STUDIES ON BOVINE MYOSIN

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CHAPTER I

INTRODUCTION

Beef products currently occupy an enviable position in the marketplace. However, considerable consumer resistance to the rising cost of beef products as well as competition from plant and "synthetic" protein foodstuffs may well ensue if current practices remain unaltered. Thus, new methods for handling and fabricating beef carcasses, which would increase marketing efficiency and enhance the quality of retail beef, must be developed and implimented. To accomplish this task, however, the need for more detailed information on the basic biochemical make-up of beef muscle and the post-mortem alterations occurring therein, has become apparent.

From the standpoint of market quality in beef, an area of primary interest centers around the various interactions of the major fibrillar protein, myosin, during the development of rigor and the subsequent post-mortem changes which occur during the "aging" process. From the practical point of view, it would be desirable to maintain myosin in the "free" state post-mortem. The importance of this is two-fold: 1) an improvement in tenderization and juiciness of retail block beef could be obtained without the necessity of prolonged aging and 2) myosin acts as the primary emulsifying agent in sausage meat items, and as such, has a strong influence on the quality of the final product.

The objectives of this study were to develop procedures which

could be used to evaluate the role of myosin in the various post-mortem quality changes occurring in beef muscle, and to investigate the effect of various chemicals on the inhibition of myosin ATPase activity. These objectives were considered to be pre-requisite to the development of methods which could lead to the control of the formation of the rigid complex, actomyosin, which occurs post-mortem.

CHAPTER II

LITERATURE REVIEW

The review to follow is divided into the following general areas: the structure of myosin; the biological function of myosin and the excitation of the contractile system.

Structure of Myosin

Molecular Weight

There has been considerable variation in the reported values for the molecular weight of myosin isolated from rabbit skeletal muscle. These values have ranged from 420,000 to 860,000 (von Hippel *et al.*, 1958, and Portzehl, 1950, respectively). It is now recognized that myosin readily undergoes spontaneous aggregation, and this phenomenon has been the primary reason for the diverse results. Holtzer (1956), working with myosin isolated from rabbit skeletal muscle, observed that the aggregation of myosin followed a step-wise process (i.e., dimers, trimers, etc.) in which the molecules joined side-to-side. The rate of aggregation was found to increase markedly with temperature and occurred about ten times as rapidly at 25°C. as at 4°C. Lowey and Holtzer (1959) conducted further studies on this aggregation reaction by investigation of the type of bonding involved and whether the reaction could be inhibited. Neither metal chelation nor intermolecular disulfide bonds were proven to be involved. Inorganic phosphate

was the most effective inhibitor tested. They suggested that the inhibitory action was caused by anion binding and the subsequent enhanced repulsion of individual protein molecules. Connell (1960), working with cod myosin, found that aggregation of myosin was minimal between pH 7.0 and 8.0. He found that the rate of aggregation of cod myosin increased at higher ionic strengths, which was in agreement with Lowey and Holtzer's (1959) observation that the rate was greater in 1.5 than in 0.6M KCl. Johnson and Rowe (1961), in their sedimentation and light-scattering experiments with rabbit skeletal muscle myosin, established evidence to support the suggestion by Holtzer (1956) that aggregation of myosin occurs through an initial side-to-side dimerization. The solubility of myosin, which was incubated at 25°C., showed rapid changes during this monomer to polymer transformation. A large salt-soluble and a smaller water soluble fraction were produced. Since the actin-combining activity was confined to the water soluble fraction, they suggested that this fraction might correspond to a native subunit.

Other factors have also been found to influence the molecular weight value obtained. In fact, the results from current research has indicated that the molecular weight for myosin in any particular experiment is dependent upon: 1) the method used in determining the molecular weight and 2) the method employed in isolating myosin.

Using the light scattering technique, Holtzer and Lowey (1959) reported a molecular weight of 493,000. Later, as a result of critical reappraisal and redetermination, Holtzer et al. (1962) obtained a value of 525,000. Using this same light-scattering technique, Brahms and Brezner (1961) determined a molecular weight of 440,000. All above values were obtained on myosin isolated from rabbit skeletal muscle by

a KCl salt extraction procedure. Gellert and Englander (1963) obtained a molecular weight of 520,000 on myosin prepared by ammonium sulfate fractionation.

Using the Archibald approach to sedimentation equilibrium, Kielly and Harrington (1960) reported a value of 619,000 for myosin isolated by ammonium sulfate fractionation. However, Lowey and Cohen (1962) reported a molecular weight of 470,000 $\pm$ 25,000 for myosin isolated by KCl salt extraction. Part of the discrepancy between the latter two groups was explained by the investigation of Mueller (1964). He demonstrated that the apparent molecular weight is properly referred to the meniscus concentration rather than to the original concentration of sample placed in the ultracentrifuge cell, as was done by Lowey and Cohen (1962). From his experiments, Mueller (1964) determined a molecular weight of 524,000 for rabbit myosin isolated by KCl salt extraction. Yet, reassessment of the 619,000 value obtained by Kielley and Harrington (1960) did not result in a large change, but did give a somewhat lower value of 597,000 (Kielley, 1965).

Thus, there was still quite a discrepancy between values obtained using the light-scattering technique as well as between the values obtained by Archibald and light-scattering techniques. However, Tominatsu and Palmer (1963) observed that the proper light reflection for the standard Brice-Phoenix cylindrical cell is quite different from the customary correction. To illustrate the importance of a proper reflection correction, Tominatsu (1964) applied the revised correction to the Gellert and Englander (1963) data, and calculated a molecular weight of 600,000. The revised data of Brahms and Brezner (1961) would increase the calculated molecular weight value to about 500,000.

However, the data of Holtzer and Lowey (1959) would not require revision since they used a different kind of light scattering cell. Tominatsu (1964) noted that values of molecular weight were now about 600,000 for all myosin preparations employing ammonium sulfate fractionation.

In other work on myosin not subjected to the ammonium sulfate precipitation step, Tonomura et al. (1966) reported a molecular weight of 470,000 - 510,000 which was based on osmotic pressure and low speed sedimentation equilibrium.

Thus, it appears that myosin isolated from rabbit skeletal muscle by the KCl salt extraction procedure yields molecular weight values of about 500,000, and that myosin prepared by ammonium sulfate fractionation has a molecular weight of about 600,000. A possible explanation for this difference may be contained in the discussion of Stracher and Dreizen (1966). They indicated that myosin prepared by the KCl salt extraction procedure usually showed a single, sharp peak on sedimentation; whereas, myosin prepared by ammonium sulfate fractionation exhibited a discrete leading peak, indicating aggregation of about 5 to 10% of the total protein. Thus, ammonium sulfate fractionation seems to enhance aggregation of myosin, and this effect presumably accounts for the higher (weight average) molecular weight values reported for myosin prepared by this method.

Substructure of Myosin

Two approaches have been taken in the study of the physical substructure of myosin. One technique has been to study the subunits derived by the use of various dissociating reagents which serve to

modify or disrupt the non-covalent bonds involved in the native myosin. Various dissociating reagents and conditions have been employed such as high concentrations of urea and guanidine hydrochloride, alkaline pH, succinylation, acetylation and carboxymethylation. The last three procedures add negatively charged groups to the free amino groups on the protein. Urea and guanidine act on water structure, hydrophobic, and hydrogen bonding; whereas, alkaline pH acts through electrostatic charging of basic residues (Kauzmann, 1959; Gordon and Jencks, 1963). The other technique has been concerned with the study of the products obtained by controlled proteolytic digestion of myosin.

Subunits Obtained with Dissociating Reagents

Kielley and Harrington (1960) reported that the molecular weight of each subunit liberated on unfolding of the parent molecule in 5M guanidine-HCl was 206,000. Based on the molecular weight for native myosin of 619,000, it was suggested that three polypeptide chains of equivalent mass were associated to form one molecule of myosin. These workers indicated that each chain was folded into the α-helical configuration and arranged side-by-side in a linearly extended fashion. They pictured the three α-helices as wound about each other as in a three-stranded rope, which was folded over at the end where the molecule thickened into a globular head. These researchers later revised their molecular weight estimates of myosin and the subunit to 597,000 and 197,000 respectively, but they did not alter their conclusion that myosin was composed of a three-stranded substructure (Kielley, 1965). Small *et al.* (1961) concluded that urea concentrations, of the order of 12M, were required to obtain complete dissociation of myosin, as judged

by ultracentrifuge patterns and gel electrophoresis. The subunit molecular weight obtained was 180,000. A slow moving component was evident in sedimentation patterns at urea concentrations of 2-8M and in 5M guanidine-HCl; however, this was considered to be a contaminant, as it could be removed by repeated ammonium sulfate fractionation.

On the other hand, Tsao (1953) using the ammonium sulfate fractionation procedure did recognize a minor component dissociated from myosin with 6.7M urea. In addition, Kominz et al. (1959) found a subunit of molecular weight 29,000 which was obtained from myosin by bicarbonate treatment at an alkaline pH, which seemed closely related to the protein released from myosin by concentrated urea. More recently, Dreizen et al. (1966 and 1967) observed that myosin dissociated in 5M guanidine-HCl into a light fraction, which represented about 10-15% of the total protein and had a molecular weight of about 20,000, and a heavy component, which had a molecular weight of about 200,000. Gershman et al. (1966) reported that myosin dissociated into a light and heavy component at a pH above 9.5. The light alkali component had a molecular weight of 20,200 and represented 11.6% of the total protein. Although the light alkali component was monodisperse in sedimentation velocity experiments, it was electrophoretically heterogeneous, usually exhibiting three bands on cellulose acetate. The heavy alkali component indicated a molecular weight of 430,000 which dissociated in 5M guanidine-HCl into subunits of about 215,000 molecular weight.

Oppenheimer et al. (1966 and 1967) have isolated a low molecular weight component from succinylated myosin which was located in the globular head portion of the molecule. Locker and Hagyard (1967) depolymerized myosin by increasing its negative charge through

acetylation and carboxymethylation. Both types of substitution dissociated 15% of the total protein into small subunits, while the main structure of the molecule remained intact. These subunits were separated by preparative gel electrophoresis and were found to consist of three components in comparable amounts, with molecular weights of 17,000, 19,000 and 20,000. Two of these subunits were determined to have their origin in the head of the myosin molecule, and it was suggested that the third was also present.

As pointed out by Perry (1967), the thickened portion of the myosin molecule could arise either from a folding back and more randomized arrangement of the same polypeptide chains of which the rod is composed or from independent shorter chains which interact with the main chain system at this point. In the former case, no minor component would be observed on dissociation; whereas in the latter it should be apparent in the ultracentrifuge. Thus, the recognition of a lower molecular weight component, in addition to the major subunit dissociated from myosin, indicates that the Kielley and Harrington (1960) model needs revision.

Subunits Obtained by Proteolysis

Gergely (1950) demonstrated that tryptic digestion converted myosin into a soluble form without the loss of ATPase activity. It was found that short tryptic digestion of myosin hydrolyzed the molecule into two major fragments, which were termed light meromyosin (LMM) and heavy meromyosin (HMM) by Szent-Gyorgyi (1953). The HMM retains the ATPase activity and actin-combining property of native myosin, and it is soluble at low ionic strengths. The LMM fragment possesses the same

solubility properties as native myosin, but possesses no biological activity (Szent-Gyorgyi, 1960). In the electron micrographs of Rice (1964), HMM appeared tadpole-shaped, being composed of the globular head region of the myosin molecule plus a rod-shaped tail segment. The LMM fragment was observed to be rod-shaped. The approximate molecular weight of HMM was 350,000 and for LMM, 150,000; however, the size of each was somewhat dependent upon the conditions of digestion (Gibbons, 1968).

Studies of the kinetics of digestion (Mihalyi and Harrington, 1959; Lowey and Cohen, 1962; Young et al., 1964) indicated that the proteolytic process consists of two, simultaneous, first-order reactions. The fast reaction produces the meromyosins and results in the cleavage of 60-90 peptide bonds per molecule. This reaction is 8-10 times faster than the slow reaction which, upon prolonged digestion, accounts for the hydrolysis of 3 to 4 times as many bonds as the fast reaction. Thus, the fast reaction is associated with the hydrolysis of the region linking the meromyosins and the slow reaction rate represents the simultaneous secondary degradation of the light and heavy meromyosins. During the meromyosin formation approximately 5 to 10% of the myosin nitrogen is converted to non-protein nitrogen, which is composed of peptides similar to those obtained by complete digestion of myosin and is presumably derived, in part, from the enzyme-sensitive area in the rod portion of the molecule (Perry, 1967).

Even though trypsin (Szent-Gyorgyi, 1953), chymotrypsin (Gergely et al., 1955), and subtilisin (Middlebrook, 1959) are proteolytic enzymes of differing specificities, they produce similar LMM and HMM fragments. These enzymes appear to induce a transverse cleavage through

the same region of the rod-shaped tail of the native myosin molecule during the early stages of digestion. This observation led Mihalyi and Harrington (1959) to suggest that the proteolysis-sensitive region resulted from an unfolded polypeptide segment embedded within the predominantly α-helical rod of myosin. More recently, Segal et al. (1967) found evidence that this unusual sensitivity to enzymatic attack is primarily dependent on the presence of an appreciable number of proline residues, which are concentrated within a narrow belt of the rod-like tail portion of the molecule. By virtue of their stereochemistry, these residues would tend to interrupt the folded α-helical conformation common to each polypeptide chain within the myosin tail segment and render this region more susceptible to proteolytic attack.

Nevertheless, Lowey et al. (1967) suggest an alternate explanation for the formation of the meromyosins based on experiments with IMET [water-insoluble copoly-(maleic acid-ethylene)-trypsin]. They suggest that the central region of the myosin molecule is α-helical, but that it has a specific amino acid sequence. Cited as evidence for this model were the findings of Kominz et al. (1965), where papain digestion of myosin resulted in the direct formation of a subfragment from HMM. Also, removal of that part of the myosin molecule responsible for the fast initial uptake of alkali in the pH-stat was not accompanied by a corresponding increase in helix content, which might be expected if an amorphous region had been degraded. Similarly, the LMM molecules from early digests have the same helix content as shorter LMM molecules which have had part of the enzyme-sensitive region digested away.

Thus, there is agreement that a region in the intact myosin molecule between LMM and HMM is unusually sensitive to proteolysis;

however, the exact nature of the proteolysis remains to be clarified.

Further studies have been made on the LMM and HMM fragments in order to gain insight into the structure of myosin. Cohen and Szent-Gyorgyi (1957) found that the LMM component of myosin could be further fractionated by alcohol treatment. About 70% of the total protein resists alcohol denaturation and redissolves in 0.6M KCl after precipitation with alcohol. This soluble fraction was called light meromyosin fraction 1 (LMM Fr. 1). It has an α-helical content approaching 100%. LMM and LMM Fr. 1 are similar in size and shape and sediment as single peaks, individually, as well as when combined. In addition to LMM Fr. 1, LMM contains other loosely associated polypeptide fragments, possibly derived from other parts of the myosin molecule, which are denatured by the alcohol treatment (Szent-Gyorgi et al., 1960). Lowey and Cohen (1962) concluded that LMM Fr. 1 is the unique α-helical fragment of myosin. However, the relationship of LMM Fr. 1 to LMM is not clear since it can not be obtained without alcohol fractionation.

By further digestion of HMM with trypsin (Mueller, 1965) and with chymotrypsin (Jones and Perry, 1966), it can be degraded into two major components. These components have been termed subfragment-1 and subfragment-2. Subfragment-1 constitutes the globular head region of the parent myosin molecule, which represents 55 to 60% of the HMM mass, and has a molecular weight of about 100,000. The enzymatic and actin-combining sites of myosin and HMM are localized within this subfragment-1. Subfragment-2 represents the rod-shaped tail segment of HMM. This subfragment-2 has also been called the 3S component by Lowey et al. (1967) and it appears similar to the 3S component obtained by Kominz and Lewis (1964). Subfragment-2 was found to be 80% α-helical with a

molecular weight of 61,000 (Lowey et al., 1967). The molecular weight of subfragment-1 and 2 are influenced to a great extent by the degree of digestion used for its preparation. In this regard, the preparation of subfragment-1 with papain rather than trypsin or chymotrypsin seems preferable due to the shorter digestion time which would reduce random degradation (Weeds and Baker, 1968).

The question as to whether myosin is composed of 2 or 3 identical subunits seems to have been clarified by the electron microscope studies of Slayter and Lowey (1967). Their findings suggest that the major portion of the myosin molecule consists of two polypeptide chains. A significant finding was that the globular head region of myosin contained two subunits. These subunits represent the HMM subfragment-1 portion of the myosin molecule; however, whether these two subfragments are identical remains uncertain.

Dimensions of the Myosin Molecule

From electron micrographs, myosin was observed to be a rod-shaped molecule which thickened to a globular "head" (Rice, 1964). The overall length of the molecule is about 1600Å with a diameter of 20Å for the rod portion of the molecule and a diameter of 30Å to 40Å for the globular "head" portion. The reported values for the length of the light meromyosin portion of the molecule fall into the range of 600-700Å. The length of the heavy meromyosin portion of the molecule is about 600Å, with the globular "head" representing about 200Å of this distance (Kielley, 1965).

Conclusions

The majority of the evidence on the subunit structure of myosin indicates that the native myosin molecule is composed of two supercoiled α-helical strands which extend along the length of the rod portion of the molecule. Each peptide chain terminates in a separate globular conformation. A schematic representation of the myosin molecule is presented in Figure 1. Further investigation is required to determine the significance of the subunits obtained from the globular portion of the myosin molecule by dissociating agents since the biological activity of myosin is lost by their use. Yet, the work of Weeds and Baker (1968) indicates that treatment of HMM subfragment-1, prepared by digestion of myosin with papain, with 0.1M Na_2CO_3 results in a 1.4S peak. This suggests that the low molecular weight subunits detected by dissociating agents are located in the subfragment-1 portion of the molecule.

Biological Function of Myosin

Two significant aspects of myosin are necessary for an understanding of the biological function of myosin. The first area of consideration is the formation of the myosin filament; and hence, its localization within the muscle cell. The second area concerns the involvement of myosin with other fibrillar proteins which pertains to its role in the muscle contraction-relaxation cycle. In order to expand on these topics, they will be considered separately.

Formation and Localization of Myosin (A filament) within the Muscle Cell

Allen and Pepe (1965) observed a morphological correlation between large polysomes and the appearance of myosin in developing muscle cells

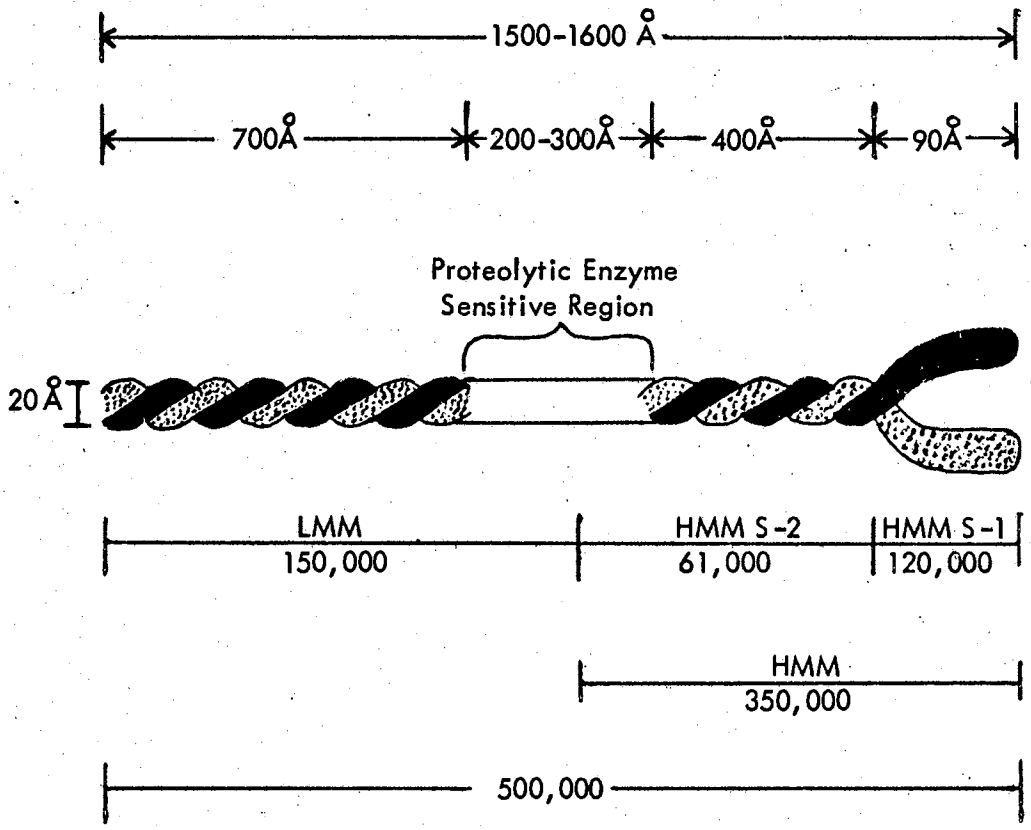


Figure 1. Proposed Model of the Myosin Molecule

from the chick embryo. Subsequently Heywood et al. (1967) from their study on embryonic chick muscle tissue, identified a class of very large polyribosomes, containing 50-60 ribosomes, as those which synthesize myosin. From the size of the polysomal cluster, they inferred that the polypeptide chain being synthesized had a molecular weight of 170,000 to 200,000. This is of interest because the subunit molecular weight of myosin has been reported to be similar to this value. Thus, these findings dispell earlier concepts which postulated that the origin of the myofibrils was from pre-existing cell organelles.

The next stage after synthesis involves the assemblage of myosin molecules into the A filament. Huxley (1963) observed that filaments, heterogeneous in length, formed from pure myosin at ionic strengths of 0.1 to 0.2 were remarkably similar to the A filament of the myofibril. From the dimensions of these filaments, he concluded that they clearly represented aggregates of myosin molecules. He suggested that the myosin filaments were formed by the scheme illustrated in Figure 2. According to this scheme, the molecules of myosin are held together by interaction between the tail-like (LMM) portion of the molecule in such a way that the globular heads (HMM) project out from the filament. In the center of the filament the direction of orientation of the molecules is reversed to give the characteristic smooth central shaft. Thus, the polarity of the myosin molecules is reversed on either side of the center, but all the molecules on the same side have the same polarity. In the studies on the formation of synthetic myosin filaments by Josephs and Harrington (1966), it was found that at pH values of 6.8 to 7.1 and ionic strengths greater than 0.35, myosin was present in sols as the monomer. As the ionic strength approached

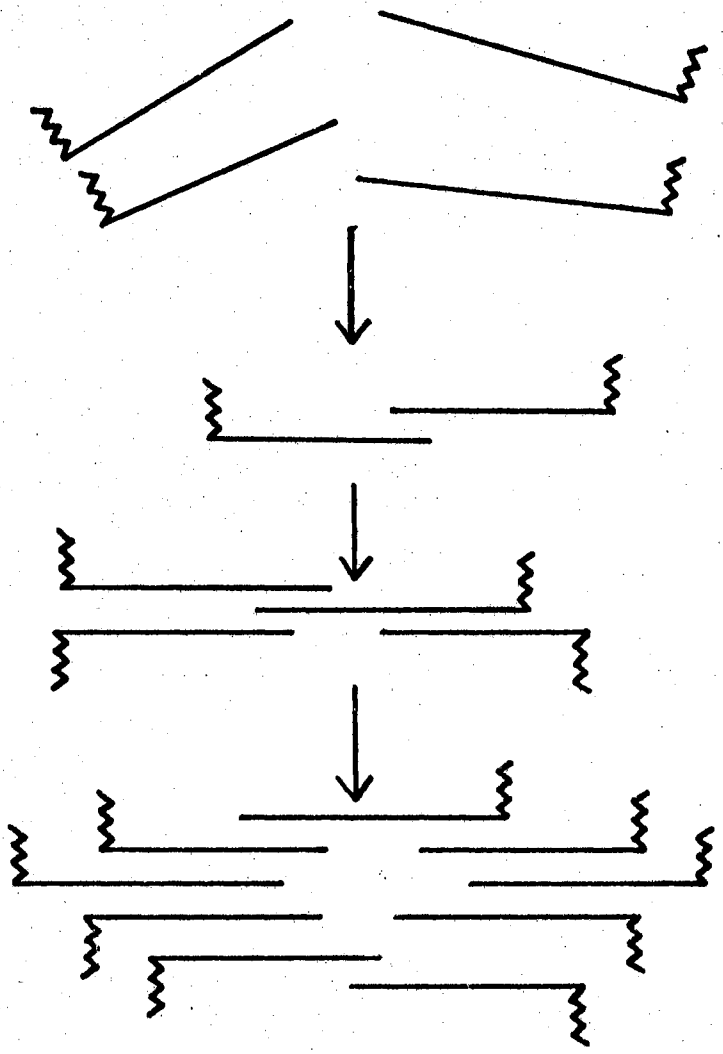


Figure 2. Scheme Illustrating the Aggregation of Myosin Molecules to Form the A Filament (Huxley, 1963)

0.3, aggregates formed and lowering the ionic strength further increased their size. In the pH range 6.2 to 7.3, they observed three classes of polymers with $S^{\circ}_{20,w}$ values of 1100S, 330S, and 180S when the ionic strength was in the range of 0.2-0.3. Since both the pH and ionic strength influenced the size of the polymer formed, this suggests that the specific ionic environment in the developing muscle cell could be a factor in determining the final length of the native thick filament. It therefore seems very probable that the A filament is formed by a process similar to myosin aggregation.

