SEPARATION, IDENTIFICATION AND CHARACTERIZATION OF SOME MYOFIBRILLAR PROTEINS

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ABSTRACT

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By

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The purposes of this investigation were to: (1) evaluate different methods for the extraction and purification of myofibrillar proteins, (2) obtain information about the interaction of myofibrillar proteins and their participation in the actomyosin complex, and (3) determine the effect of common meat spoilage bacteria on myofibrillar proteins.

The salt soluble proteins from skeletal muscle were separated and characterized by gel filtration, density gradient centrifugation, ion exchange chromatography and disc gel electrophoresis. Gel filtration and density gradient centrifugation afforded only crude fractionation, while both ion exchange chromatography and disc gel electrophoresis in the presence of 7 M urea separated Weber-Edsall extract into 8-11 principal fractions. Isolation and purification of known myofibrillar proteins by several methods demonstrated that many so-called "pure preparations" contained significant amounts of contaminants requiring special techniques for removal.

Myosin, actin and tropomyosin were each prepared by four different methods, and the purest preparations utilized in subsequent studies. The purest preparation of myosin was obtained by chromatography on DEAE-Sephadex A-50. Disc gel electrophoresis indicated that myosin gave several bands between R_m values of 0.00 and 0.15--the monomers and aggregates apparently remaining at or near the origin and the dissociated polypeptide

subunits occurring between 0.05 and 0.15. The purest preparation of actin was isolated directly from myofibrils, and gave a single diffuse band at R_m = 0.39 on disc gel electrophoresis. The purest preparation of tropomyosin was obtained by ammonium sulfate fractionation and isoelectric precipitation followed by further purification with DEAE-cellulose chromatography in the presence of 0.01 M EDTA. Disc gel electrophoresis of tropomyosin prepared in this way gave two bands at R_m = 0.34 and 0.50, the slow moving component being identified as the oxidized form and the other as reduced tropomyosin.

Tropomyosin was prepared by two methods, while α-actinin, β-actinin, inhibitory factor and the extra protein group were each prepared by one method. None of these fractions could be identified by the disc gel system. However, results tentatively indicate that extra protein Fraction I A and troponin may be identical..

Weber-Edsall extracts of washed muscle residue or of prepared myofibrils contained myosin, actin, oxidized and reduced tropomyosin, extra protein Fraction I A, and probably α-actinin. Specific staining of electrophoretic gels from Weber-Edsall extracts indicated that nucleic acids are complexed with tropomyosin, extra protein Fraction I A, and probably with actin.

Actomyosin preparations contained myosin, actin, reduced tropomyosin, varying amounts of extra protein Fraction I A, and probably α-actinin. Gel filtration of actomyosin gave two peaks with apparent molecular weights of 6,000,000 and 50,000,000. The former peak contained mainly actin, myosin, reduced tropomyosin and an unidentified component at R_m = 0.60, while the latter peak consisted mainly of actin and myosin, apparently aggregated together. Pyrophosphate decreased the sedimentation

rate of all detectable protein moieties of actomyosin. Sedimentation behaviors indicated that pyrophosphate actually dissociated myosin from the actomyosin complex, yet left actin and tropomyosin in a partially dissociated or otherwise unnatural state. EDTA appeared to have a similar action to pyrophosphate on actomyosin, resulting in dissociation of myosin and partial dissociation of actin and tropomyosin. Further, EDTA influenced the properties of extra protein Fraction I A.

Microbial growth caused no apparent changes in the myofibrillar proteins. However, storage at temperatures above freezing and bacterial growth both decreased the concentration of certain non-protein ultraviolet absorbing components detectable by density gradient centrifugation of Weber-Edsall extract.

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James Henry Rampton

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INTRODUCTION

Both the meat scientist and muscle chemist are concerned with identifying and characterizing the proteins of the myofibril. In meat science these proteins may play a key role in determining meat quality, while in muscle chemistry they appear to form the link in the transfer of chemical energy into muscle contraction.

Banga and A. Szent-Györgyi (1942) distinguished between myosin (myosin A) and actomyosin (myosin B). This was followed by the discovery and description of actin by Straub (1942). Since that time myofibrillar proteins have been the subject of intensive study, however, fundamental questions about their identity, properties, and interactions remain unanswered.

In recent years, all of the supposedly pure preparations of myofibrillar proteins have been found to contain considerable amounts of contaminating substances, as well as lesser amounts of previously unknown factors. Furthermore, the highly complex interactions of the myofibrillar proteins and the different physical properties of impure preparations have renewed interest in the true properties of the pure proteins. Isolation of the various pure preparations and studying their interactions should prove beneficial in elucidating their relationships to the physical properties of meat and meat products. Since muscle proteins may play a key role in the spoilage of meat, an understanding of the proteins involved and the nature of the changes could lead to better methods of preventing meat spoilage.

The present investigations were undertaken to separate, identify, and purify the myofibrillar proteins, including special studies on the actomyosin complex and its behavior in the presence of pyrophosphate and EDTA. In addition, the nature and extent of degradation of the constituents of the myofibril by the common microbial spoilage flora were also investigated.

REVIEW OF LITERATURE

Structure of Skeletal Muscle

The structure of skeletal muscle and its relationship to myofibrillar proteins has been discussed by Bendall (1964) and Lawrie (1966). They stated that skeletal muscle is surrounded by an outer layer of connective tissue called the epimysium. Branching inward from the epimysium are septa of connective tissue, which penetrate the muscle and separate the fibers into bundles. According to Lawrie (1966) these septa are collectively called the perimysium and carry the larger blood vessels and nerves into the muscles. He further stated that the endomysium originates at the perimysium and is composed of septa, which run throughout the muscle bundle to eventually surround each fiber.

Lawrie (1966) stated that the muscle fiber is the essential structural unit of all muscles. He then described the muscle fiber as a long, narrow, multinucleated cell, which may run from one end of the muscle to the other. He further stated that a fiber may be 10-100μ in diameter and up to 34 cm. long. Fibers at the end of the muscle blend in with the endomysium, perimysium, and epimysium, which in turn converge to form tendons.

A double membrane called the sarcolemma surrounds each muscle fiber just beneath the endomysium (Robertson, 1957).

The myofibrils are bathed by the sarcoplasm, which contains soluble substances, mitochondria, nuclei, and a complex of internal membranes (Bloom and Fawcett, 1968). Porter (1961) described the complex of

of internal membranes as consisting of two main systems: (1) the sarcoplasmic reticulum, which is a branching network of thin, irregularly shaped vesicles found between and enveloping the fibrils; and (2) the T-system, a set of transverse tubules about 0.3μ in diameter, which originates at or near the sarcolemma and projects inward into the muscle fibers. He further stated that the sarcoplasmic reticulum seems to function in controlling the relaxation process.

The myofibrils have been described as the contractile elements of striated muscle by Huxley (1960, 1965, 1967). He stated that myofibrils measure 1-2μ across and run parallel to the long axis of the fiber. He further stated that the cross striations are due to regularly arranged myofilaments which are composed of the three principal myofibrillar proteins, myosin, actin, and tropomyosin.

Bloom and Fawcett (1968) listed and described the main bands of the myofibril which are summarized below:

(1) The A band contains myosin filaments measuring about 100Å x 1.5μ. When viewed under the polarizing microscope, the A band appears bright or anisotropic. On the other hand, it stains dark with iron-hematoxylin.

(2) The I band contains actin filaments, which are 50Å thick and extend about 1μ from each side of the Z line. These filaments, which may also contain some tropomyosin, are often called "thin" filaments in contrast with the thicker myosin filaments in the A band. The thin filaments appear dark or isotropic under the polarizing microscope and remain essentially unstained with iron-hematoxylin.

(3) The Z line contains tropomyosin and runs across the myofibril to bisect the I band. The Z line is visible with dark phase contrast microscopy and appears dark with many stains. One sarcomere (the

distance in which the striation pattern of the myofibril repeats itself) is defined as the distance between two successive Z lines.

(4) The M line runs across the middle of the A band, bisecting it and apparently holding the thick filaments together at their midpoint. The clarity of this line varies with the degree of contraction of the myofibril and with the method of preparing the histological section. Thus, at times it is hardly detectable.

According to Huxley (1960, 1965, 1967), a cross section of the myofibril taken in the region of overlap between the myosin and actin filaments shows each myosin filament to be surrounded by six actin filaments in hexagonal array. Each actin filament is in turn shared by the six neighboring myosin filaments. He further showed that each thick filament has distributed along its length many short lateral projections or cross-bridges. These appear to extend outward and touch the thin filaments. Huxley (1960, 1965, 1967) postulated that the cross bridges interact with the thin filaments, and that the myosin-actin interaction corresponds to the formation of actomyosin during muscle contraction.

Proteins of the Myofibril

All but a small percentage of myofibrillar material has been found to be protein (Perry, 1951; Perry, 1967b), and consists mainly (80-90%) of myosin, actin and tropomyosin (Poglazov, 1966; Perry, 1967b). The contribution of the various myofibrillar proteins as reported by different investigators is summarized in the following table:

The Protein Composition of the Myofibril

Protein	Percent of total myofibrillar protein (Ebashi, 1966)	(Perry, 1965)	(Hanson & Lowy, 1964)
myosin	55	50-55	54
actin	20	20-25	21
tropomyosin	7	10-15	15
troponin	2	other 5-10	unknown 10
α-actinin	10		
β-actinin	2		

Perry (1967b) recently pointed out that myofibrillar proteins interact strongly with each other, thus during purification one gets incomplete extraction and persistent impurities. Consequently, he has indicated that composition data for the myofibril is only approximate. He has also listed several minor components of the myofibril, which have not yet been well defined. In addition to the myofibrillar proteins listed by Ebashi (1966), Perry (1967b) also included the inhibitory factor, fibrillin, and ribonucleoprotein.

Other proteins have been found in the myofibril, which have different properties than those mentioned by Perry (1967b) and Ebashi (1966). Poglazov (1966) lists them as contractin, γ myosin, metamyosin, Y protein, and Δ protein. Although these constituents have certain distinguishing features, they are similar in many respects, and probably consist of complexes of the already known proteins (Poglazov, 1966). This agrees with Ebashi (1966), who stated that the well-defined myofibrillar proteins make up 96% of the total.

Myosin:

Extraction of myosin has been reviewed by Huxley (1960), who stated that two solutions have commonly been used for extracting myosin. 1) The Guba-Straub solution, which consists of 0.3 M KCl and 0.15 M phosphate

buffer at pH 6.5 (Guba and Straub, 1943), usually extracts some actin along with myosin. Its effectiveness can be increased by adding 5 x 10^4 M ATP (Huxley, 1960). 2) The Hasselbach-Schneider solution, which is composed of 0.47 M KCl, 0.1 M phosphate buffer, and 0.01 M sodium pyrophosphate at pH 6.5 (Hasselbach and Schneider, 1951), extracts myosin with almost no contamination from actin. Its effectiveness can be increased by adding 10^{-3} M $MgCl_2$ (Huxley, 1960).

Huxley and Hanson (1957) have developed a solution which gives excellent selective extraction of myosin. It consists of 0.6 M KCl, 0.1 M phosphate buffer, 0.01 M sodium pyrophosphate and 10^{-3} M $MgCl_2$ at pH 6.5.

According to Huxley (1960) and Bendall (1964), extracted myosin remains soluble if the ionic strength is lowered to 0.2-0.3. Thus, removal of contaminating actin can be achieved by adjusting the ionic strength to 0.3, which precipitates any actomyosin present (Bendall, 1964). Similarly, myosin can be separated by adjusting the ionic strength to 0.15 and adding 10^{-3} M ATP, thus superprecipitating the actomyosin (Huxley, 1960). By lowering the ionic strength to 0.05, the myosin can be precipitated, leaving the more soluble proteins in solution (Huxley, 1960). The widely used method of Perry (1955) for purification of myosin utilizes the above technique to eliminate impurities, however, myosin prepared in this manner is far from pure (Perry, 1967a).

Richards et al. (1967) pointed out that myosin preparations have been plagued by the presence of myokinase, AMP deaminase, nucleic acids, myosin aggregates, myosin-nucleic acid complexes, unidentified proteins, and decreased ATPase activity. They indicated that attempts to purify myosin have all yielded impure preparations or reduced myosin ATPase activity. Thus, they outlined a procedure using DEAE Sephadex A-50 for

obtaining monomeric myosin essentially free of contaminating substances and having high ATPase activity.

They reported that myokinase was the only contaminant, and that 50% of the original myokinase activity could be removed during purification.

Myosin has been studied extensively (Poglazov, 1966), yet much confusion persists about the size of the myosin monomer (Dreizen, 1967). Perry(1967a) explained that the tendency of myosin to aggregate as well as difficulty in purification have caused errors in determining its molecular weight. Dreizen (1967), in reviewing molecular weight studies on myosin, indicated that reported values have ranged from 420,000 - 1,500,000, but stated that most workers now agree on a value of approximately 500,000. Richards et al. (1967) have also reported a molecular weight for highly purified myosin of about 500,000.

After reviewing measurements on the size of the myosin molecule, Perry (1967a) stated that it is about 1550 Å long, from 200 - 400 Å in width at the head and about 20 Å wide along the tail.

Some workers have attempted to simplify the myosin molecule by breaking it down into subunits (Poglazov, 1966). Perry (1967a) pointed out that the only real subunits of the myosin molecule are those obtained by using dissociating agents. He stated that the subunits usually have a molecular weight of 160,000 - 260,000 and have never been known to exhibit biological activity. According to Perry (1967a), the myosin molecule has also been split up by controlled proteolytic digestion to yield "fragments" of the myosin molecule. He stated that light meromyosin (LMM) and heavy meromyosin (HMM) are fragments rather than subunits "since they are produced by the breaking of peptide bonds and do not preexist as units from which the molecule is made up". HMM has a molecular

weight of 380,000, is more soluble than myosin, contains the ATPase activity and the actin-combining ability of myosin (Perry, 1967a; Poglazov, 1966). LMM has a molecular weight of 120,000, retains similar solubility properties to myosin, has no known biological activity, and seems to be only structural in function (Perry, 1967a; Poglazov, 1966).

According to Bendall (1964), the isoelectric point of myosin in KCl solutions is 5.4, however, on addition of Mg^{++} or Ca^{++} ions it increases to 9.3 due to the unusual affinity of myosin for divalent ions. Lawrie (1966) stated that the affinity for divalent ions is due to a high content of glutamic, aspartic, and the dibasic amino acids.

Poglazov (1966) stated that the two most important chemical properties of myosin are its ATPase activity, and its ability to combine with actin. Bendall (1964) stated that myosin ATPase is activated by Ca^{++}, inhibited by Mg^{++}, and has two pH optima (pH 6.4 and 9.3). He also stated that myosin ATPase is very heat and acid sensitive; its activity is influenced by sulfhydryl groups in the molecule; and its behavior is modified by the presence or absence of actin.

Bendall (1964) pointed out that since myosin complexes with actin to form actomyosin, the term "actin-modified ATPase" really refers to actomyosin ATPase. He also stated that the behavior of actomyosin ATPase depends on the ionic strength of the medium. At high ionic strength (0.6MKCl), the addition of ATP causes dissociation of actomyosin so that its ATPase now behaves much like myosin ATPase, i.e., it is activated by Ca^{++} and inhibited by Mg^{++}. At low ionic strength (0.15 M KCl), the addition of ATP superprecipitates actomyosin, causing it to "contract" isodimensionally. In the superprecipitated state, actomyosin ATPase is activated by Mg^{++} if trace amounts of Ca^{++} are present.

The localization of myosin in the sarcomere has been reviewed by Huxley (1960). He stated that Hasselbach (1953) and Hanson and Huxley (1953, 1957) used high ionic strength solutions of KCl, sodium pyrophosphate and $MgCl_2$ to study the localization of myosin in the myofibril. Since such solutions remove the A band completely, these authors suggest that myosin is located in the A band. Seifter and Gallop (1966) reviewed the use of antibodies in localizing myosin. Since antisera prepared against myosin generally precipitate at the A band (Seifter and Gallop, 1966), antibody studies further confirm localization of myosin at this point.

Actin

Actin is firmly attached to the muscle structure, so that severe treatments are needed to extract it (Huxley, 1960), i.e., acetone treatment (Straub, 1942) or 0.6 M KI (A.G. Szent Györgyi, 1951b). Treatment with 0.6 M KI has been found to slowly denature actin (Lewis et al., 1963). The extraction method of Straub (1942), or variations thereof, has been the starting point for most actin preparations (Bendall, 1964). Preparation involves: 1) pre-extraction of myosin from the muscle; 2) acetone treatment to denature various remaining proteins and to free the actin from the muscle structure as well as removal of the lipids; and 3) extraction with neutral distilled water to obtain globular actin (G-actin).

Actin can exist in two forms, globular (G-actin) and fibrous (F-actin) Bendall, 1964). In vivo it is believed to exist as F-actin (Huxley, 1960). According to Briskey (1967), the only successful attempt in isolating F-actin directly was carried out by Hama et al. (1965). They began by pre-extracting myosin from the muscle, then performed short, mild tryptic digestion on the residue to release F-actin from the muscle structure.

Once extracted, actin has usually been purified by repeated polymerization-depolymerization (Adelstein et al., 1963). Conventional techniques, however, have failed to purify actin sufficiently for studying its molecular parameters (Hayashi, 1967). According to Hayashi (1967), tropomyosin and myokinase are probably the most persistent impurities found in actin preparations. He further stated that extraction of actin at 0°C reduces the amount of tropomyosin, yet even under these conditions enough tropomyosin remains to alter the properties of actin.

