyrosine	23.22±2.83	19.2±1.3	21.4	22.2	23.2
Phenylalanine	30.91±1.66	34.9±1.5	32.2	31.5	33.6
Lysine	93.97±5.52	99.5±9.4	101.1	103.8	106.6
Histidine	16.14±1.82	21.4±1.0	17.9	18.7	18.5
Arginine	51.70±1.54	53.0±1.7	48.7	50.1	49.8
Tryptophan	N.C.[5]	3.7	4.7	7.8	
Cystine/2[3]		7.8±0.6	10.8	10.3	10.2
PEC[4]	5.51±1.24				
(NH_2)	N.C.[5]	(175.0±6.0)	(102.3)	(112.0)	(106.6)

A = Myosin from rabbit back muscle, two samples in duplicate.
B = Bodwell et al. (1971).
C = Kominz et al. (1954).
D = Bárany et al. (1964).
E = Lowey and Cohen (1962).
[1]Mean values plus or minus the standard deviation.
[2]Values were recalculated per 1000 residues.
[3]All derivatives of cysteine expressed as cystine-2 equivalents.
[4]PEC is s-β-4 (pyridylethyl)-L-cysteine
[5]N.C. indicates no calculation.

Table 4 presents the amino acid composition for whole myosin, large subunits and small subunits after reduction and alkylation. Columns A, B and C show whole myosin, large subunits and small subunits, respectively. The amino acid composition of whole myosin is similar to the values for whole myosin given in table 3. The myosin large subunits are also similar in amino acid composition to the whole myosin values. This would be expected since the two large subunits compose approximately ninety percent of the myosin molecule.

The small subunit amino acid composition (table 4, column C) shows several striking features, including a high phenylalanine to tyrosine ratio (3:1), a high proline content in comparison to whole myosin, and low values for most of the basic amino acids. The myosin small subunit data is in general agreement with that of Oppenheimer et al. (1966) and Kominz et al. (1959).

Chromatographic Separation of Myosin Subunits

Figure 11 shows an example of a Sephadex G-100 column with a 5 M LiCl buffer for separation of small from large myosin subunits. Reduced and alkylated whole myosin was chromatographed and resulted in three separate peaks. After column separation, each peak was dialyzed against an appropriate buffer and electrophoreses as a check for purity. Peak 1 (large subunits) contained some small subunits. Peak 2 (small subunits) contained a high molecular weight

Table 4. Amino Acid Composition of Reduced and Alkylated Whole Myosin, Large Subunits and Small Subunits Expressed per 1,000 Residues.

	A[1]	B[2]	C[1]
Aspartic Acid	118.05	90.25	94.86
Glutamic Acid	184.83	195.50	163.86
Threonine	51.16	59.02	56.52
Serine	47.11	48.31	53.37
Proline	26.98	22.89	48.90
Alanine	87.33	91.94	111.59
Glycine	45.83	52.00	91.22
Valine	46.50	51.93	63.68
Cystine	---	---	---
Methionine[5]	27.53	18.92	32.85
Isoleucine	49.84	49.42	52.02
Leucine	93.20	97.29	83.65
Tyrosine	21.71	22.34	11.59
Phenylalanine	32.17	31.02	35.44
Lysine	90.1[4]	92.3[4]	75.33
Histidine	18.59	18.60	6.94
Arginine	51.11	51.91	19.05
PEC[6]	7.92	6.11	N.C.[3]

A = Whole myosin.
B = Large subunits of myosin.
C = Small subunits of myosin.
[1]Data presented as average values.
[2]Data presented as average values from large subunits alkylated in different dissociation agents.
[3]N.C. indicates no calculation.
[4]Based on an assumed lysine value of 90.1 and 92.3 for calculations.
[5]Problems were encountered in determining methionine.
[6]S-β-4(pyridylethyl)-L-cysteine