An interesting area of research suggested by these findings would be to investigate the use of various chemicals on the dissociation of this myosin A filament at physiological pH and ionic strength. This could be studied by the use of the analytical ultracentrifuge as well as by other techniques. This kind of basic information could have a definite bearing on developing methods for enhancing meat quality (tenderness) and the emulsifing capacity of myosin.

Although this review has been limited to the formation of the A filament, additional steps are involved in obtaining the final orderly array of filaments within the myofibrils of skeletal muscle. For a more detailed discussion of the stages involved in myofibrillogenesis, the reader is referred to the review of Spiro and Hagopian (1967).

In regard to the localization of the A filament within the myofibril, a brief review of the microstructure of the myofibril is in order. Each skeletal muscle cell (fiber) is composed of many myofibrils which serve as the contractile machine. The myofibrils are characterized by regularly repeating units, sarcomeres, which give rise to the striated appearance of skeletal muscle fibers as seen in a longitudinal

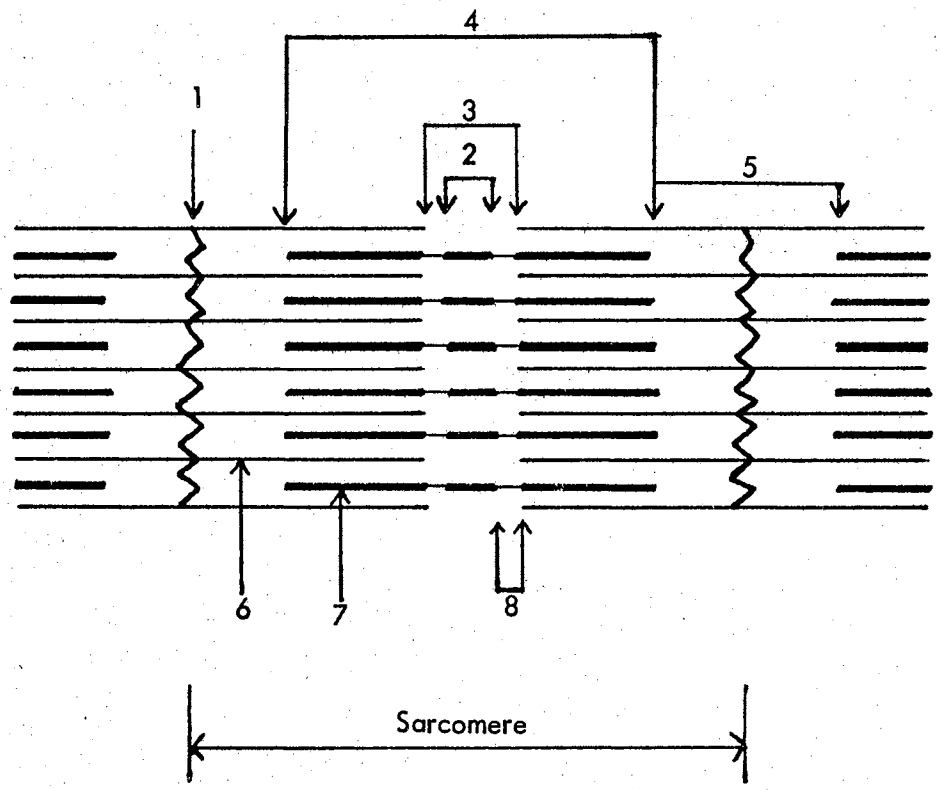
section with the electron microscope. The sarcomere is defined as that portion of a myofibril from one Z line to the next Z line and represents the smallest functional contractile unit within the myofibril. All sarcomeres are further subdivided into the A and I bands. Other bands such as the M-line, L zone and H zone may also be present as illustrated in Figure 3. The A band contains the thick (myosin) filaments plus partially overlapping thin (actin) filaments. The thin, actin, filaments are connected to the Z lines and course through the I band into the A band. In the central portion of the A band, the thick filament exhibits a narrow zone, referred to as the L line, which is of lower electron density. This results from the absence of both the thin filaments which terminate at this line, as well as from the absence of cross-bridges (Spiro and Hagopian, 1967). The mid-portion of the pseudo H zone or H zone, the M-line, is more electron dense because of the presence of cross-bridges, known as M-line bridges, which interconnect the thick filaments from opposite sides of the A band in the center of the sarcomere (Pepe, 1966).

Muscle Contraction-Relaxation Cycle

The function of myosin is concerned with its ATPase activity and interaction with actin during muscle contraction. The role of myosin has been studied from both a morphological and biochemical point of view in an attempt to understand the mechanisms and chemical reactions involved.

Morphological Studies

The sliding-filament theory is the most widely accepted description



1: Z line (tropomyosin, troponin, α-actinin)
2: M-line
3: H or pseudo H zone
4: A band
5: I band
6: Thin filament (actin, α-actinin, β-actinin, tropomyosin, troponin)
7: Thick filament (myosin)
8: L line

Figure 3. Proposed Structure of the Sarcomere

of the mechanism by which striated muscle contracts (Huxley and Hanson, 1954; A. F. Huxley and Hiedergerke, 1954). This theory is based on observations which have shown that the A bands remain constant in length while the I bands either shorten or lengthen depending on whether the sarcomere length is decreasing or increasing. Corresponding changes were observed in the width of the H zone. From these observations it was concluded that the filaments remained at least approximately constant in length, and simply slid past each other during changes in muscle length (Huxley, 1965). According to the sliding-filament theory, contraction results from the cyclic formation and dissociation of cross-bridges between myosin and actin filaments. The splitting of ATP, located on the cross-bridges, is believed to be linked to the development of a relative force between the myosin and actin filaments, causing the actin filament to slide past the myosin filament (Huxley, 1965). The tension generated by the cross-bridges along a given thick (myosin) filament adds up in parallel, so that the tension generated by the system as a whole varies according to the extent of overlap of the filaments, and also according to the number of cross-bridges which have time to attach to the active sites of the actin molecules alongside them (Huxley, 1967). The significance of the reverse polarity structure of the A filament becomes apparent in order that the force generated by the cross-bridges acts in the appropriate direction. That is, all elements of force in one half of the A band must act in one direction and those in the other half must act in the reverse direction, so that the actin filaments are moved toward the center of the A band from each side. When the muscle is no longer activated, the cross-bridges detach from the thin filaments, which are then free to be drawn

out of the A bands again, and the muscle thereby is re-extended to its resting length (Huxley, 1967).

The way in which the interaction of actin and myosin at the cross-bridges could produce movement has been a matter of some conjecture. Pepe (1967) proposed that the HMM portion of the myosin molecule must bend back from the filament axis and form a small angle with the actin filament. In order to do this, the enzyme-sensitive region in a random-coil conformation serves as a hinge between the meromyosins permitting motion of the HMM subunit. Alternatively, Lowey et al. (1967) suggested that if the α-helical coiled-coil conformation extends throughout the rod-like portion of myosin, this entire part of the molecule would act as an elastic element with uniform bending. The findings of Slayter and Lowey (1967) that the two globular subunits (subfraction-1) of myosin have the potential for a high degree of flexibility may well prove to be involved in the movement of the cross-bridges. Although the exact mechanism is still vague, these workers believe that the cross-bridges physically move the thin filament along. In contrast, Metherell (1967) suggests that instead of the force for movement being transferred through the cross-bridges, it is transferred through viscous shearing in the sarcoplasmic fluid that surrounds the filaments. The viscous shearing can be set up by gradients in interface tension caused by the release of energy at the cross-bridge contact sites. Thus, it seems that further experiments must be conducted before this question is settled.

Biochemical Studies

The complex, actomyosin, formed by the interaction of myosin and

actin in the presence of ATP has received considerable attention in an attempt to elucidate the chemical reactions associated with the physical events described by the sliding-filament theory. The nature of the complex and the precise effects of ATP upon it can be broadly distinguished into two groups based on ionic strength (Perry, 1967). At high ionic strength (greater than 0.3), the interaction results in the formation of a viscous complex, actomyosin. The addition of ATP results in a number of changes which have been interpreted as the dissociation of actomyosin into actin and myosin. This interpretation is based on the findings that viscosity and light-scattering values decrease to those equivalent to the additive contribution of the two proteins. The fast sedimenting actomyosin, in the presence of ATP, gives a peak in the ultracentrifuge which sediments at the same rate and has the same distribution as pure myosin. Although ATP brings about dissociation, its hydrolysis is not necessary since dissociation also results from the addition of inorganic pyrophosphate. At low ionic strength (0.05-0.15) the actomyosin complex, like myosin, is insoluble. The addition of ATP to the actomyosin suspension results in synaeresis, becoming denser and settling rapidly. This effect is known as superprecipitation which was defined by Szent-Gyorgyi (1947) as a reaction in which the "abundantly hydrated actomyosin molecules become a shrunken and dehydrated particle". Hydrolysis of ATP is an essential requirement for this response since inorganic phosphate does not cause superprecipitation. Under certain conditions after the addition of ATP and to a lesser extent with other nucleotide triphosphates, but not with pyrophosphate, an immediate reduction in viscosity of the suspension occurs, termed clearing by Spicer (1952),

which is then followed by superprecipitation. Both ATP and Mg++ are specific requirements for the clearing phase to occur, although its appearance also depends on ionic strength and the presence or absence of relaxing factors (Maruyama and Gergely, 1962a). Physicochemical measurements have shown that the actomyosin is dissociated into F-actin and myosin during the clear phase and that the ATPase activity of the system is low during this stage, but rises sharply with the onset of superprecipitation (Maruyama and Gergely, 1962b). The superprecipitation and clearing reactions of actomyosin gels in vitro are considered analogous to muscle contraction and relaxation in vivo with the obvious exception that there is greater structural integrity of actomyosin in vivo.

As previously mentioned, the biologically active sites of myosin are associated with the HMM portion of the molecule and localized on subfragment-1. The weight of evidence now favors the existence of two different sites responsible for biological activity. The conclusion that different sulfhydryl residues in myosin are involved in ATPase activity and in actin binding came from the findings that the actin-combining activity of myosin could be retained even though the ATPase activity was destroyed (Stracher and Dreizen, 1966; Perry, 1967). Likewise in actin, Bailin and Barany (1967) have concluded that different parts of the actin molecule are responsible for the actin-actin interaction and the actin-myosin interaction. In addition, it appears that these different sites are associated with sulfhydryl groups.

The enzymic properties of myosin are altered by its interaction with actin. In the absence of actin, myosin ATPase activity is

stimulated by Ca++, but is inhibited by Mg++ regardless of the ionic strength of the incubation system. However in the presence of actin, myosin ATPase is activated by Mg++ in low ionic strength incubation media, termed actomyosin ATPase.

Several reports indicate that Mg-ATP is the moiety that controls dissociation of actin and myosin by altering the critical level of Ca++ needed for association, however, the mechanism involved is a matter of conjecture (Gergely, 1966). From the results of Davies (1965), it is now clear that ATP breakdown occurs during contraction, and therefore is the source of energy driving contraction. The source of ATP for this reaction comes primarily from the resynthesis of ATP by the creatine kinase and myokinase enzyme systems. In addition, studies by Barany et al. (1966) have shown that the bound ADP of F-actin is not involved in its interaction with myosin to form actomyosin, thus dispelling the concept that the bound nucleotide of actin was a source of energy for contraction. However, Hayoshi (1967) concludes that the nucleotide of actin is necessary to bring about contraction in the actin-myosin interaction, but its specific role in the process is uncertain.

Evidence for the functional relationship between ATPase activity and contraction has been obtained in several studies. Gergely et al. (1965) have reported that myosin from red skeletal muscle has a lower specific ATPase activity than myosin from white skeletal muscle of the same species. This was in keeping with the slower contraction-relaxation cycle of red muscles versus white muscles. Barany (1967) has shown that the ATPase activity of actomyosin and myosin was approximately proportional to the shortening speed of the muscle. Thus,

it appears that hydrolysis of ATP and contraction are tightly coupled processes allowing contraction to be controlled through the enzyme center. In vitro studies have shown that when the enzymic interaction between actin and myosin is inhibited, contraction is also inhibited (H. Weber, 1964). Certainly from the standpoint of the palatibility characteristics of muscle as food, improvement in tenderness and juiciness could well result from the development of methods to maintain myosin and actin dissociated post-mortem. In considering this aspect, a fruitful approach to the problem would be to investigate methods of inhibiting myosin ATPase activity.

Role of the Other Myofibrillar Proteins

In recent years additional myofibrillar proteins have been found to be involved in the muscle contraction-relaxation cycle. A perplexing question arose from the results of Perry and Grey (1956) and A. Weber and Winicur (1961) which was manifest in the variability of response of actomyosin preparations to Ca++. While superprecipitation of natural actomyosin (myosin B) was consistently inhibited by chelating agents, synthetic or reconstituted actomyosin (prepared from the separately purified proteins) showed erratic behavior. Subsequently, this question was resolved by the finding that if actin was contaminated by another protein or if a purified fraction of a protein resembling tropomyosin (termed "native tropomyosin") was added to the preparation made with pure actin, the presence of Ca++ in the medium was required for superprecipitation to occur (Ebashi and Ebashi, 1964). Since the presence of this protein factor in actomyosin preparations caused clearing upon the addition of EGTA [ethylenedioxbis (ethyleneamino)

tetraacetic acid], the terms EGTA sensitizing factor, calcium-sensitizing factor, and relaxing protein have all been used in reference to this protein. This native tropomyosin was found to consist of two proteins, tropomyosin and troponin (Ebashi and Kodama, 1966). In addition, it was found that both proteins were required to make actomyosin sensitive to Ca++. Purified tropomyosin (tropomyosin B) as prepared by Bailey (1948) was found to be devoid of calcium-sensitizing activity. The observation that calcium is not required for superprecipitation of actomyosin in the absence of "native" tropomyosin (Schaub et al., 1967) has been used as an argument against the direct role of calcium ion in cross-bridge attachment as suggested by Davies (1963). In addition, Schaub et al. (1967) found that the addition of "native" tropomyosin to purified actomyosin modified its enzymic properties by inhibiting Ca^{++}-activated ATPase activity while leaving Mg^{++}-activated activity intact. Yasui et al. (1968) found that troponin was a potent calcium binding substance, possessing high affinity calcium-binding sites which were not present in tropomyosin. They hypothesized that the binding of Ca++ to these sites inactivates troponin and that this is the mechanism whereby Ca++ activates myofibrillar contraction. The protein termed crude metin, which consists of tropomyosin plus a minor component metin, appears to be analogous to "native" tropomyosin, or tropomyosin combined with troponin (Peachey, 1968).

Another protein which can be isolated from usual actin preparations, called α-actinin, was found by Ebashi and Ebashi (1965). They found that it was similar to actin in amino acid composition, and it promoted the superprecipitation of purified actin and myosin. This effect was

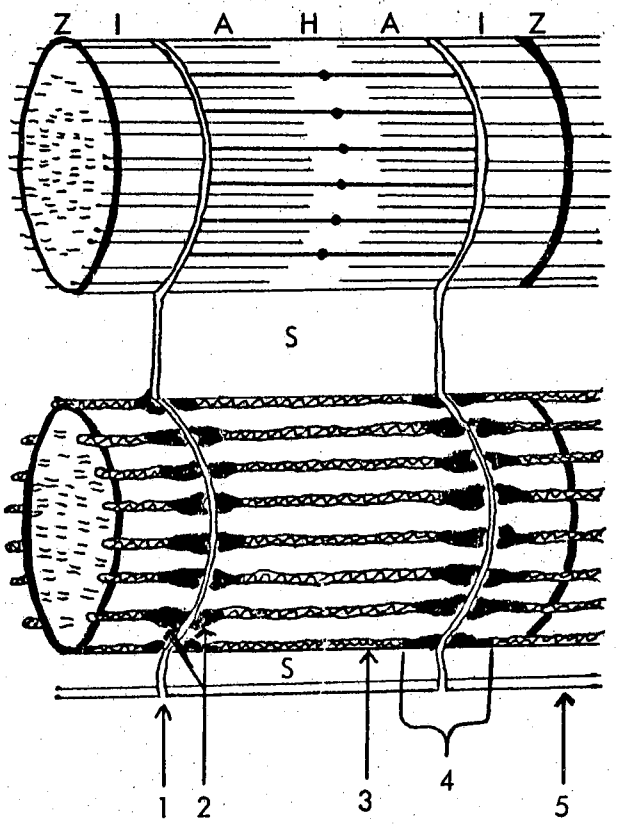
originally interpreted to mean that α-actinin was required for contractility. Other investigations have shown that α-actinin is not required for contraction although it can cause superprecipitation under specific conditions (Briskey, 1967).

The limitation of F-actin particle length to a range similar to that at which they occur in vivo has been attributed to another protein, called β-actinin, with an amino acid composition like that of actin (Maruyama, 1965).

Thus, muscle contraction represents a complex scheme which may well contain other proteins unknown to date.

Excitation of the Contractile System

The sarcoplasmic reticulum network is the system responsible for conducting a nervous impulse to the muscle fiber which results in excitation of the contractile system. The terminology and location of the sarcoplasmic reticulum complex is illustrated in Figure 4. The junction of the transverse tubules and the lateral sacs or vesicles of the longitudinal tubules, called triads, occurs at the A-I junction of the sarcomere in mammalian muscle; whereas in frogs, fish and reptiles, they occur at the Z band (Bendall, 1966). It is now well established that the transverse tubules are continuous with the cell membrane (sarcolemma), and this provides the means by which the nerve impulse is conducted into the interior of the muscle fiber (Peachey, 1968). A dense material has been found to be located in the lateral sacs of the sarcoplasmic reticulum of frog and fish body muscle, however, in most other muscles it is spread throughout the sarcoplasmic reticulum. It has been suggested that this dense material contains calcium-binding



Z: Z line
I : I band
A: A band
H: H zone
S: Sarcoplasm

1: Transverse tubule
2: Lateral sacs or vesicles, or Terminal cisternae
3: Longitudinal tubule
4: Triad
5: Sarcolemma

Figure 4. Two Myofibrils to Illustrate the Sarcotubular System (Bendall, 1966)

sites (Peachey, 1968). Continuity of the transverse tubules and lateral sacs has never been reported. In fact, there is a space of about 250Å separating the membranes of the two systems (Rostgaard and Behnke, 1966). The results of Winegrad (1968) have been interpreted to mean that while the terminal sacs are the site of calcium storage and release, the longitudinal tubules are the site of rapid calcium uptake.

The mechanism of calcium release and uptake has been the subject of considerable research. Ebashi (1965) cites two plausible mechanisms for the "Ca-release system". One mechanism assumes that the release of calcium is due to leakage of stored calcium from the lateral sacs as free ions, resulting from an increase in the permeability of the vesicular membrane to calcium ions. According to this mechanism, the release and uptake of calcium are mediated by different mechanisms analogous to the active and passive transport system of sodium. The other mechanism is to assume that the site of calcium release is the same as that of calcium binding. In this case, calcium release is a result of the loss of binding capacity of the site for calcium.

An active transport system is involved in the uptake of calcium since it is transported against a concentration gradient. A current view is that the mechanism of calcium uptake consists of the transportation across the membrane of the longitudinal tubule by the formation of a Ca-carrier complex within the membrane which is specific for calcium. The hydrolysis of ATP occurs either for the formation of this complex or for the release of calcium from this complex and results in the accumulation of calcium within the longitudinal system (A. Weber, 1966).

Calcium accumulation by the reticulum is sufficiently rapid to

account for the decrease in calcium concentration from $\sim 10^{-6}$M to 10^{-7}M during the period of relaxation. In addition, the only well documented instance of calcium reversible relaxation by reticulum is relaxation by calcium removal rather than by the presence of a soluble relaxing substance (A. Weber, 1966).

Summary

To establish a degree of continuity to the previously reviewed structures and functions, the following consists of a description of the muscle contraction-relaxation cycle. The nervous impulse, a wave of depolarization, is conducted via the transverse tubules within the muscle fiber. When the action potential arrives at the triads, Ca^{++} is released, either by a change in the permeability of the membrane of the lateral sacs or by an inactivation of the Ca-binding sites, and diffuses into the sarcoplasm. When the intracellular Ca^{++} level increases to about 10^{-6}M, the contraction response is initiated. Once in the sarcoplasm, Ca^{++} interacts with "native" tropomyosin (tropomyosin + troponin) which results in inactivating the relaxing effect of these proteins. This activates the enzymic centers located on the projections from the thick filament, HMM subfragment-1 portion of the myosin molecule, resulting in Mg^{++} activated hydrolysis of ATP. At this point, it will be recalled that pure myosin ATPase activity is activated by Ca^{++} and inhibited by Mg^{++} at low ionic strengths. However, it is believed that it is the actomyosin type ATPase activity (which is activated by Mg^{++} as well as by Ca^{++} at low ionic strength *in vivo*) which is responsible for cleaving ATP. The reason that Mg^{++} is considered to be the major participating divalent cation *in vivo*

is that concentrations of 10^{-3}M Ca^{++} rather than the observed requirement of $\sim 10^{-6}$M would be necessary to produce significant enzymic activity (Perry, 1965). The mechanism by which actin is able to confer Mg^{++} activated properties to the enzymic active site in vivo is not clear, however, it may well be associated with the interaction of Ca^{++} with the "native" tropomyosin. At any rate, the hydrolysis of ATP results in a cross-bridge between actin in the thin filament and HMM subfragment-1 of the myosin in the thick filament. Due to the opposite polarity of the projections in the thick filament, the thin filaments slide towards the center of the sarcomere (indicated by the decrease in the H zone) by a series of make and break cross-bridges. The synchronization between sarcomeres within the same myofibril occurs from the actin molecules on one side of the Z line having a reverse polarity to the actin molecules on the other side (Huxley, 1963). The exact nature of the bonds formed in the interaction of actin with the actin-combining sites of HMM subfragment-1 is unknown, however, they do possess some electrostatic character since the binding constant and rate of formation is reduced with increasing ionic strength (Perry, 1967). Upon removal of the stimulus, Ca^{++} is actively removed from the sarcoplasm to the longitudinal tubules of the sarcoplasmic reticulum. The level of Ca^{++} falls to the 10^{-7}M which results in the "native" tropomyosin factor again being active which inhibits enzymic activity. The ATP levels are rapidly returned to normal ($\sim 10^{-3}$M) primarily from the creatine kinase system. ATP then exerts its "plasticizing effect" allowing the thin filaments to freely extend to their original positions in the sarcomere; hence, relaxation. The dual function of ATP (that is, if hydrolyzed, contraction occurs and if not hydrolyzed,

relaxation occurs) comes from the findings that interaction inhibitors only exert dissociating or relaxing effect on actomyosin in the presence of ATP (H. Weber, 1964).