Ebashi (1966) pointed out that actin extracted from acetone powder (Straub-type actin) usually contains α-actinin as an impurity. Ebashi and Ebashi (1965) were able to remove essentially all of the α-actinin by raising the KCl concentration to 3.3 M. They concluded that this treatment precipitates α-actinin and leaves actin in solution.

β-actinin is present in KI-extracted actin solutions, but seems to be absent in most Straub-type actin preparations (Maruyama, 1965 a, b). Troponin, although quite instable to acetone treatment, is present in some actin preparations (Ebashi and Ebashi, 1964; Briskey, 1967).

Tropomyosin, α-actinin, and β-actinin all interact with actin to change its behavior (Hayashi, 1967; Maruyama and Ebashi, 1965; and Maruyama, 1965 a, b), thus it is important to remove these contaminants before studying the properties of actin. Adelstein et al. (1963) purified actin on a column of Sephadex G-200 to obtain actin free of smaller molecules and certain enzymes. They made no statement concerning the amount of tropomyosin or other impurities commonly found in actin preparations. Seraydarian et al. (1967) obtained so-called "pure" actin by extraction at 0°C, treatment with 3.3 M KCl and other modifications.

The molecular weight of the G-actin monomer is not known with certainty due to difficulty in obtaining purified actin, and the tendency

of actin to polymerize (Huxley, 1960; Hayashi, 1967). In four recent reviews (Bendall, 1964; Poglazov, 1966; Briskey, 1967; and Hayashi, 1967), values from 50,000 to 150,000 were cited for the molecular weight of G-actin, with most of the values falling between 56,000-70,000. On the other hand, there is no agreement on the molecular weight of F-actin since it behaves as either a polymer or a dimer (Huxley, 1960; Poglazov, 1966; and Briskey, 1967).

Briskey (1967) stated that the G-actin molecule measures approximately 55Å in diameter. He described F-actin as a double helical polymer of G-actin monomers, making a complete turn every 700 Å and measuring 80Å in diameter.

The actin molecule has 450 amino acid residues (Laki and Standaert, 1960), with large amounts of glutamic and aspartic acids (Carsten, 1963). According to Briskey (1967), actin has more proline than most proteins. He suggests that the proline content may account for the low percentage of helical structure (30%) as compared to myosin (56%) and tropomyosin (96%).

According to Poglazov (1966), the two most important properties of actin are the G-F transformation and the interaction between actin and myosin. The latter has already been discussed.

Hayashi (1967) has represented the G-F transformation by the following equation:

$$n\ \text{G-ATP} \xrightarrow{0.1\text{M KCl}} \text{F-ADP} + nP_i$$

where: G-ATP is G-actin containing ATP, F-ADP is F-actin containing ADP and P_i is inorganic phosphate. Poglazov (1966), in reviewing the G-F transformation, stated that G-actin is converted to F-actin, when the ionic strength of the medium is raised to 0.01 - 0.15. The change is enhanced by Mg^{++} and is inhibited by Ca^{++} in the presence of monovalent ions. He

further stated that polymerization likely occurs due to hydrogen bonding between sulfhydryl and amino groups and between hydroxyl and amino groups. ATP also seems to be involved in polymerization (Poglazov, 1966). Recently, G-actin has been found to polymerize in the absence of nucleotides and divalent ions (Kasai et al., 1964; Hayashi, 1967). Thus, theories on the role of the G-F transformation in muscle contraction should be re-evaluated (Mommaerts, 1966; Hayashi, 1967).

The localization of actin in the myofibril was studied by Rozsa et al. (1950), who reported that synthetic actin fibers resemble the thin filaments of the I band when viewed under the electron microscope. Hence, A.G. Sentz-Györgyi (1951a) concluded that the I band contained actin. This was later confirmed by Hanson and Huxley (1955), who used 0.6M KI to extract actin from myofibrils containing no A band or M line. The thin filaments were removed by this treatment. Szentkiralyi (1961) prepared HMM, then added this to isolated myofibrils and observed that the I band became darker in appearance as the HMM became attached to the actin filaments. This further confirms the presence of actin filaments in the I band.

Endo et al. (1960) used the fluorescent antibody technique to show that tropomyosin and troponin are capable of complexing with the material in the A band. Seifter and Gallop (1966) later reviewed the use of antibodies in localizing actin, and suggested such studies indicated actin may be present in both the A and I bands. However, they concluded that caution must be used in interpreting results obtained with antibody techniques, especially since impurities in the antigens alter the antibodies.

Tropomyosin B

Tropomyosin was first isolated and studied by Bailey (1946, 1948). Tropomyosin prepared by his method is usually called tropomyosin B to distinguish it from paramyosin (tropomyosin A), which is found in certain muscles capable of prolonged tetanic contraction (Poglazov, 1966; Seifter and Gallop, 1966). The method of Bailey (1948) involves : 1) extraction of muscle with distilled water; 2) treatment with organic solvents (ethanol followed by ether) to remove the lipids and denature the unwanted components; 3) extraction of tropomyosin B with 1M KCl; and 4) purification by repeated isoelectric precipitation and ammonium sulfate fractionation.

Tropomyosin B has been reported to contain some impurities. For example, it is often isolated as a complex containing nucleic acids, which are difficult to remove (Hamoir, 1951; Needham and Williams, 1963; Carstens, 1968). Other contaminants found in tropomyosin B preparations include: actomyosin (Hamoir and Laszt, 1962), tryptophan, which is not present in tropomyosin B (Kominz et al., 1954), and up to 5% of low molecular weight material (Woods, 1967). On using free boundary electrophoresis Davey and Gilbert (1968) found tropomyosin B to be about 70% pure, but were able to achieve further purification using DEAE-cellulose chromatography.

Tropomyosin B preparations usually do not contain troponin unless prepared without the use of organic solvents (Ebashi, 1963; Perry 1967a). However, tropomyosin B prepared in the presence of a sulfhydryl reducing agent or EDTA has been shown to have troponin activity (Mueller, 1966), or a tendency to aggregate (Woods, 1967), which also indicates troponin contamination (Ebashi and Kodama, 1965).

As with other myofibrillar proteins, there is uncertainty regarding the molecular weight of tropomyosin B. Although reported values range between 53,000 - 140,000, until recently the generally accepted molecular weight has been 54,000 (Seifter and Gallop, 1966; Poglazov, 1966). However, evidence now indicates that the molecular weight of tropomyosin B may be around 70,000 (Holtzer et al., 1965; Woods, 1965, 1967). Furthermore, tropomyosin B can be dissociated into two identical subunits with a molecular weight of 34,000, which points to a molecular weight of approximately 68,000 for tropomyosin B (Woods, 1965, 1967). The molecule is believed to measure 340-385Å in length and 14Å in diameter (Tsao et al., 1951).

Due to its low content (16%) of glycine, alanine, and serine residues, as well as a high content (40%) of amino acids with free acidic or basic groups, tropomyosin B contains the highest zwitterion charge density of any known protein (Seifter and Gallop, 1966). Tropomyosin B has two free sulfhydryl groups and a very low proline content, which is consistent with its high percentage of α-helix (Seifter and Gallop, 1966). Tropomyosin B has no free N-terminal groups (Bailey, 1951) and the polypeptide chain may be folded back on itself (Huxley, 1960; Poglazov, 1966).

Poglazov (1966) stated that tropomyosin B has the following properties: 1) It is soluble in distilled water and dilute salt solutions at all pH values outside the range of 4.5 - 6.5. 2) It shows a considerable increase in viscosity below 0.01 M KCl. 3) It has an isoelectric point of 5.1. 4) It resists denaturation by heat, acid and organic solvents, while urea and surfactants have only a slight effect.

Tropomyosin B has no known enzymatic properties and does not combine with myosin (Bendall, 1964). Further, it has no effect on actomyosin

(Ebashi, 1966), but does, however, combine with actin (Hayashi, 1967).

The amount of protein in the I band exceeds the amount of extractable actin (Hanson and Huxley, 1957; Huxley and Hanson, 1957). This residual protein is thought to be in part tropomyosin B (Huxley, 1953; Huxley and Hanson, 1957). Electron microscope studies have shown that the residual material in the Z and I bands has a crystal lattice similar to that of tropomyosin B (Huxley, 1963; Knappeis and Carlsen, 1962; Cohen, 1966). Furthermore, the thin filaments appear to be attached to the Z band by a network of tropomyosin (Huxley, 1957; Knappeis and Carlsen, 1962). Thus, tropomyosin appears to be localized mostly in the I band with a small amount in the Z line (Hansen and Lowy, 1963; 1964). The presence of tropomyosin B, as well as troponin, in the I band has recently been confirmed by antibody techniques (Endo et al., 1966; Pepe, 1966).

Troponin

Troponin has been called the EGTA-sensitizing factor (ESF) by Perry (1967a) and the relaxing protein by Watanabe and Staphrans (1966). The discovery and description of troponin has been described by Perry (1967a) and Ebashi (1966). They indicated that natural actomyosin is usually relaxed by calcium chelators, whereas, synthetic actomyosin is often unaffected by the presence or absence of Ca^{++}. Ebashi (1963) discovered a factor, which would restore the ability of synthetic actomyosin to relax in the absence of Ca^{++}. His preparation resembled tropomyosin B, but was subsequently shown to be a complex of tropomyosin B plus another factor, which he called troponin (Ebashi and Kodama, 1965, 1966). Simultaneously, A. Szent-Györgyi and Kaminer (1963) isolated a preparation, that they called metin. This was subsequently shown to be a

complex of tropomyosin B and troponin (Azuma and Watanabe, 1965 a, b), and is closely related to the preparation isolated by Ebashi (1963).

Perry (1967a) has stated that all ESF preparations except that prepared by Perry et al. (1966) have contained tropomyosin as the major component. On attempting to isolate ESF from "extra protein" by chromotography on DEAE-cellulose, Perry et al. (1966) detected ESF activity in several fractions throughout the chromatogram. The reason ESF is not cleanly separated from other components is not apparent. (Perry et al., 1966).

The properties of troponin have not yet been well described (Perry, 1967a). Troponin is a globular protein, which will complex with tropomyosin B, thus increasing the tendency of tropomyosin B to aggregate (Ebashi, 1966). The aggregated complex is called native tropomyosin and has a higher viscosity, greater flow birefrigence and a larger sedimentation constant than does tropomyosin B (Ebashi and Kodama, 1965). There is a relationship between ESF activity, the E_{278}/E_{260} ratio, and the thiol content of tropomyosin (Perry, 1967a).

Perry (1967a) indicated that little is known regarding the mode of action of ESF. However, it affects the Mg^{++} activated actomyosin ATPase but not the Ca^{++} activated myosin ATPase. Ebashi (1966) suggested that troponin influences the actin moiety of actomyosin. In the same investigations, he presented evidence that troponin can not act alone, but requires tropomyosin B. On the other hand, Perry et al.,(1966) and Perry (1967a) suggested that troponin may not require tropomyosin B.

Endo et al. (1966) used antibody techniques to localize native tropomyosin in the myofibril. Results suggested that tropomyosin B and troponin are both distributed along the entire length of the thin filaments.

According to Peachey (1968), two roles are thus implied for tropomyosin: 1) as a structural part of the thin filaments, and 2) as a means of controlling the interaction of actin by the presence or absence of Ca^{++}.

α-actinin

Ebashi and Ebashi (1964, 1965) first isolated and studied α-actinin. Since this protein complexes with actin (Maruyama and Ebashi, 1965; Briskey et al., 1967b), the contaminating actin must be removed from all purified preparations. Ebashi and Ebashi (1965) and Ebashi and Maruyama (1965) found that α-actinin could be precipitated from the contaminating actin by treatment with 3.3 M KCl. Seraydarian et al. (1967)prepared purified α-actinin by employing the method of Ebashi and Ebashi (1965) and Ebashi and Maruyama (1965) along with their own modifications. Their method involved: 1) extraction of myosin from muscle with a KCl-phosphate solution; 2) washing of the muscle in a low ionic strength medium; 3) extraction of α-actinin with a low ionic strength medium for several hours at 20°C; 4) partial purification by ammonium sulfate fractionation; and 5) final purification by precipitating the α-actinin with 3.3 M KCl.

Preparations of α-actinin obtained by ammonium sulfate and potassium chloride fractionation have been found to contain 3 components, with sedimentation constants of about 6S, 10S and 25S (Ebashi, 1966). According to Ebashi (1966) all three components have the same physiological activity. He concluded that the 25S component is probably an artifact formed during preparation, while either the 6S or the 10S component is found in the myofibril.

The amino acid composition of α-actinin resembles that of actin, thus it has been suspected of being denatured actin (Ebashi, 1966). Purified α-actinin does not complex with myosin, but forms a complex with

F-actin (Maruyama and Ebashi, 1965; Briskey et al., 1967b). If α-actinin is added to a suspension of F-actin under proper conditions, it will form a gel (Ebashi, 1966).

Ebashi (1966) pointed out that the presence of α-actinin is known to enhance the superprecipitation of actomyosin. Thus, he postulated that α-actinin plays a direct role in muscle contraction. On the other hand, Briskey et al. (1967a, b) presented evidence that the α-actinin-actin interaction may be unrelated to muscle contraction.

Goll et al. (1967) were able to detect α-actinin in the Z band by a tryptic digestion and subsequent treatment of the solublized fration with 3.3 M KCl. They concluded that α-actinin is at least partly located in the Z band. Similarly, Ebashi (1966) presented preliminary results of antibody studies, which indicated that α-actinin is located in the Z band, and perhaps in the M band.

β-actinin

Maruyama (1965a, b) isolated a protein factor which inhibited network formation in Straub-type F-actin and restricted the fiber length to 1-2μ. He named the factor β-actinin and prepared it as follows (Maruyama et al., 1965; Maruyama, 1965a, b): 1) Myofibrils were prepared according to the method of Perry and Zydowo (1959a). 2) Myosin was extracted from the myofibrils with a solution of KCl and potassium phosphate. 3) Actin was extracted with a solution of KI. 4) β-actinin was separated from actin by ammonium sulfate fractionation.

According to Maruyama (1965b), β-actinin prepared by the above method has an amino acid composition similar to that of actin, and a molecular weight of 300,000 in 0.1 M KCl. Maruyama (1965b) also stated that β-actinin restricts the fiber length of F-actin in vitro to that of

F-actin filaments in vivo (1-2μ). Thus, Ebashi (1966) suggests that β-actinin may function in muscle development rather than in muscle contraction.

Inhibitory Factor

Hartshorne et al. (1966) isolated a factor from skeletal muscle, which they called the inhibitory factor (IF). This factor, in the absence of EGTA, inhibits the Mg^{++}-activated ATPase of desensitized actomyosin (natural actomyosin from which ESF has been removed). IF is probably a component of the myofibril (Perry, 1967b).

Hartshorne et al. (1967) prepared IF by extracting myofibrils with a high ionic strength solution at pH 8.6, then lowering the ionic strength to precipitate the salt-soluble components, and finally isolating IF from the supernatant by ammonium sulfate fractionation. IF prepared in this manner appears to be protein in nature, since it is precipitated by ammonium sulfate, destroyed by trypsin and heat, and loses activity on prolonged storage at 0°C (Hartshorne et al., 1966).

The significance of IF is not yet clear (Hartshorne et al., 1966). It is often found associated with ESF activity, and sometimes a decrease in ESF activity is accompanied by a rise in IF activity (Perry, 1967a). However, the low levels of tropomyosin in IF preparations, as well as differences in their properties suggest that the two factors may not be the same (Hartshorne et al., 1966).

Extra Protein Group

On extracting myosin with high ionic strength salt solutions and ATP, A.G. Szent-Györgyi et al. (1955) found that other proteins are also solublized. On lowering the ionic strength to about 0.05, the myosin precipitated, leaving the so-called "extra protein" in solution (Poglazov, 1966). The extra protein fraction is heterogeneous (Perry & Zydowo, 1959a) and probably accounts for about 7% of the total myofibrillar protein.

Perry and Zydowo (1959a) separated extra protein into four components by means of DEAE-cellulose chromatography. Fraction I was shown to consist mostly of sarcoplasmic components, which could not be removed from the myofibrils by extensive washing. Fraction II was found to consist of a water-soluble and a water-insoluble component, and has not been identified. Fraction III consisted of tropomyosin B plus some other protein. Fraction IV contained considerable bound ribonucleic acid. Perry and Zydowo (1959b) characterized the ribonucleoprotein further, and postulated that it may be associated with protein synthesis.

Nature of the Actomyosin Complex

Composition

Perry (1967a) stated that natural actomyosin, which is extracted and purified directly from muscle, is different from synthetic actomyosin, which is prepared from purified actin and myosin. He indicated that natural actomyosin is usually relaxed by calcium chelators, whereas, synthetic actomyosin is not similarly relaxed. It has been shown that minute amounts of Ca^{++} regulate the contraction-relaxation cycle, thus synthetic actomyosin may not represent the complete fundamental system of muscle contraction (Ebashi, 1966).

The discovery of several new myofibrillar proteins (Ebashi and Ebashi, 1964, 1965; Maruyama, 1965 a,b) and of their effect on actomyosin suggested that natural actomyosin may contain other proteins besides actin and myosin (Ebashi, 1966). Briskey (1967) stated that natural actomyosin probably contains myosin, actin, tropomyosin, α-actinin, β-actinin and troponin, as well as other unknown proteins.