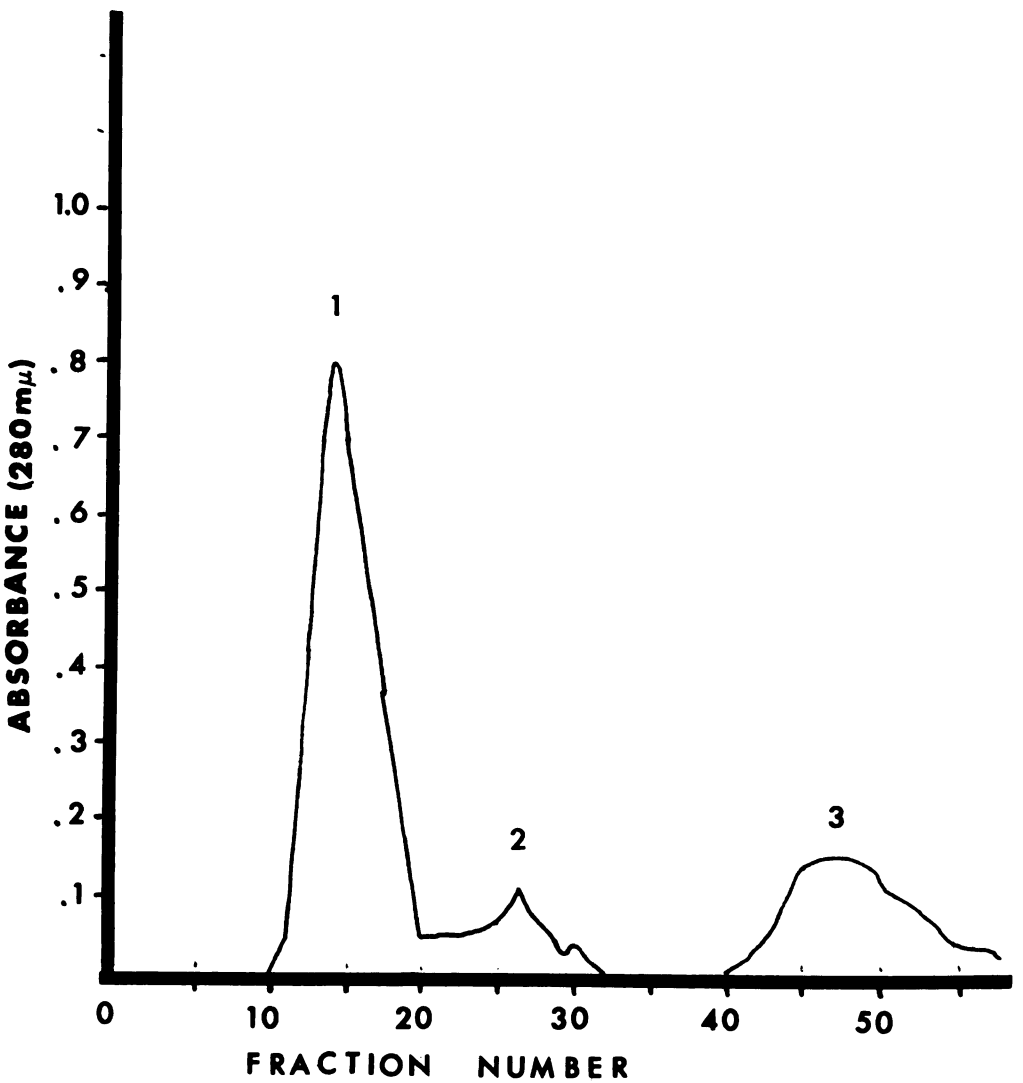


Figure 11. Elution profile from reduced and alkylated whole myosin from a Sephadex G-100 column using a 5 M LiCl, 0.025 M tris, 0.001% ETDA, pH 7.5 buffer. Solid LiCl was added to the sample to make 5 M. Peaks are designated numerically.

protein, while peak 3 contained an unidentified compound. Results with several different columns (Sephadex G-100, 5 M LiCl buffer) indicated that complete separation of large and small subunits did not occur and are in agreement with those of Paterson and Strohman (1970). However these results are in contrast to those of Stracher (1969) who reported that myosin large and small subunits could be separated by a Sephadex G-200 column with a 5 M LiCl buffer.

In the present study some problems with aggregation of protein were encountered with the LiCl buffer. It is also questionable whether separation with 5 M LiCl on a Sephadex-200 column can be achieved rapidly enough to preserve ATPase activity after recombination of the subunits.

Several attempts were made to dissociate myosin subunits on Sephadex G-150 columns with an 8 molar urea, β-mercaptoethanol buffer. This system was difficult to handle due to the high concentrations of urea and problems caused with the baseline by β-mercaptoethanol. Electrophoresis of the peaks indicated that some small subunits were not separated from the large subunits.