To be sure, this scheme is by no means complete in its minute detail. Yet, an understanding of the events now known provides the fundamental knowledge on which improved approaches can be taken in the study of the changes in myofibrillar proteins post-mortem.

CHAPTER III

ISOLATION OF MYOSIN

Due to the complexity and number of biochemical determinations made on each steer, it was not possible to study more than one animal at a time. The steers used in this study were obtained from the Oklahoma Agriculture Experiment Station. The criterion for selection was that the steers be of the Hereford breed and approximately 408 kg. live weight.

Feed and water were withheld for 24 hours prior to slaughter. Care was taken to avoid any violent handling of the steers in order to prevent any undue stress or excitation of the animals.

The longissimus dorsi muscle from the right side was excised as rapidly as possible after the steer was bled. After the muscle was removed, it was taken to the cold room where all subsequent operations were conducted at $0^{\circ}C$. The muscle was freed of surrounding fat and epimysial connective tissue, then minced twice through a 3 mm. plate in a stainless steel grinder. Approximately 20 minutes were required to obtain and prepare the muscle sample. A random 500 gm. aliquot of the muscle mince was taken for the isolation of myosin. The pH of the muscle was also determined at this time. This was accomplished by mixing 50 ml. of glass distilled water with a 10 gm. aliquot of the minced muscle. The pH was determined with a Corning model 10 pH meter. The purpose of obtaining the pH reading was to provide some measure of

the rapidity of sample preparation post-mortem. The pH values obtained were in the range of 6.89 to 6.95 which indicates that sample preparation was accomplished before a large reduction from *in vivo* pH (assuming 7.0) had occurred. In addition, there was no visual indication of "abnormal" muscle characteristics (i.e. bruises, dark cutter).

Myosin was isolated from the minced muscle by the salt extraction procedure outlined in Figure 5. This procedure is a modification of the one developed by Szent-Gyorgyi (1943). The average yield of myosin per 500 gm. aliquot of minced muscle was approximately 4 gm., after recrystallization. The purity of the myosin preparation was assessed by ultracentrifuge sedimentation, electrophoresis, and Mg-activated ATPase activity which will be discussed in detail in the subsequent chapters.

1. Extract minced muscle with three volumes of cold buffer: 0.3M KCl + 0.09M KH_2PO_4 + 0.06M K_2HPO_4; pH 6.5. Stir for 15 min. and extract is obtained by centrifugation at 10,000 x G for 10 min. Supernatant is strained through cheese cloth to remove fat.

2. Muscle extract is dialyzed for 18 hr. without stirring against 10 volumes of distilled water to an ionic strength of 0.05. Add 300 ml. of distilled water to dialyzed extract and centrifuge: 10,000 x G for 10 min.

3. Discard supernatant fluid.

   Dissolve precipitate in buffered 2M KCl (pH 6.8) to a final volume of 400 ml., ionic strength: 0,5.

   Add 300 ml. of distilled water while stirring rapidly, then centrifuge: 10,000 x G for 15 min.

4. Dilute supernatant fluid with distilled water to 4 liters. Add water slowly with rapid stirring. Centrifuge: 10,000 x G for 15 min.

   Discard precipitate.

5. Discard supernatant fluid.

   Dissolve precipitate by the addition of 75 ml. of 2M KCl and water to 300 ml. Centrifuge: 10,000 x G for 15 min.

6. Dilute supernatant fluid with distilled water to 3 liters. Add water slowly with rapid stirring. Centrifuge: 10,000 x G for 15 min.

   Discard precipitate.

7. Discard supernatant fluid.

   Recrystallization: Take resultant precipitate and repeat steps 5 and 6.

8. Dissolve final recrystallized myosin in 0.5M KCl.

Figure 5. Isolation of Myosin from Bovine Muscle

CHAPTER IV

ULTRACENTRIFUGE SEDIMENTATION STUDIES ON BOVINE MYOSIN

Introduction

In assessing the purity of a particular protein isolation, one of the most commonly used methods is to determine its sedimentation characteristics in the analytical ultracentrifuge. Accordingly, the objectives of this study were to determine the purity of the myosin preparation as isolated by procedures refined in our laboratory and to define suitable ultracentrifuge conditions for evaluating the sedimentation characteristics of bovine myosin.

Materials and Methods

Isolation of Myosin

Myosin was isolated according to the procedure outlined in Chapter III. After the recrystallization step, the myosin preparation was dissolved in 0.5M KCl and dialyzed against 50 volumes of 0.5M KCl + 0.01M Tris-HCl buffer (pH 7.1) for 12 hours. Three aliquots of the dialyzed myosin were diluted to a final protein concentration of 3 mg. per ml. by the addition of one of the following solvents: unbuffered 0.5M KCl (pH 6.8); 0.5M KCl + 0.05M phosphate (pH 7.1) and 0.5M KCl + 0.05M phosphate (pH 7.1) + sucrose. When sucrose was used in this study, it was added in an amount equal to twice the protein

concentration (6 mg. per ml.).

Protein Concentration

Protein (myosin) concentration was determined via the biuret method of Layne (1957). Crystalline bovine serum albumin was used as the standard.

Ultracentrifugation

The sedimentation pattern of each sample was obtained on a Spinco model E analytical ultracentrifuge which was equipped with a phase plate and RTIC temperature control. All runs were conducted using the analytical D rotor and 2° sector cell. This cell had a sample volume of 0.4 ml. and was equipped with an epoxy centerpiece. The specific conditions for each run are described in the figure legends. The plates were measured with a Nikon comparator. The observed sedimentation coefficient was calculated and corrected to standard conditions according to the procedures outlined by Schachman (1957). In correcting the observed sedimentation coefficient to standard conditions, the value for the partial specific volume of myosin was taken as 0.728 (Parrish and Mommaerts, 1954). Ultracentrifuge sedimentation patterns were obtained after the samples had been stored for 7 days at 0°C.

Results and Discussion

The initial ultracentrifuge sedimentation study was conducted on the myosin preparation dissolved in 0.5M KCl. For this run, the ultracentrifuge was operated at a speed of 42,040 r.p.m. and at a temperature of 20°C. The pH of the myosin sample solution was 6.8.

The sedimentation patterns obtained at various time intervals are presented in Figure 6. These results show that the myosin sedimented as a rather symmetrical, homogeneous entity until approximately 16 min. after operating speed had been attained. After this time, however, a number of minor peaks could be observed in the sedimentation patterns. Moreover, these minor peaks tended to increase in magnitude as the centrifugation time was prolonged.

These observations led to a question as to whether the minor peaks resulted from actual impurities in the myosin preparation or whether they were due to the formation of aggregates of myosin molecules into higher molecular weight entities, i.e. dimers, trimers, etc., during prolonged ultracentrifugation.

To investigate this question, another run was conducted, using the myosin preparation dissolved in 0.5M KCl (pH 6.8), in which the temperature was maintained at 2°C. throughout the time course of the run. In addition, care was taken to avoid exposure of the myosin sample to temperatures above 2°C. during the ultracentrifuge preparatory steps. In this run the ultracentrifuge was operated at a speed of 59,780 r.p.m. From the sedimentation patterns presented in Figure 7, it can be seen that a significant reduction in the number of minor peaks as well as a much sharper major peak was obtained when sedimentation was performed at this lower temperature. This indicates that the heterogenity observed in the previous run (Figure 6) was largely due to formation of aggregates of myosin molecules, and that by lowering the centrifugation temperature from 20° to 2°C. the tendency of myosin to form aggregates was significantly lessened.

These data suggest an interesting corollary concerning the

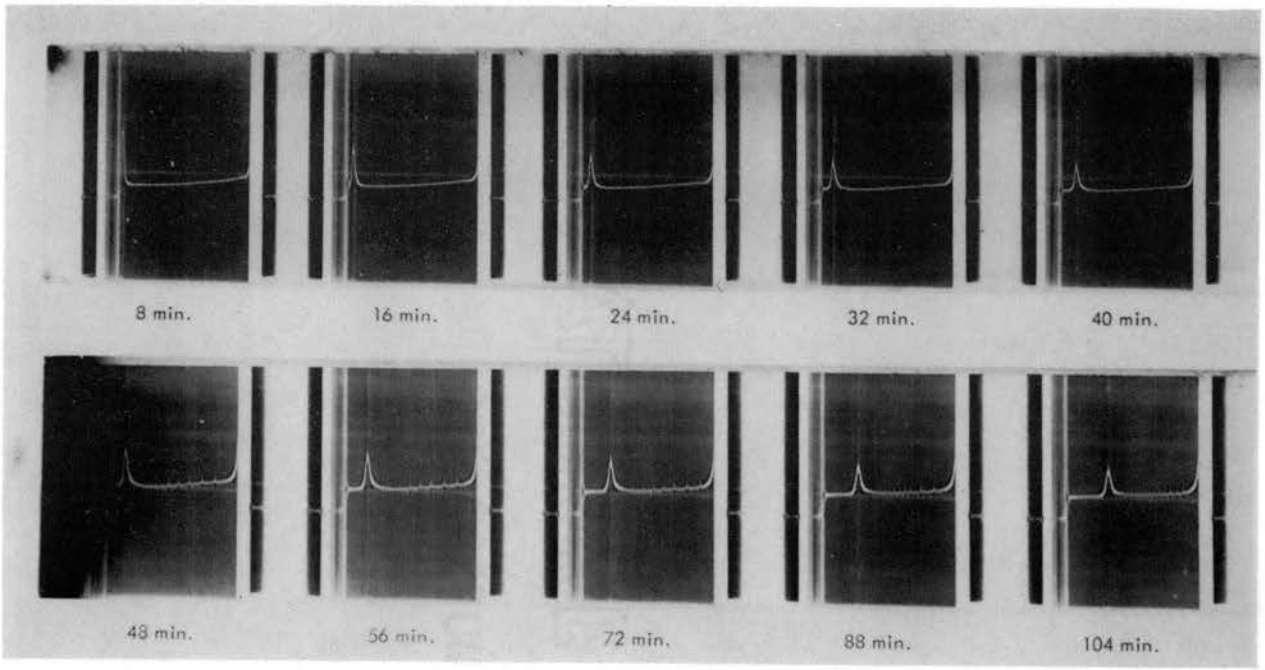


Figure 6. Ultracentrifuge Sedimentation of Bovine Myosin Dissolved in 0.5M KCl (pH 6.8). Protein Concentration, 3 mg./ml.; Speed, 42,040 r.p.m.; Temperature, 20°C.; Direction of Sedimentation, Left to Right; Time Intervals Measured After Rotor Reached Operating Speed. Top Plate: Diaphragm Angle, 70°; Bottom Plate: Diaphragm Angle, 60°. $S_{20,w} = 5.89$.

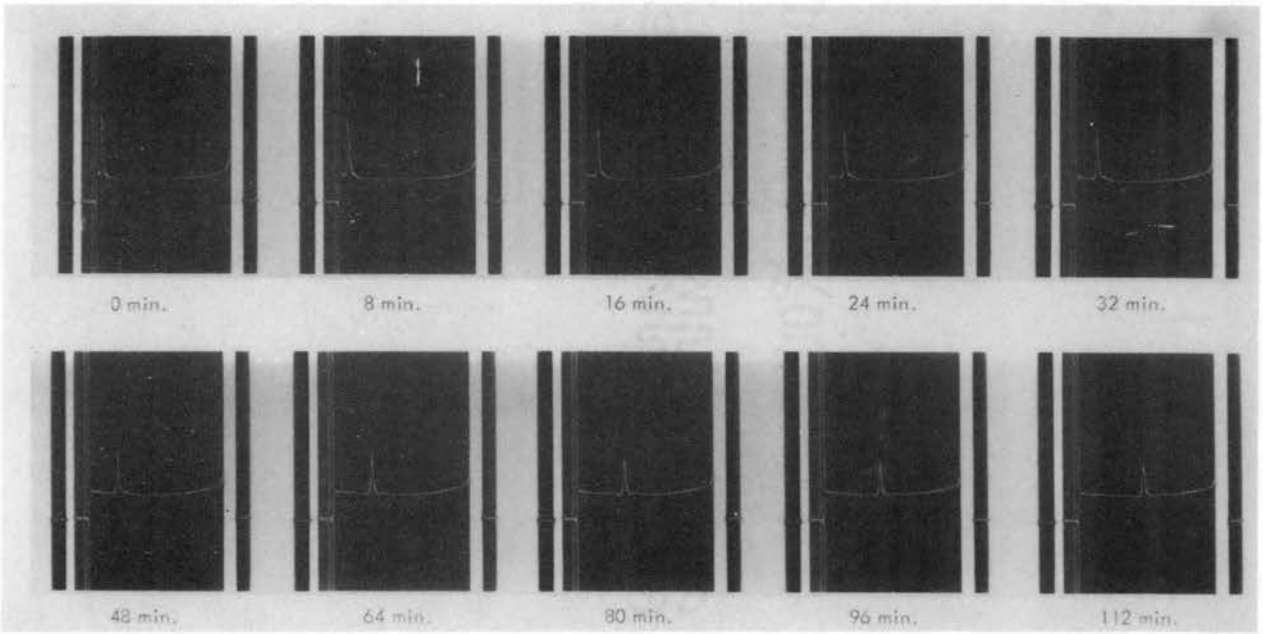


Figure 7. Ultracentrifuge Sedimentation of Bovine Myosin Dissolved in 0.5M KCl (pH 6.8). Protein Concentration, 3 mg./ml.; Speed, 59,780 r.p.m.; Temperature, 2°C.; Diaphragm Angle, 70°; Direction of Sedimentation, Left to Right. Time Intervals Measured After Rotor Reached Operating Speed. $S_{20,w} = 5.89$.

response of bovine and rabbit myosin to temperature. Several investigators (Holtzer, 1956; Johnson and Rowe, 1960; Laki and Carroll, 1955) have found that the rate of aggregation of rabbit myosin increased as temperature was increased. In addition, they found that the amount of aggregation of rabbit myosin was dependent upon the duration of exposure to the elevated temperature. The present results with bovine myosin (Figure 6 and 7) show that as duration of centrifugation at 20°C. was prolonged, the amount of spontaneous aggregation increased, and that when the ultracentrifugation temperature was lowered to 2°C., a significant reduction in the amount of aggregation occurred. Based on these results, all subsequent runs were conducted at 2°C. in order to avoid any spontaneous temperature induced aggregation. Also, all subsequent sedimentations were run at 59,780 r.p.m.

To determine if the observed aggregation could be reversed or further reduced, sucrose was added, 24 hr. prior to the run and in an amount equal to twice the protein concentration (6 mg. /ml.) to the remainder of the myosin sample used for the previous study (Figure 7). Results show that with the exception of a discrete, leading peak, which became readily apparent 45 min. after operating speed was attained, no other leading peaks or "aggregation" could be observed (Figure 8).

These data suggest that sucrose has a stabilizing effect on the myosin molecule. This conclusion is based on the two observations: 1) the addition of sucrose eliminated the aggregation previously obtained and 2) the sedimentation coefficient ($S_{20,w}$) was reduced from 5.89 to 5.63.

Lowey and Holtzer (1959) reported that the addition of inorganic phosphate tended to stabilize the myosin molecule and retard the

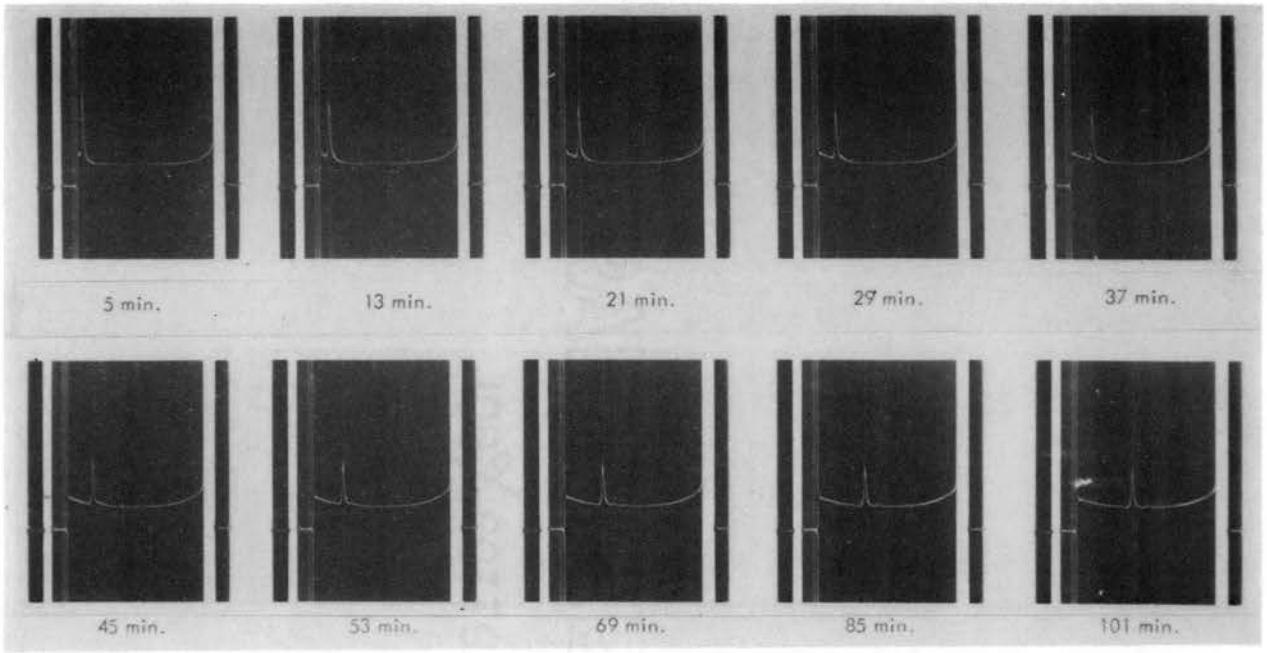


Figure 8. Ultracentrifuge Sedimentation of Bovine Myosin Dissolved in 0.5M KCl (pH 6.8), Sucrose Added 24 Hours Prior to Run. Protein Concentration, 3 mg./ml.; Speed, 59,780 r.p.m.; Temperature, 2°C.; Diaphragm Angle, 70°; Direction of Sedimentation, Left to Right. Time Intervals Measured After Rotor Reached Operating Speed. $S_{20,w} = 5.63$.

formation of aggregates in myosin isolated from rabbit muscle. To determine if a similar response occurred in bovine myosin, the myosin preparation was dissolved in 0.5M KCl + 0.05M phosphate (pH 7.1) immediately upon completion of the isolation procedure. From the results obtained (Figure 9), it appears that the addition of phosphate to bovine myosin did not significantly lower the spontaneous aggregation observed in the no phosphate run at 2°C. (Figure 7).

To test the effect of added sucrose to the 0.5M KCl + 0.05M phosphate (pH 7.1) dissolved bovine myosin, sucrose was added 24 hr. prior to centrifugation to the remainder of the sample used in the previous run (Figure 9). From the results presented in Figure 10, all aggregation, save the discrete leading peak detected at the 53 min. interval, was eliminated.

The results in Figures 8 and 10, suggest that the reduction in aggregation of bovine myosin was due to the protective effect of sucrose rather than the phosphate ions.

To determine if this protective effect of sucrose could be expanded to eliminate the minor irreversible aggregation (discrete leading peak in Figures 8 and 10) which occurred during storage at 0°C., sucrose was added, immediately after isolation, to the 0.5M KCl + 0.05M phosphate (pH 7.1) dissolved myosin sample.

Since the sedimentation patterns in Figure 11 show a single, sharp, symmetrical peak and are devoid of any aggregation, including the discrete leading peak, it can be concluded that sucrose, added to the myosin sample immediately after isolation, precluded any spontaneous aggregation which can occur during the 7 day storage period or subsequent ultracentrifugation.

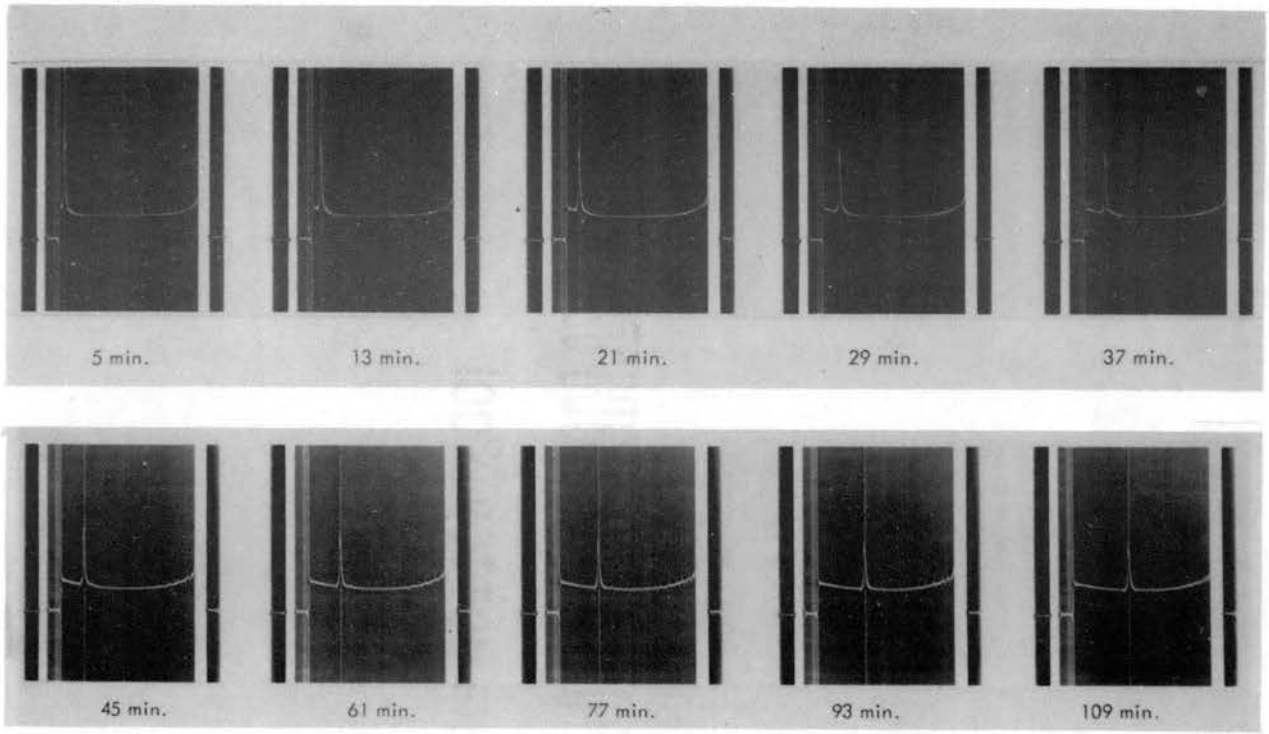


Figure 9. Ultracentrifuge Sedimentation of Bovine Myosin Dissolved in 0.5M KCl + 0.05M Phosphate (pH 7.1). Protein Concentration, 3 mg./ml.; Speed, 59,780 r.p.m.; Temperature, 2°C.; Diaphragm Angle, 70°; Direction of Sedimentation, Left to Right. Time Intervals Measured After Rotor Reached Operating Speed. $S_{20,w} = 5.57$.