Effect of ATP and EDTA

On the basis of viscosity, light scattering, and sedimentation in

the ultracentrifuge, several workers (A. Weber, 1956; Barany and Jaisle, 1960) have shown that ATP appears to cause dissociation of actomyosin at high concentrations (0.6M) of KCl (H.H. Weber, 1964). Under suitable conditions, ATP is rapidly broken down by actomyosin ATPase, so that the viscosity and light scattering soon return to their former values (Bendall, 1964). Pyrophosphate in the presence of Mg^{++} seems to dissociate actomyosin in the same manner as ATP (Azzone and Dobrilla, 1964), except that pyrophosphate is not hydrolyzed by actomyosin ATPase. Actomyosin, therefore, cannot eliminate the pyrophosphate and remains dissociated (H.H. Weber, 1964; Bendall, 1964).

Little is known about the mechanism by which ATP and its analogs affect actomyosin (Azzone and Dobrilla, 1964), but according to H. H. Weber (1964) it is likely not a simple dissociation of actin and myosin. Although the majority of workers have concluded that ATP dissociates actomyosin (Johnson and Rowe, 1964), some have suggested that actomyosin undergoes shape changes without dissociating (Blum and Morales, 1953; Morales et al., 1955; von Hippel et al., 1957). Johnson and Rowe (1964) presented evidence showing that ATP may induce several changes in the actomyosin molecule. They pointed out that if the simple dissociation theory is correct, analytical ultracentrifugation of actomyosin in the presence of ATP should produce two peaks corresponding to F-actin and myosin. Instead, a "slow diffuse" peak appeared as well as a "myosin-like" peak, which seemed to contain varying amounts of G-actin. Thus, they concluded that the action of ATP on actomyosin is more than a simple dissociation.

According to Perry (1967a), the ATPase and actin-combining properties of myosin are probably controlled through separate active centers, although the mechanism by which ATP influences the two separate centers

is not clear.

By studying the effect of EDTA on individual glycerinated muscle fibers, Watanabe and Sleator (1957) showed that EDTA is capable of relaxing contracted fibers. Subsequently, Weiner and Pearson (1966) found that a lethal intravenous injection of EDTA in rabbits inhibited the post-mortem shortening and the inextensibility associated with development of rigor mortis. Similarly, H. H. Weber (1964) listed EDTA as an inhibitor of the myosin-actin interaction. On the other hand, Azzone and Dobrilla (1964) stated that EDTA does not dissociate the actomyosin complex as does ATP and its analogs.

The effect of EDTA on actomyosin likely results from the chelation of metal ions, as EDTA has been shown not to interact with actomyosin (H. H. Weber, 1964). Furthermore, the inhibitory effect of EDTA is abolished on adding excess Ca^{++} to the system (H.H. Weber, 1964).

Bacterial Action on Meat Proteins

Frazier (1958) indicated that temperature is the most important factor in determining the type of microorganisms that will grow, and thus determines the type of spoilage. He further stated that psychrophiles are favored under proper refrigeration. According to Evans and Niven (1960), the type of bacteria accumulating in fresh meat stored at 10°C or below are various strains of Pseudomonas, Achromobacter, Lactobacillus, Microbacterium and Micrococcus. On the other hand, mesophiles such as coliform bacteria and species of Bacillus and Clostridium will proliferate at intermediate temperatures (Frazier, 1958).

Early workers assumed proteolysis to be a major process in the bacterial spoilage of beef, poultry, and fish (Lerke et al., 1967). Jay (1966, 1967) and Jay and Kontou (1967) showed that the ability of certain

bacteria to spoil meat is not related to their proteolytic activity, but the primary proteins of meat are attacked only in advanced stages of bacterial spoilage. The fact that non-proteolytic strains are capable of spoiling meat would suggest that substances other than proteins are attacked by the bacteria (Jay, 1967; Jay and Kontou, 1967).

Jay and Kontou (1967) investigated possible sources of bacterial nutrients and suggested that the low molecular weight compounds in the sarcoplasm may be used by bacteria as sources of nitrogen. On this basis, Jay and Kontou (1967) tested for disappearance of free amino acids and nucleotides during bacterial growth. They found that the amounts and type of amino acids and nucleotides decreased during storage.

Lerke et al. (1967) studied the bacterial spoilage of fish muscle. By separating the soluble nitrogenous components of fish muscle into protein and non-protein fractions, they showed that spoilage of fish (as measured by the usual chemical and organoleptic tests) is probably not due to the breakdown of soluble proteins. Instead, bacterial spoilage occurred only in the presence of non-protein nitrogen components.

EXPERIMENTAL METHODS

Unless otherwise specified, all work was performed at 3°C. Distilled water was run through a Barnstead mixed bed ion exchanger before use. Solutions were prepared at room temperature and final pH adjustments were made at 3-5°C. Reagent grade chemicals were used unless otherwise stated.

Cellulose ion exchangers utilized were washed with 0.5 N NaOH, briefly with 0.5 N HCl, again with 0.5 N NaOH, and distilled water before packing into columns (Sober and Peterson, 1962). Aminoethyl-cellulose was washed with 0.1 N NaOH, 0.1 N HCl, 0.1 N NaOH, and water. Cellulose columns were packed under nitrogen pressure beginning at 0 psi and reaching a maximum of 10 psi at the completion of the column. The columns were equilibrated with buffer in the cold before use.

Gel filtration media were allowed to swell in water or eluting buffer before packing into columns. Gel columns then were equilibrated with eluting buffer and tested for uniformity with Blue Dextran before using. Column void volumes were also determined using Blue Dextran.

Centrifugations are described in terms of the relative centrifugal force developed at the tip of the centrifuge tube.

Sample Preparation

Rabbit Longissimus Dorsi

Female rabbits (3-5 lbs.) were obtained locally, killed by exsanguination, and transferred immediately to a cold room at 2-4°C. The left longissimus dorsi muscle was removed, trimmed free of connective

tissue and the desired amount of muscle was weighed.

Removal of Sarcoplasmic Fraction

The muscle was homogenized with 12 volumes of a solution containing 0.25 M Sucrose, 1 mM EDTA and 0.05 M tris (hydroxymethyl) amino methane (Tris buffer) at pH 7.6 (Czok and Bucher, 1960; Goll and Robson, 1967). After standing 10 minutes, the slurry was centrifuged for 15 minutes at 20,000 x g. The supernatant was discarded and the residue was washed a second time. The remaining material, which is referred to as the washed muscle residue, was used in preparation of myofibrils or myofibrillar proteins.

Myofibrils

Myofibrils were prepared according to the method of Perry and Zydowo (1959a). The washed muscle residue was suspended in 9 volumes (based on original weight of muscle) of 0.1 M KCl containing 0.039 M sodium borate buffer, pH 7.1, then centrifuged 15 minutes at 600 x g. The supernatant was discarded and the residue was washed again. The loose upper layer of sedimented material was removed by adding a little KCl-borate solution, gently swirling and decanting. The firmly sedimented material was discarded, and the decanted portion was diluted with KCl-borate solution to a volume 10 times that of the original muscle sample. The slurry was spun for 3 minutes at 400 x g. and the sediment was discarded. The myofibrils remaining in suspension were washed eight times in KCl-borate solution, centrifuging each time for 15 minutes at 600 x g. After the last washing the slurry was centrifuged 3 minutes at 400 x g. The myofibrils remaining in suspension were decanted and concentrated by centrifuging 15 minutes at 600 x g. The sedimented myofibrils were stored at 0°C.

Salt-Soluble Fraction

Perry (1953) reported Weber-Edsall solution (0.6 M KCl, 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3) to be a very efficient extractant of myofibrillar proteins. Accordingly, the Weber-Edsall solution was used to extract the total soluble myofibrillar proteins.

The washed muscle residue was homogenized with 60 ml of Weber-Edsall solution per 10 gm of original muscle, or else the prepared myofibrils were concentrated by centrifuging for 20 minutes at 15,000 x g, weighed, and extracted with 45 ml of Weber-Edsall solution for every 10 gm of the myofibril preparation. Extraction was allowed to proceed 20-24 hours, after which the viscous mass was diluted with 180 ml of Weber-Edsall solution per 10 gm of original muscle or 135 ml per 10 gm of myofibril preparation. The suspension was centrifuged for 1 hour at 25,000 x g. The supernatant containing the salt-soluble proteins was saved, and the residue and loosely-sedimented gel were discarded. The supernatant was used for preparation of actomyosin, or for studies of salt-soluble proteins.

Actomyosin

Actomyosin was prepared after the method of Morita and Tonomura (1960). Salt-soluble proteins of muscle or of myofibrils were prepared as previously described. The salt-soluble proteins were brought to an ionic strength of 0.2 by addition of 2 volumes of distilled water. The pH was adjusted to 6.5, and the precipitate formed was collected by centrifugation for 15 minutes at 2,000 x g. The supernatant was discarded, and the actomyosin precipitate was dissolved in 0.6 M KCl at pH 7. The sample was further purified by repeating the precipitation and dissolution cycle twice as described above. The purified actomyosin in 0.6 M KCl was centrifuged 1 hour at 25,000 x g. before utilization.

Natural Actomyosin

Natural actomyosin was prepared after the method of Perry and Corsi (1958) with modifications as suggested by Schaub et al. (1967). Salt-soluble proteins of myofibrils were prepared as previously described. The salt-soluble protein was brought to pH 7.0 with 2 N HCl, and 1400 ml of distilled water (pH 7) were added per 100 ml of protein solution. The precipitate was collected by centrifugation for 15 minutes at 2,000 x g. The supernatant was discarded and the precipitate was dissolved in 0.6 M KCl (pH 7). Natural actomyosin was precipitated again as described above and was then washed twice with 0.05 M KCl. After each washing, the precipitate was collected by centrifuging 15 minutes at 1,200 x g. The natural actomyosin was dissolved in 0.6 M KCl (pH 7) and characterized or used for further studies.

Individual Myofibrillar Proteins

Myosin

Crude myosin was prepared after the method of Perry (1955). Washed muscle residue was extracted with 30 ml of a solution of 0.3 M KCl, 0.10 M KH_2PO_4, and 0.05 M K_2HPO_4 (pH 6.5) per 10 gm of original muscle. After stirring for 15 minutes, the mixture was centrifuged for 20 minutes at 20,000 x g. and the sediment was discarded. The volume of the supernatant was measured, and 14 volumes of distilled water were added with constant stirring. The precipitated myosin was allowed to settle, and the supernatant discarded. Sufficient KCl was added to bring the ionic strength to 0.5, after which 0.67 volumes of water were added to adjust the ionic strength to 0.3. Centrifugation for 20 minutes at 20,000 x g removed any precipitated actomyosin. The ionic strength was adjusted to 0.04 by slow addition (over 10-15 minutes) of distilled water

with stirring, whereupon the myosin became insoluble. The precipitate was collected by centrifugation, redissolved by addition of solid KCl to give an ionic strength of 0.5 and reprecipitated by addition of distilled water to bring the ionic strength to 0.04. The crude myosin was dissolved in 0.5 M KCl as before and stored at 2-4°C. for use or further purification.

Crude myosin was purified as described by Harris and Suelter (1967). It was dialyzed at least 24 hours against several changes of 0.2 M KCl containing 0.02 M Tris-HCl (pH 7.8). It was then passed through a combination cellulose phosphate and DEAE-cellulose column, which had been previously equilibrated with the same buffer. The combination column consisted of an upper column (2.5 x 10 cm) packed with cellulose phosphate, coupled in series to a lower column (2.5 x 12 cm) packed with DEAE-cellulose. The purified myosin was eluted with the same buffer.

For purification by the method of Richards et al. (1967), crude myosin was dialyzed 24 hours against 0.15 M potassium phosphate, pH 7.5. The myosin was applied to a 1.5 x 7 cm column of DEAE-Sephadex A-50, previously equilibrated with the same buffer. The purified myosin was then eluted with a linear gradient using 30 ml of 0.15 M potassium phosphate at pH 7.5 and 30 ml of 0.5 M KCl-0.15 M potassium phosphate, pH 7.5.

Actin

Actin preparations were usually made from the acetone powder of muscle prepared after the method of Seraydarian et al. (1967). The first step in preparing acetone powder was homogenization of 100 gm of muscle with 330 ml of the Guba-Straub solution (0.3 M KCl - 0.15 M KH_2PO_4 at pH 6.5), after which the homogenate was allowed to stand 15 minutes. The slurry was mixed with 1,330 ml of distilled water, and was centrifuged for 15 minutes at 2,000 x g. The supernatant and those obtained from subsequent washings were discarded. The residue was

washed for 20 minutes with 500 ml of 0.05 M $NaHCO_3$, after which it was centrifuged for 15 minutes at 2,000 x g. The residue was washed for 10 minutes with 100 ml of a solution of 0.05 M $NaHCO_3$ and 0.05 M Na_2CO_3, and then was dispersed in 1000 ml of 5×10^{-5} M $CaCl_2$. After 10 minutes, the solid material was centrifuged down as before, and mixed with 300 ml of acetone. The mixture was allowed to stand for 5 minutes, and was then recentrifuged under the same conditions. After two additional acetone washes, the residue was spread on a sheet of filter paper and dried at room temperature. The powder was stored in a tightly sealed container for no more than 2 weeks (Mommaerts, 1952).

G-Actin was prepared by the method of Mommaerts (1952). Acetone powder of muscle was dispersed in 30 volumes of distilled water using a teflon homogenizer. After extraction for 30 minutes, the viscous mass was centrifuged for 30 minutes at 35,000 x g. The supernatant was saved, KCl was added to a concentration of 0.04 M, and polymerization was allowed to proceed for 6 hours. Centrifugation for 2 hours at 100,000 x g. sedimented the F-actin. The supernatant was discarded and the F-actin pellet was dissolved in a minimal amount of a solution containing 125 mg ATP (pH 8.2) per liter. This step usually required stirring for 2 hours. The polymerization cycle was repeated by adding KCl, allowing polymerization to proceed, then collecting the F-actin by centrifugation. The pellets were then dispersed in a solution containing 250 mg ATP per liter at pH 8.2. Depolymerization of actin was brought to completion by dialysis for 2 days against several changes of 10^{-4} M ATP (pH 8.2) under a nitrogen atmosphere. The G-actin solution was centrifuged 2 hours at 100,000 x g and stored at 0°C. for characterization.

The method of Adelstein et al. (1963) was also used to prepare G-actin. The dried powder was extracted with distilled water as in the method of Mommaerts (1952). After addition of ATP to give a concentration of 5×10^{-4} M in the G-actin extract, the actin was passed through a column of Sephadex G-200, which had previously been equilibrated with 5×10^{-4} M ATP (pH 8.1). G-actin was eluted with the same buffer.

KI-extracted F-actin was prepared by the method of Maruyama et al. (1965). Myofibrils were prepared by the method of Perry and Zydowo (1959a), which has been previously described. Myofibrils were dispersed in three volumes of a solution containing 0.6 M KCl, 0.1 M phosphate buffer (pH 6.4), 10 mM sodium pyrophosphate and 1 mM $MgCl_2$. The mixture was then centrifuged for 15 minutes at 1,500 x g, and the residue was extracted twice more with the same solution. The insoluble residue was rinsed three times with a five-fold volume of 0.5 mM $NaHCO_3$, and was then dispersed in three volumes of a solution containing 0.6 M KI, 6 mM sodium thiosulfate, 5 mM β-mercaptoethanol, 1 mM ATP and 30 mM Tris buffer (pH 7.5). Extraction was allowed to proceed for 15 minutes and the slurry was then centrifuged for 30 minutes at 15,000 x g. The supernatant was collected and dialyzed overnight against a solution containing 0.1 M KCl, 0.5 mM ATP and 5 mM Tris buffer (pH 7.5). Any precipitate present was removed by centrifugation for 10 minutes at 15,000 x g. The crude F-actin preparation thus obtained was partially purified by sedimentation in the ultracentrifuge for 2 hours at 100,000 x g. The supernatant was saved for preparation of β-actinin. For characterization, the F-actin pellet was dispersed in a minimum amount of a solution containing 0.1 M KCl, 0.5 mM ATP and 5 mM Tris buffer (pH 7.5).

The method of Hama et al. (1965) was used for preparation of F-actin directly from muscle without depolymerization or acetone treatment.

Myofibrils were prepared as already described. Myofibrils obtained from 10 gm of muscle were extracted for 30 minutes with 200 ml of a solution of 0.6 M KCl, 0.1 M phosphate buffer buffer (pH 6.4), 10 mM pyrophosphate and 1 mM $MgCl_2$. The insoluble material was collected by centrifugation for 15 minutes at 2,000 x g, and was extracted twice again in the same manner. The residue was then rinsed in 150 ml of 0.05 M histidine-HCl buffer (pH 7.1), centrifuged as before, and re-suspended in 15 ml of histidine-HCl buffer (pH 7.5) at 25°C. As soon as the suspension reached 25°C, 2.5 mg of trypsin were added with gentle stirring. After 15 minutes, digestion was terminated by addition of 4.0 mg of soybean trypsin inhibitor. The solid material was collected by centrifugation (15 minutes at 2,000 x g) and was rinsed 3-4 times with 0.05 M histidine-HCl buffer as before. In order to free the F-actin from the muscle structure, the residue was dispersed in 25 ml of 0.1 M KCl by means of a teflon homogenizer. Centrifugation for 15 minutes at 8,000 x g sedimented the coarse material, leaving the F-actin in the supernatant. The F-actin was collected by ultracentrifugation for 2 hours at 100,000 x g, re-suspended in 0.1 M KCl, and if necessary, was clarified by centrifuging for 10 minutes at 41,000 x g. The supernatant contained the "natural F-actin".