A clean separation of myosin large and small subunits was achieved on a Sephadex G-150 column using a 5 M guanidine-HCl solvent. Figure 12 shows whole myosin chromatographed in the 5 M guanidine-HCl solvent. The leading portion of peak 1 was dialyzed and electrophoresed. Results indicated that it contained pure large

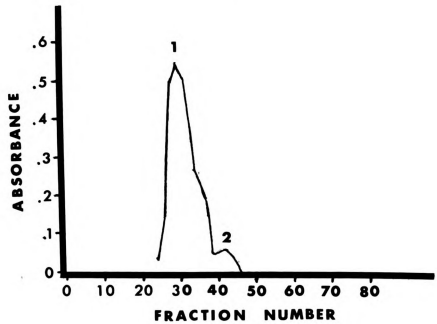


Figure 12. Elution profile of reduced and alkylated whole myosin on a Sephadex G-150 column with a 5 M guanidine-HCl, 0.1% β-mercaptoethanol, 0.005 M EDTA, 0.05 M KCl, pH 7.0 buffer. Protein assayed by the TCA precipitation method of Davison (1969). Peaks are designated numerically.

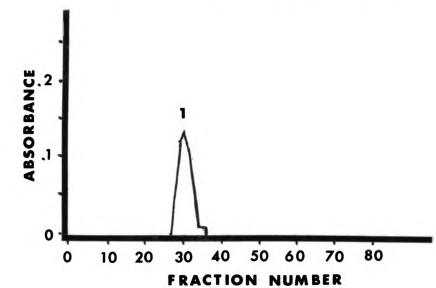


Figure 13. Elution profile of the leading edge of peak 1 figure 12. Conditions are identical to those in figure 12.

subunits, and was not contaminated by small subunits. The second half of peak 1 contained a fast migrating contaminant, while peak 2 contained a minimum of three bands on 7% electrophoretic gels.

Figure 13 shows the large subunits from the leading edge of peak 1 (figure 12) after they had been rechromatographed on the same column under identical conditions. The resultant peak is more symmetrical, while peak 2 is missing in figure 13. These results indicate that pure large subunits of myosin can be obtained by using guanidine-HCl chromatography.

Although guanidine-HCl column chromatography can be used to produce pure myosin subunits, several major problems were encountered. First, the chromatography procedure is time consuming. Secondly, enzymatic activity is destroyed due to the dissociating properties of the solvent. Thirdly, artifacts may be produced and gradient chromatography is eliminated due to the high ionic strength of the solvent.

The results of this study indicate that the 5 M guanidine-HCl chromatographic procedures of Gazith *et al*. (1970) and Kuehl and Adelstein (1970) yielded pure large subunits. Other columns employed during this study did not result in purified large subunits. In addition, the various precipitation methods of separating large and small myosin subunits did not result in a purified large subunit fraction. Although the recycling pH precipitation method of purifying

large subunits removed almost all of the small subunits, only the guanidine-HCl column resulted in a preparation that did not contain any small subunits as shown by electrophoresis on a 7% disc gel. The guanidine-HCl method removed aggregated small subunits from large subunits by dissociation (guanidine-HCl and β-mercaptoethanol) and subsequently separating the subunits on the basis of size.

Purification of myosin small subunits is much easier to achieve than that of large subunits. This is accomplished by dissociating the myosin complex, followed by adjustment of ionic strength so that the large subunits, whole myosin and aggregated small subunits can be precipitated. The unaggregated small subunits are left in solution. Several cycles of this procedure yielded a small subunit fraction void of large molecular weight contaminants. Further ion exchange gradient chromatographic purification of the small subunit fraction is shown in figure 14. The small subunit fraction used for purification contained three small subunits when electrophoresed on a 7% gel. Peak 1 contained two bands, while peak 2 contained the third band.

Since the ion exchange gradient contained small amounts of protein, the eluate was analyzed by a quantitative ninhydrin test. The resultant gradient was uniform as indicated by the linear conductivity readings. The two myosin subunits were eluted as one peak. Column results indicated that gradient chromatography was a useful