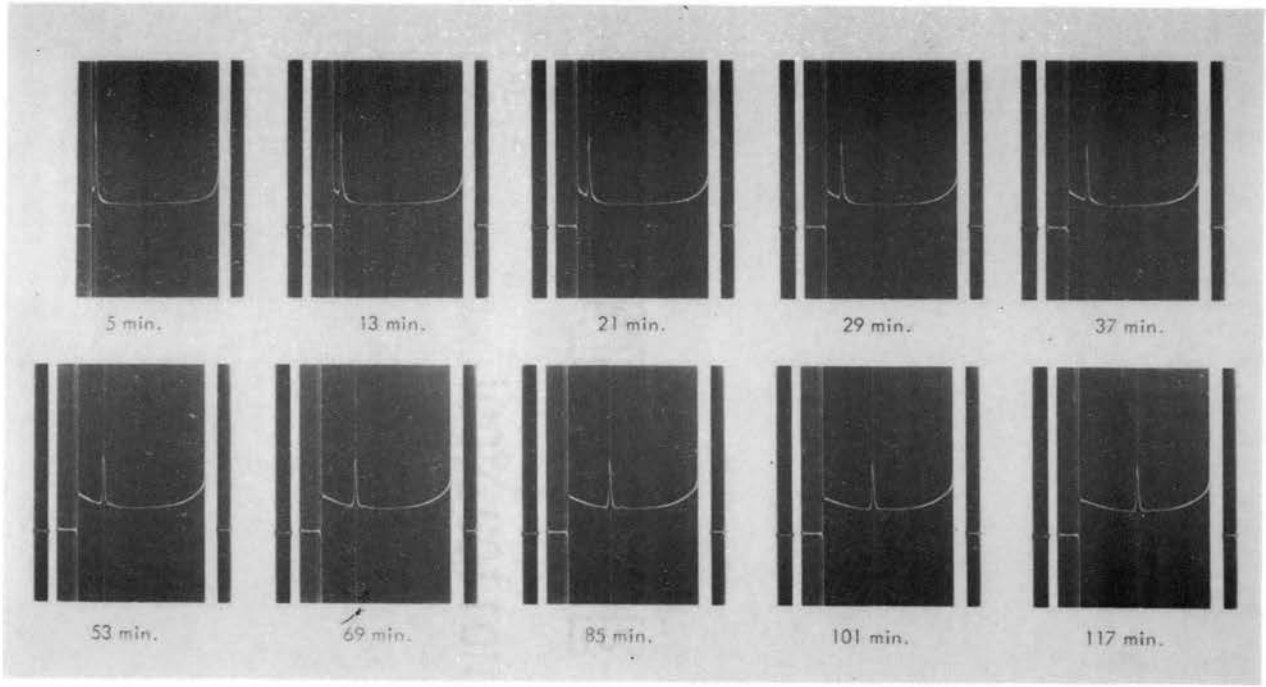


Figure 10. Ultracentrifuge Sedimentation of Bovine Myosin Dissolved in 0.5M KCl + 0.05M Phosphate (pH 7.1), Sucrose Added 24 Hours Prior to Run. Protein Concentration, 3 mg./ml.; Speed, 59,780 r.p.m.; Temperature, 2°C.; Diaphragm Angle, 70°; Direction of Sedimentation, Left to Right. Time Intervals Measured After Rotor Reached Operating Speed. $S_{20,w} = 5.58$.

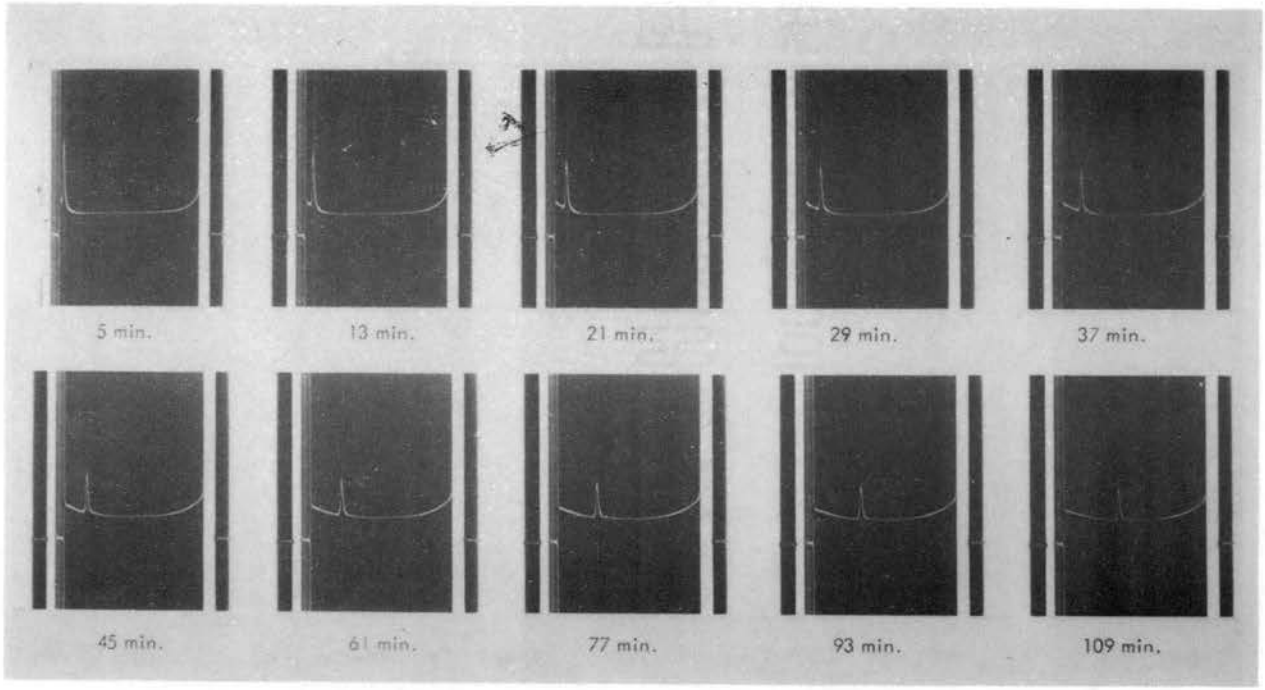


Figure 11. Ultracentrifuge Sedimentation of Bovine Myosin Dissolved in 0.5M KCl + 0.05M Phosphate (pH 7.1) + Sucrose. Protein Concentration, 3 mg./ml.; Speed, 59,780 r.p.m.; Temperature, 2°C.; Diaphragm Angle, 70°; Direction of Sedimentation, Left to Right. Time Intervals Measured After Rotor Reached Operating Speed. $S_{20,w} = 5.58$.

The above studies indicate that from the standpoint of ultracentrifuge sedimentation characteristics, the isolation procedure outlined in Chapter III yielded a pure, homogenous myosin preparation. Also these studies emphasize the importance of continuing to examine the sedimentation pattern for extended time intervals after operating speed has been attained in order to avoid missing significant observations. For example, the discrete leading peak, which occurred in two of the above runs after sedimentation proceeded 45 min., would not have been observed if the centrifugation had been terminated prior to this time.

Since the same protein concentration (3 mg./ml.) was used throughout these experiments, the $S_{20,w}$ values could not be extrapolated to zero concentration. In view of the results obtained with rabbit myosin (Johnson and Rowe, 1961; Baril et al., 1966) and cod myosin (Connell, 1963) where it has been found that the sedimentation coefficient ($S_{20,w}$) decreases as protein concentration increases, the $S_{20,w}$ values obtained in this study probably represent conservative estimates. However, it is interesting to note that the $S_{20,w}$ value of 5.89 obtained on myosin dissolved in 0.5M KCl at 2°C. is in close agreement with the value of 5.85 obtained on rabbit myosin by Johnson and Rowe (1961) under the same conditions.

CHAPTER V

ELECTROPHORETIC STUDIES ON BOVINE MYOSIN

Introduction

The purpose of this study was to establish the electrophoretic characteristics of purified bovine myosin. It was believed that this would be an important first step in developing standard objective techniques to evaluate the role of myosin in the various post-mortem quality changes occurring in beef muscle.

Materials and Methods

Isolation of Bovine Myosin

Myosin was isolated according to the procedure outlined in Chapter III. After the recrystallization step, an aliquot of the myosin preparation was dissolved in 0.5M KCl. It was then dialyzed against 50 volumes of 0.01M Tris-HCl buffer (pH 7.1) for 12 hours. Following dialysis, the sample was lyophilized and stored at 0°C. until used.

Electrophoretic Analysis

The electrophoretic behavior of bovine myosin was studied by the use of a disc electrophoresis unit (model 12), manufactured by Canalco Instrument Company. Aqueous and 8M urea solutions of the lyophilized

myosin were prepared and electrophoresed on polyacrylamide gels according to procedures outlined by the manufacturer (Canalco, 1962). The spacer and separating gels were polymerized at a temperature of 25°C., whereas the sample gel was polymerized at 2°C. The electrode buffers contained 14.4 gm. glycine and 3.0 gm. Tris in 1 liter of distilled water (pH 8.4). All electrophoretic tests were performed at a temperature of 2°C. The current setting for protein separation was 5 ma. per tube, and the dye front was allowed to migrate (cathode to anode) 5.7 cm. in about 45 minutes. Gels were stained with 1% Amido-Schwartz in 11% acetic acid:45% methanol and destained electrophoretically.

Results and Discussion

The electrophoretic pattern obtained on an aqueous solution of the myosin preparation exhibited seven faint, but distinct bands, which could be grouped into three zones along the gel. Zone 1, 2 and 3, contained 2, 3 and 2 bands, respectively (Figure 12).

From a preliminary experiment, it was found that high inorganic salt solutions could not be successfully electrophoresed on polyacrylamide gels. Hence, for the electrophoretic studies the myosin preparation could not be dissolved in its usual (0.5 M KCl) solvent. Considerable difficulty was encountered in getting sufficient lyophilized myosin to dissolve in water. Consequently, the electrophoretic patterns obtained on myosin in aqueous solutions were always rather faint (Figure 12).

Experimentation revealed that the lyophilized myosin preparation was readily soluble in 8M urea. This observation led to the comparison

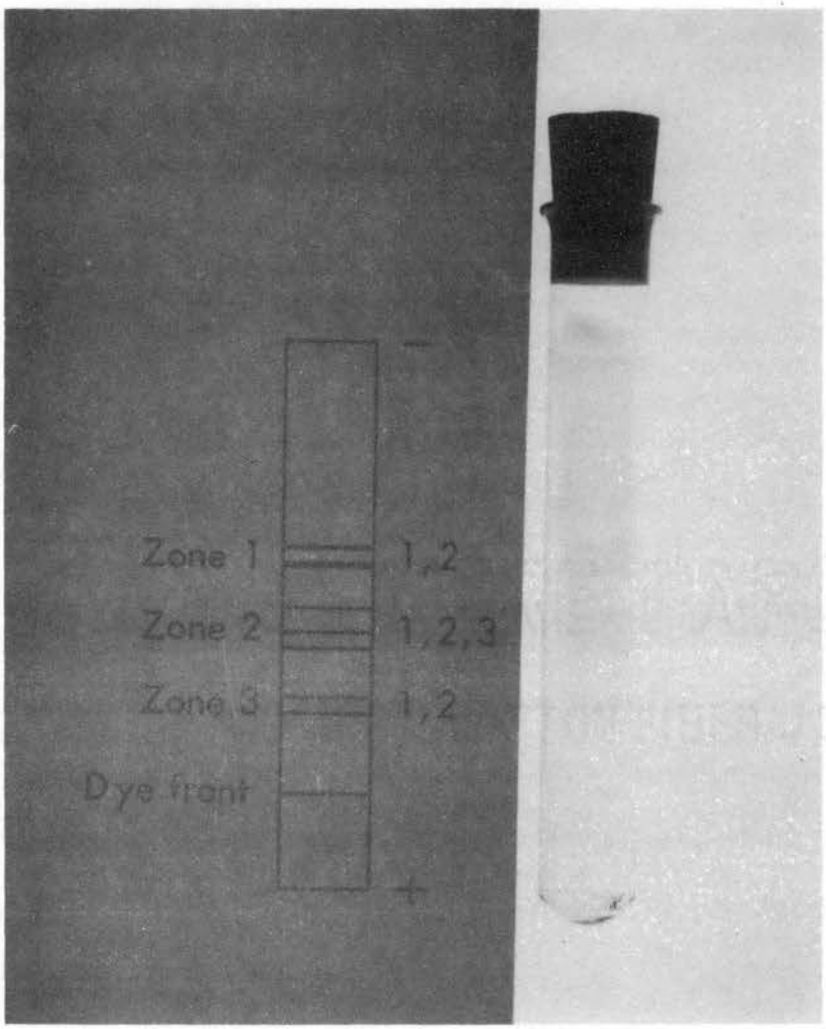


Figure 12. Electrophoretic Separation of Bovine Myosin in Aqueous Solution

of the electrophoretic patterns of myosin dissolved in water and in 8M urea. The results presented in Figure 13 showed that the myosin-urea electrophoretogram had greater band density and definition. Also, it can be noted that the myosin-water and myosin-urea electrophoretograms were very similar in terms of the number of zones, bands within each zone and overall mobility with the exception that band 2 in zone 2 showed a little more aniodic migration on the 8M urea gel (Figures 12 and 13).

Recent studies with polyacrylamide gel electrophoresis have indicated that the ammonium persulfate catalyst, an oxidizing agent used in polymerizing the separating gel, can leave this gel in an oxygen-rich state which could result in increased electrophoretic heterogeneity of the separated compounds (Brewer, 1967). Since myosin has a high sulfhydryl content, it was considered possible that this phenomenon might have an adverse effect on the electrophoretic behavior of myosin. To test this condition, sodium thioglycolate, an anionic reducing agent, was layered in front of the sample gel (0.7μM Na-thioglycolate in 50% sucrose per tube). Due to the similarity between the water and urea dissolved myosin electrophoretograms, and to avoid the solubility problem, this condition was tested on myosin dissolved in urea. The results obtained indicated that myosin still migrated as three primary zones with the same number of bands within each zone (Figure 14). Since a greater length of time was required to destain the gel when sodium thioglycolate was used, the two bands in zone 3 became quite faint. It should be pointed out that the two stained bands at the sample end of the gel (cathode end) represents the portion of the gel length occupied by the added reducing agent. Consequently,

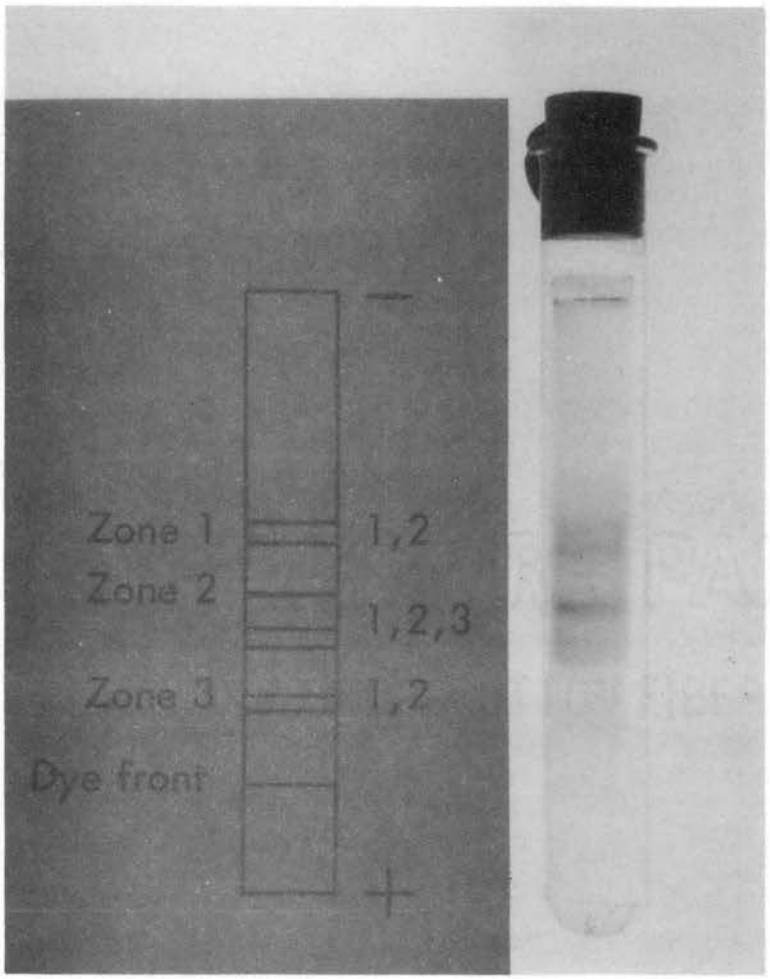


Figure 13. Electrophoretic Separation of Bovine Myosin in 8M Urea

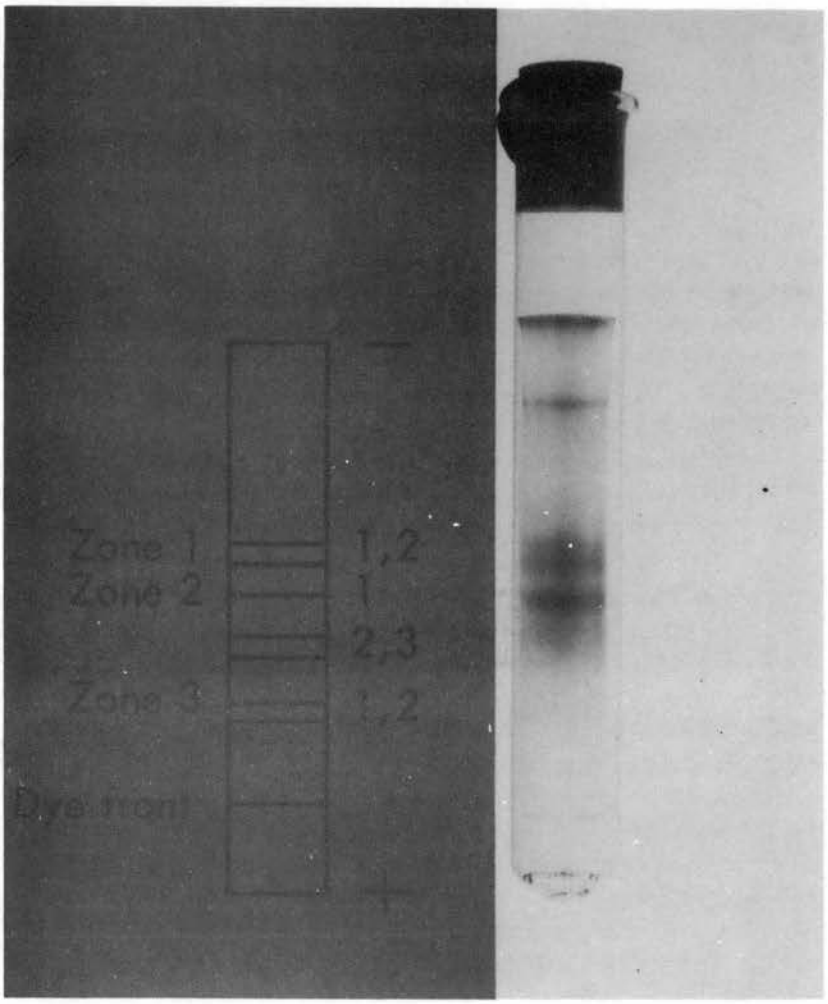


Figure 14. Electrophoretic Separation of Bovine Myosin in 8M Urea with Na-thioglycolate Layered in Front of the Sample Gel

these two bands simply represent immobile protein. Since the addition of the reducing agent to the gel did not significantly alter the myosin electrophoretogram, it can be concluded that the ammonium persulfate effect was negligible in this test.

When the molecular sieving effect of the separating gel was reduced by decreasing its acrylamide concentration from 7% to 3.5%, the electrophoretic pattern obtained on an aqueous solution of the myosin preparation showed only one band in each of the three zones (Figure 15). As expected, greater migration of the protein occurred in the 3.5% gel. These results emphasized the influence of gel concentration upon the sensitivity of this technique.

It is interesting to note that while the myosin preparation sedimented as a homogeneous entity in the ultracentrifuge, it exhibited considerable micro-heterogenity when electrophoresed on polyacrylamide gels. This lends weight to the sensitivity of the latter analytical technique.

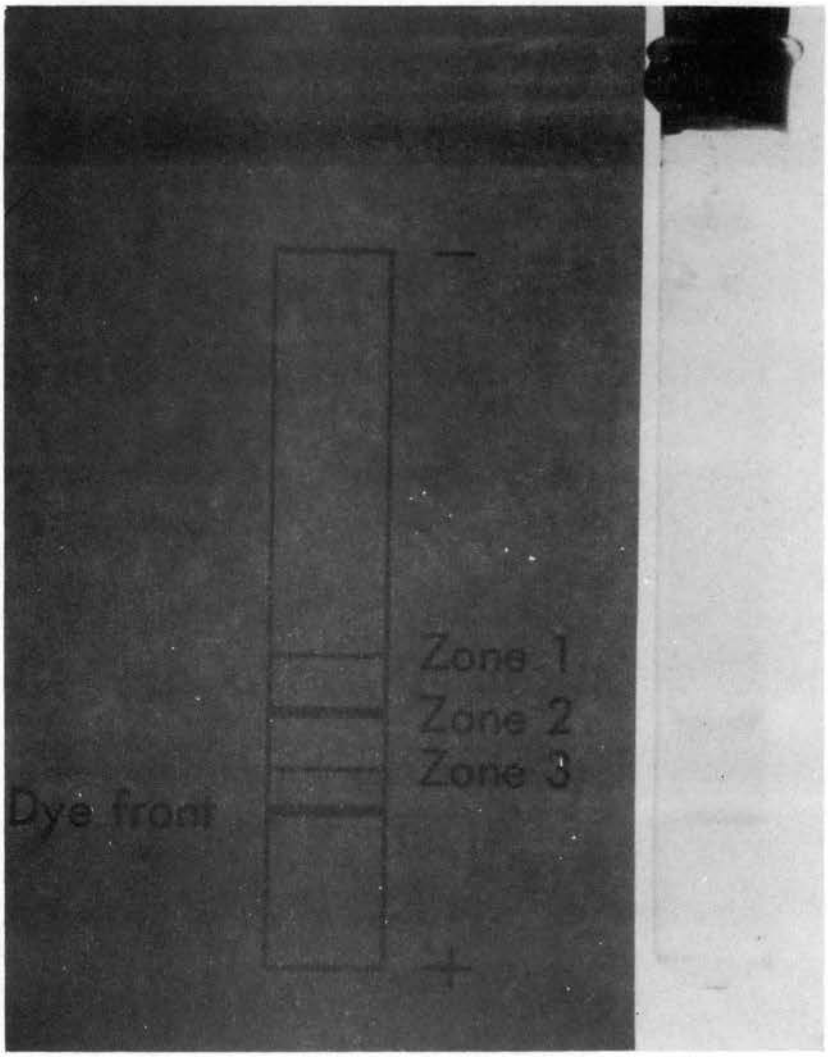


Figure 15. Electrophoretic Separation of Bovine Myosin in Aqueous Solution Using a 3.5% Separating Gel

CHAPTER VI

ELECTROPHORETIC CHARACTERISTICS OF THREE PROTEIN FRACTIONS ISOLATED FROM BOVINE LONGISSIMUS DORSI MUSCLE

Introduction

Considering the fact that the procedure for the isolation and purification of myosin is very tedious and time consuming, it is obvious that a rapid method for fractionating this protein would greatly facilitate the assessment of various treatment effects on myosin. In addition, this would remove the limitation on the number of samples which could be handled in such studies and permit a more accurate evaluation of the inherent variation which might exist between animals, muscles, etc., in the basic characteristics of this protein.