Tropomyosin B

Tropomyosin B was prepared according to the method of Bailey (1948). Preparation of the muscle powder was performed at room temperature and all subsequent steps at 2-4°C. Fresh rabbit longissimus dorsi muscle was homogenized with 2 volumes of water for 1 minute in a Waring Blender. After standing 30 minutes, the mass was centrifuged for 15 minutes at 2,000 x g, and the supernatant was discarded. The residue was washed with an equal volume of ethanol, then with 4 volumes of ethanol-water (1:1), twice

with 95% ethanol, and then twice with ether, with centrifuging for 10 minutes at 2,000 x g after each treatment. The fibrous material was allowed to become semi-dry by evaporation, and was then stored for not more than 2 days in a tightly sealed container at -20°C.

The ether-damp muscle powder was extracted with 10 volumes (w/v) of 1 M KCl (pH adjusted to 7.0 with 1 M NaOH) in the cold. After 12 hours, the viscous mixture was centrifuged 20 minutes at 2,000 x g. The residue was re-extracted with a small volume of 1 M KCl (pH 7.0), and the two extracts were combined. The combined extracts were adjusted to pH 4.3 with 1 N HCl, allowed to stand for 1 hour, and the precipitate was centrifuged down for 10 minutes at 1,500 x g. The precipitate was then dispersed in 5 volumes of distilled water, and the pH was adjusted to 7.0. The total volume was measured and saturated $(NH_4)_2SO_4$ (containing 1% of freshly added concentrated ammonium-hydroxide) was slowly added with stirring to give 41% saturation. Centrifugation for 10 minutes at 1,500 x g removed the precipitate, which was discarded and saturated $(NH_4)_2SO_4$ was slowly added to the supernatant with stirring to give 70% saturation. The precipitated tropomyosin B was centrifuged for 15 minutes at 15,000 x g, then dialyzed for 24 hours against several changes of distilled water. The isoelectric precipitation was repeated at pH 4.5, and the product was refractionated with ammonium sulfate, saving only the fraction precipitating between 47-70% saturation. The precipitated tropomyosin B was subjected to the purification cycle again and then finally dialized against distilled water to remove the ammonium sulfate. The subsequent ethanol-ether treatment for storage of the protein was not carried out, but instead the tropomyosin B was characterized immediately.

Tropomyosin B was also isolated by the method of Mueller (1966), which was the same as that of Bailey (1948) except that: 1) All reagents

after the ether denaturation contained 0.5 mM dithiothreitol. 2) The first extraction with 1 M KCl lasted 15 hours, and the second lasted 3 hours. 3) The protein was purified first by isoelectric precipitation at pH 4.3, a second isoelectric precipitation at pH 4.9, a third isoelectric precipitation at pH 5.2, and dialysis against 0.5 mM dithiothreitol. 4) The second purification phase consisted of an ammonium sulfate fractionation, collecting the precipitate formed between 40-55% saturation; then fractionation a second time, saving the tropomyosin B precipitated between 47-55% saturation.

Tropomyosin B was also prepared by the method of Bailey (1948) with modifications as suggested by Bodwell (unpublished method) and Woods (1967). The procedure was actually that of Bailey (1948) with the following modifications; 1) Glass distilled ethanol was used. 2) Reagent grade ammonium sulfate was recrystallized twice from 10^{-3} M EDTA by addition of glass-distilled ethanol. 3) All work except denaturation with organic solvents was done at 2-4°C. 4) All solutions used for extraction and fractionation of tropomyosin B, except the saturated ammonium sulfate solution, contained 0.01 M EDTA (pH 7.0). The saturated ammonium sulfate solution was prepared by saturating 0.2 M EDTA (pH 8.0) with solid ammonium sulfate. 5) An extra wash with 1 volume of ethanol-water (1:1) was included as the initial step in denaturation with organic solvents. 6) The purification cycle consisted of isoelectric precipitation at pH 4.4-4.8, centrifugation (15 minutes at 1,500 x g) of the re-dissolved protein, precipitation of tropomyosin B between 41-65% $(NH_4)_2SO_4$ saturation, isoelectric precipitation at pH 4.4-4.8, precipitation of the protein in the range of 45-65% $(NH_4)_2SO_4$ saturation, and two final precipitations of tropomyosin B between 50-65% $(NH_4)_2SO_4$ saturation.

Further attempted purification of tropomyosin B was based on the method of Davey and Gilbert (1968). Tropomyosin B prepared by the method of Bailey (1948) with modifications utilized by Bodwell (unpublished method) and Woods (1967) was dialyzed for 48 hours against several changes of a solution containing 0.01 M EDTA brought to pH 8.2 by addition of solid Tris buffer. The protein was then applied to a 2.5 x 11 cm column of DEAE-cellulose, which had been previously equilibrated with the same buffer. Tropomyosin B was eluted with a linear gradient composed of 100 ml of the starting buffer and 100 ml of the starting buffer containing 1 M KCl.

Troponin (ESF)

Preparation of ESF by the method of Katz (1966) was initiated by extracting acetone-dried muscle powder prepared as already described with 30 volumes (w/v) of 0.1 mM ATP (pH 7.6) for 1 hour at 25°C. The mixture was centrifuged 20 minutes at 35,000 x g, and the supernatant containing G-actin was saved. The volume of the supernatant was measured, and 1.1 ml of 0.15 M Tris-nitrate buffer pH 7.6, were added per 10 ml. Solid KCl and $MgCl_2$ were added with stirring to give final concentrations of 0.1 M and 0.1 mM, respectively. Polymerization was allowed to proceed for 16 hours at 2-4°C. The F-actin formed was collected after centrifugation for 2 1/2 hours at 105,000 x g. The supernatant was discarded, and the pellet was dispersed in 15 volumes of 0.1 mM ATP at pH 7.6 using a teflon homogenizer. Centrifugation of this suspension for 1 hour at 105,000 x g sedimented the unwanted material and left the G-actin in solution. Formation of F-actin was again induced, this time by addition of $MgCl_2$ to a level of 0.6 mM. This step prevented incorporation of contaminating tropomyosin and ESF into the F-actin polymer. Centrifugation for 2 1/2 hours at 105,000 x g removed the unwanted F-actin from

solution, after which the supernatant was fractionated by adding saturated-ammonium sulfate solution containing 1% of freshly added ammonium hydroxide. The protein precipitating in the range of 40-70% was collected by centrifugation for 20 minutes at 35,000 x g, and is believed to contain tropomyosin and ESF. The precipitate was dialyzed against several changes of distilled water to remove the ammonium sulfate before characterization.

ESF was also prepared by the method of Azuma and Watanabe (1965b). The supernatant obtained from the first ammonium sulfate fractionation utilized in the preparation of α-actinin according to the method of Seraydarian et al. (1967) was the starting point for preparing ESF by this method. This supernatant was brought to 55% saturation with ammonium sulfate and was centrifuged for 10 minutes at 10,000 x g. The precipitate obtained was dissolved in distilled water and dialyzed against 0.1 M KCl buffered at pH 7.0 with 0.02 M potassium phosphate. The protein solution containing ESF was applied to a 4.5 x 40 cm column of Sephadex G-200, which had previously been equilibrated with the same buffer. ESF emerged at the void volume and was stored at 0°C. until removed for characterization.

α-actinin

The method of Seraydarian et al. (1967) was employed for preparation of α-actinin. Rabbit longissimus dorsi muscle was homogenized with 3.3 volumes of a solution containing 0.3 M KCl and 0.15 M potassium phosphate (pH 6.5). After stirring for 15 minutes, 1,330 ml of distilled water per 100 gm of muscle were slowly added with stirring. The slurry was centrifuged 15 minutes at 2,000 x g. The residue was washed twice (10 minutes each time) with 4 volumes of 0.02 M KCl - 0.2 mM $NaHCO_3$, and then twice (5 minutes each time) with 4 volumes of distilled water. The solids were extracted for 4 hours at 20°C with 1 volume of distilled

water. The gelatinous mass was allowed to drain through Whatman No. 41 filter paper for 4 hours at 20°C. The filtrate was collected and stored at 0°C. In the same manner, the solids were extracted 5 hours with 1 volume of 1 mM $NaHCO_3$ and then drained for 4 hours. The filtrate was collected and combined with the previous filtrate.

After cooling to 2-4°C, the volume of the combined extracts was measured. For each 100 ml of extract, 22.5 gm of recrystallized $(NH_4)_2SO_4$ were added slowly with stirring. The solution was allowed to stand for 20 minutes and then was centrifuged 10 minutes at 5,000 x g. The supernatant was saved for preparation of troponin according to the method of Azuma and Watanabe (1965b), while the precipitate was dissolved in 50 ml of 1 mM $NaHCO_3$ per 100 gm of original muscle. The solution was clarified for 30 minutes by centrifugation. Afterwards, 10 gm of solid recrystallized $(NH_4)_2SO_4$ were added per 100 ml of solution, while stirring constantly. The mixture was allowed to stand for 20 minutes and then centrifuged 10 minutes at 5,000 x g. The precipitate was dissolved in a minimum of 1 mM $NaHCO_3$ and centrifuged at 20,000 x g for 30 minutes. It was then dialyzed against 1 mM $NaHCO_3$ for 10 hours to remove $(NH_4)_2SO_4$. Any precipitate formed during dialysis was removed by centrifugation for 1 hour at 20,000 x g. Solid KCl was added to the supernatant to bring the concentration to 3.3 M, and the α-actinin was allowed to precipitate for 20 minutes. The α-actinin was collected by centrifugation for 10 minutes at 5,000 x g, after which it was dissolved in a minimum amount of 1 mM $NaHCO_3$ and dialyzed against 1 mM $NaHCO_3$ for 10 hours. Any precipitate formed during dialysis was removed by centrifuging for 1 hour at 20,000 x g. Greater purification of α-actinin was achieved by repeating the precipitation with 3.3 M KCl and following all subsequent steps.

β-actinin

The method of Maruyama (1965b) was used for preparation of β-actinin. The final supernatant obtained in the preparation of KI-extracted F-actin according to the method of Maruyama et al. (1965) as described earlier herein, was used as the starting material. The volume of the supernatant was measured and solid $KHCO_3$ was added to bring the concentration to 5 mM. Saturated $(NH_4)_2SO_4$ solution was added till 40% saturation was attained. The supernatant was clarified by centrifugation for 10 minutes at 10,000 x g, and the precipitate was discarded. Addition of saturated $(NH_4)_2SO_4$ to the supernatant to give 60% saturation caused the β-actinin to precipitate. After standing for 10 minutes, the turbid solution was centrifuged for 20 minutes at 20,000 x g. The precipitate was dissolved in a small amount of 5 mM $KHCO_3$, and dialyzed against 5 mM $KHCO_3$ prior to characterization.

Inhibitory Factor (IF)

Inhibitory factor was prepared by the method of Hartshorne et al. (1967). Myofibrils prepared as previously described were extracted for 15 hours with an equal volume of a solution containing 1.2 M KCl, 0.08 M $NaHCO_3$ and 0.02 M $NaCO_3$ (pH 8.7). The slurry was exhaustively dialyzed against 0.1 M KCl containing 0.02 M Tris-HCl buffer (pH 7.6). The precipitate formed was removed by centrifugation for 15 minutes at 20,000 x g and discarded. Saturated $(NH_4)_2SO_4$ solution was added slowly with stirring until the supernatant was 30% saturated. The precipitate, containing IF, was collected by centrifugation for 15 minutes at 30,000 x g and dissolved in 20 volumes of 0.01 M Tris-HCl buffer, pH 7.6. The protein was exhaustively dialyzed against the same buffer. After centrifugation for 1 hour at 100,000 x g, the IF preparation was characterized.

Extra Protein

Extra protein was prepared by the method of Perry and Zydowo (1959a). Myofibrils prepared as already described were extracted for 20 minutes with 3 volumes of a solution containing 0.47 M KCl, 0.1 M potassium phosphate buffer (pH 6.5), 1 mM $MgCl_2$ and 0.01 M sodium pyrophosphate. The insoluble material was centrifuged for 30 minutes at 600 x g, and the turbid supernatant was dialized against 0.04 M KCl containing 6.7 mM potassium phosphate buffer (pH 7.2). After precipitation was complete the extra protein solution was clarified by centrifuging for 20 minutes at 40,000 x g. Extra protein preparations were perevaporated to one-fifth of their volume before characterization.

Preliminary Experiments

Salt-Soluble Fraction

Early work was hampered by difficulty in distinguishing between the salt-soluble and salt-insoluble components. Extraction of rabbit skeletal muscle with 6 volumes of Weber-Edsall solution (0.6 M KCl, 0.04 M $KHCO_3$ and 0.01 M K_2CO_3) for 24 hour yielded a viscous mass, which was not easily clarified by centrifugation. Definite boundaries failed to form between the solution, gel and sediment, which made it extremely difficult to prepare consistent samples. Dilution with additional Weber-Edsall solution prior to centrifugation increased the optical clarity of the supernatant solution, increased the volume of the supernatant, decreased the volume occupied by the gelatinous material, and established a clear boundary between the supernatant and residue.

Gel Filtration

Various gel filtration media were explored in fractionation of the salt-soluble proteins from muscle. Based on several buffers previously

used in various studies on salt-soluble proteins (Tonomura, 1961; Fujimaki et al., 1965; Haga et al., 1965; Oppenheimer, 1967; Richards et al., 1967; Davey and Gilbert, 1968), an eluting solution composed of 0.6 M KCl and 0.05 M Tris-HCl buffer (pH 7.4) was chosen. The salt-soluble proteins were fractionated on 2.5 x 37 cm columns packed with either Bio-Gel P-30, Bio-Gel A-1.5m, Bio-Gel A-15m or Bio-Gel A-50m, which had previously been equilibrated with the eluting buffer. The best separations were attained with Bio-Gel A-50M, which gave four discernible fractions. (Figure 1). When using an eluting solution composed of 1.0 M KCl and 0.05 M Tris-HCl buffer (pH 7.6) five fractions were sometimes discernible, although most preparations of salt-soluble proteins separated into only four fractions.

Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation was investigated as a means of separating and characterizing the salt-soluble proteins. Linear gradients of 12 ml total volume were utilized. Gradients were composed of 6 ml of a light component (5% sucrose containing 0.6 M KCl and 0.05 M Tris-HCl buffer at pH 7.6) and 6 ml of a heavy component (40% sucrose containing 0.6 M KCl and 0.05 M Tris-HCl at pH 7.6). Figure 2 shows the results obtained with this system.

The nature of the components was investigated by incorporating them into acrylamide gels and staining the gels with biological dyes. Fractions were incorporated into the gels by two methods. In the first method, density gradient separations were performed with 5% Cyanogum throughout the above gradient (Jolley et al., 1967). Following centrifugation, the intact separation was photo-polymerized in the centrifuge tube. In the second method, density gradient separations were performed without Cyanogum in the gradient described above. The separation pattern

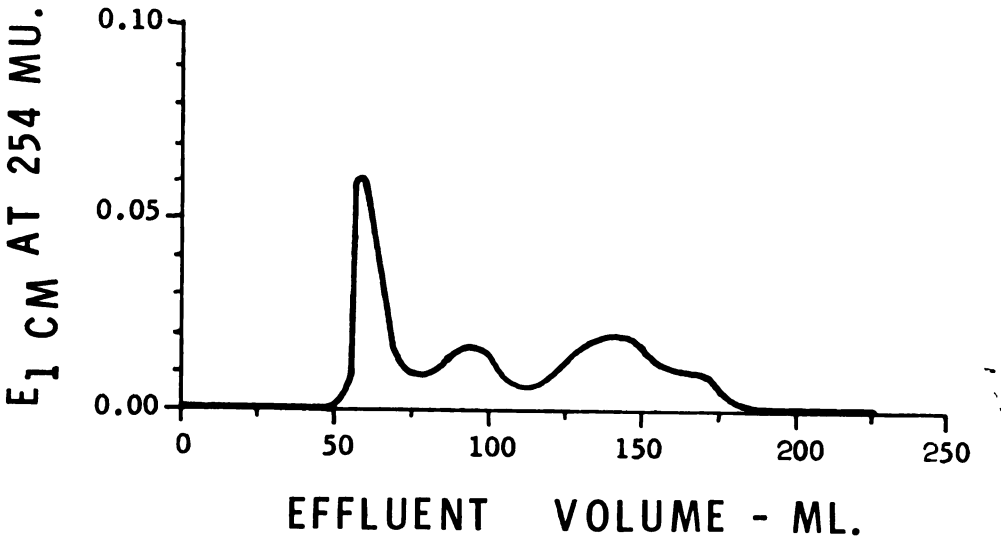


Figure 1. Gel filtration of Weber-Edsall extract. A 2 ml sample of extract containing 1.5 mg protein/ml was applied at 0 ml effluent volume.