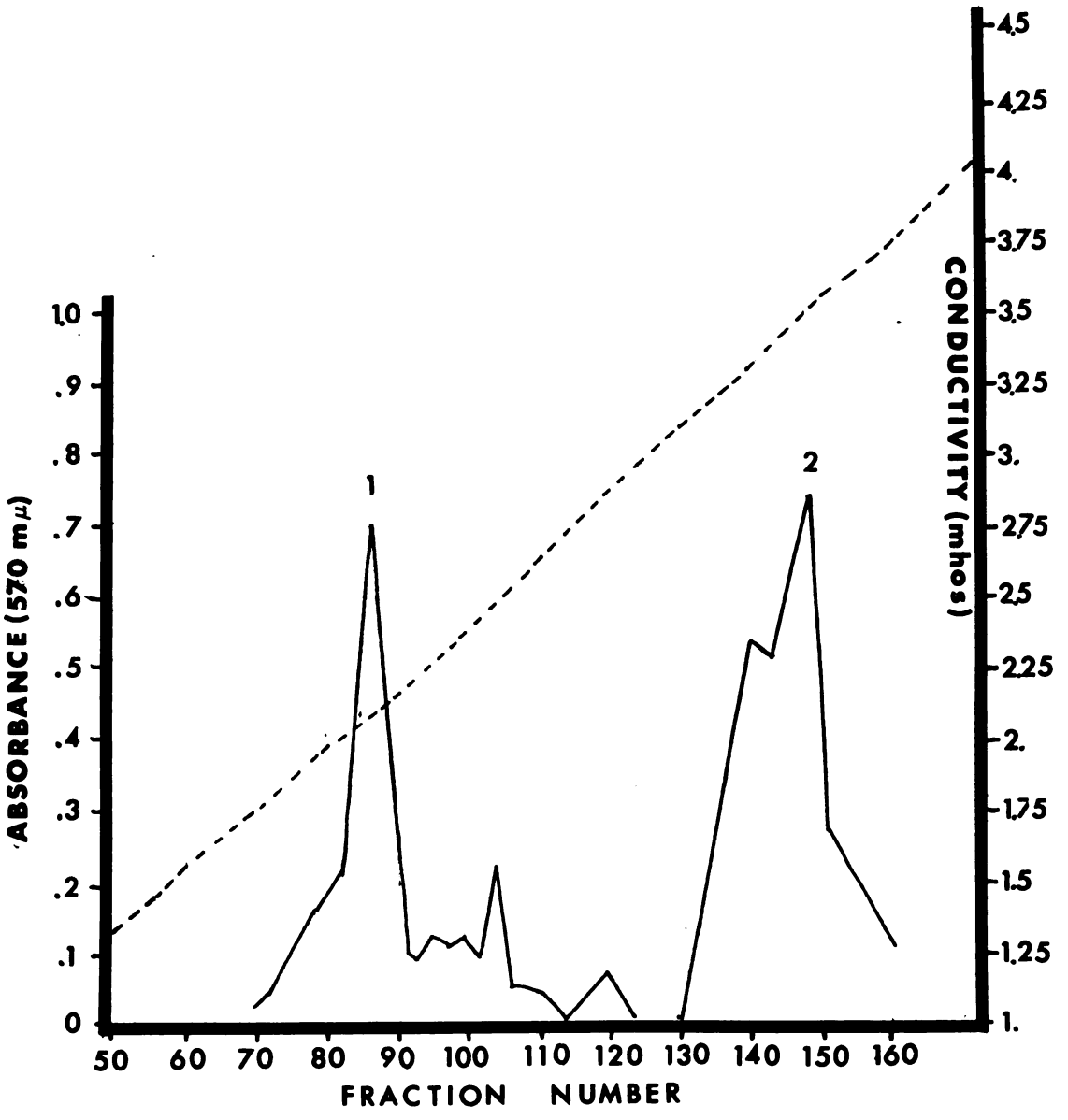


Figure 14. Elution profile from myosin small subunits. Gradient is from 0.05 to 0.75 MKCl on a Sephadex DEAE A-25 matrix. Protein assayed by quantitative ninhydrin reaction. Peaks designated numerically.

method for purification of small subunits, which agreed with reports by Weeds (1969), Perrie and Perry (1970), Locker and Hagyard (1967a) and Gaetjens et al. (1968). Due to different gradients, buffers, pHs and protein preparations, however, it is difficult to compare the subunit contents of the various peaks from different studies.

End Group Analysis of Myosin

Hydrazinolysis experiments were carried out using the basic procedure of Fraenkel-Conrat and Tsung (1967). Table 5 gives the results of hydrazinolysis of myosin large subunits and shows the resultant amino acids and two unknown components. In an attempt to identify the unknown fractions, a portion of the sample was subjected to electrophoresis on thin layer plates. Unknowns 1 and 2 migrated rapidly and were obviously not free amino acids. Although the two unknown components were not identified, it is probable that they were hydrazides.

Table 5 shows that hydrazinolysis of myosin large subunits resulted in the release of threonine, serine and glycine. All three of these amino acids increased with increasing periods of hydrazinolysis, although only in submolar amounts. The low yields of free amino acids suggests that the C-terminus of myosin large subunits is not composed of a single amino acid. Inconsistencies in the data made it difficult to ascertain the exact composition of the carboxy terminal group.