Towards this goal, disc electrophoresis on polyacrylamide gels was employed to determine the electrophoretic characteristics of three protein fractions from bovine muscle, classified as Total, Sarcoplasmic and Myofibrillar. The objective of this investigation was to study the electrophoretic behavior of proteins contained in these three fractions and to compare their electrophoretograms with that of purified myosin.

Materials and Methods

Protein Fractionation

The longissimus dorsi muscle was excised and minced as previously

described for the isolation of myosin (see Chapter III). A random 100 gm. aliquot of the muscle mince was homogenized in a Sorval Omni-mixer. Random 4 gm. aliquots were taken from this muscle homogenate for the isolation of the respective protein fractions. The Total protein fraction was isolated by the procedure outlined in Figure 16. The Sarcoplasmic and Myofibrillar protein fractions were obtained according to the procedure presented in Figure 17. The resultant supernatants, containing the respective protein fractions, were dialyzed against 50 volumes of distilled water, then lyophilized. All operations in the isolation procedure were conducted at 0°C. The lyophilized samples were stored at 0°C. until used.

Myosin Isolation

Myosin was isolated according to the procedure outlined in Chapter III. The lyophilized myosin sample was prepared as described in Chapter V.

Electrophoretic Analysis

Aqueous solutions of the respective protein fractions were prepared and electrophoresed on polyacrylamide gels as described in Chapter V. All runs were accomplished within two weeks after the respective protein fractions were isolated.

Results and Discussion

In studying the electrophoretic patterns obtained on the Total, Sarcoplasmic and Myofibrillar fractions, it was observed that the various protein "bands" separated could be uniformly grouped into four

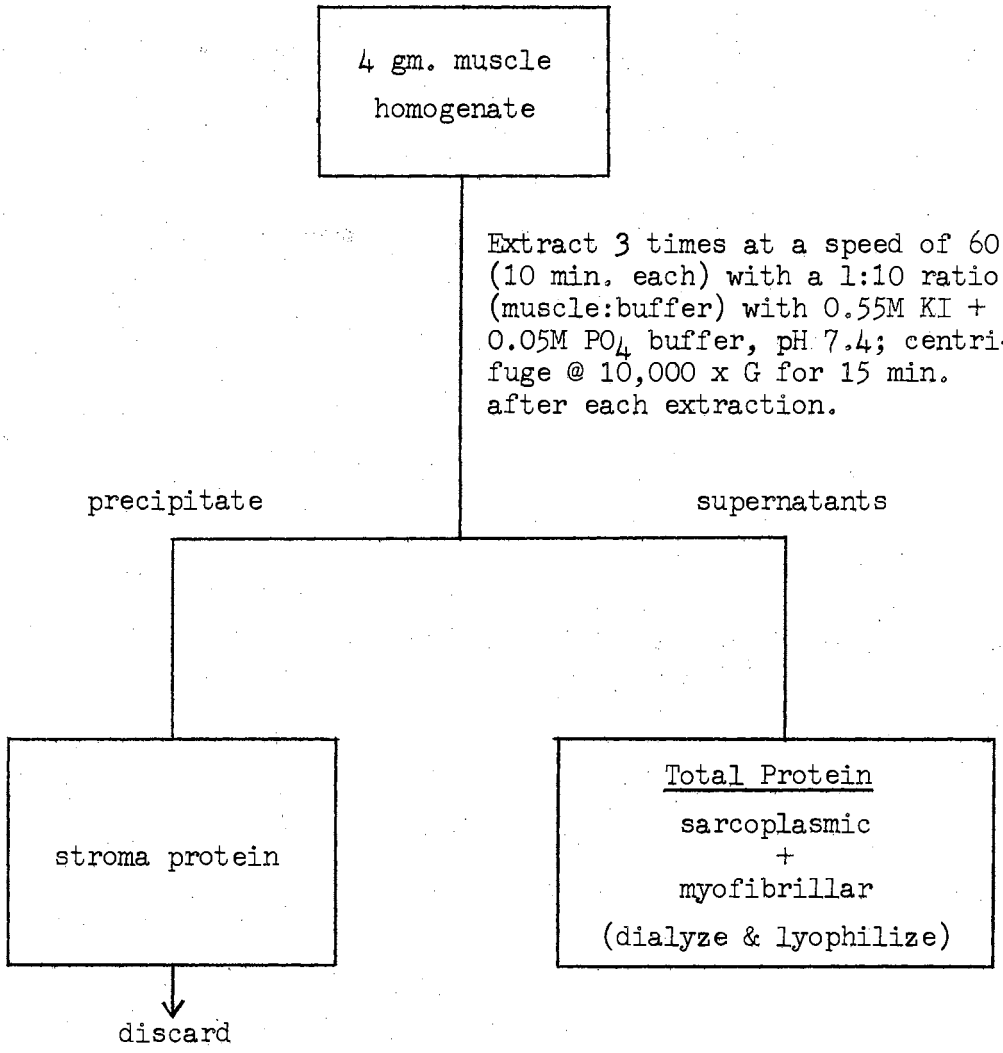


Figure 16. Extraction Procedure for the Total Protein Fraction

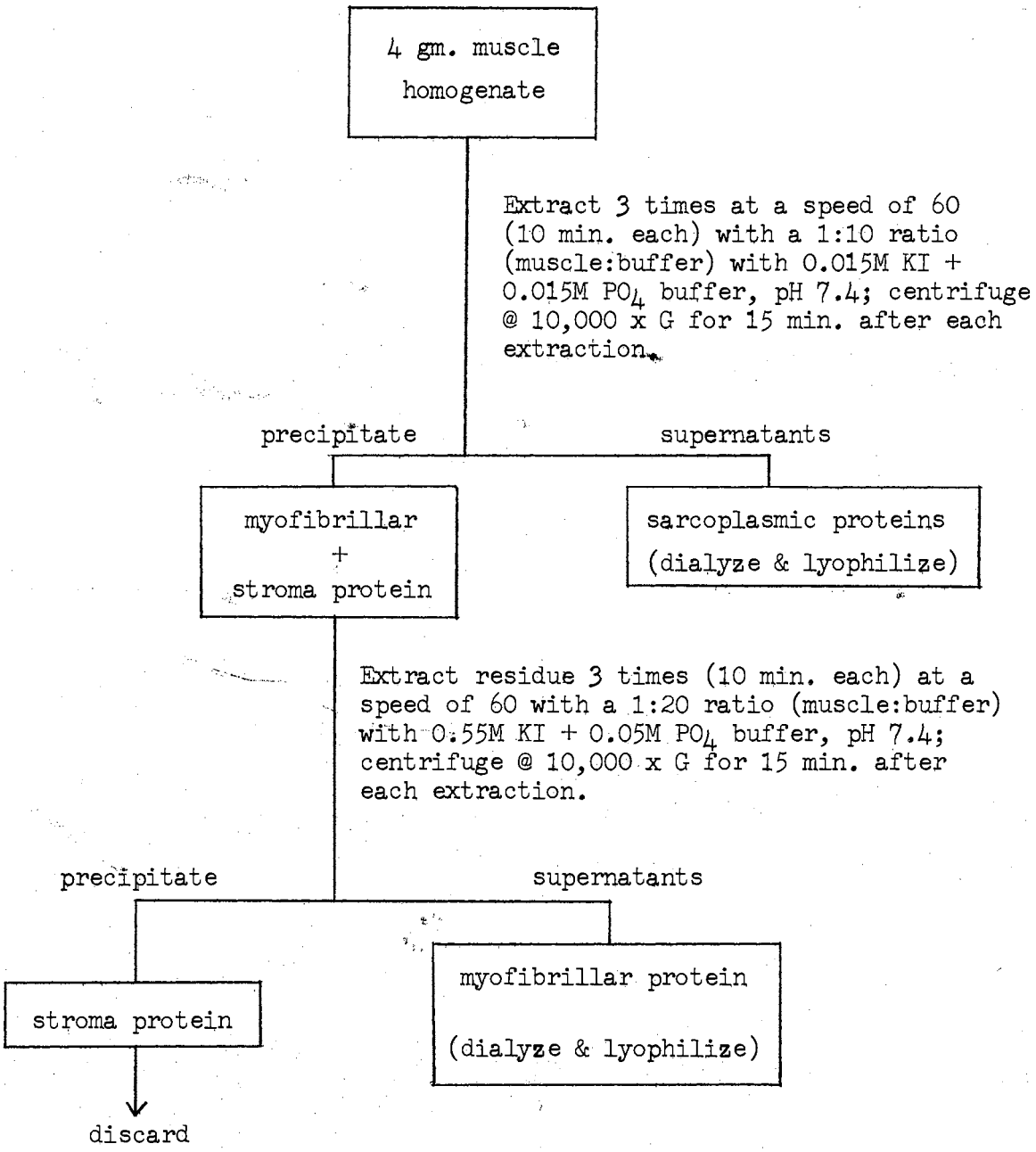


Figure 17. Extraction Procedure for the Sarcoplasmic and Myofibrillar Protein Fractions

distinct zones (which were designated as A, B, C and D) along the gel. This permitted the classification of the "bands" separated according to the order of their appearance in one of the major zones. This also provided a means for making systematic comparisons between the respective protein fractions. In Figure 18, it can be seen that the initial band in the A, B, C and D zones of the Total and Sarcoplasmic patterns was very distinct, being much larger than the other bands within their respective zones. In the Myofibrillar pattern, however, these major bands were not as distinct, yet they could be delineated.

The electrophoretic pattern obtained on each of the three protein fractions is presented in Figure 18. In the Total protein pattern, 3, 7, 2 and 7 protein bands were discernible in the A, B, C and D zones, respectively; while the Sarcoplasmic and Myofibrillar patterns exhibited 3, 4, 2, 2 and 3, 3, 2, 5 bands in these four respective zones. Thus, in terms of the number of bands, the major differences in the electrophoretic patterns of these protein fractions occurred in the B and D zones. Of greater significance was the distribution of the protein along the gel for the Sarcoplasmic and Myofibrillar fractions in relationship to the Total protein fraction. The majority of the protein components of the Sarcoplasmic fraction was contained in the A and B zones; whereas, the Myofibrillar proteins separated predominantly in the C and D zones. In addition, it was noted that the C_1 band in the Total and Sarcoplasmic fraction was always red in color prior to staining; whereas the C_1 band in the Myofibrillar fraction did not exhibit any color in the unstained state. It was concluded that the C_1 band in the Total and Sarcoplasmic fractions contained a portion of the proteinaceous pigment, myoglobin. These

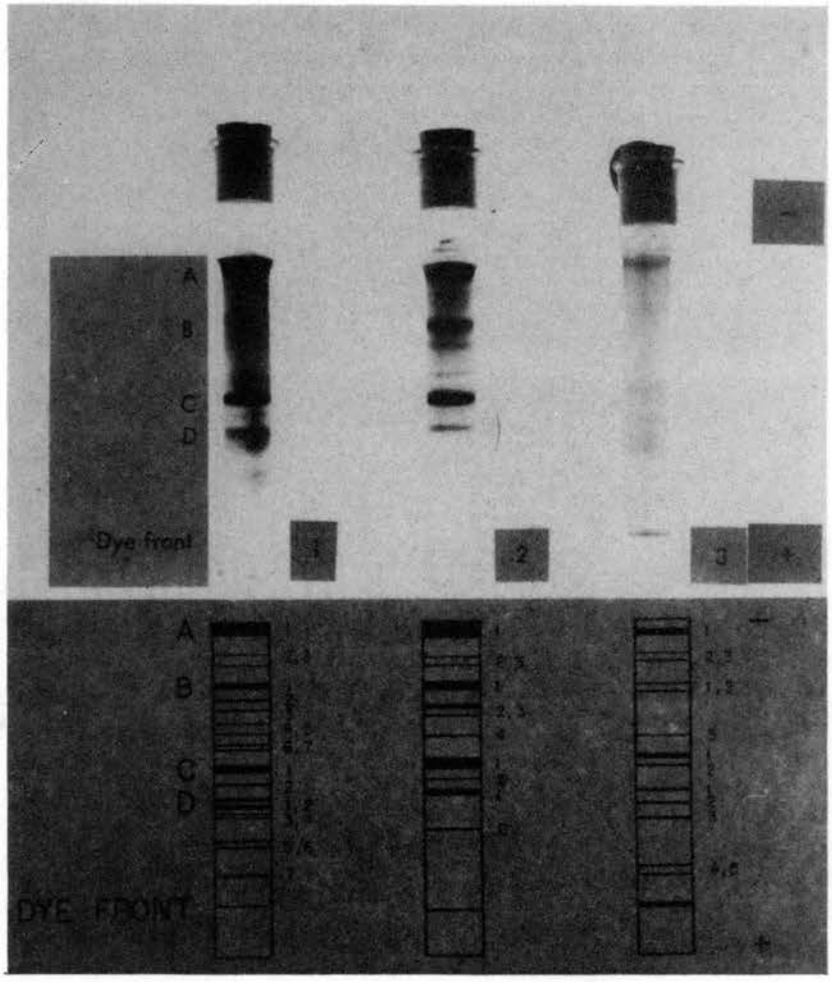


Figure 18. Electrophoretic Separation of Three Protein Fractions. 1) Total 2) Sarcoplasmic and 3) Myofibrillar

results indicate the possible methods by which standards could be developed to identify the various protein "bands" obtained during electrophoresis of whole muscle extracts.

Figure 19 shows the electrophoretic patterns of the Myofibrillar fraction and purified myosin dissolved in 8M urea. In comparing these patterns, a rather striking similarity was observed in the number and character of bands in the C and D zones. Each pattern contained 2 bands in the C zone and 5 bands in the D zone, however, there were some differences in the mobilities of the bands within the D zone. In addition, the electrophoretogram obtained with the purified myosin was devoid of protein in the A and B zones. These results strongly suggest that myosin can be rapidly isolated via polyacrylamide electrophoresis from semi-purified muscle extracts.

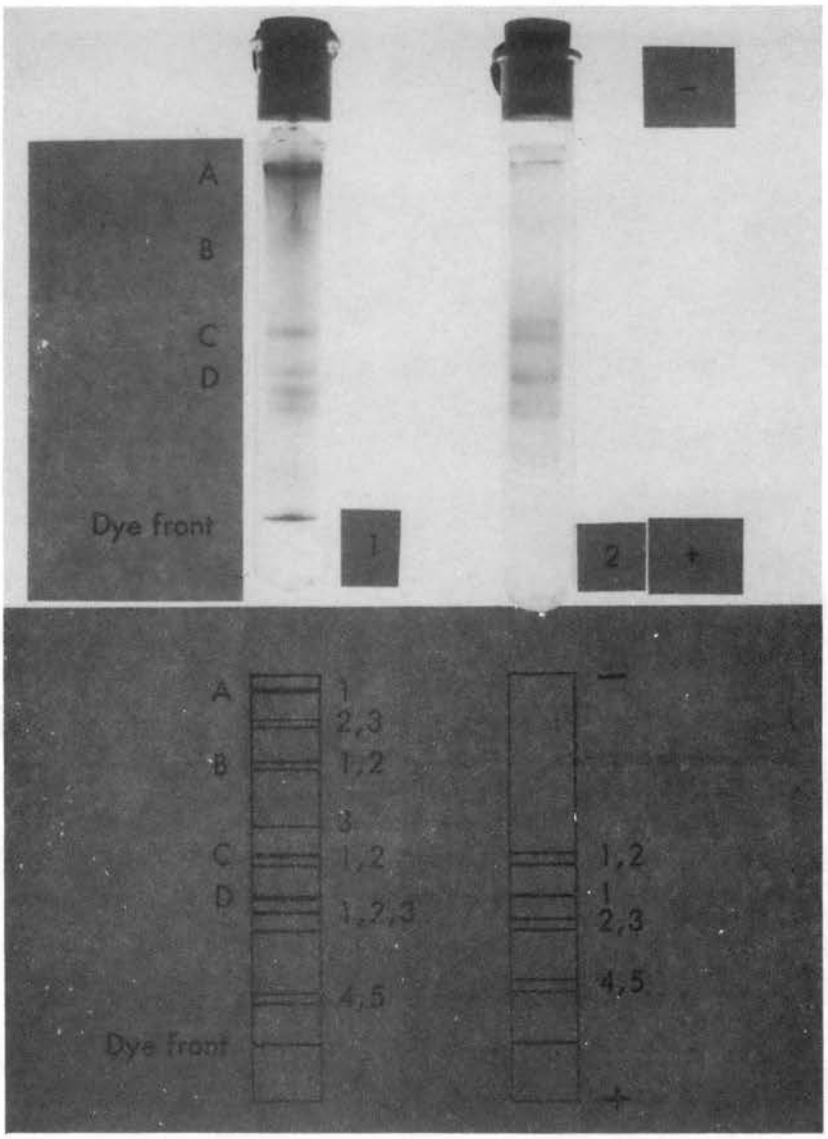


Figure 19. Electrophoretic Separation
1) Myofibrillar Fraction
2) Myosin in 8M Urea

CHAPTER VII

STUDIES ON MYOSIN ADENOSINETRIPHOSPHATASE ACTIVITY

Introduction

The primary biologically important aspects of myosin are its ATPase and actin-combining properties. Recent evidence favors the existence of two different sites on the myosin molecule responsible for the enzymic activity and its actin-combining ability, nevertheless, these two centers are closely related (Perry, 1967). Evidence for a functional relationship between myosin ATPase activity and contraction has been obtained in several studies. Gergely et al. (1965) have reported that myosin from red skeletal muscle has a lower specific ATPase activity than myosin from white skeletal muscle of the same species. This is in keeping with the slower contraction-relaxation cycle of red versus white muscles. Also, Barany (1967) has shown that myosin ATPase activity is related to the speed of muscle contraction. In addition, he indicates that it is the myosin molecule which plays the key role in the interaction between actin, myosin, and ATP. Thus, it seems reasonable to suppose that if the enzymic center of myosin is inhibited, the interaction between actin and myosin, in the formation of the complex actomyosin, would also be inhibited.

Certainly from the standpoint of the palatibility characteristics of muscle as food, improvement in tenderness and juiciness could

well result from the development of methods to keep myosin and actin dissociated post-mortem. In consideration of this aspect, the purpose of this study was to investigate methods of inhibiting bovine myosin ATPase activity. An exploratory approach was taken in this study in order to determine the influence of a combination of factors on myosin ATPase activity. This would serve as a logical first step in defining conditions for future experiments dealing with the effect of various chemicals on the actin-binding capacity of myosin.

Materials and Methods

Chemicals Studied

The selection of the chemicals studied was based on the type of reaction they cause and the sensitive group with which they react (Neilands and Stumpf, 1955). Ethylenediaminetetracetic acid (EDTA) functions as a chelating agent, which would result in divalent cation inactivation. Alkylating agents such as iodoacetic acid or iodoacetamide react with sulfhydryl or amino groups. Para-chloromercuribenzoate (PCMB) ties up free sulfhydryl groups by the formation of mercaptides. N-ethylmaleimide (NEM) reacts with sulfhydryl groups by addition.

Isolation of Myosin

Myosin was isolated from the longissimus dorsi muscle of two Hereford steers according to the procedures described in Chapter III. The final recrystallized myosin precipitate was dissolved in 0.5M KCl and diluted to a final protein concentration of 15 mg. per ml. It was then dialyzed against 50 vol. of 0.5M KCl + 0.01M Tris-HCl buffer

(pH 7.1) for 12 hours. This step was included to remove the free divalent cations, Ca^{++} and Mg^{++}, from the myosin solution.

Sucrose has been found to protect the enzyme active center of the myosin molecule against inactivation by various physical or chemical operations such as thawing, lyophilizing, and shifting the pH of the solution to the acid or alkaline side (Hashimoto and Yasui, 1966). Thus, it was postulated that sucrose might counteract any inhibitory effect of myosin ATPase activity caused by the various added chemicals. It was reasoned that this could be of practical importance since sucrose is used in the preparation of cured meat items. Consequently, another factor included in these experiments was the addition of sucrose to the myosin sample. This was accomplished by dividing the final, dialyzed myosin preparation into two equal aliquots and adding sucrose to one of these aliquots. Sucrose was added in an amount equal to twice the weight of the protein present.

The myosin preparations were stored at $0^{\circ}C$. until used.

Protein Determination

The protein concentration of the myosin solution was determined using the biuret method (Layne, 1957). Crystalline bovine serum albumin was used as the standard.

Calcium and Magnesium Determination

After the addition of sucrose, duplicate 2 ml. aliquots were taken from both the sucrose and non-sucrose myosin solutions. The samples were dried and then ashed at $600^{\circ}C$. for 24 hours. The ash was dissolved in 1 ml. of 12N HCl. The Ca^{++} and Mg^{++} content was deter-

mined using a Perkin-Elmer Absorption Spectrophotometer, by methods set forth by the manufacturer.

Enzyme Assay Conditions

Three incubation systems were employed in the determination of myosin ATPase activity. Based on the cation used and the ionic strength of the assay media, they were termed: 1) $CaCl_2$ high ionic strength system ($\mu = 0.6$), 2) $CaCl_2$ low ionic strength system ($\mu = 0.06$) and 3) $MgCl_2$ low ionic strength system ($\mu = 0.06$). Since Mg^{++} inhibits the ATPase activity of pure myosin, but activates actomyosin ATPase activity, this latter system was used only as a test of purity of the myosin preparation. The composition of the reaction mixtures for the high and low ionic strength systems was based on those described by Baril et al. (1966) and Kielley (1955), respectively, with modifications. The contents of the reaction mixture for each of the three systems were as follows: 1) $CaCl_2$ high ionic strength system: 1.5 ml. of 0.6M KCl + 0.01M Tris-HCl (pH 7.0), 0.2 ml. of 0.05M $CaCl_2$ in 0.6M KCl (unbuffered), 0.2 ml. of 0.02M ATP in 0.6M KCl buffered with 0.01M Tris-HCl (pH 7.0), and 0.1ml. myosin in 0.5M KCl (ca. 15 mg./ml.); 2) $CaCl_2$ low ionic strength system: 0.6 ml. of 0.15M KCl + 0.2M Tris-HCl (pH 7.0), 0.2 ml. of 0.05M $CaCl_2$, 0.2 ml. of 0.02M ATP + 0.01M Tris-HCl (pH 7.0), 0.9 ml. of deionized, distilled water, and 0.1 ml. of myosin in 0.5M KCl (ca. 15 mg./ml.); 3) $MgCl_2$ low ionic strength system; 0.2 ml. of 0.05M $MgCl_2$ was substituted for the $CaCl_2$ in the previous procedure. The total volume for each assay was 2 ml.

These above reaction mixtures served as the controls in deter-

mining the effect of the various added chemicals on myosin ATPase activity. The different levels of the added chemicals were obtained by replacing a portion of the buffer volume with the chemical, in question, dissolved in 0.6M KCl for the $CaCl_2$ high ionic strength system. Replacement of a portion of the distilled water with the desired level of the chemical dissolved in distilled water was used for the $CaCl_2$ low ionic strength system. Only in the EDTA experiment was $CaCl_2$ omitted from the reaction mixture. This was accomplished by adding buffer or distilled water to replace $CaCl_2$ in the high and low ionic strength systems, respectively. In this manner, the 2 ml. total volume of the reaction mixture as well as the proper ionic strength of the respective systems were maintained.

All reaction mixtures, for the respective systems and level of added chemical, were prepared in quadruplicate. To one set of duplicates, trichloroacetic acid was added prior to the addition of myosin and was termed the zero time sample. The other set of duplicates was used for the timed assays. This procedure allowed for the correction of the duplicate timed assays for inorganic phosphate not derived from the myosin catalyzed hydrolysis of ATP.