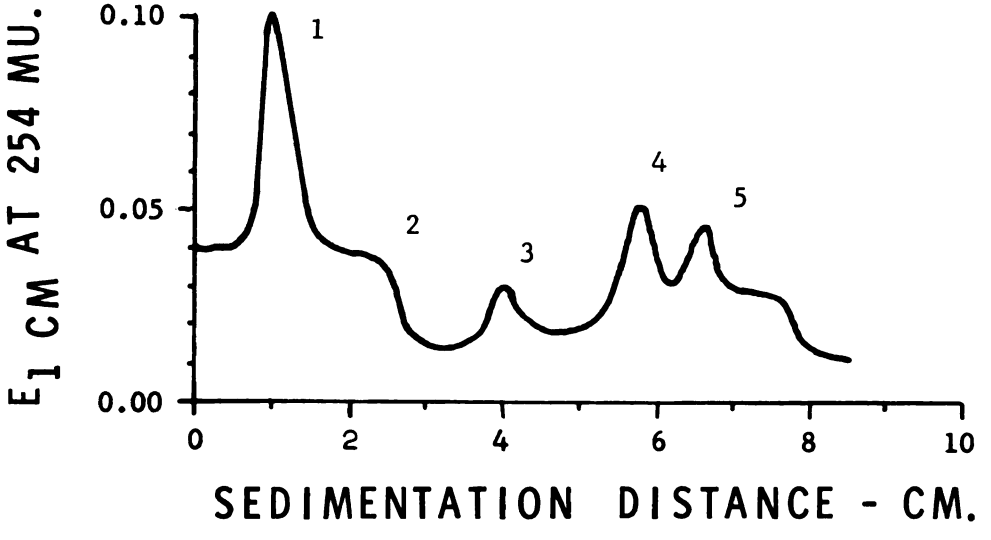


Figure 2. Density gradient centrifugation of Weber-Edsall extract. Sample = 0.6 ml of Weber-Edsall extract containing 1.5 mg protein/ml.

was analyzed at 254 mμ in an Isco density gradient fractionator and 0.5 ml fractions were collected. The fractions were then mixed with gel components and photopolymerized.

The gels were stained for proteins with Amido Black and Coomassie Blue, for nucleoproteins utilizing Pyronin Y and Acridine Orange, for lipoproteins with Sudan Black B and Oil Red O, and for glycoproteins by means of Periodic Acid-Schiff stain and Alcian Blue. The majority (96%) of the protein was found to reside in fractions 1 and 2, with a barely noticeable amount in fractions 3, 4, and 5. Nucleo-proteins were detected in fraction 1 and to a small extent in fraction 3. Staining indicated that lipoproteins were absent. The periodic Acid-Schiff stain showed no positive reaction, while Alcian Blue showed a definite affinity for fraction 1 and a slight affinity for fraction 3.

Subsequent experiments indicated that fractions 3-5 may be sarcoplasmic in origin. These fractions decreased with more thorough washing of the muscle residue prior to salt extraction. They were usually not present in the salt extracts of well-washed myofibrils. Further, purified preparations of known myofibrillar proteins usually sedimented similarly to fractions 1 or 2.

Electrophoresis

Many attempts were made at using zonal electrophoretic techniques for the separation and study of salt-soluble proteins. The use of urea was initially avoided, since it is known to irreversibly denature myosin at concentrations over 2 M (Takashina and Kasuya, 1967). Over 140 different buffer systems were explored, using disc gel electrophoresis, column electrophoresis in a sucrose gradient or in ethanolyzed cellulose, cellulose acetate membrane electrophoresis, or electrophoresis on glass paper. In every system not employing urea, the majority of the protein,

as determined by staining or by monitoring the column contents at 254 mμ, remained at the point of sample application.

Column Chromatography

Considerable effort was devoted to separating the salt-soluble proteins by column chromatography without the use of urea. By employing 6 M glycerol in the chromatography buffers, it was usually possible to reduce the ionic strength below 0.3 without precipitation of actomyosin. In all systems not containing urea, however, the tendency for some samples to precipitate persisted. Dialysis of salt-soluble proteins against various buffer systems containing sucrose or glycerol indicated that approximately 1 M urea was required to prevent precipitation of all samples.

Trials with DEAE-, TEAE-, AE-, ECTEOLA- and CM-cellulose or with cellulose phosphate indicated that those exchangers, which hold most of the salt-soluble proteins in the column, also tend to bind them irreversibly. Through the use of ECTEOLA-, AE-cellulose and cellulose phosphate columns, in that order, the salt-soluble proteins were separated into 8-11 fractions. About 95% of the ultraviolet-absorbing material was recovered from the columns. Although only one separation was performed, dialysis experiments indicated that the buffer system was compatible with all preparations of salt-soluble proteins tested. Time did not permit further work along this line, so the procedures and results are briefly outlined below.

Salt-soluble proteins of muscle were prepared as previously described. Solid KCl was added to bring the concentration to 1 M. The volume of the solution was measured and an equal weight (w/v) of sucrose was added. Stirring for 20-30 minutes was sufficient to dissolve the sucrose. The sample was stored at -20°C until chromatographed.

Chromatography buffers were made from a stock solution of sucrose and urea, which had been passed over a bed of washed DEAE-cellulose to remove the colored material and turbidity. Ion-exchange columns were composed of a series of 4 short columns of decreasing diameter as suggested by Hagdahl (1954). This type of column was found to sharpen the eluted fractions, thus permitting the use of a much smaller total volume of buffer during elution.

The sample was thawed, and then 20 ml were applied to a 4.5 x 35 cm column of Sephadex G-15, which had previously been equilibrated with the chromatography starting buffer (1.45 M sucrose, 1.2 M urea, 0.1 M KCl and 0.03 M Tris brought to pH 9.2 with concentrated H_3PO_4).

The sample was collected as it emerged from the column and was then passed through a column of ECTEOLA-cellulose previously equilibrated with the starting buffer. Elution was accomplished with a linear gradient composed of 100 ml of the starting buffer and 90 ml of a solution, containing 1.45 M sucrose, 1.2 M urea, 1.5 M KCl and 0.03 M Tris-H_3PO_4 buffer at pH 9.2. After completion of the gradient, elution was continued using an additional 100 ml of the final buffer. The first fraction emerging from the ECTEOLA-cellulose column was applied to an AE-cellulose column equilibrated with the starting buffer. Separation was accomplished in the same manner as for the ECTEOLA-cellulose column.

The first fraction emerging from the AE-cellulose column was quite dilute, being dispersed in a volume of about 100 ml. The first fraction from the AE-cellulose column was adjusted to pH 6.0 with 1 M H_3PO_4, and dialyzed 2 days against several changes of a solution containing 0.03 M KCl and 0.01 M KH_2PO_4 (pH 6.0). The volume of the protein solution was reduced to 25 ml by perevaporation. The solution was dialyzed against

0.03 M KCl containing 0.01 M KH_2PO_4 buffer (pH 6.0) and then applied to a column of cellulose phosphate, which had previously been equilibrated with the same buffer. Elution was accomplished using 100 ml of starting buffer followed by 200 ml of a solution containing 1.5 M KCl and 0.01 M KH_2PO_4 (pH 6.0). Additional elution of each column with its final eluting buffer, to which 0.1 M K_3PO_4 had been added, failed to yield any additional ultraviolet-absorbing material. Separation patterns obtained are shown in Figure 3.

Techniques Utilized in Studying Myofibrillar Proteins

Gel Filtration

A 2 ml sample of the protein to be fractionated was applied to a 2.5 x 37 cm column of Bio-Gel A-50m, which had previously been equilibrated with a solution of 1 M KCl and 0.05 M Tris-HCl (pH 7.6). The protein was eluted with the same solution. Gel filtration patterns were monitored at 254 mμ with an Isco column monitor-recorder, and 10 ml fractions were collected.

Sucrose Density Gradient Centrifugation

Linear gradients of 12 ml total volume were produced in 14.5 x 96 mm centrifuge tubes by means of a two-chamber gradient device. Gradients were composed of 6 ml of a light component (5% sucrose containing 0.6 M KCl and 0.05 M Tris-HCl at pH 7.6) and 6 ml of a heavy component (40% sucrose containing 0.6 M KCl and 0.05 M Tris-HCl at pH 7.6). Results indicated that the gradients could be used immediately or allowed to stand in the cold several hours without any noticeable influence. The protein solution to be analyzed was carefully layered over each gradient. The gradient and sample were spun for 9.5 hours at 256,000 x g in an International, model B-60, ultracentrifuge equipped with an International

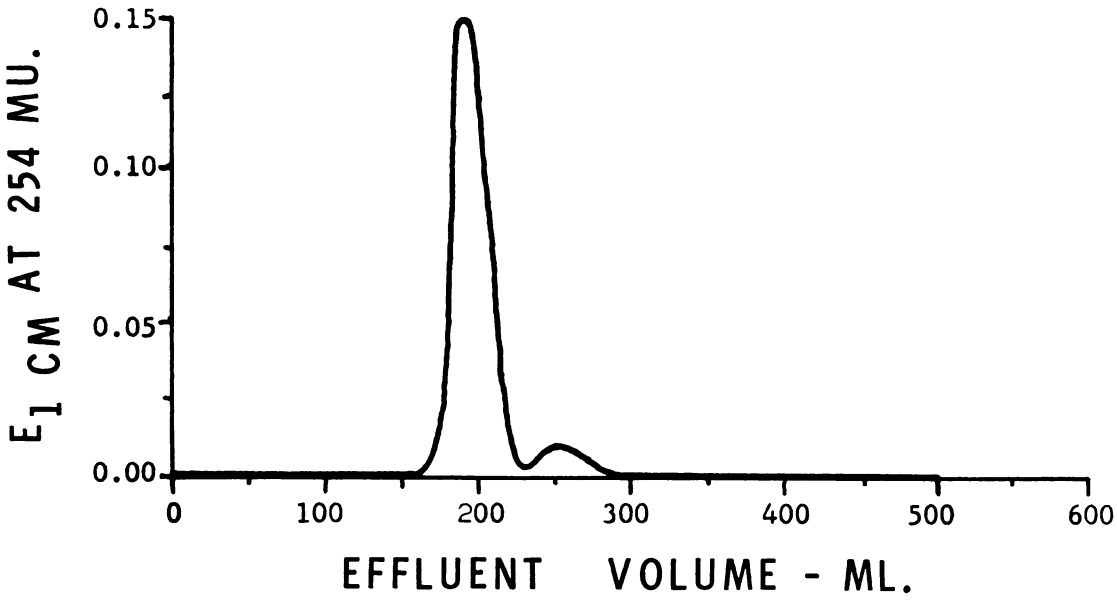


Figure 3A. Gel filtration of 24 ml of Weber-Edsall preparation on Sephadex G-15. Experimental conditions are described in the text. Sample was applied at 0 ml effluent volume.

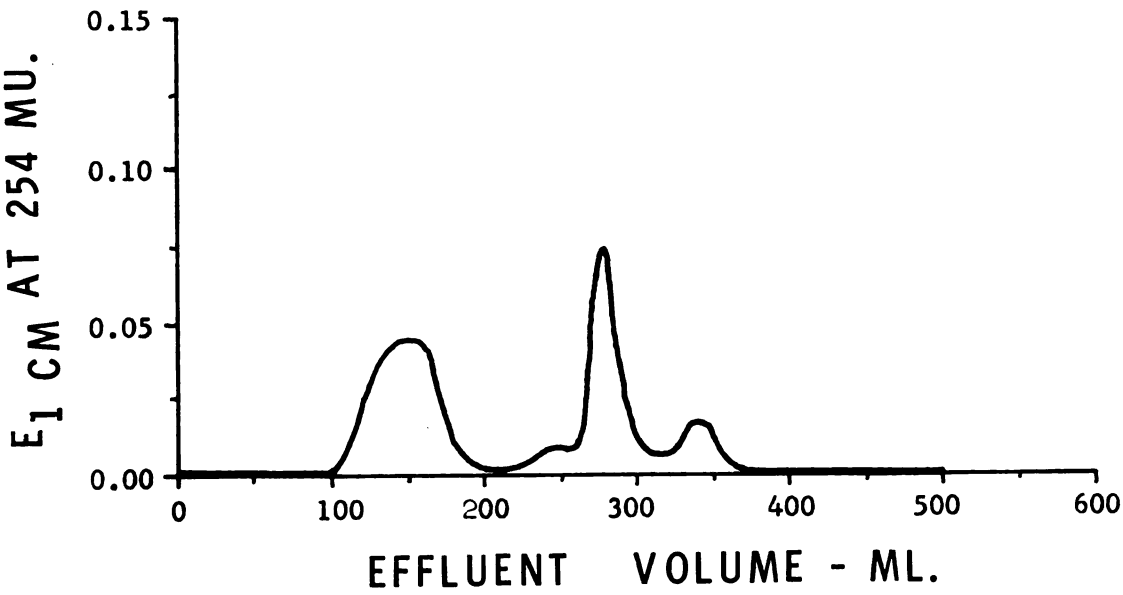


Figure 3B. Chromatography of breakthrough peak from Figure 3A on ECTEOLA-cellulose. Sample was applied at 0 ml effluent volume. Experimental conditions are described in the text.

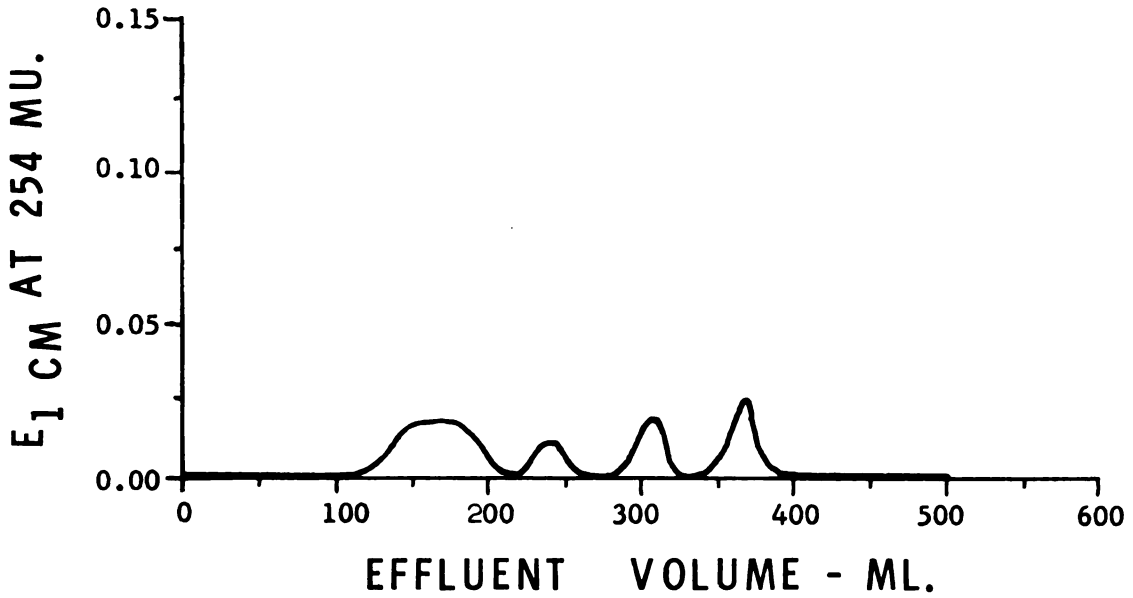


Figure 3C. Chromatography of breakthrough peak from Figure 3B on AE-cellulose. Sample was applied at 0 ml effluent volume. Experimental conditions are described in the text.

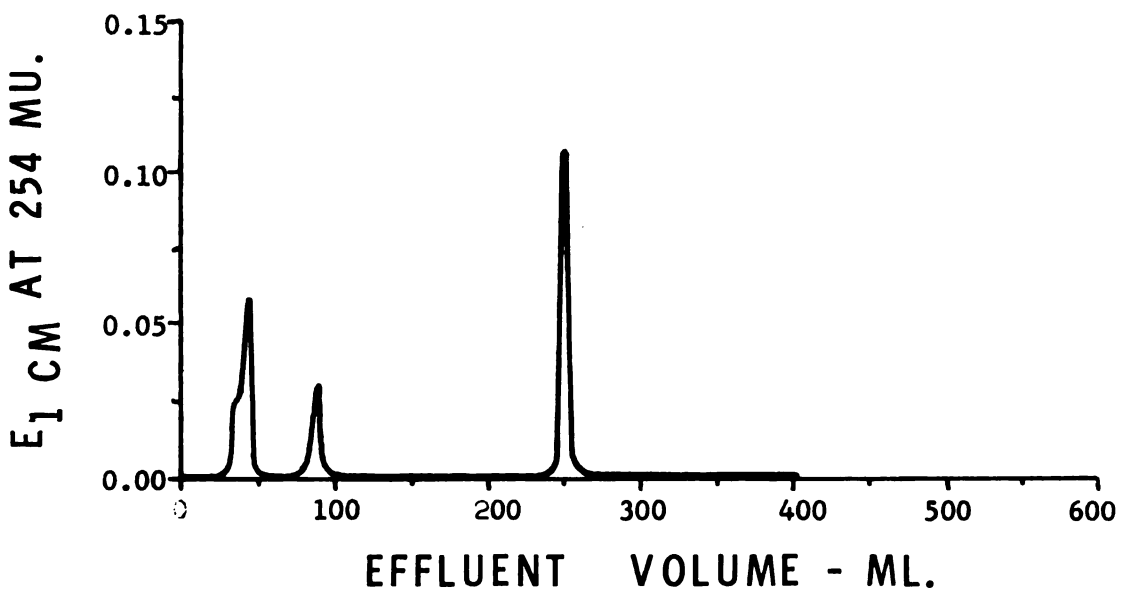


Figure 3D. Chromatography of breakthrough peak from Figure 3C on cellulose phosphate. Sample was applied at 0 ml effluent volume. Experimental conditions are described in the text.

SB-283 rotor. The tube contents were then analyzed at 254 mμ with an Isco density gradient analyzer. Fractions of 0.5 ml were collected for further analysis.

Disc Acrylamide Gel Electrophoresis

The basic disc gel electrophoresis system of Davis (1964) was used with appropriate modifications for fractionation of the salt-soluble proteins of the myofibril. All gels contained 7 M urea and were photopolymerized. Acrylamide was added in the form of Cyanogum. The running gel contained 6.5% acrylamide, and the spacer gel 5.0% acrylamide. A 10 M urea stock solution was deionized by passing through a bed of Amberlite MB-3 mixed bed resin before use.