Table 5. Yields of Amino Acids on Hydrazinolysis of Alkylated Myosin Large Subunits at 100°C at Different Times[1]

	8 hr	16 hr	24 hr
Unknown 1[2]	(0.26)	(0.57)	(0.76)
Threonine	0.08	0.11	0.18
Serine	0.14	0.23	0.81
Glycine	0.01	--	0.41
Unknown 2[3]	(0.12)	(0.25)	(0.46)

[1]Yields corrected for blanks (using 24 hr value) and adjusted with norleucine. They are expressed as moles of amino acid per mole of protein assuming a molecular weight of 210,000 for myosin large subunits.
[2]Unknown in the approximate position of glutamic acid on chromatograph; values calculated using "C" value for glutamic acid.
[3]Unknown eluting near the "cysteine" position and having absorption profile similar to cysteine. Cysteine "C" values were used for calculations.

The amino acid analysis on hydrazinolysis of whole myosin is shown in table 6. Analysis of the sample indicated the presence of two unknown peaks and some seven amino acids. In order to ascertain the nature of the unknown peaks, they were subjected to thin layer electrophoresis. Results showed that the unknowns migrated more rapidly than the corresponding free amino acids and suggested they were not amino acids.

The isoleucine data was of interest since it became larger as time at 100°C was increased (figure 6). The theoretical value of two moles of isoleucine per mole of myosin was not obtained. The low value for isoleucine was probably produced from incomplete reaction

Table 6. The Yield of Amino Acid on Hydrazinolysis of Alkylated Whole Myosin at 100 and 110°C[1]

	100°C			110°C
	8 hr	16 hr	24 hr	8 hr
Unknown 1	0.48[4]	---[3]	0.49[4]	0.24[4]
Glutamic Acid[2]	(0.09)	(0.08)	---	(0.27)
Threonine	---	---	0.05	---
Serine	0.13	0.12	0.18	---
Alanine	0.12	0.12	0.15	---
Glycine	0.13	0.09	0.18	---
Unknown 2	0.73	0.64	0.80	1.37
Valine	---	---	0.11	---
Isoleucine	0.13	0.19	0.38	---

[1]Yields corrected for blanks (using 24 hr value) and adjusted with norleucine. They are expressed as moles of amino acid per mole of protein assuming a molecular weight of 480,000 g per mole.
[2]Glutamic acid region had 2 or 3 peaks, one of which was calculated as glutamic acid.
[3]No calculation, two peaks.
[4]Single peak, calculations using glutamic acid "C" values.

of the protein with hydrazine, and the lack of a full compliment of small subunits upon initiation of the reaction. Furthermore, the 110°C sample was probably contaminated with hydrazides making analysis difficult.

Locker (1954) used the hydrazinolysis procedure to determine the C-terminal group of whole myosin. His results were not quantitated but revealed small amounts of glutamic acid, serine, alanine, isoleucine and traces of glycine. Results of the present hydrazinolysis study agreed for the most part with that of Locker (1954). Although the present investigation attempted to quantitate the results

of hydrazinolysis, no single amino acid was consistently hydrolyzed from the carboxy terminus of myosin large subunits. However, small amounts of glutamic acid, threonine, serine, alanine and glycine were all found. The hydrazinolysis procedure is specific for the C-terminal of myosin (Fraenkel-Conrat and Tsung, 1967), but has a number of drawbacks, such as complicated time-temperature relationships, the necessity for imposing anhydrous conditions, low yields, difficulties in complete removal of hydrazides and incomplete reaction with the proteins.

Carboxypeptidase A and B were used to determine the C-terminal end group for whole myosin and its large subunits utilizing the procedure of Ambler (1967). DFP (diisopropylfluorophosphate) treated enzymes were purchased to eliminate tryptic and chymotryptic activity. Both whole myosin and myosin large subunits were reduced, alkylated and succinylated. The succinylation step was necessary to solubilize myosin in low ionic strength solutions.