The reactions were initiated by the addition of myosin and allowed to proceed for 5 minutes at $0^{\circ}C.$ at pH 7.0. Initially, incubation times of 5, 10, 15, and 30 minutes were used with all control enzyme systems, and the 5 minute reaction time was selected since this was in the region where ATPase activity was a linear function of reaction time. A pH of 7.0 was chosen since this closely approximates the pH of bovine skeletal muscle at the time of slaughter. The temperature of $0^{\circ}C.$ was used. The reactions were terminated by the addition of 1 ml.

of 20% trichloroacetic acid. After the samples were centrifuged at 2000 x G at 0°C., 2 ml. aliquots were taken for inorganic phosphate analysis.

Inorganic Phosphate Determination

Inorganic phosphate was determined by the method of Fiske and Subbarow (1925). In a preliminary experiment, considerable variation was encountered between duplicate inorganic phosphate determinations. This led to an investigation of color development and color stability as measured by the Fiske and Subbarow method. It was found that the optical density of the samples increased with time of reading after the addition of the Fiske-Subbarow reagent. The ATPase activity calculated from an optical density reading taken after 5 hours color development was 36% higher than that determined on the same sample read after 10 minutes. This response was probably due to the catalytic effect of molybdate on the hydrolysis of the ATP left after termination of the enzymic reaction (Weil-Malherbe and Green, 1951). It was also observed that centrifugation at room temperature, with no temperature control, often resulted in erroneously high inorganic phosphate values. Further, it was found that storage of samples for 24 hours at 25°C., prior to the inorganic phosphate determination, resulted in values which were as much as 110% higher than that of the control values. This would suggest that the unreacted ATP underwent spontaneous hydrolysis to yield inorganic phosphate.

To circumvent these sources of error and to improve the repeatability between runs made on different days, the following procedure was followed: 1) After the reactions were terminated, samples were

centrifuged at 2000 x G in the cold room at 0°C. 2) a 2 ml. aliquot from the supernatant of each sample was pipetted into pre-chilled test tubes in the cold room at 0°C. 3) The volume of the sample was made to 3.6 ml. with distilled water at 25°C. This served to bring the sample to room temperature without prolonged standing at 25°C. 4) Then the following reagents were added in rapid succession to one tube at a time in order to maintain a constant time of 10 minutes for color development: a) add 0.4 ml. of 10N H_2SO_4. b) add 0.8 ml. of 2.5% ammonium molybdate, c) add 0.4 ml. of Fiske-Subbarow reagent, and d) make to total volume of 10 ml. with distilled water. 5) Color was allowed to develop for 10 minutes in a 25° C., temperature-controlled, water bath. 6) The % transmittance was read at a wavelength of 660 mu, using the Bausch and Lomb Spectronic 20. The % transmittance readings were converted to optical density (O.D.) by the formula: O.D. = 2 - log %T. The standard was prepared to include the concentration range of 0.1 μ mole to 1.0 μ mole inorganic phosphate from a stock solution of KH_2PO_4. The procedure for the standard was the same from step 3 on. A fresh standard was prepared for each day's run.

Calculations of ATPase Activity

The mean O.D. of the duplicate zero time samples was subtracted from each of the duplicate 5 minute timed reactions in the calculation of myosin ATPase activity. This enabled the calculation of the experimental error term in the statistical analysis of the data. Myosin ATPase activity, expressed as micromoles of inorganic phosphate per milligram protein per minute, was calculated from the following formula:

$$\mu M \text{ Pi/min./mg. protein} = \frac{(K)\ (1.5)\ (d.f.)\ (O.D._{5 \text{ min.}} - O.D._{0 \text{ time}})}{(5 \text{ min.})\ (\text{mg. protein/ml. of fraction})} ;$$

where the proportionality constant K = O.D./1μM Pi as calculated from the phosphate standard, 1.5 is the fraction of the total volume from which the 2 ml. aliquot for phosphate analysis was taken, d.f. is the dilution factor to put the myosin fraction on a 1 ml. basis, and mg. protein/ml. of fraction refers to the protein concentration of the myosin preparation.

Statistical Analysis

Due to the different solubilities of the various chemicals used in this study, they could not all be prepared in equal concentrations. Therefore, the results obtained with each chemical were analyzed as a separate experiment. The data from each experiment were analyzed as a completely randomized experiment with treatments arranged factorially on a within steer basis. Analysis of variance and calculation of standard errors were conducted according to the methods outlined by Steel and Torrie (1960).

The experimental layout for the EDTA experiment is presented in Table I. The four factors studied were designated as: A) ionic strength of the incubation system, B) $CaCl_2$ level, C) sucrose added to the myosin sample, and D) level of EDTA.

The experimental layout for the other chemicals studied is presented in Table II. The three factors considered were: A) ionic strength of the incubation system, B) sucrose added to the myosin sample, and C) level of the chemical under investigation.

Since these experiments were designed to find the factor or

TABLE I

EXPERIMENTAL LAYOUT FOR THE EDTA EXPERIMENT

	Animal							
A:	High Ionic Strength System				Low Ionic Strength System			
B:	No $CaCl_2$		+ $CaCl_2$		No $CaCl_2$		+ $CaCl_2$	
C:	No Sucrose	+ Sucrose	No Sucrose	+ Sucrose	No Sucrose	+ Sucrose	No Sucrose	+ Sucrose
D:								
Control	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples
0.01mM	"	"	"	"	"	"	"	"
0.02mM	"	"	"	"	"	"	"	"
0.04mM	"	"	"	"	"	"	"	"
0.08mM	"	"	"	"	"	"	"	"

A: Ionic strength of the incubation system.

B: $CaCl_2$ level.

C: Sucrose added to the myosin sample.

D: Level of EDTA added (mM/tube).

TABLE II

EXPERIMENTAL LAYOUT FOR THE OTHER CHEMICALS TESTED

	Animal			
A:	$CaCl_2$ High Ionic Strength System		$CaCl_2$ Low Ionic Strength System	
B:	No Sucrose	+ Sucrose	No Sucrose	+ Sucrose
C:				
0	2 samples	2 samples	2 samples	2 samples
1	"	"	"	"
2	"	"	"	"
3	"	"	"	"
4	"	"	"	"

A: Ionic strength of the incubation system.

B: Sucrose added to the myosin sample.

C: Level of the respective added chemicals (μM/tube):

Level	PCMB	NEM	Iodoacetic Acid	Iodoacetamide
0	control	control	control	control
1:	$.5 \times 10^{-4}$mM	.01mM	.1mM	.01mM
2:	1×10^{-4}mM	.02mM	.2mM	.02mM
3:	2×10^{-4}mM	.04mM	.4mM	.04mM
4:	4×10^{-4}mM	.08mM	.8mM	.08mM

combination of factors which cause the greatest variation, only the critical regions of $\alpha = .01$ and $\alpha = .005$ were used in determining the significance of the results.

Results and Discussion

Purity of the Myosin Preparation

As shown in Chapter IV, the myosin sample appeared homogeneous in the ultracentrifuge. In addition, no detectable ATPase activity was found for the myosin preparation isolated from steer 1 or steer 2, either in the presence or absence of sucrose, using the $MgCl_2$ low ionic strength system. These results indicate that the isolated myosin was free from any actin or actomyosin contamination since Mg^{++} serves as an activator of myosin ATPase activity only when actin is also present (Perry, 1955). Thus, myosin of a high degree of purity was obtained using the isolation procedure outlined in Chapter III.

Calcium and Magnesium Determination

The results of the calcium and magnesium determinations are presented in Table III. The addition of sucrose to the myosin solution after the dialysis step did not result in an appreciable increase in Ca^{++} or Mg^{++} contamination. The Ca^{++} and Mg^{++} concentrations for the myosin isolated from steer 1 were in close agreement. Also, the Mg^{++} concentration for myosin isolated from steer 1 and 2 were similar. The most striking difference occurred in the Ca^{++} content of the myosin preparations from the two steers. The two possible explanations for this are: 1) myosin from steer 2 contained more bound

TABLE III

CALCIUM AND MAGNESIUM CONTENT OF MYOSIN[1,2]

	Calcium	Magnesium
Animal 1:		
Myosin (No Sucrose)	0.24	0.23
Myosin (+ Sucrose)	0.28	0.28
Animal 2:		
Myosin (No Sucrose)	0.55	0.22
Myosin (+ Sucrose)	0.58	0.23

[1]Values represent the mean of two determinations.
[2]Values are expressed in terms of μg./mg. protein.

Ca^{++} and 2) greater Ca^{++} concentrations existed in the solvents used in the isolation of myosin in steer 2, and might have been removed if dialysis had been prolonged. No distinction can be made between bound or free calcium since the determinations were made on aliquots of the myosin dissolved in 0.5M KCl. Thus, it appears that dialysis was more effective in the removal of free Ca^{++} in the myosin preparation from steer 1 than from steer 2.

Effect of Ethylenediaminetetraacetic Acid (EDTA) on Myosin ATPase Activity

The results of the analysis of variance on the four factors studied are presented in Table IV. Three of the four main effects: ionic strength of the incubation system, sucrose addition, and level of EDTA, had a highly significant effect ($P<.005$) on myosin ATPase activity. However, several interactions were also significant. The fact that two of the interaction terms that were non-significant in animal 1,

TABLE IV

ANALYSIS OF VARIANCE ON THE EFFECTS OF IONIC STRENGTH, $CaCl_2$ LEVEL, SUCROSE ADDITION, AND LEVEL OF EDTA ON MYOSIN ATPase ACTIVITY

Source	d.f.	Animal 1 Mean Square	Animal 2 Mean Square
Total	79	--	--
Treatments[1]	39	.00032041	.00039444
A	1	.00742858**	.00901213**
B	1	.00002679	.00001540
C	1	.00066528**	.00014018**
D	4	.00024585**	.00020743**
AB	1	.00002893	.00035913**
AC	1	.00032603**	.00009397**
AD	4	.00031556**	.00051170**
BC	1	.00000118	.00003991
BD	4	.00027151**	.00045889**
CD	4	.00002586	.00000809
ABC	1	.00000610	.00000599
ABD	4	.00011187**	.00019890**
ACD	4	.00000962	.00001289
BCD	4	.00000074	.00002547*
ABCD	4	.00002246	.00000577
Error	40	.00001001	.00000607

[1] A: Ionic strength of the incubation system
B: Level of $CaCl_2$
C: Sucrose added to myosin sample
D: Level of EDTA

*$P < .01$

**$P < .005$

but were significant in animal 2, indicates the existence of variation between animals and/or the inability to duplicate procedures. No distinction can be made between these two sources of variation since the data was collected from each steer at different times.

The cause of these significant interactions becomes more apparent from the plots of the simple effects for animal 1 and animal 2 presented in Figures 20 and 21, respectively. The significant ionic strength x $CaCl_2$ interaction found for animal 2 resulted from the greater myosin ATPase activity, averaged over all levels of EDTA and sucrose addition, obtained using the high ionic strength incubation system without $CaCl_2$ than with $CaCl_2$; whereas the reverse occurred using the low ionic strength incubation system (see Figure 21). Although this same trend occurred with animal 1, it was not statistically significant, probably because the response with the high ionic strength incubation system was not as pronounced (see Figure 20). The significant ionic strength x sucrose addition interaction found for both animals was due to the larger depression in ATPase activity, averaged over all levels of EDTA and $CaCl_2$, caused by the addition of sucrose when measured by the high ionic strength over the low ionic strength systems (see Figures 20 and 21 for animals 1 and 2, respectively). A more obvious significant interaction, ionic strength x level of EDTA, resulted from EDTA functioning more as an activator in the high ionic strength system, but as an inhibitor in the low ionic strength system. The significant $CaCl_2$ x level of EDTA interaction is apparent from the inhibitory action of 0.01 mM EDTA in the presence of $CaCl_2$, but activates ATPase activity in the absence of $CaCl_2$ at this same concentration (see Figures 20 and 21 for animals 1 and 2, respectively). The significance of the three-

way interaction, ionic strength x $CaCl_2$ x level of EDTA, implies that the effect on myosin ATPase activity of EDTA is influenced by both the ionic strength of the incubation system as well as the presence or absence of $CaCl_2$. In addition, for animal 2, the three-way interaction between $CaCl_2$ x sucrose addition x level of EDTA was significant ($P<.01$). The significance of this interaction implies that the effect of EDTA on myosin ATPase activity is governed by the presence or absence of both $CaCl_2$ or sucrose. Also, this interaction was not significant for animal 1 which illustrates the between animal variation.

The fact that several interactions were found highly significant, clearly emphasizes the complexity of the problem in terms of defining a set of conditions for the maximum inhibition of myosin ATPase activity. Yet, there are some very interesting and similar trends apparent from the data plotted in Figures 20 and 21. These results show that the effect of EDTA on myosin ATPase activity was strongly influenced by the ionic strength of the incubation system.

Using the high ionic strength incubation system with no added $CaCl_2$, the maximum rate of ATPase activity occurred at the 0.01mM concentration of EDTA; whereas in the presence of $CaCl_2$, maximum acceleration did not occur until the concentration of EDTA exceeded that of $CaCl_2$. The inhibition of the ATPase activity, which occurred when the added EDTA and $CaCl_2$ concentrations were equivalent (0.01mM), was probably due to the preferential binding of EDTA to Ca^{++}. It is also interesting to note that EDTA, at a concentration equivalent to $CaCl_2$ (0.01mM), had a greater activating effect on myosin ATPase activity than did Ca^{++}. This response is illustrated by comparing the $CaCl_2$ controls (0 level of EDTA in Figures 20-A and 21-A) with the

0.01mM level of EDTA in Figures 20-B and 21-B. Since Mg^{++} was present in the final myosin preparation and is a known inhibitor of myosin ATPase activity, a logical explanation for this effect is that EDTA combined with the Mg^{++} present, thereby causing the added stimulation of myosin ATPase activity. Although the specific values obtained with the sucrose myosin sample were lower, the general trend in response was similar for both the non-sucrose and sucrose myosin samples in the high ionic strength systems. When the sucrose added myosin sample was assayed in the high ionic strength $CaCl_2$ activated system, the 0.01mM concentration of EDTA was the most effective in inhibiting myosin ATPase activity over the control (Figures 20-A and 21-A). When $CaCl_2$ was omitted, only the 0.08mM concentration of EDTA caused a depression in activity that approached or was lower than the control value since the response was different in magnitude between the two animals (Figures 20-B and 21-B).

In the low ionic strength systems, EDTA had, in general, a suppressing effect on myosin ATPase activity. For the $CaCl_2$ low ionic strength system, maximum inhibition over the control was obtained at the 0.01mM concentration of EDTA for the sucrose myosin sample and at 0.02mM concentration for the non-sucrose myosin sample (Figures 20-A and 21-A). This implies that sucrose also complexed with Ca^{++}, which would result in this added inhibition. The addition of EDTA had little effect on myosin ATPase activity in the low ionic strength system without $CaCl_2$ (Figures 20-B and 21-B). In fact, with this incubation system, very little ATPase activity occurred.

In contrasting the control values in Figures 20-B and 21-B, it can be noted that myosin ATPase activity was obviously stimulated by

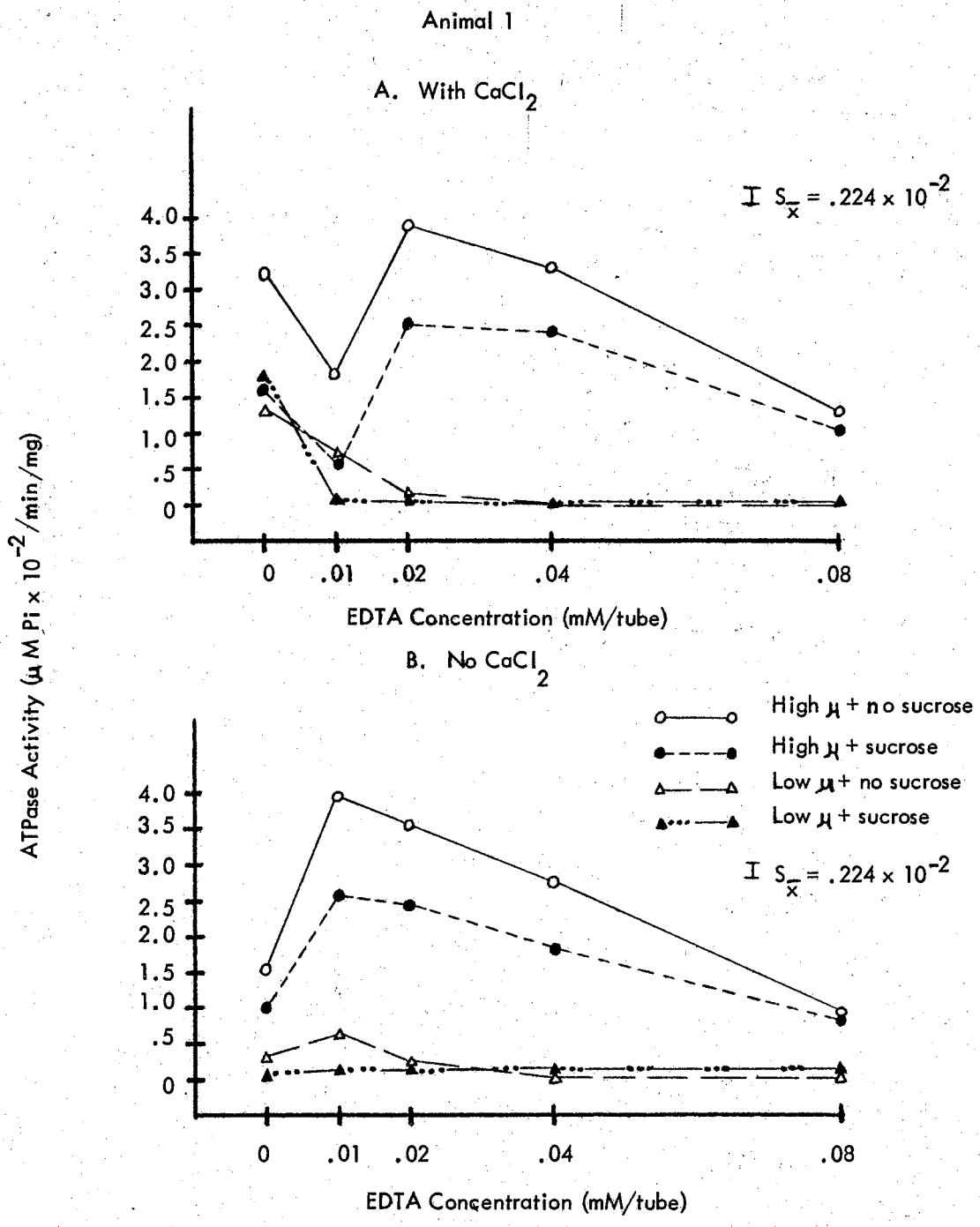


Figure 20. The Influence of Ionic Strength, Sucrose Addition and Level of EDTA on Myosin ATPase Activity

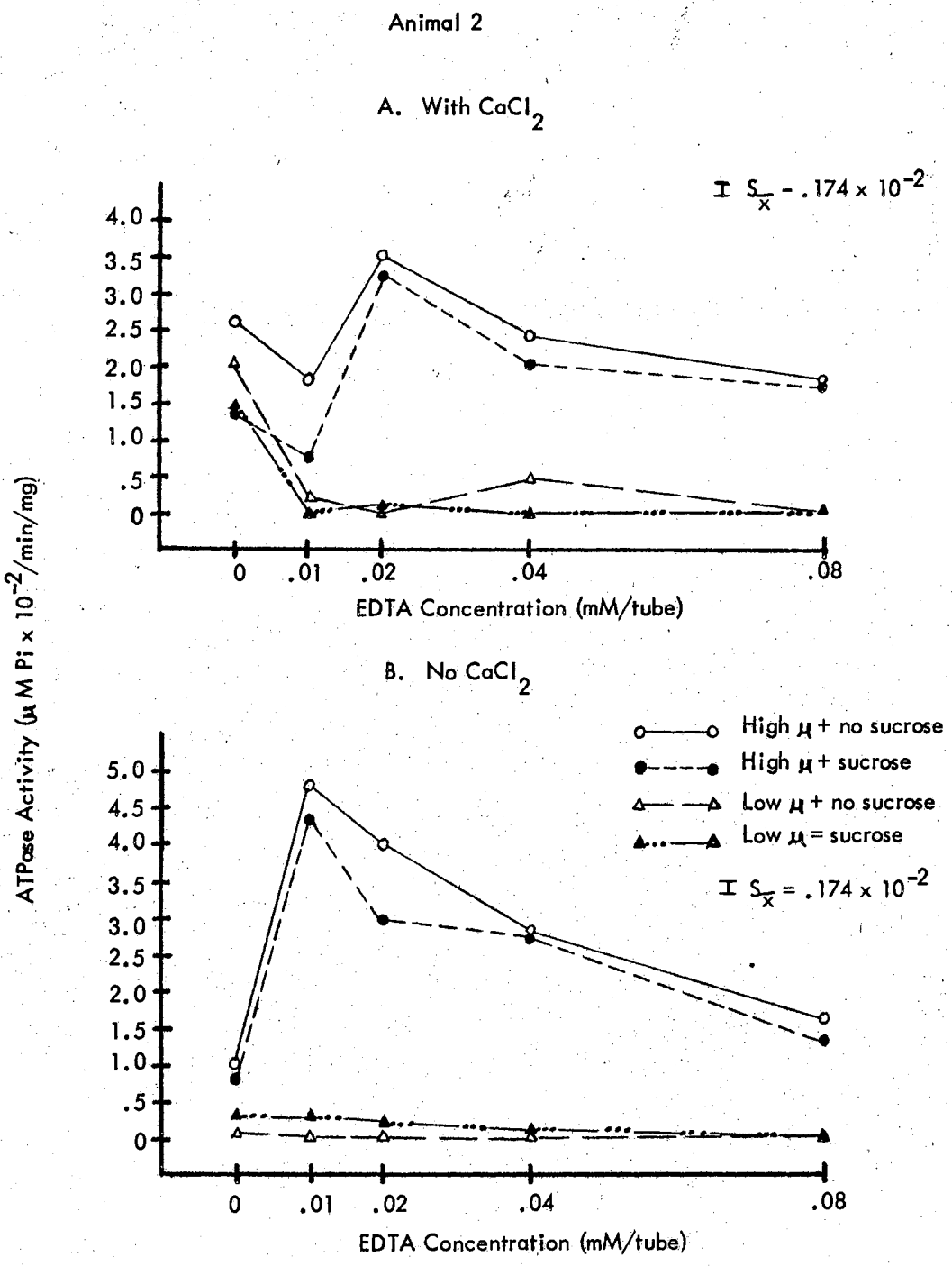


Figure 21. The Influence of Ionic Strength, Sucrose Addition and Level of EDTA on Myosin ATPase Activity

the K^+ ion. This is indicated by the significantly higher activity obtained in the high ionic strength controls over the low ionic strength controls in the absence of both $CaCl_2$ and EDTA.

EDTA activation of myosin ATPase, under certain conditions, has been recognized for myosin isolated from other species. Friess (1954), using rabbit skeletal muscle myosin, found that EDTA accelerated ATPase activity in 0.6M KCl, but inhibited ATPase activity in 0.05M KCl. Porcine myosin ATPase was also activated by EDTA in 0.5M KCl (Quass and Briskey, 1968). These results, coupled with those obtained in this experiment, indicate that there is some similarity in response to EDTA in myosin isolated from different species.