The running gel was prepared from three stock solutions (Jolley et al., 1967). Stock solution 1 contained 4.0 ml of 2 N HCl, 6.1 gm of Tris, 0.08 ml of N, N, N', N'-Tetramethyl-ethelynediamine (TEMED), 65 ml of 10 M urea and enough distilled water to bring the total volume to 80 ml. Stock solution 2 contained 43.3 gm of Cyanogum, 25 ml of 10 M urea, and enough distilled water to bring the total volume to 100 ml. Stock solution 3 contained 1 mg of riboflavin, 35 ml of 10 M urea, and distilled water to bring the total volume to 50 ml. To prepare the running gel, 8.0 ml of stock solution 1 were mixed with 2.0 ml of stock solution 2 and 3.3 ml of stock solution 3 in a 20 ml beaker. The mixture was dispensed into a series of 0.5 (I.D.) x 6.5 cm glass tubes, which were mounted on end in a transparent plastic holder. The tubes were filled to a height of 5.0 cm, after which the meniscus of the gel solution was carefully layered over with 0.5 cm of distilled water. Photopolymerization of the running gel was carried out immediately by placing the tubes under a fluorescent lamp for 15 minutes.

The spacer gel was prepared from three stock solutions. Stock solution 1 contained 4 ml of 2 N HCl, 1.0 gm of Tris, 0.06 ml TEMED, 65 ml of 10 M urea, and adequate distilled water to bring the total volume to 80 ml. Stock solution 2 contained 33.3 gm of Cyanogum, 25 ml of 10 M urea, and enough distilled water to bring the total volume to 100 ml. Stock solution 3 contained 1 mg of riboflavin, 35 ml of 10 M urea, and distilled water to bring the total volume to 50 ml. To prepare the spacer gel, 4.0 ml of stock solution 1 were mixed in a 20 ml beaker with 1.0 ml of stock solution 2 and 1.67 ml of stock solution 3. The glass tubes were removed from the fluorescent lamp. The shallow layer of water on top of the gel was decanted, and the spacer gel mixture was layered on top of the running gel to an additional height of 0.75 cm. A 0.5 cm layer of water was carefully applied to the upper surface of the spacer gel mixture, and photopolymerization was carried out as described earlier.

After 15 minutes, the tubes were removed from the fluorescent lamp and the water layer on top of the spacer gel was decanted. The glass tubes were removed from the plastic holder and mounted on the bottom of the upper buffer reservoir. They were arranged so that the spacer gel would make contact with the buffer in the upper reservoir, and the running gel with the buffer in the lower reservoir. The upper and lower reservoirs were charged with a solution containing 0.6 gm Tris and 2.88 gm glycine per liter. Two drops of a 0.01% aqueous solution of Bromphenol Blue were then stirred into the buffer in the upper reservoir.

The protein sample, which had previously been dialyzed against 16 volumes of 8 M deionized urea, was layered beneath the buffer on top of the spacer gel. The negative lead of a Heathkit Model PS-4 power supply was connected to the carbon electrode of the upper reservoir, and the

positive lead was connected to the carbon electrode of the lower reservoir. Electrophoresis was started by applying 100 V d-c across the two electrodes. The current was maintained at 2 ma per tube by gradually increasing the voltage as electrophoresis proceeded.

Electrophoresis was terminated when the blue colored band of Bromphenol Blue reached the lower end of the gel tubes. If the blue band on any of the tubes indicated completion ahead of the others, the early tubes were terminated individually. Thus, all gels reached the same stage of completion. Gels were removed from their respective tubes by rimming them with a 26 gauge hypodermic needle. Injection of water through the needle during the rimming process aided in extracting the gels. Staining of the gels was carried out as described later herein.

Absorption Spectra

Protein solutions to be analyzed were scanned automatically in a Beckman Model DB-G Recording Spectrophotometer. A sample of the protein solution in a 1 cm silica cell was placed in the sample slot and scanned against a buffer blank containing no protein. Samples were scanned from 340 to 240 mμ.

Nitrogen Determination

Since Kjeldahl samples of several key analyses were inadvertently discarded, protein concentrations of the various preparations were estimated by absorbance at 254 mμ calibrated by micro Kjeldahl nitrogen determination performed on total salt-soluble extracts of purified myofibrils. Readings were corrected for nucleotide content by deproteinization with perchloric acid (Kasai et al., 1964). Nitrogen analysis was carried out by the micro Kjeldahl method as outlined by the American Instrument Company (1961). Protein concentrations were calculated assuming that myofibrillar proteins contain 16.15% nitrogen (Mihalyi and Rowe, 1966).

Identification of Fractions

The proteins of the myofibril were prepared and their behavior was studied by gel filtration, density gradient centrifugation and disc gel electrophoresis. The separation patterns from known protein preparations were used to identify the components in the various separation patterns obtained from unknown samples.

In gel filtration patterns, each fraction was assigned the value of the ratio, $\frac{Ve}{Vo}$, where Ve is the number of milliliters eluted between the point of sample application and the maximum peak height of that fraction; and Vo is the number of milliliters required to elute a sample of Blue Dextran from the column. The value of $\frac{Ve}{Vo}$ is theoretically constant for a given protein and a given gel porosity (Largier and Polson 1964).

In density gradient centrifugation separations, each fraction is given in terms of the distance in centimeters from the top of the gradient through which that component sedimented. Sedimentation distances were determined by analysis using an Isco Model D density gradient fractionator.

In disc gel electrophoretic separations, the relative mobility (Rm) of each band was used for identification. The Rm value of a band is expressed as the ratio of the distance of migration of the band to the distance of migration of the buffer front. Distances in the present study were determined by direct measurement in centimeters. The most reproducible R_m values were obtained by measuring from the top of the spacer gel to the trailing edge of a given protein band. Difficulty was experienced in producing consistent acrylamide gels due to the instable nature of the gel stock solutions. In order to relate different disc gel separations with each other, tropomyosin was employed as an internal standard. The migration distance of other bands was then related to the tropomyosin standards. R_m values thus calculated were consistent within $\pm$ 0.02 R_m units.

Staining

Disc gel electrophoretic separations were stained for the detection of proteins, free sulfhydryl groups, nucleoproteins, lipoproteins and glycoproteins.

Amido Black staining for proteins was carried out as follows: 1) Gels were immersed for 2 hours in a solution containing 250 ml of distilled water, 50 ml of glacial acetic acid, 250 ml of methanol and 2 gm of Amido Black dye. 2) Gels were then destained electrophoretically in 7% acetic acid solution.

The Coomassie Blue stain for proteins was used according to the method of Crambach et al. (1967). The gels were immersed for 30 minutes in a 12.5% solution of trichloracetic acid. They were then transferred to a freshly mixed solution, containing 1 part of 1% aqueous Coomassie Blue and 20 parts of 12.5% aqueous trichloroacetic acid solution. After 30 minutes the gels were placed in 10% trichloroacetic acid solution for destaining.

The DDD (2,2'-dihydroxy-6,6'-dinaphthyldisulfide) method of Zwann (1966) was used for detection of free sulfhydryl groups in disc gel separations of the myofibrillar proteins. The gels were fixed for 30 minutes in a solution of ethanol-glacial acetic acid-distilled water (70:5:25). They were then placed in 0.04 M sodium barbital buffer brought to pH 8.5 with acetic acid for 1 hour. They were incubated for 4 hours at 50°C in a freshly made solution prepared by mixing 25 mg of DDD in 15 ml absolute ethanol, and then adding 35 ml of barbital-acetate buffer (pH 8.5). Gels were washed twice for 15 minutes each time in distilled water (pH 4.5). Washing was repeated in 60% ethanol, after which the gel were washed once in 90% ethanol and again in distilled water. Gels were stained 40-60 minutes in a solution made by dissolving

50 mg of Fast Black K salt in 50 ml of 0.04 M sodium barbital adjusted to pH 7.0 with glacial acetic acid. They were then destained in distilled water.

Staining for ribonucleoproteins utilized the Acridine Orange method described by Richards et al.(1965). The staining solution consisted of 1% lanthanum acetate, 2% Acridine Orange and 15% acetic acid. It was prepared by mixing 5 gm of lanthanum acetate with 75 ml of glacial acetic acid and heating on a steam bath to 90°C. An equal volume of water at 90°C was added with stirring. Clearing of the solution indicated formation of lanthanum acetate. The solution was cooled and diluted to 490 ml with distilled water, after which 10 gm of Acridine Orange were added with stirring. Gels were immersed in the staining solution overnight and destained in 7% acetic acid.

The Methylene Blue stain for ribonucleic acid was carried out according to the method of Peacock and Dingman (1967). Gels were immersed for 15-20 minutes in 1 M acetic acid, then transferred for 2 hours to a solution containing 0.2% Methylene Blue, 0.2 M sodium acetate and 0.2 M acetic acid. The gels were then destained in water.

The method of Gifford and Yuknis (1965) was used to stain for lipoproteins. Gels were fixed for 30 minutes in 15% acetic acid, then stained overnight in a solution of 40% ethanol saturated with Sudan Black B. Destaining was carried out in 40% ethanol.

Oil Red O was also utilized to stain for lipoproteins by the method of Beaton et al. (1961). The stain was made by saturating warm methanol with Oil Red O, cooling and filtering. The mixture was then added to an equal volume of aqueous 20% trichloroacetic acid and stirred thoroughly. Gels were immersed overnight in the staining solution and then transferred to a mixture of methanol-distilled water-acetic acid (50:50:10) for destaining.

The method of Gifford and Yuknis (1965) was used for detection of glycoproteins. Gels were stained overnight in a solution of 0.2% Alcian Blue in 15% acetic acid. Destaining was done in 15% acetic acid.

Bacterial Action on Myofibrillar Proteins

Sample Preparation

Aseptic muscle samples were prepared according to Borton (1966). Pigs used in this study were stunned electrically, the neck was scrubbed with pHisoHex bacteriocidal soap, and they were stuck in a conventional manner using a sterile knife. After bleeding, the hogs were scalded and dehaired. Further cleaning of the carcasses was accomplished in a normal manner, except that the carcasses were not split. The carcasses were then rinsed with alcohol and placed in the cooler for 20 hours. The longissimus dorsi muscle was then aseptically excised from each carcass and placed in a sterile container. The muscle was ground in a sterile grinder equipped with a 2 mm plate. The grinder had been previously autoclaved for sterilization.

Bacteria obtained from known stock cultures were inoculated into sterile APT broth (Difco), and then subcultured in sterile APT broth. The organisms were tested for viability by pipetting an appropriate dilution of the inoculum into sterile petri dishes and incubating in APT agar (Difco) for 48 hours at 25°C. The inoculum was diluted to contain 10^4-10^5 organisms per ml, after which 1 ml was added to each 100 gm of sterile muscle sample. The inoculum was aseptically mixed throughout the ground muscle sample. Inoculated samples were covered with a loose-fitting lid and were incubated at 3 or 10° C for 0, 8 or 20 days. After the desired incubation time, the salt-soluble proteins were prepared from the muscle as previously described. The protein preparations were then analyzed by sucrose

density gradient centrifugation, gel filtration and disc gel electrophoresis as outlined earlier herein.

RESULTS AND DISCUSSION

Characterization and Analysis of Known Proteins.

In order to identify the various components in unknown preparations of myofibrillar proteins, the known proteins of the myofibril were isolated and purified by conventional methods. Behavior of the known components was studied by density gradient centrifugation and disc gel electrophoresis. Although gel filtration was also used for studying myofibrillar proteins, it was less useful for studying the known proteins. Reproducible and meaningful gel filtration patterns were difficult to obtain, apparently due to the tendency of myofibrillar proteins to aggregate (Huxley, 1960; Poglazov, 1966; Ebashi, 1966; Perry, 1967a; Dreizen et al., 1967; Hayashi, 1967).

Myosin

Four separate samples of myosin were prepared by the method of Harris and Suelter (1967). Figure 4 depicts a typical chromatogram obtained during the purification of myosin by this method. The portion of the myosin peak emerging at 80-110 ml was designated as HM (Harris' Myosin) and was characterized for purity by density gradient centrifugation and disc gel electrophoresis. A typical density gradient centrifugation pattern obtained from HM is shown in Figure 5. HM preparations produced only one peak, which sedimented at 1.0-1.1 cm.

Disc gel electrophoresis of HM usually revealed the presence of 7-8 components. Serial disc gel separations (Figure 6) performed on the fractions eluted during purification of HM indicated that the myosin peak

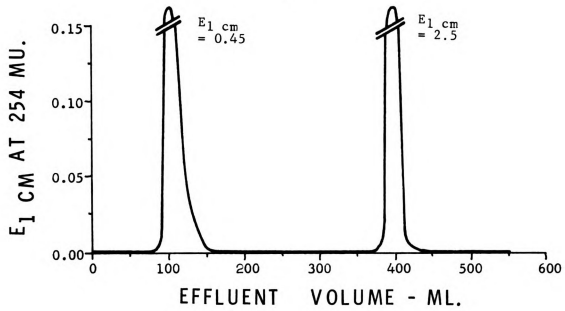


Figure 4. Purification of myosin on a combination column of DEAE-cellulose and cellulose phosphate (Harris and Suelter, 1967). Column dimensions were 2.5 x 12 cm. Sample = 18 ml of myosin containing 3.3 mg protein/ml. Sample was applied at 0 ml elution volume. Myosin was eluted with 0.2 M KCl containing 0.02 M Tris-HCl (pH 7.8).

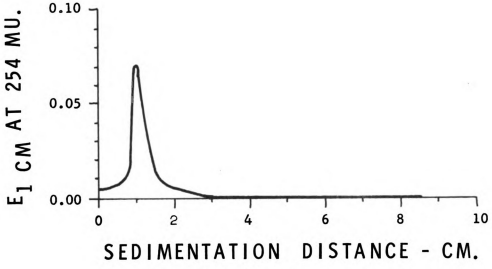


Figure 5. Density gradient centrifugation of HM. Sample = 0.6 ml of HM preparation containing 0.7 mg protein/ml. Sedimentation distance of myosin peak = 1.0 cm.

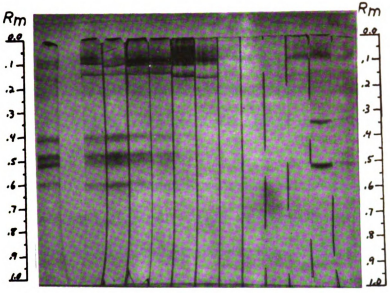


Figure 6. Serial disc gel electrophoretic analyses of the myosin purification shown in Figure 4. On the far left is shown the separation pattern obtained from 0.05 ml of the unpurified myosin sample, which was subsequently applied to the column. Then, proceeding from left to right 0.05 ml samples taken at effluent volumes of 80, 90, 100, 110, 120, 130, 160, 190, 360, 370, 380 and 390 ml were analyzed.

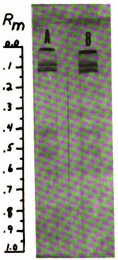


Figure 7. Disc gel electrophoresis of myosin. A, disc gel pattern from 0.01 ml of PM containing 2.1 mg protein/ml. B, Disc gel pattern from 0.01 ml of EM containing 1.3 mg protein/ml.

(Figure 4) was not uniform in composition. The protein eluted at 80-110 ml contained several slow-moving components (R_m = 0.00-0.15) and four additional faster-moving components having R_m values of 0.39, 0.46, 0.50, and 0.58. The tail of the HM peak emerging from the column at 120-130 ml gave a simpler disc gel pattern composed almost entirely of slower moving components (R_m = 0.00-0.15).

The nature of the components retained on the column during chromatography of HM was investigated. After elution of the HM peak, the column was washed with the eluting buffer containing 1 M KCL (Figure 4), An additional peak then emerged from the column at 370-420 ml. Disc gel electrophoretic analysis (Figure 6) of samples taken from the second peak at 370-390 ml revealed that the principal impurities removed from HM during chromatography possessed R_m values of 0.06, 0.33 and 0.52. Since myosin prepared by other methods was more homogeneous, HM was not utilized in subsequent studies.

In a separate series of experiments, myosin prepared by the method of Perry (1955) was further purified by two methods. The first method was based on the finding of Baril *et al.* (1966) that EDTA may remove certain impurities during the purification of myosin. Thus, PM (Perry's Myosin) was subjected to two additional purification cycles in the presence of 0.05 M EDTA (pH 6.5). This was followed by an additional purification cycle (Perry, 1955) without added EDTA. The product was designated as EM (EDTA-purified myosin).

The EM preparation was compared with the original PM preparation by disc gel electrophoresis, and the results are shown in Figure 7. Densitometric analysis of the disc gel patterns showed that EM contained most of the impurities present in the original preparation (PM). Consequently, EM was not utilized in subsequent characterization studies.

The second method for further purifying PM utilized chromatography on DEAE-Sephadex A-50 as outlined by Richards et al. (1967). Figure 8 shows the elution pattern obtained.

The asymmetry of the myosin peak (Figure 8) suggested heterogeneity. Therefore, the material eluted at 50-54 ml was designated as RM (Richard's Myosin) Fraction 1, while the material emerging at 54-60 ml was designated as RM Fraction 2.

Disc gel electrophoresis showed the RM preparation to be substantially purer than any of the other myosin preparations analyzed in this work. Disc gel electrophoretic patterns (Figure 9) of the original PM preparation and subsequent RM preparations were scanned in the densitometer. Comparison of densitometer values from PM and RM Fraction 1 showed that 70, 100, 50 and 40% of the components having R_m values of 0.39, 0.46, 0.50 and 0.58, respectively, were removed from PM by chromatography on DEAE-Sephadex A-50. A similar comparison between PM and RM Fraction 2 showed that 95, 100, 80 and 95% of the same components, respectively, had been removed during chromatography.