Whole myosin was subjected to simultaneous carboxypeptidase A and B cleavage, and aliquots were removed at various time intervals as shown in figure 15. Initially isoleucine appeared to be released from myosin in greater amounts than the other amino acids. Approximately two moles of isoleucine were released per mole of whole myosin in the initial stages of cleavage. Although alanine, leucine, valine, phenylalanine and tyrosine were released from whole myosin,

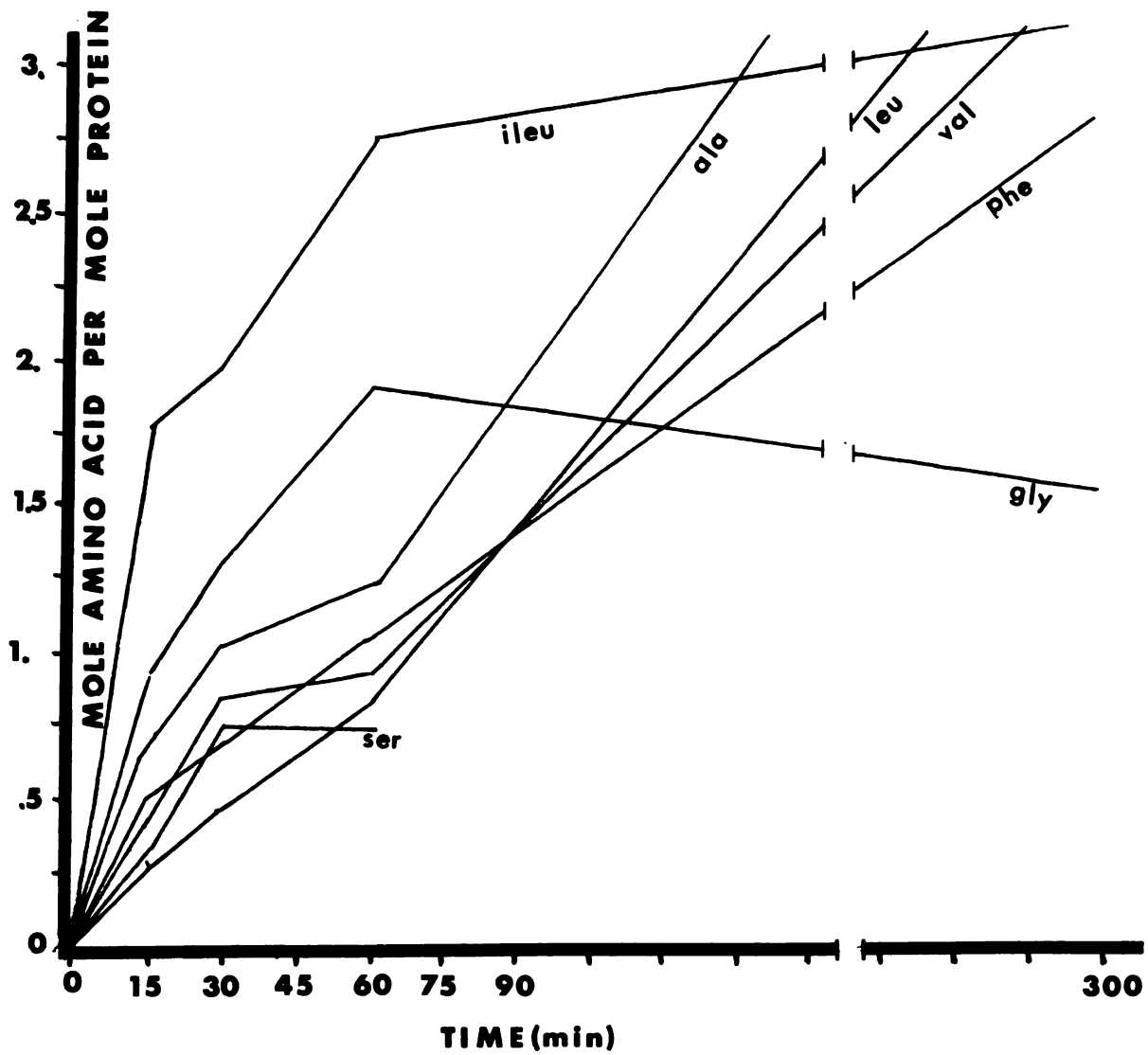


Figure 15. Amino acids released by carboxypeptidase A and B enzymic action on reduced, alkylated and succinylated whole myosin. Results were corrected for both the protein and enzyme blanks. The enzyme to protein ratios (w/w) were 1:25 for carboxy peptidase A and 1:100 for carboxypeptidase B. A molecular weight of 460,000 g per mole of myosin was assumed.

they were present in submolar quantities. By 300 min these amino acids had been released in large quantities, possibly from the action of endopeptidases.

The release of carboxy terminal amino acids from myosin large subunits is shown in figure 16. Upon initiation of the carboxypeptidase A and B reaction, the amino acids were released very slowly. After 60 min, however, valine, alanine, leucine, serine and glycine were all present in molar quantities.