A number of experiments have been conducted in an attempt to understand the mechanism of EDTA activation. Ebashi et al. (1960) indicated that the activation of myosin ATPase in 0.6M KCl followed the order of the Mg-chelating capacity of the reagents tested within a series of EDTA analogues. Martonosi and Meyer (1964) suggested that EDTA activation was due to the removal of Mg^{++}. The consideration of other factors, such as conformational changes or allosteric effects, has been suggested by Sekine (1965). The results of Seidel (1969) indicate that the ATPase activity in the presence of EDTA is actually a K^+ - activated ATPase. Despite these efforts to understand this interesting phenomenon, there is still no general agreement concerning the mechanism of EDTA activation.

The results obtained in this experiment fit well with both the Mg^{++} removal and K^+ dependence hypotheses for EDTA activation. From the practical point of view, a knowledge of the ionic strength as well as the $CaCl_2$ content is necessary in the planned use of EDTA as a means

of inhibiting myosin ATPase activity.

Effect of Alkylating Agents on Myosin ATPase Activity.

A. Iodoacetic Acid (IAA)

After this experiment was completed, it was found that the high ionic strength incubation media contained insufficient buffer to maintain a constant pH of 7.0 when the selected levels of IAA were added. Consequently, the depression of ATPase activity observed with the high ionic strength system could have been due to this increasing acidity of the media rather than the direct involvement of IAA with myosin (Figure 22). Thus, the significance of the ionic strength term and its interactions obtained in the analysis of variance was probably due to this lowering of pH (Table V).

This finding does serve to emphasize a very important point. In literature concerned with the effects of various chemicals on myosin ATPase activity, rarely is any mention made as to whether or not a specified chemical was neutralized or buffered prior to its inclusion in a given incubation system. It is left to the reader to assume that the buffering capacity of the incubation system was sufficient to offset any resultant change in pH. Yet, as observed in this experiment, this assumption can lead to a misinterpretation of the data.

For the low ionic strength system, the buffering capacity of the incubation media was sufficient to maintain the desired pH of 7.0. Thus, valid assessments of the effect of IAA could be made in this system.

The curves in Figures 22-A and B show that for the non-sucrose samples, the lower levels of IAA caused an elevation in ATPase activity.

TABLE V

ANALYSIS OF VARIANCE ON THE EFFECT OF IONIC STRENGTH, SUCROSE ADDITION, AND LEVEL OF IODOACETIC ACID ON MYOSIN ATPase ACTIVITY

Source	d.f.	Animal 1 Mean Square	Animal 2 Mean Square
Total	39	--	--
Treatment[1]	19	.00026047	.00068243
A	1	.00102718**	.00715295**
B	1	.00042706**	.00048651**
C	4	.00032712**	.00061038**
AB	1	.00001428	.00016362*
AC	4	.00043301**	.00044485**
BC	4	.00005734*	.00012330**
ABC	4	.00005266*	.00011222**
Error	20	.00001073	.00001773

[1]A: Ionic strength of the incubation system

B: Sucrose added to myosin sample

C: Level of Iodoacetic Acid

*P $<$ .01

**P $<$ .005

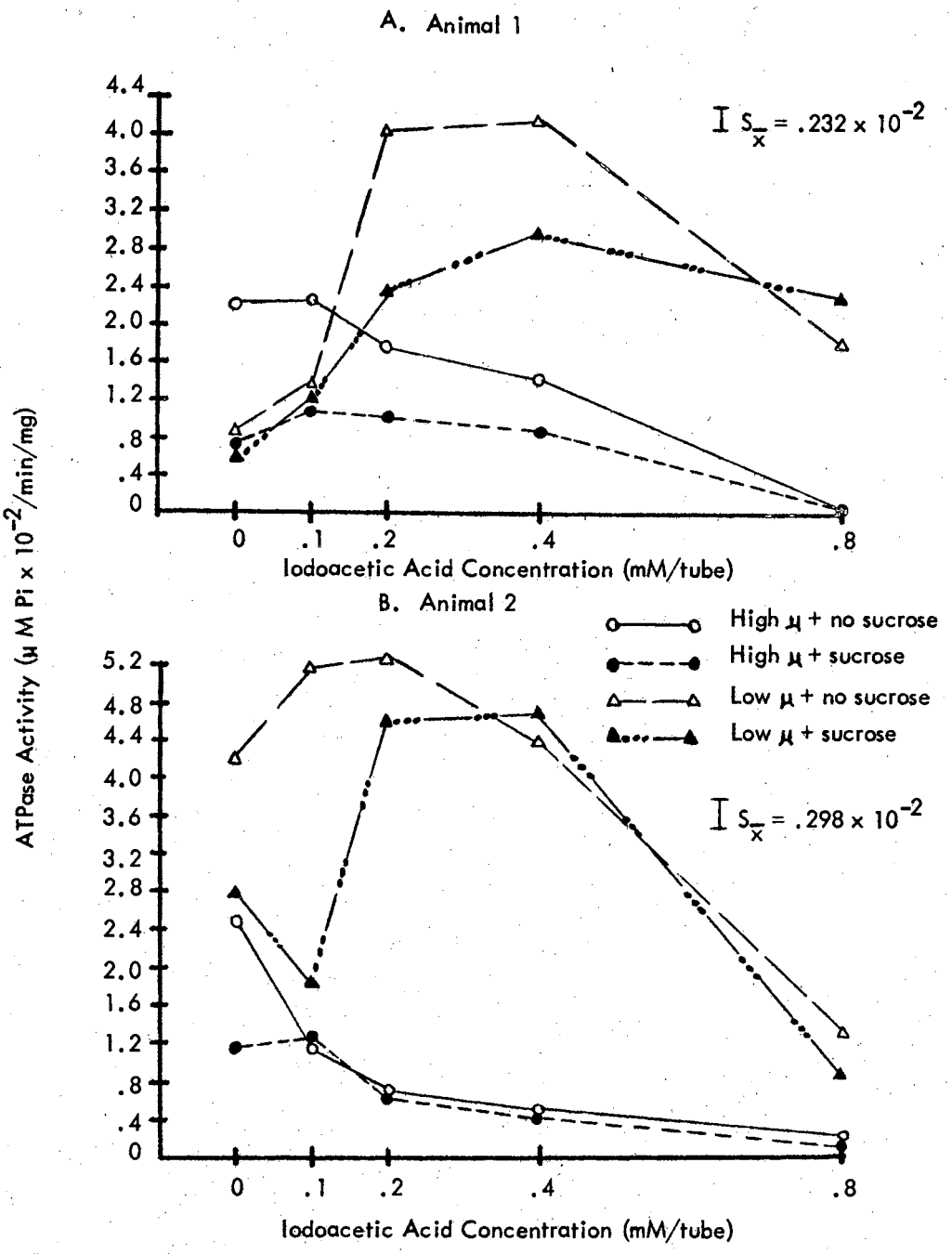


Figure 22. The Effect of Iodoacetic Acid on Myosin ATPase Activity as Influenced by the Addition of Sucrose to the Myosin Sample and the Ionic Strength of the Incubation System

When the IAA level was increased beyond 0.4mM, ATPase activity was depressed, becoming significantly lower than that of the control in animal 2, at the 0.8mM concentration. With one exception (0.1mM concentration), the sucrose samples followed a similar trend in response to IAA. The difference in level of response between animals 1 and 2 may be partly explained by the difference in the magnitude of the control values for these two animals.

A surprising response was the acceleration of myosin ATPase activity caused by the lower levels of added IAA. A possible explanation for this observation is that IAA reacted with a few of the "free" sulfhydryl groups which resulted in activation. This seems reasonable in view of the results of Kielley and Bradley (1956). These authors found that partial titration of thiol groups with sulfhydryl reagents caused an increase in Ca^{++}-activated rabbit myosin ATPase. This may not be the only factor involved since IAA is not specific for thiol groups. It can also react with amino groups at physiological pH values, however, this reaction occurs very slowly (Barron, 1951). In this experiment, no distinction can be made as to which functional groups reacted. Yet, the pH and reaction time used in this experiment would favor the slow rate of reaction between IAA with amino groups, therefore, the reaction with sulfhydryl groups seems more logical. Certainly this point warrants further investigation.

B. Iodoacetamide

The mean squares for this experiment are given in Table VI. A highly significant ($P<.005$) effect on myosin ATPase activity was obtained for the three main effects: ionic strength of the incubation

TABLE VI

ANALYSIS OF VARIANCE ON THE EFFECT OF IONIC STRENGTH, SUCROSE ADDITION, AND LEVEL OF IODOACETAMIDE ON MYOSIN ATPase ACTIVITY

Source	d.f.	Animal 1 Mean Square	Animal 2 Mean Square
Total	39	--	--
Treatment[1]	19	.00012142	.00011522
A	1	.00069139**	.00092930**
B	1	.00077352**	.00036361**
C	4	.00007370**	.00013536**
AB	1	.00027510**	.00000002
AC	4	.00003562	.00006108
BC	4	.00000380	.00002492
ABC	4	.00002864	.00000270
Error	20	.00001012	.00001803

[1]A: Ionic strength of the incubation system

B: Sucrose added to myosin sample

C: Level of Iodoacetamide

**P < .005

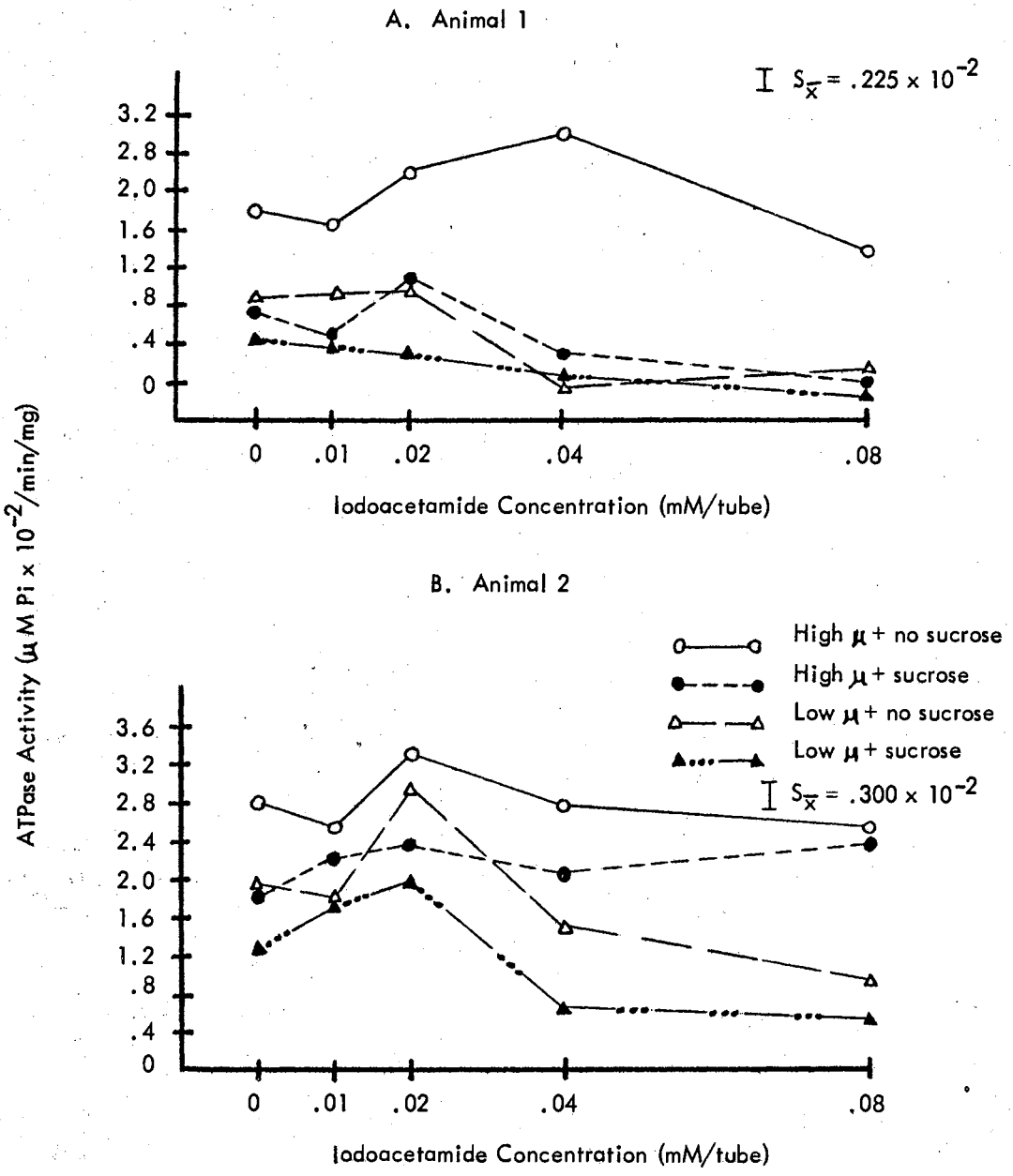


Figure 23. The Effect of Iodoacetamide on Myosin ATPase Activity as Influence by the Addition of Sucrose to the Myosin Sample and the Ionic Strength of the Incubation System

system; sucrose addition, and level of iodoacetamide. In addition, the ionic strength x sucrose addition interaction was also highly significant ($P<.005$) for animal 1. This interaction was due to sucrose having a greater inhibitory effect on myosin ATPase activity at the high over the low ionic strength systems, when averaged over all levels of iodoacetamide.

The results for the low ionic strength system plotted in Figures 23-A and B indicate that the response to Iodoacetamide was similar to that observed for IAA (Figures 22-A and B). However, the concentration of Iodoacetamide to obtain inhibition of myosin ATPase was considerably lower than that required for IAA, implying that Iodoacetamide is a more effective inhibitor than IAA.

Although there was variation in the magnitude of response to Iodoacetamide, in the high ionic strength system, between the two animals, the results suggest that lower concentrations of Iodoacetamide are required to obtain inhibition in the low versus the high ionic strength systems (see Figure 23).

Effect of Sulfhydryl Reagents on Myosin ATPase Activity

A. p-Chloromercuribenzoate (PCMB)

Although all levels of PCMB tested resulted in the inhibition of myosin ATPase activity over the respective controls, the concentration at which maximum inhibition occurred was influenced by both ionic strength and sucrose addition (Figure 24). This accounts for the significance of the interactions found from the analysis of variance for PCMB (Table VII). Even the lowest concentration of PCMB caused a

TABLE VII

ANALYSIS OF VARIANCE ON THE EFFECT OF IONIC STRENGTH, SUCROSE ADDITION, AND LEVEL OF p-CHLOROMERCURIBENZOATE ON MYOSIN ATPase ACTIVITY

Source	d.f.	Animal 1 Mean Square	Animal 2 Mean Square
Total	39	--	--
Treatment[1]	19	.00007354	.00011089
A	1	.00000015	.00001232
B	1	.00007209**	.00023233**
C	4	.00025283**	.00035155**
AB	1	.00002739**	.00000017
AC	4	.00001618**	.00003960**
BC	4	.00002269**	.00005403**
ABC	4	.00003270**	.00002033**
Error	20	.00000141	.00000182

[1]A: Ionic strength of the incubation system

B: Sucrose added to myosin sample

C: Level of p-Chloromercuribenzoate

**P < .005

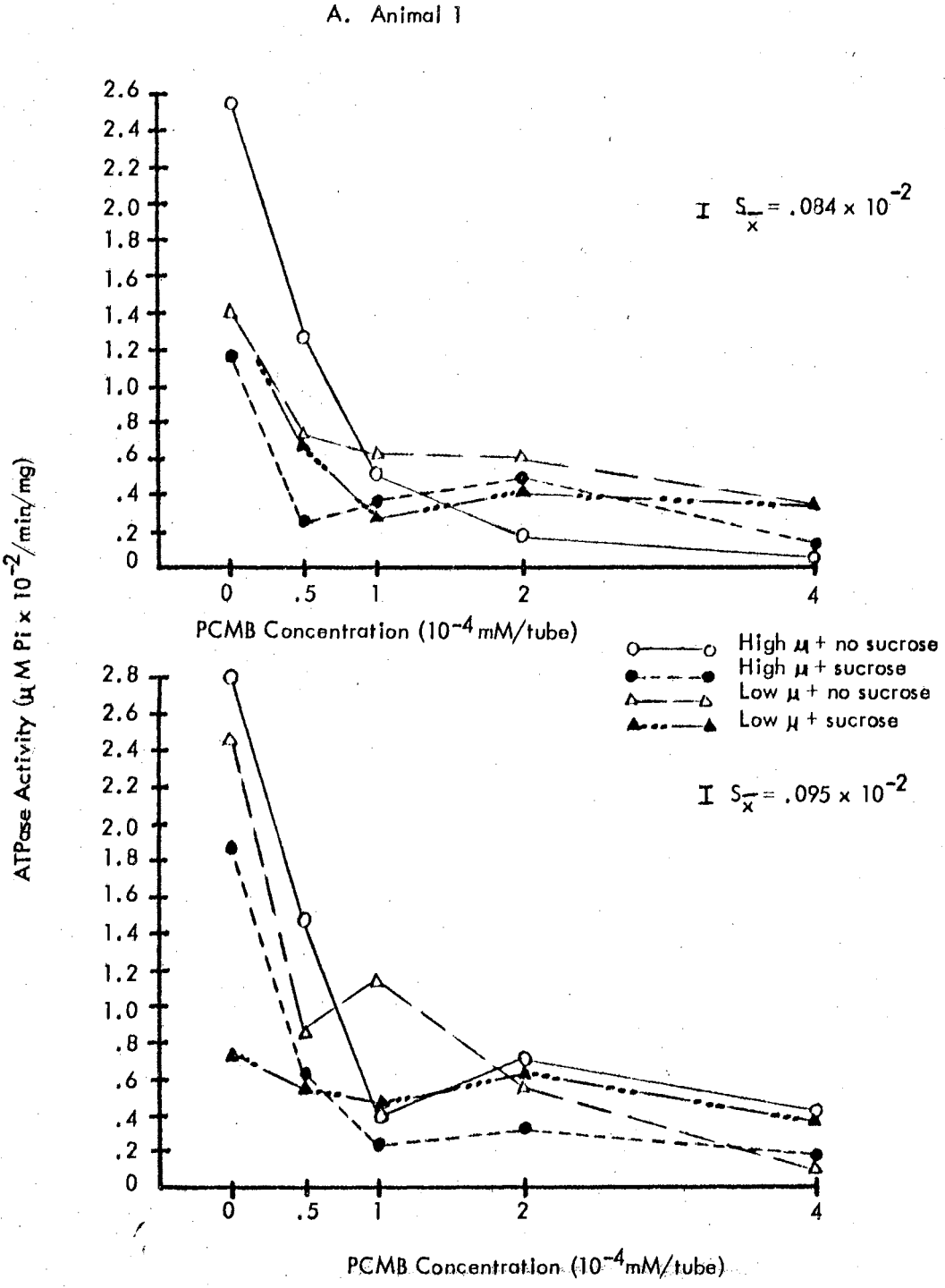


Figure 24. The Effect of p-Chloromercuribenzoate on Myosin ATPase Activity as Influenced by the Addition of Sucrose to the Myosin Sample and the Ionic Strength of the Incubation System

sharp, significant depression in ATPase activity over the control.

These results suggest that even lower concentrations of PCMB might be effective in causing an inhibition of myosin ATPase activity. An interesting adjunct to this experiment would be to determine the sulfhydryl content of the myosin preparation in conjunction with the study of ATPase activity. Certainly, a source of variation between myosin preparations from different animals could be reflected in the sulfhydryl content; and hence, influence ATPase activity.

B. N-ethylmaleimide (NEM)

From the results of the analysis of variance (Table VIII), all factors tested, with the exception of the ionic strength x sucrose interaction in animal 1, were highly significant ($P<.005$). The significance of these interactions was due to the variation in the amount of NEM required for maximum inhibition of myosin ATPase activity over the respective controls (Figure 25). Nevertheless, all of the selected levels of NEM caused a significant decrease in ATPase activity over the controls. These results augment the findings obtained with PCMB. However, PCMB appears to be a more effective inhibitor than NEM since much lower concentrations of PCMB were required for inhibition.

These results obtained with PCMB and NEM clearly emphasize the importance of the sulfhydryl groups on bovine myosin ATPase activity. This is in agreement with findings obtained on myosin isolated from other species. Based on the results obtained in these experiments, it was interesting to note that the concentration of PCMB and NEM required to inhibit bovine myosin ATPase activity were both less than the amount found necessary for the inhibition of rabbit myosin ATPase by

TABLE VIII

ANALYSIS OF VARIANCE ON THE EFFECT OF IONIC STRENGTH, SUCROSE ADDITION, AND LEVEL OF N-ETHYLMALEIMIDE ON MYOSIN ATPase ACTIVITY

Sucrose	d.f.	Animal 1 Mean Square	Animal 2 Mean Square
Total	39	--	--
Treatment[1]	19	.00008270	.00011048
A	1	.00004973**	.00001809**
B	1	.00009181**	.00014938**
C	4	.00028545**	.00038152**
AB	1	.00000449	.00007868**
AC	4	.00002814**	.00002913**
BC	4	.00002684**	.00002818**
ABC	4	.00001588*	.00002443**
Error	20	.00000343	.00000170

[1] A: Ionic strength of the incubation system

B: Sucrose added to myosin sample

C: Level of N-Ethylmaleimide

*$P < .01$

**$P < .005$

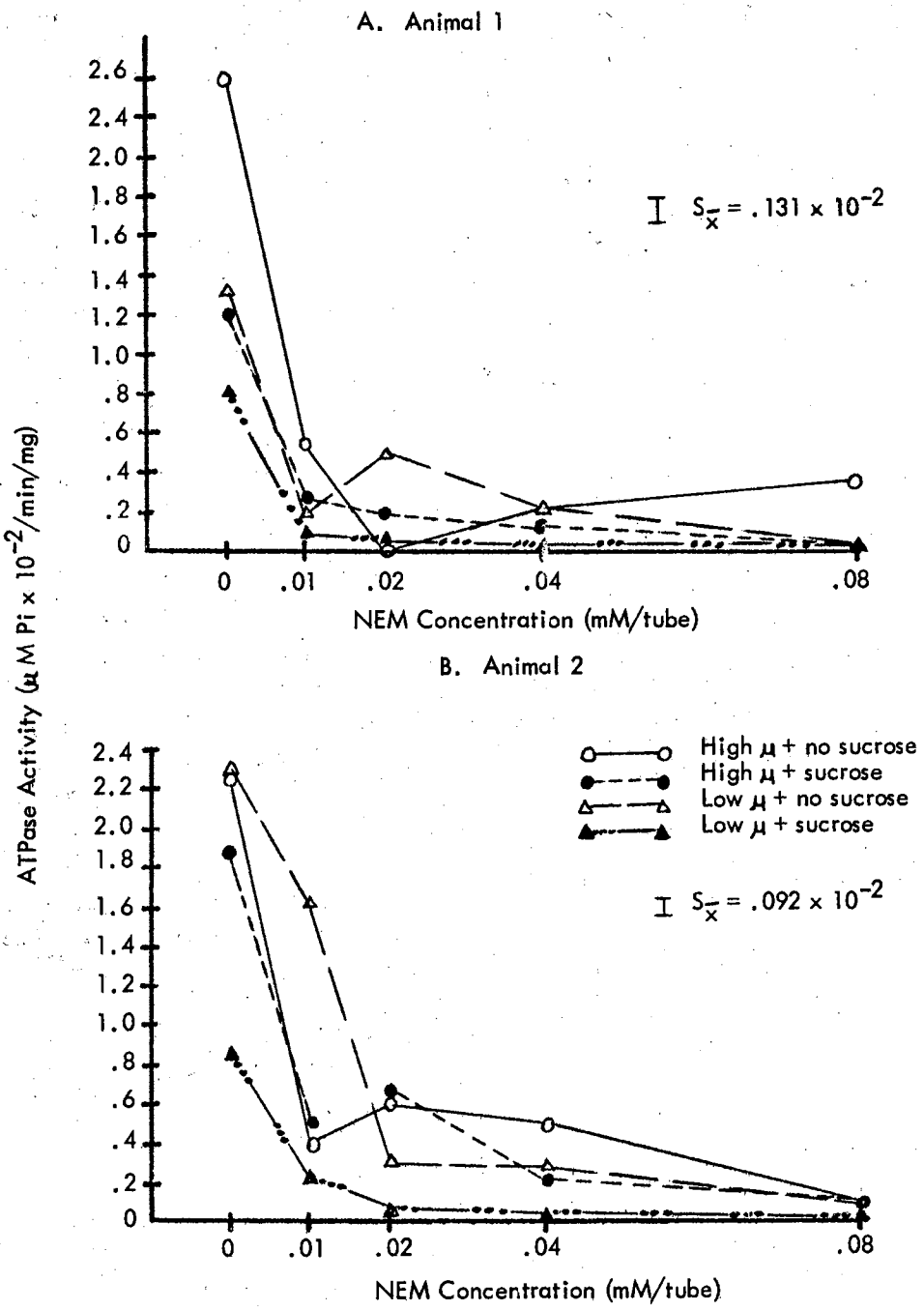


Figure 25. The Effect of N-ethylmaleimide on Myosin ATPase Activity as Influenced by the Addition of Sucrose to the Myosin Sample and the Ionic Strength of the Incubation System

Kielley and Bradley (1956). This could mean that the sulfhydryl groups associated with the active center of bovine myosin are either fewer in number or more reactive than those for rabbit myosin. However, in the Kielley and Bradley paper, the temperature used for ATPase assay was not clearly stated. The results of more recent experiments have indicated that the amount of PCMB or NEM required to inhibit Ca^{++}-activated myosin ATPase can be influenced by the temperature used for assay (Gilmour, 1960; Sekine and Kielley, 1964).