From the preceding studies on myosin, the single peak, which sedimented at 1.0-1.1 cm during density gradient centrifugation (Figure 5), appears to be myosin. Results of disc urea-gel electrophoresis are more difficult to interpret, because the action of urea on the myosin molecule must be considered. High concentrations of urea are known to break the myosin molecule down into two sub-units having molecular weights of 16,000 and 165,000-180,000 (Perry, 1967a). The formation of the smaller species of sub-unit is a very slow process, at least 2 weeks being required to detect the first traces (Wetlaufer and Edsall, 1960) and several months to achieve equilibrium (Tsao, 1953).

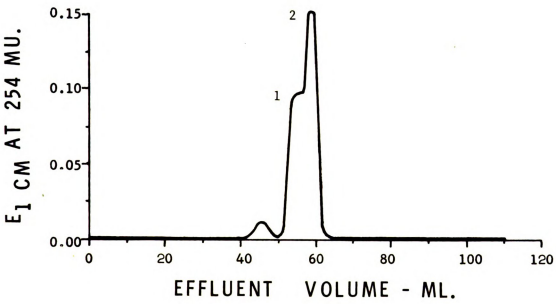


Figure 8. Purification of myosin on DEAE-Sephadex A-50 (Richards et al., 1967). Column dimensions were 1.5 x 7 cm. Sample = 2 ml of crude myosin containing 1.8 mg protein/ml. Sample was applied at 0 ml effluent volume. Myosin was eluted with a linear gradient made from 30 ml of 0.15 M potassium phosphate (pH 7.5) and 30 ml of 0.5 M KCl containing 0.15 M potassium phosphate (pH 7.5).

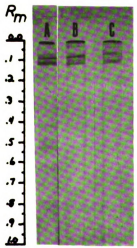


Figure 9. Disc gel electrophoresis of PM, RM fraction 1 and RM fraction 2. A, sample = 0.01 ml of PM preparation containing 2.1 mg protein/ml. B, sample = 0.03 ml of RM fraction 1 containing 0.3 mg protein/ml. C, sample = 0.03 ml of RM fraction 2 containing 0.3 mg protein/ml.

The immediate effect of urea on myosin appears to be a shift in chemical equilibrium toward dissociation into individual myosin polypeptide sub-units of about 180,000 molecular weight (Small et al. 1961). Below 4M urea, myosin tends to form high molecular weight aggregates and does not noticeably dissociate into polypeptide sub-units. Beginning at 4M urea, however, dissociation into individual sub-units begins. Dissociation then increases with increasing urea concentration until complete dissociation is obtained at 12M urea (Small et al. 1961). Thus, between 4 and 12M urea an association--dissociation system exists with the relative amounts of myosin aggregates, myosin monomers and myosin polypeptide sub-unit chains being dependent upon the concentration of urea.

Small et al. (1961) showed that, if myosin is electrophoresed in a weak acrylamide gel, 8M urea and a continuous Tris-glycine buffer system at pH 9.5, the myosin polypeptide sub-units migrate very slowly as a diffuse, apparently heterogeneous band. Undissociated myosin molecules and myosin aggregates remain at the origin. On this basis, the material remaining at the origin in Figures 6, 7 and 9 probably consists of myosin monomers and myosin aggregates. The bands with R_m = 0.05-0.10 (Figures 6, 7 and 9) likely represent the myosin polypeptide sub-unit chains, which Small et al. (1961) has also observed at this position.

The apparent heterogeneity of the myosin bands shown in Figures 6, 7 and 9 was also observed by Small et al.(1961). These same authors also observed that the multiple species were likely the result of the association-dissociation system, which exists in the presence of urea. They concluded that the multiple myosin bands all represent the same myosin sub-unit. No further experiments were undertaken to determine the reason for more than one band in some of the disc gels. The components obtained from HM and PM preparations at R_m = 0.39, 0.46, 0.50 and 0.59 (Figures 6

and 7) appear to have been impurities, since they were largely removed by purification using the method of Richards et al. (1967).

A final observation on the behavior of myosin deserves comment. Some preparations of myosin, actomyosin and Weber-Edsall extract failed to show the dark bands at R_m = 0.05-0.10, which are characteristic of myosin. This behavior seemed to depend more on the individual variation between muscle samples than on the methods used in preparing the protein. Nevertheless, preparations showing weak myosin bands consistently produced dark myosin bands after ion-exchange chromatography, gel filtration or ultracentrifugation. An example of this behavior is seen in Figure 6. The HM preparation showed very weak myosin bands before chromatography. However, after chromatography the eluted myosin produced much darker bands at R_m = 0.05-0.10, as well as some previously unobservable material at R_m = 0.10-0.15. The reason for this behavior is not clear, but presumably stems from changes in the aggregational state of myosin (Perry, 1967a; Dreizen, 1967).

Actin

Actin was the most difficult protein to analyze, since G-actin polymerizes to form F-actin when exposed to ionic strengths of approximately 0.1 or more (Seifter and Gallop, 1966). Thus, interpretation of density gradient experiments was more difficult. Further, the Amido Black stain used to visualize the disc gel electrophoretic separations seemed to have a low affinity for actin. Consequently, actin bands lack intensity and clarity.

Preparation of G-actin by the method of Adelstein et al. (1963) utilized chromatography on Sephadex G-200 at low ionic strength. Actin purified in this manner was designated as AA (Adelstein's Actin). The

elution profile obtained during gel filtration on Sephadex G-200 is depicted in Figure 10, and shows at least three different fractions. Serial disc gel electrophoretic analyses of the different fractions (Figure 10) are shown in Figure 11. The disc gel patterns obtained (Figure 11) indicate that the protein contained in peak 1 (160-220 ml) consists mainly of one component with an R_m value of 0.50. Beginning with an effluent volume of 280 mls, the electrophoretic component at and R_m = 0.50 decreases in amount and two new components appear. The newly appearing components are somewhat diffuse and possess relative mobilities of about 0.39 and 0.59.

Peak 3 (Figure 11) contained no material detectable by disc gel electrophoresis. This agrees with the findings of Adelstein et al (1963), who reported that the third peak is not proteinaceous as shown by a negative buiret test.

G-actin was also prepared as described by Mommaerts (1952). The product was designated as MA (Mommaerts' Actin). Density gradient centrifugation performed on the MA preparation revealed the presence of two components (Figure 12A). Peak 1 remained at the top of the gradient and peak 2 sedimented at 4.3 cm.

In order to further investigate the nature of peaks 1 and 2, a sample of MA was dialyzed 24 hours against 1 M KCl to convert G-actin to F-actin and to remove any excess unbound nucleotides. The density gradient centrifugation pattern obtained from the dialyzed F-actin sample is illustrated in Figure 12B. Peak 1 remained at the top of the gradient, although it was greatly reduced. Peak 2 from G-actin (Figure 12A) was no longer present (Figure 12B), indicating that it had been converted to F-actin. Disc gel electrophoretic analysis of the MA preparation (Figure 13)

AT 254 MU

Fi
19
e
e
c

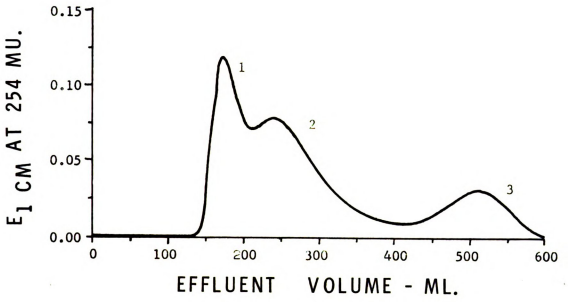


Figure 10. Purification of actin on Sephadex G-200 (Adelstein et al., 1963). Column dimensions were 4.5 x 38.0 cm. Sample = 28 ml of water extract of acetone powder of muscle. Sample was applied at 0 ml effluent volume, and was eluted with 5 x 10^{-4} M ATP (pH 8.1). V_o of column was 160 ml.

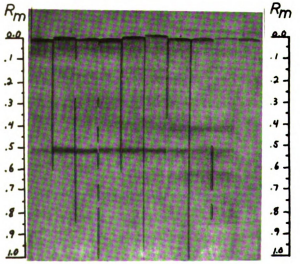
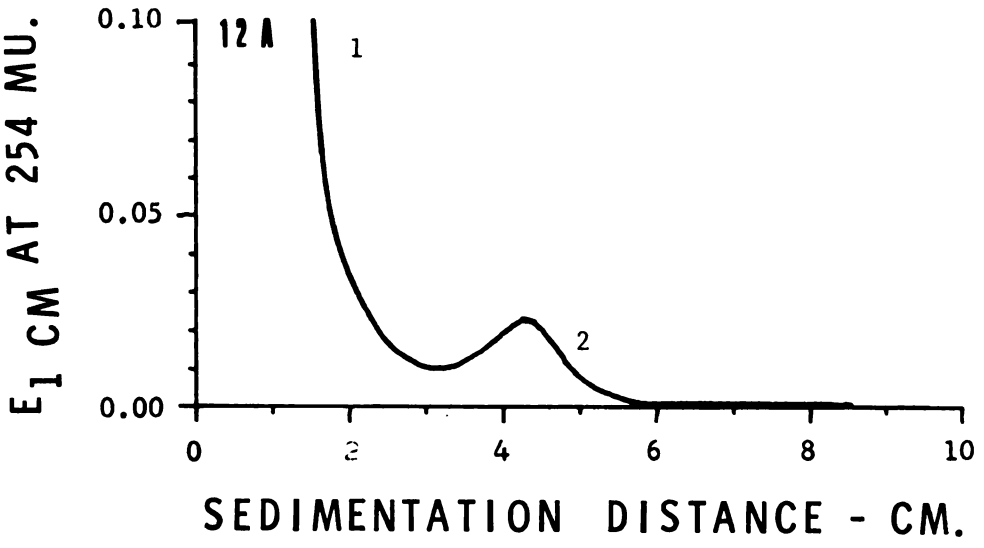


Figure 11. Serial disc gel electrophoretic analysis of the actin purification shown in Figure 10. From left to right, 0.05 ml samples taken at effluent volumes of 160, 180, 200, 220, 240, 260, 280, 300, 320 and 510 ml were analyzed.



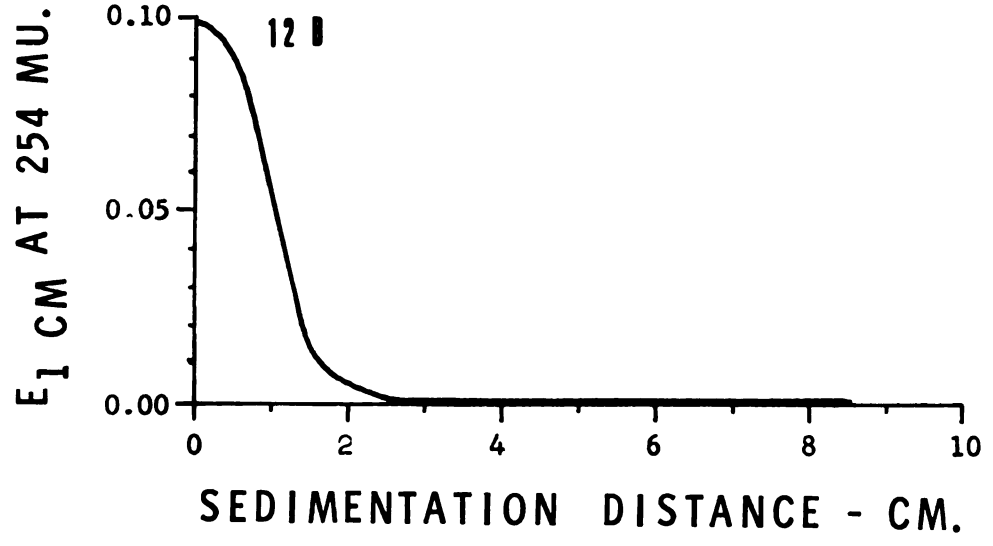


Figure 12. Density gradient centrifugation of actin. A, sample = 0.6 ml of MA preparation containing 0.3 mg protein/ml. B, sample = 0.6 ml of MA preparation which had been dialyzed against 1.0 M KCl. Sedimentation distances of peaks 1 and 2 = 0.0 and 4.2 cm, respectively.

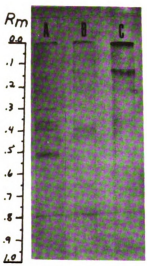


Figure 13. Disc gel electrophoresis of actin. A, sample = 0.06 ml of MA containing 0.3 mg protein per ml. B, sample = 0.06 ml of NFA containing 0.3 mg protein/ml. C, sample = 0.06 ml of KIA containing 1.0 mg protein/ml.

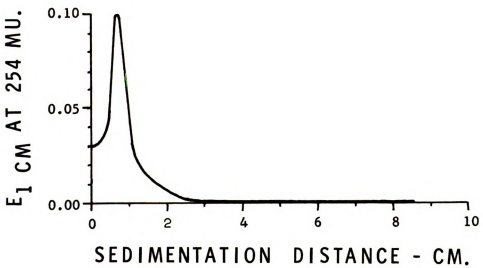


Figure 14. Density gradient centrifugation of tropomyosin. Sample = 0.6 ml of BAT preparation containing 3.3 mg protein/ml. Sedimentation distance of tropomyosin peak = 0.7 cm.

resulted in three principal bands at R_m values of 0.31, 0.37 and 0.51.

"Natural F-actin" (NFA) was prepared according to the method of Hama et al. (1965). Disc gel electrophoresis of the NFA preparation (Figure 13) resulted in a diffuse band with an R_m value of 0.38.

The method of Maruyama et al. (1965) was employed to prepare KI-extracted F-actin (KIA). Disc gel electrophoretic analysis of KIA is shown in Figure 13. Two main bands are apparent, one of which is clearly localized and possesses an R_m value of 0.14. The fastest moving material in the KIA electrophoretic pattern is quite diffuse and appears to possess an R_m value of 0.37.

Based on the foregoing experiments with actin, peak 1 from density gradient centrifugation of MA (Figure 12) is probably due to nudleotides, which are known to be present in conventional actin preparations (Poglazov, 1966). This fraction may also contain tropomyosin B, which is a common contaminant of MA preparations (Hayashi, 1967), and is shown later herein to sediment at a distance of less than 1 cm. The fact that peak 2 (Figure 12) was completely removed during conversion of G-actin to F-actin suggests that this peak may be G-actin. The 0.6M KCl present in the density gradient centrifugation system would be expected to convert G-actin to F-actin during centrifugation. If, however, the nucleotides remained at the top of the gradient while the G-actin sedimented, the G-F transformation of actin would be inhibited (Poglazov, 1966). Thus, G-actin is possibly the fraction sedimenting at 4.3 cm.

Adelstein et al. (1963) stated that the first gel filtration peak in the purification of actin (Figure 10, peak 1) probably contains tropomyosin, F-actin and inactive aggregates of denatured actin. He further concluded that in common with the present investigation, the second peak (Figure 10, peak 2) contains G-actin with some F-actin present in the

leading portion. This information suggests that the trailing portion of the second peak (Figure 10) beginning at an effluent volume of approximately 270 ml, probably contains the purest G-actin. On this basis, the sample taken at an elution volume of 300 ml was selected for use in identification of actin. Disc gel electrophoresis of the 300 ml eluate can be seen in Figure 11.

Since it is known that 30% (5M) urea completely depolymerizes F-actin (Poglazov, 1966), disc gel electrophoresis of G- or F-actin preparations should show a G-actin band and no F-actin band. Comparison of disc patterns obtained from the protein eluted at 300 ml in the purification of AA (Figure 11), and from the MA, NFA and KIA preparations (Figure 13) shows that all actin preparations produce a diffuse, lightly colored band with an R_m value of 0.38 ± 0.01. Furthermore, the NFA preparation was the most homogeneous and produced only one diffuse band with $R_m = 0.38$. Since all actin preparations exhibited common bands at R_m values of 0.38 ± 0.01, this is believed to be actin.

Tropomyosin B

Tropomyosin B was prepared according to the method of Bailey (1948). The product was designated as BAT (Bailey's Tropomyosin). Analysis of the BAT preparation by density gradient centrifugation showed a single, symmetrical peak 0.7 cm from the top of the gradient (Figure 14). The results of disc gel electrophoresis of BAT are shown in Figure 15. The two principal bands are quite broad and possess R_m values of 0.34 and 0.51. Several other minor bands are apparent, the two larger ones having R_m values of 0.26 and 0.91.

The method of Mueller (1966) was also utilized for preparing tropomyosin. The product was designated as MT and produced a single peak during

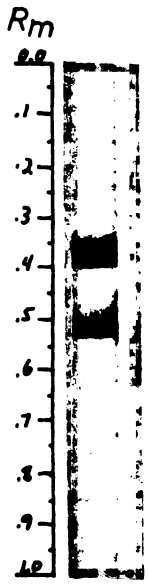


Figure 15. Disc gel electrophoresis of tropomyosin. Sample = 0.01 ml of BAT preparation containing 3.3 mg protein/ml.