Results from the carboxypeptidase C-terminal analysis indicated that whole myosin contained approximately two moles of isoleucine per mole of protein. Since isoleucine is the C-terminal residue of the myosin small subunits (Weeds, 1967), the present data indicated that myosin contains two small subunits in each molecule.

C-terminal analysis of myosin large subunits using carboxypeptidase A and B failed to yield interpretable data. Combined results from several carboxypeptidase experiments indicated that submolar quantities of alanine, glycine, leucine, valine, serine and phenylalanine were released. The fact that molar quantities of these amino acids were not released when the reaction was initiated may indicate that the C-terminal is not accessible to the enzymes. Other possible explanations could be the release of ninhydrin negative compounds, a cyclic structure, or a C-terminal proline residue, all of which would not be identified by the methods used.

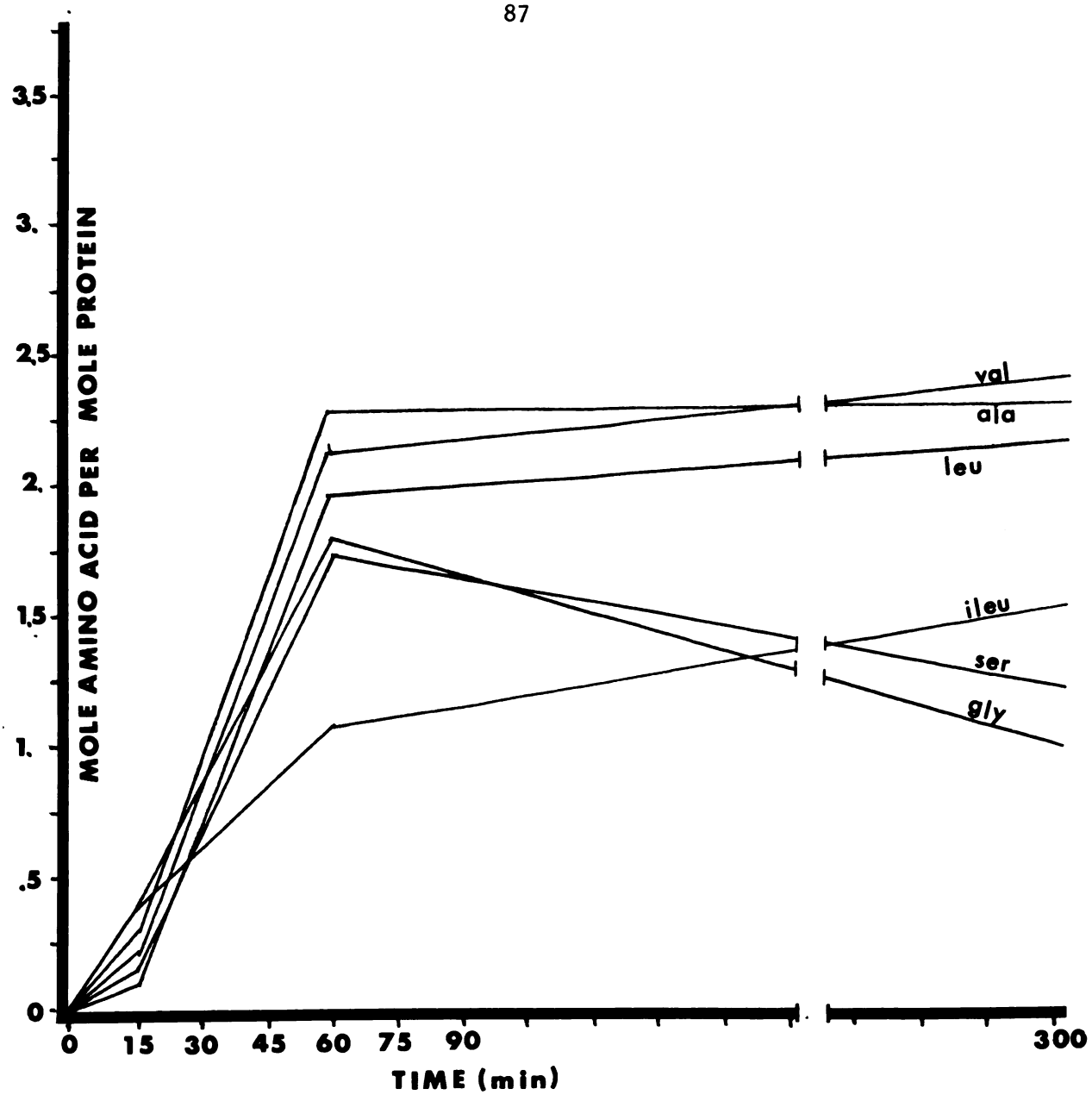
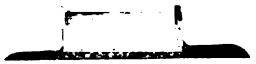


Figure 16. Amino acids released by carboxypeptidase A and B from the reduced, alkylated and succinylated myosin large subunits. Results were corrected for both the protein and enzyme blanks. The enzyme to protein ratios (w/w) were 1:25 for carboxypeptidase A and 1:100 for carboxypeptidase B. A molecular weight of 210,000 g per mole of myosin large subunits was assumed.