Effect of Sucrose on Myosin ATPase Activity

In studying the results of the previous experiments, a rather consistent trend towards lower ATPase activity was observed for the sucrose added myosin samples when compared to its non-sucrose counterpart. These observations led to the further analysis of the data in order to determine the effect of sucrose, per se, on myosin ATPase activity. This was accomplished by comparing the control values obtained from the four primary treatment combinations (i.e., $CaCl_2$ high and $CaCl_2$ low ionic strength incubation systems, with the sucrose and non-sucrose myosin samples).

Due to the complexity of these experiments, it was not possible to complete the chemical assays for all chemicals tested within a given day. Consequently, the controls used for this comparison were from separate runs. Therefore, another factor included in this analysis of the data was time. This provided the means for evaluating any variation which might have resulted from day-to-day differences and/or from myosin denaturation. The time factor has relevance only on a within steer basis because the data from each animal were collected at

different times.

From the analysis of variance (Table IX), it can be seen that both ionic strength and sucrose addition had a highly significant ($P<.005$) effect on myosin ATPase activity. In addition, for animal 1 only, the interaction between ionic strength and sucrose addition was highly significant ($P<.005$). The time factor was non-significant ($P>.1$), indicating that the between-run differences, as well as any possible physico-chemical alterations which might have occurred in the myosin preparations during storage, were minimal and hence did not significantly influence the assessment of ATPase activity over the period of time required to complete all chemical analyses. Moreover, this was taken as justification for pooling the values obtained from the different runs.

The pooled mean, for each of the four treatment combinations was plotted, on a within animal basis, in Figure 26. As illustrated in Figure 26-A the significant interaction, noted in animal 1, between ionic strength and sucrose addition resulted from sucrose having a greater suppressing effect on ATPase activity in the high ionic strength system than it did in the low system. For animal 2, the degree of inhibition due to sucrose addition was approximately the same regardless of the ionic strength of the incubation system (Figure 26-B). Though there were differences in the magnitude of ATPase activity due to animal effect, the order of response to the various treatment combinations was the same for both animals.

Duncan's New Multiple Range Test was used to test the simple effects (i.e. the four treatment combinations) on a within animal basis. All four treatments means from each animal were significantly different

TABLE IX

ANALYSIS OF VARIANCE ON THE EFFECT OF SUCROSE ADDITION, IONIC STRENGTH, AND TIME OF ASSAY ON MYOSIN ATPase ACTIVITY

Source	d.f.	Animal 1 Mean Square	Animal 2 Mean Square
Total	47	--	--
Treatment[1]	23	.00009494	.00008859
A	1	.00058590**	.00025346**
B	1	.00122311**	.00157209**
C	5	.00001625	.00000877
AB	1	.00017366**	.00000063
AC	5	.00000739	.00001139
BC	5	.00000666	.00001016
ABC	5	.00000989	.00001195
Error	24	.00000902	.00001709

1
A: Ionic strength of the incubation system

B: Sucrose added to myosin sample

C: Time of assay

**P < .005

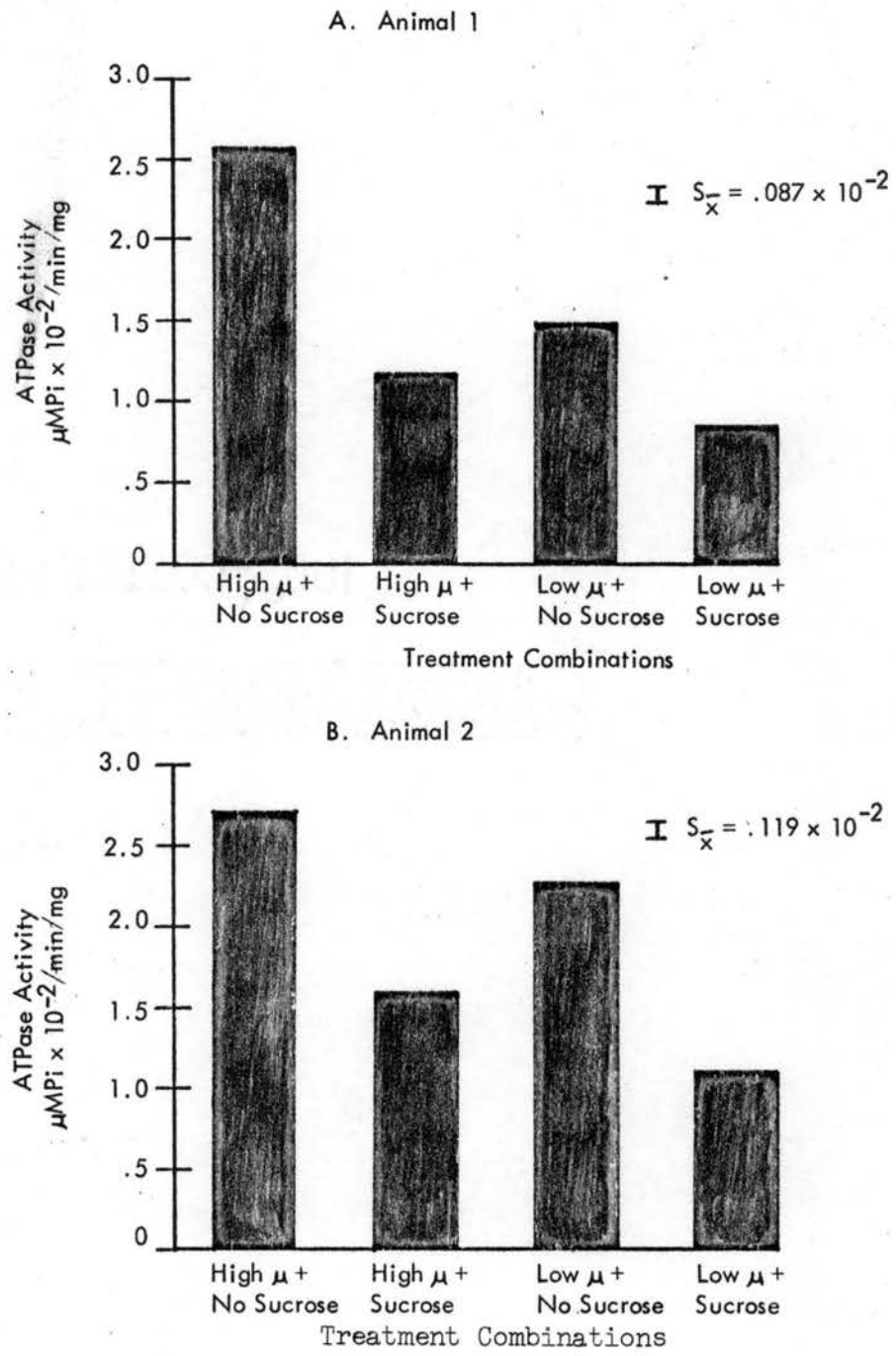


Figure 26. The Effect of Ionic Strength and Sucrose Addition on Myosin ATPase Activity as Measured in the Presence of $CaCl_2$

($P<.05$). The ranking from highest to lowest ATPase activity was as follows: $CaCl_2$ high μ + no sucrose > $CaCl_2$ low μ + sucrose > $CaCl_2$ high μ + sucrose > $CaCl_2$ low μ + sucrose. These results clearly indicate that sucrose, as added in this experiment, caused a significant inhibition of myosin ATPase activity.

These results prompted further investigation in order to gain insight into the possible reasons for the inhibitory action of sucrose on myosin ATPase activity. It was reasoned that a good starting point would be to examine the effect of sucrose using the same four treatment combinations, but without added $CaCl_2$.

The analysis of variance of the results from this experiment are shown in Table X. Ionic strength of the incubation system was found to have a highly significant ($P<.005$) effect on myosin ATPase activity for both animals. Sucrose did not have a statistically significant effect on myosin ATPase activity in this experiment, which was in contrast to the results obtained when $CaCl_2$ was included in the incubation system.

As can be seen in Figure 27 the addition of sucrose did cause some inhibition of myosin ATPase activity in the high ionic strength system, and the effect was more pronounced for animal 1 than animal 2. However, the degree of inhibition was not large enough to be statistically significant for either animal. Although the response to sucrose when assayed by the low ionic strength system was reversed between the two animals, the difference in either case was not significant.

Thus, in the absence of $CaCl_2$, sucrose did not cause a significant depression in myosin ATPase activity. In contrast, when assayed in the presence of $CaCl_2$, a significantly lower rate of activity was obtained when sucrose was added. These data suggest that the primary action of

TABLE X

ANALYSIS OF VARIANCE ON THE EFFECT OF IONIC STRENGTH AND SUCROSE ADDITION ON MYOSIN ATPase ACTIVITY AS MEASURED IN THE ABSENCE OF $CaCl_2$

Source	d.f.	Animal 1 Mean Square	Animal 2 Mean Square
Total	7	--	--
Treatment[1]	3	.00009622	.00004011
A	1	.00025538**	.00011250**
B	1	.00002964	.00000024
AB	1	.00000365	.00000761
Error	4	.00000768	.00000168

[1]A: Ionic strength of the incubation system

B: Sucrose added to myosin sample

**$P < .005$

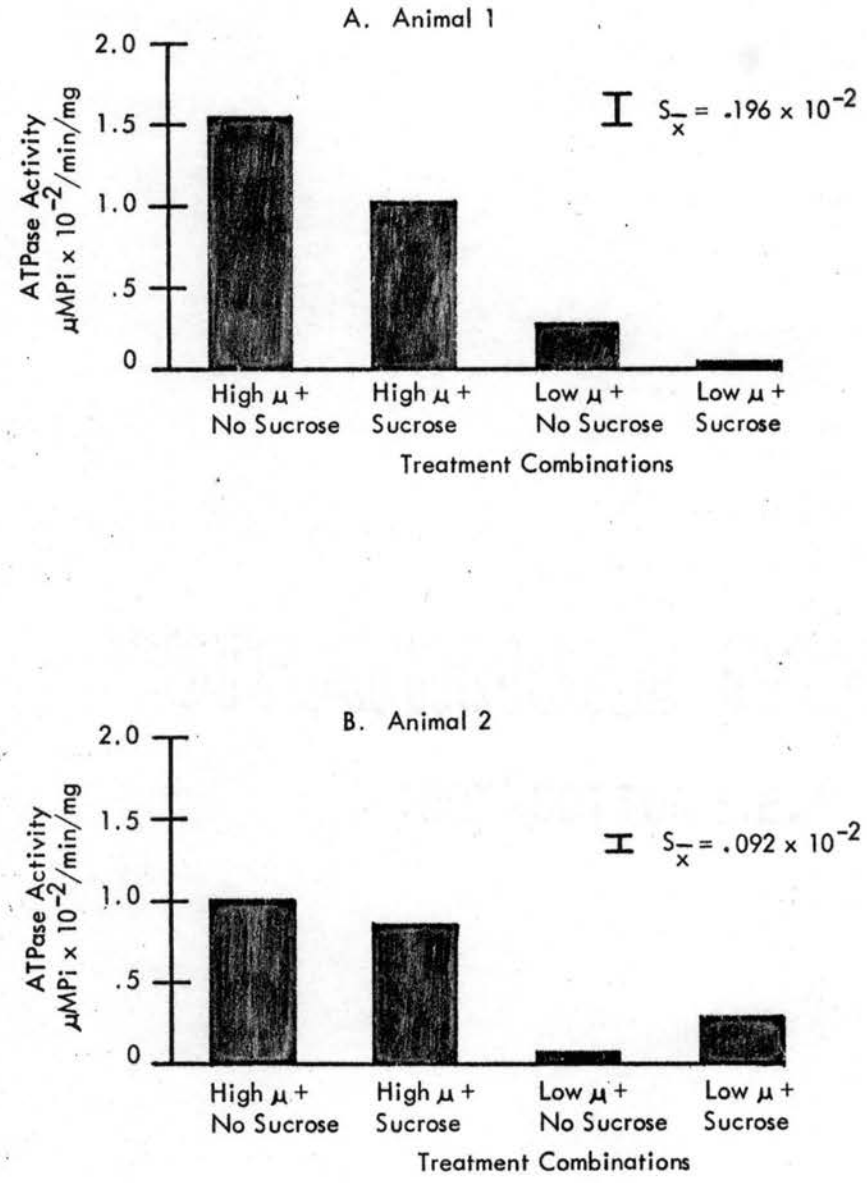


Figure 27. The Effect of Ionic Strength and Sucrose Addition on Myosin ATPase Activity as Measured in the Absence of $CaCl_2$

sucrose in effecting a suppression of the Ca^{++} activated myosin ATPase, was to combine with or chelate the activating Ca^{++} ion.

In addition to the important effect of sucrose, the results from this experiment have brought to light other factors concerning myosin ATPase. A discussion of these results involves the cross-reference of data portrayed in Figures 26 and 27.

In considering the no sucrose systems, it can be seen that $CaCl_2$ added to the low ionic strength system resulted in a greater increase in ATPase activity than when added to the high ionic strength system. Also the data show that in both the high and low ionic strength systems, animal 2 responded to a much higher level to the added $CaCl_2$ than did animal 1.

By examining the data for the no sucrose systems for both animals in Figure 27, it can be observed that in the absence of $CaCl_2$ the dominant factor influencing ATPase activity is the ionic strength of the system. Since the ionic strength of the high and low systems simply reflects the amount of KCl present, it can be concluded that the increased activity of the high ionic strength system is a direct result of the effect of the K^+ ion. Again, the data show an animal difference regarding the magnitude of response to the K^+ ion, with animal 1 having the greater ATPase activity in both the high and low ionic strength systems.

The difference in individual animal response to $CaCl_2$ and KCl could be related to the effect of the divalent cation, Mg^{++} on myosin ATPase activity. It is well established that Mg^{++} inhibits ATPase activity of pure myosin. Consequently, the presence of any Mg^{++} contamination in the reaction mixture would lead to the suppression in

the rate of myosin ATPase (in animal 2). The inhibitory effect of Mg^{++} would be more critical when assayed in the absence of $CaCl_2$, particularly at the low ionic strength, since both $CaCl_2$ addition and/or high KCl concentration would counteract the suppressing effect of Mg^{++}. In addition, the complexity of the analyses conducted on the myosin preparations necessitated the preparation of fresh reagents in order to complete the ATPase assay for animal 2. As a result, the possibility exists that the reaction mixtures used for animal 2 contained more Mg^{++} contamination.

It is implied in the data obtained from animal 2 (Figure 27-B) that Mg^{++} contamination was partly responsible for the suppression in ATPase activity. This would account for some of the differences observed between the two animals. Thus, the chelation of Mg^{++} by sucrose could account for the small rise in ATPase activity observed in the low ionic strength system for animal 2 (Figure 27-B).

It is pointed out that the above discussion, though largely theoretical, is interjected here to partially explain the differences in animal response to the various treatment combinations and in no way alters the conclusions as to the primary effect of sucrose, and Ca^{++} and K^{+} ions on myosin ATPase activity. Finally these results emphasize the importance of the "total ionic milieu" (added, as well as inherent) in incubation systems used to assess ATPase activity of myosin.

CHAPTER VIII

GENERAL SUMMARY AND CONCLUSIONS

The objectives of this study were to develop procedures which could be used to evaluate the role of myosin in the various post-mortem quality changes occurring in beef muscle, and to investigate the effect of various chemicals on the inhibition of myosin ATPase activity. These objectives were considered to be pre-requisite for the development of methods to control the formation of the rigid complex, actomyosin, which occurs port-mortem.

The experiments included in this study were done on myosin isolated from bovine longissimus dorsi muscle. The isolation and purification of myosin was accomplished by use of a salt extraction procedure perfected in this laboratory. The longissimus dorsi muscle was excised, immediately post-mortem, from Hereford steers of approximately 408 kg. live weight.

The experiments contained in this study were concerned with: 1) the isolation and purification of bovine myosin, 2) establishing the electrophoretic characteristics of bovine myosin, 3) comparing the electrophoretic characteristics of three protein fractions, classified as Total, Sarcoplasmic and Myofibrillar with those obtained for bovine myosin, and 4) the effect of various chemicals (Ethylenediaminetetraacetic acid, EDTA; Iodoacetic acid, IAA; Iodoacetamide; p-chloromercuribenzoate, PCMB; N-ethylmaleimide, NEM) on myosin ATPase

activity. The enzyme assay studies were designed to evaluate the influence of a number of factors on myosin ATPase activity. The factors included were: A) Ca^{++}-activated high and low ionic strength incubation systems (μ=0.6 and μ=0.06, respectively), B) sucrose and non-sucrose myosin samples and C) level of added chemical under investigation. Another factor, $CaCl_2$ omitted from the incubation system, was included in the EDTA study.

Initial research efforts were concerned with the purity of the myosin preparation. Two methods were used in assessing the purity of the myosin preparation: 1) Mg^{++}-activated ATPase activity and 2) sedimentation characteristics in the analytical ultracentrifuge. No detectable ATPase activity was obtained with the $MgCl_2$ low ionic strength system, indicating that the myosin preparations were free of any actin or actomyosin contamination. Also, the sedimentation pattern of myosin showed a single, sharp, symmetrical peak in the analytical ultracentrifuge. These two criteria indicate that myosin of a high degree of purity was obtained by the procedure developed in these studies.

The ultracentrifuge studies showed that both temperature and duration of centrifugation were of considerable importance in the interpretation of the sedimentation patterns of bovine myosin. A significant reduction in the amount of heterogenity observed in the sedimentation patterns occurred when the temperature of the run was lowered from 20° C. to 2°C. The addition of sucrose to myosin solutions which had been stored in 0.5M KCl or 0.5M KCl + 0.05M PO_4 (pH 7.1) for 7 days at 0°C. led to further reduction in the number of leading peaks. A discrete leading peak became apparent in some of the sedimentation patterns when centrifugation proceeded for 45 minutes after reaching

operating speed. Thus, had the run been terminated prior to this time, this significant observation would not have been detected. When sucrose was added to the myosin preparation immediately after isolation, the sedimentation patterns showed a single, sharp, symmetrical peak indicating that the heterogenity observed in other patterns was due to myosin aggregation and not to impurities. Also, the data obtained suggest that sucrose has a stabilizing effect on the myosin molecule which results in reducing the tendency of myosin to undergo spontaneous aggregation.

The electrophoretic characteristics of bovine myosin were investigated by means of disc electrophoresis on polyacrylamide gels. Under the conditions employed in this study, myosin migrated as an anionic entity. The electrophoretic pattern obtained on an aqueous solution of myosin exhibited seven faint, but distinct bands, which could be grouped into three zones along the gel. Zone 1, 2 and 3 contained 2, 3 and 2 bands, respectively. Results indicated that the major solubility problem encountered in getting sufficient lyophilized myosin to dissolve in water could be circumvented by using 8M urea as a solvent. The electrophoretograms of myosin dissolved in 8M urea showed greater band density and definition and were very similar to those obtained with myosin dissolved in water in terms of the number of zones and bands within each zone. The addition of a reducing agent (Na-thioglycolate) to the gel did not significantly alter the electrophoretic pattern of myosin dissolved in 8M urea. Based on this test, it was concluded that the ammonium persulfate catalyst used to polymerize the separating gel did not have an adverse effect on the electrophoretic behavior of myosin. When the molecular sieving effect

of the separating gel was reduced by decreasing its acrylamide concentration from 7% to 3.5%, the myosin pattern showed only one band in each of the three zones, and as expected, greater mobility.

In studying the electrophoretic results obtained on the three protein fractions isolated from bovine longissimus dorsi muscle, it was found that the various protein "bands" separated could be uniformly and logically grouped into four distinct zones, designated as A, B, C and D, along the gel. Consequently, all "bands" separated, from the three protein fractions isolated, were classified according to their appearance in a particular zone. In the Total protein pattern, 3, 7, 2 and 7 protein bands were discernible in the A, B, C and D zones, respectively. The Sarcoplasmic and Myofibrillar patterns exhibited 3, 4, 2, 2 and 3, 3, 2, 5 bands in these four respective zones. Thus, in terms of the number of bands, the major differences between the electrophoretic patterns of these protein fractions occurred in the B and D zones. In comparing the Sarcoplasmic and Myofibrillar patterns with the electrophoretogram obtained for the Total protein fraction, it was found that the majority of the protein components in the Sarcoplasmic fraction was contained in the A and B zones; whereas, the Myofibrillar proteins separated predominantly in the C and D zones. A striking similarity was observed between the myosin in 8M urea and the Myofibrillar electrophoretograms. They were very comparable with respect to the number of bands within the C and D zones; however, there was some difference in the mobility of the bands within the D zone.

In regard to the effect on myosin ATPase activity of the various chemicals studies, it is pointed out that many of the interactions

between the various factors tested were found to be highly significant when analyzed statistically. Consequently, only the general trends are summarized herein.

The effect of EDTA on myosin ATPase activity was strongly influenced by the ionic strength of the incubation system. In general, EDTA caused activation if present in concentrations above the Ca^{++} ion concentration, when assayed in the high ionic strength system. In contrast, for the $CaCl_2$ low ionic strength system, EDTA had a suppressing effect on myosin ATPase activity. The addition of EDTA had little effect on activity in the low ionic strength system without $CaCl_2$.

For the $CaCl_2$ low ionic strength system, the lower levels of IAA caused an elevation in myosin ATPase activity. The lower levels of Iodoacetamide also tended to enhance activity. The results indicate that lower concentrations of Iodoacetamide are required to cause inhibition in low versus high ionic strength media.

All levels of PCMB and NEM tested resulted in inhibition of myosin ATPase activity. These results emphasize the importance of sulfhydryl groups on bovine myosin ATPase activity. Of the chemicals studied, PCMB was the most effective inhibitor.

The addition of sucrose to the myosin sample resulted in a suppression of myosin ATPase activity. The data suggest that the primary action of sucrose, in effecting this response, was to combine with or chelate the Ca^{++} ion.

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VITA 2

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