Carboxypeptidase end group analysis was complicated by the large amounts of myosin required, and the large amounts of enzymes necessary to obtain a reactive system. In addition, separation problems were encountered due to the high molecular weight of myosin.

Two methods of determining the C-terminal end group of myosin large subunits were utilized. Both studies failed to yield any single amino acid as the major constituent of the C-terminal group of myosin large subunits. Gershman et al. (1969) also attempted to elucidate the C-terminal residue of myosin large subunits using carboxypeptidase A, but failed to identify possible C-terminal residues. They did, however, refer to some unpublished data indicating that several amino acids were found in submolar quantities. Similarly, in the present study myosin large subunits yielded submolar quantities of some six amino acids.



CONCLUSIONS

Isoelectric focusing on polyacrylamide gel was used for determining the isoelectric points and for detecting heterogeneity of whole rabbit myosin and its small and large subunits. Electrofocusing was done in the presence of 12 M urea at 43°C using either 3.5 or 5% acrylamide as a supporting matrix. The large subunits focused as one or two bands, however, the composition of the bands was not determined. The large subunits had isoelectric points between pH 6.4 and 7.2. Small subunits focused as three or more bands, having isoelectric points between pH 4.9 and 5.3. When whole myosin was isoelectrofocused, it dissociated into both large and small subunits, which focused at their respective isoelectric points.

Myosin large subunits were separated from the small myosin components by a cycle pH adjustment and precipitation. Myosin large subunit separation by column chromatography on Sephadex G-100 using either a 5 M LiCl or an 8 M urea buffer did not yield pure subunits. Pure large subunits were obtained only by using a 5 M guanidine-HCl solvent on a Sephadex G-100 column.

Since large subunits did not electrophoretically migrate on the conventional 7% gel matrix, a 3.5% acrylamide matrix was used with an SDS urea solvent. In this system the dissociated myosin large subunits migrated as one band. Since the electrophoretic mobility of proteins in a SDS solvent is proportional to molecular

weight, and the two myosin large subunits migrated as one band, they appear to have similar molecular weights.

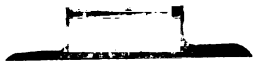
Myosin large subunits were then subjected to electrophoresis on a 3.5% acrylamide gel in a 12 M urea solvent. The large subunits migrated as a single band. Since the electrophoretic mobility in a 12 M urea solvent is proportional to the charge on the proteins and the myosin large subunits migrated as a single band, the two large subunits appear to have similar charges.

End group analysis of myosin large subunits was investigated using both hydrazinolysis and cleavage with carboxypeptidase A and B. Neither hydrazinolysis nor carboxypeptidase cleavage yielded any significant quantities of C-terminal amino acids. However, submolar quantities of alanine, leucine, valine, glycine, serine, and phenylalanine were released. Attempts to identify the end groups of the myosin large subunits were unsuccessful.

Disc gel electrophoresis of reduced and alkylated myosin small subunits on a conventional 7% acrylamide gel resulted in three distinct bands. Alkylation of the small subunits with different agents did not alter the bands. On the 7% acrylamide gel system, the large subunits appeared to bind two of the small subunits more tightly during cyclic preparation.

Electrophoresis of myosin small subunits in the presence of SDS resulted in three bands. It was, therefore, concluded that these bands had different molecular weights.

The amino acid composition of myosin small subunits was different from that of either whole myosin or the large subunits. The small subunits had a high phenylalanine to tyrosine ratio (3:1) and an appreciable proline content. Amino acid analysis of products released from the end groups by carboxypeptidase A and B cleavage of whole myosin yielded two moles of isoleucine per mole of myosin. Since the C-terminal residue to the small subunits is isoleucine, and the carboxypeptidase A and B cleavage of large subunits did not release appreciable quantities of isoleucine, it was concluded the isoleucine was derived from the small subunits. These results indicated that each mole of myosin contains two moles of small subunits.



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