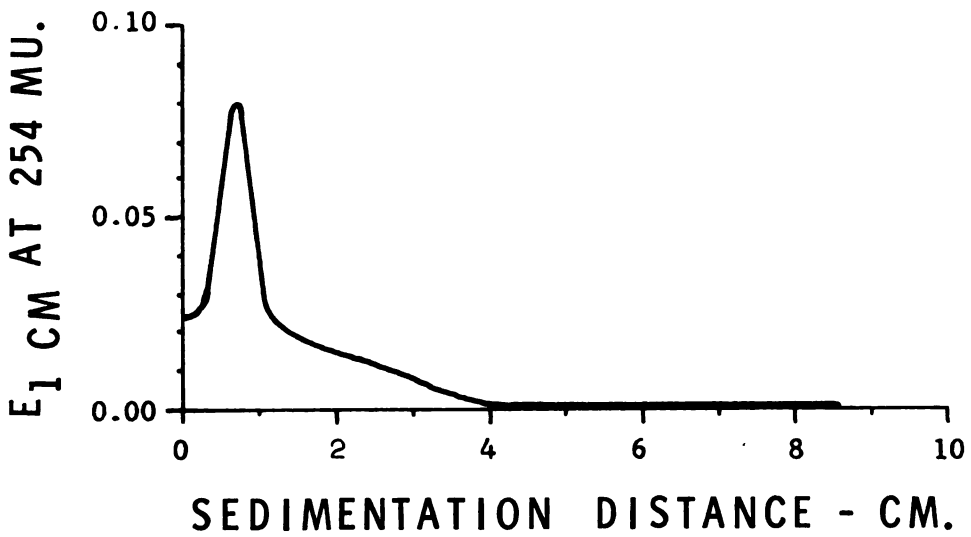


Figure 16. Density gradient centrifugation of tropomyosin. Sample = 0.6 ml of MT preparation containing 2.2 mg protein/ml. Sedimentation distance of tropomyosin peak = 0.7 cm.

density gradient centrifugation (Figure 16). The MT peak has a sedimentation distance of 0.7 cm, which agrees with that of BAT (Figure 14). Figure 17 shows the disc gel electrophoretic separation of MT. The principal band has an R_m value of 0.34 and appears to be identical with that of BAT. Minor bands also occurred at R_m values of 0.26, 0.51 and 0.91, likewise corresponding with those obtained from BAT. Smaller amounts of other minor bands are also evident.

Tropomyosin prepared by the method of Bodwell (unpublished method) was designated as BOT (Bodwell's Tropomyosin). Analysis of BOT by means of density gradient centrifugation yielded the pattern shown in Figure 18. A single peak was produced, which sedimented at 0.6 cm. This value is in fairly good agreement with the corresponding values for BAT and MT preparations.

Disc gel electrophoretic analysis of the BOT preparation resulted in the pattern shown in Figure 19. Similar to patterns for BAT and MT (Figures 15 and 17, respectively), two main bands are present having R_m values of 0.34 and 0.50. Contrary to BAT and MT preparations, however, the band at an R_m value of 0.51 was the most intense.

The BOT preparation was subjected to further purification by DEAE-cellulose chromatography according to the method of Davey and Gilbert (1968) except for the addition of 0.01 M EDTA. The product was designated as DT (Davey's Tropomyosin). A typical chromatogram is shown in Figure 20. An artificial peak (also shown in Figure 20) was eluted ahead of the tropomyosin peak. It was found to be caused by the EDTA in the buffer system. The true chromatogram (Figure 20) was ascertained by the difference between values obtained with and without a sample on the column.

Density gradient centrifugation of DT showed a single component sedimenting at 0.6 cm as previously shown in Figure 18. Disc gel

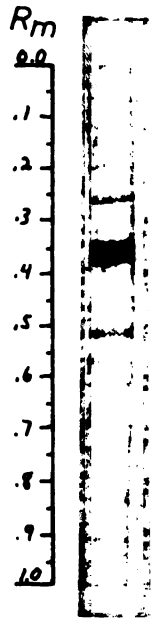


Figure 17. Disc gel electrophoresis of tropomyosin. Sample = 0.01 ml of MT preparation containing 2.2 mg protein/ml.

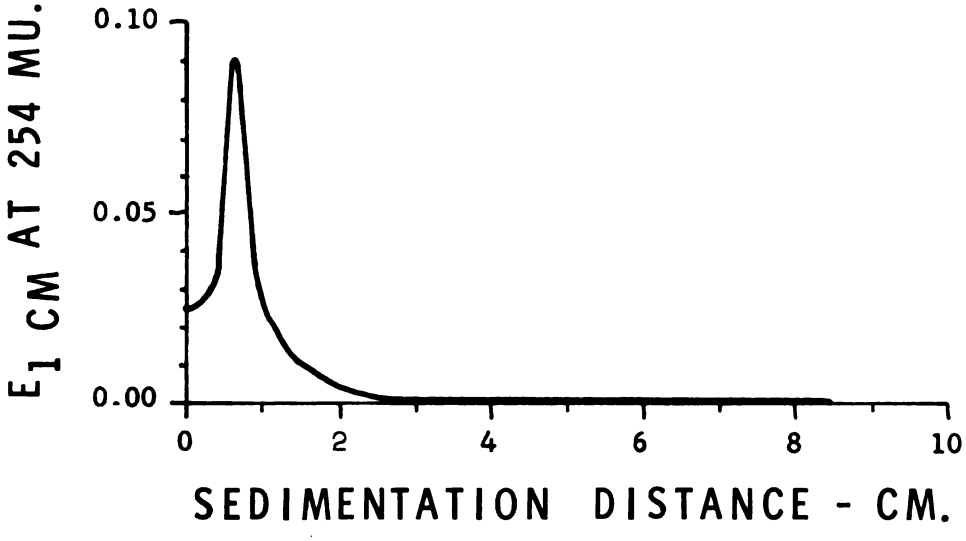


Figure 18. Density gradient centrifugation of tropomyosin. Sample = 0.6 ml of BOT preparation containing 3.3 mg protein/ml. Sedimentation distance of tropomyosin peak = 0.6 cm.

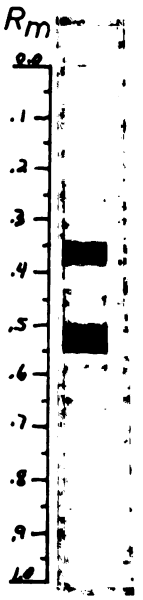


Figure 19. Disc gel electrophoresis of tropomyosin. Sample = 0.01 ml of BOT preparation containing 3.3 mg protein/ml.

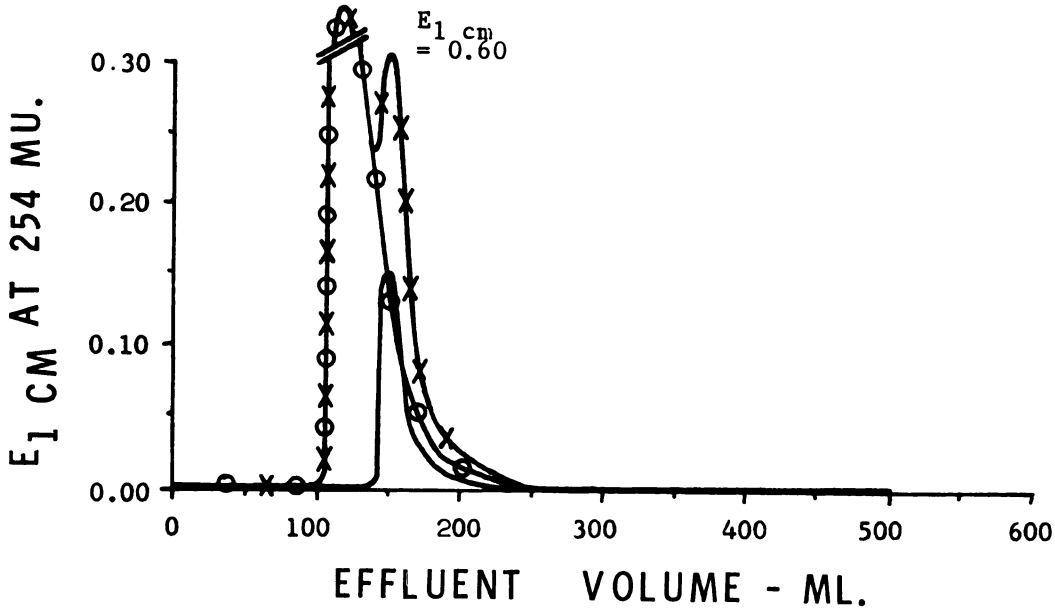


Figure 20. Purification of tropomyosin on DEAE-cellulose as adapted from the method of Davey and Gilbert (1968). Column dimensions were 2.5 x 12 cm. Sample = 21 ml of BOT preparation containing 3.3 mg protein/ml. Sample was applied at 0 ml effluent volume. Tropomyosin was eluted with a linear gradient composed of 150 ml of 0.01 M EDTA adjusted to pH 8.2 with solid Tris buffer and 150 ml of 0.5 M KCl containing 0.01 M EDTA adjusted to pH 8.2 with solid Tris buffer.
✕—✕ denotes pattern obtained during chromatography of tropomyosin.
o—o denotes pattern obtained without protein sample on column.
——— denotes difference between patterns obtained with and without tropomyosin sample applied to column.

electrophoretic analysis of the DT preparation is presented in Figure 21. Examination of the gel pattern showed that the band with an R_m value of 0.51 contained most of the protein, just as it did before chromatography. Densitometric analysis conducted on the gels in Figures 19 and 21 indicated that chromatography on DEAE-cellulose removed 70, 0 and 70% of the minor components having R_m values of 0.25, 0.57 and 0.91, respectively.

The preceding experiments with tropomyosin have shown that BAT, MT and BOT preparations all produced a single peak sedimenting at 0.6-0.7 cm during density gradient centrifugation (Figures 14, 16 and 18). Thus, it is probable that tropomyosin sediments at 0.6-0.7 cm in the density gradient system employed.

Disc gel electrophoresis of BAT, MT, BOT and DT preparations (Figures 15, 17, 19 and 21) indicate that tropomyosin preparations contain two components, which are present in varying amounts, and which possess two species of tropomyosin observed by Woods (1967) during disc gel electrophoresis in the presence of 8 M urea. He presented evidence that the formation of the slower-moving species is caused by oxidation of -SH groups. Woods (1967) also showed that disc gel electrophoretic analysis of several tropomyosin preparations produced varying relative amounts of the fast- and slow-moving species. However, complete conversion of tropomyosin to the slow-moving species could be effected by electrophoresing in the presence of a reducing agent, such as 0.001 M thioglycolate.

Woods (1967) concluded that classical tropomyosin B consists mainly of the oxidized species with a molecular weight of 68,000, which he called monomeric tropomyosin. On the other hand, Woods (1967) showed that if precautions are taken to prevent oxidation of tropomyosin, 8 M urea can cause almost complete dissociation into the reduced species, which he

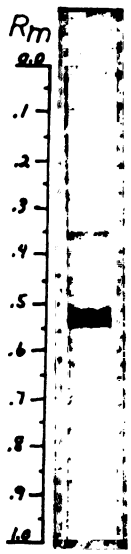


Figure 21. Disc gel electrophoresis of tropomyosin. Sample = 0.01 ml of DT preparation containing 1.8 mg protein/ml.

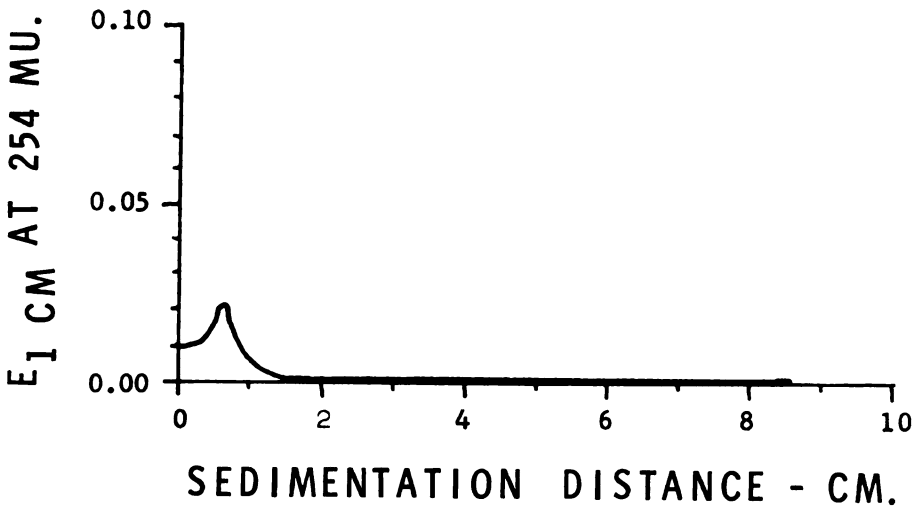


Figure 22. Density gradient centrifugation of ESF. Sample = 0.4 ml of KESF preparation containing 0.2 mg protein/ml. Sedimentation distance of KESF peak = 0.6 cm.

reported to have a molecular weight of 34,000. He described this species as being composed of two identical polypeptide sub-units, which made up the tropomyosin. In the present investigation it appears that the band with an R_m value of 0.34 (Figures 15, 17, 19 and 21) corresponds to the tropomyosin monomer, while that with an R_m of 0.51 represents the two identical polypeptide sub-units of tropomyosin.

Troponin (ESF), ∝-Actinin, β-actinin and Inhibitory Factor (IF)

Interpretation of studies on ESF, ∝-actinin, β-actinin and IF was difficult, owing to the lack of information about these proteins. Due to the tendency of these proteins toward spontaneous aggregation, (Ebashi and Ebashi, 1964; Maruyama, 1965b; Ebashi, 1966), their true molecular weights are not known with certainty (Ebashi, 1966; Briskey et al., 1967b). Very little is known about the action of urea on ESF, ∝-actinin, β-actinin and IF. In addition, the tendency of ∝-actinin and β-actinin to assume various aggregates (Ebashi, 1966; Maruyama, 1965b) suggests that these proteins might appear as more than one electrophoretic species. The various bands obtained during electrophoresis are, therefore, referred to as the "ESF group", the "∝-actinin group", the "β-actinin group" and the "IF group".

ESF was prepared according to the method of Katz (1966). The product was designated as KESF (Katz' ESF). Density gradient centrifugation of KESF produced a single peak sedimenting at 0.6 cm (Figure 22). Analysis by disc gel electrophoresis produced the pattern shown in Figure 23. Two bands are visible with R_m values of 0.32 and 0.51.

A second sample of ESF was obtained as a by-product during preparation of ∝-actinin according to the method of Seraydarian et al. (1967). Impurities were removed by ammonium sulfate precipitation and gel

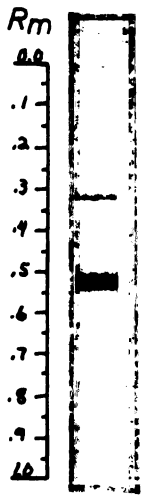


Figure 23. Disc gel electrophoresis of ESF. Sample = 0.05 ml of KESF preparation containing 0.2 mg protein per ml.

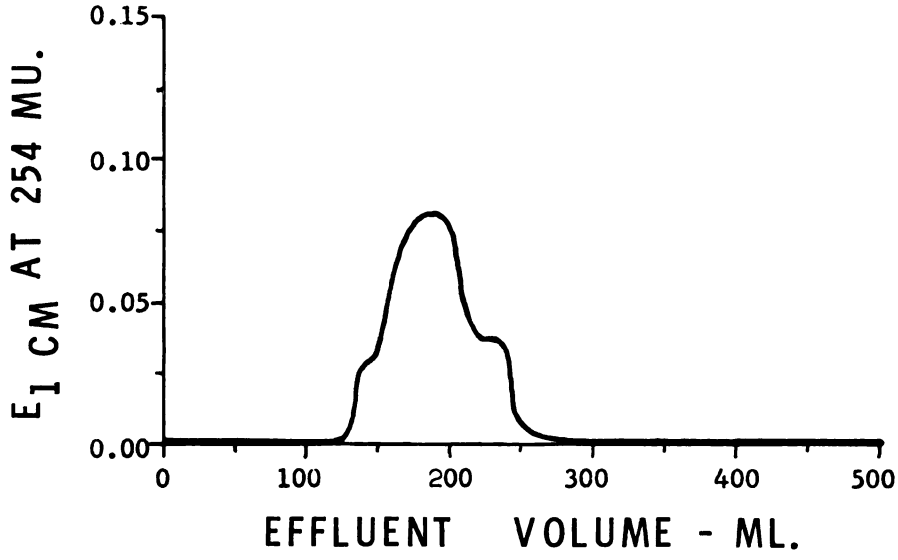


Figure 24. Purification of ESF on Sephadex G-200 (Azuma and Watanabe, 1965 b). Column dimensions were 4.5 x 36 cm. Sample = 11 ml of supernatant from the first ammonium sulfate fractionation utilized in the preparation of α-actinin according to Seraydarian *et al.* (1967). Sample was applied at 0 ml elution volume, and was eluted with 0.1 M KCl buffered at pH 7.0 with 0.02 M potassium phosphate. V_o of column was 140 ml.

filtration as described by Azuma and Watanabe (1965b). The product was designated as AESF (Azuma's ESF). Purification of AESF by gel filtration (Azuma and Watanabe, 1965b) yielded the chromatogram shown in Figure 24. Serial disc gel electrophoretic analyses of the fractions collected during gel filtration of AESF (Figure 24) are presented in Figure 25. Four main bands are evident, having R_m values of 0.20, 0.34, 0.51 and 0.92. In addition, two minor bands are present with R_m values of 0.25 and 0.54.

At this point, the fact that KESF produced only tropomyosin bands (R_m = 0.34 and 0.51) suggests that ESF is not separated from tropomyosin in the disc gel system, or else it is non-protein in nature and is not stained by Amido Black. Further discussion of this point is presented later herein.

α-Actinin was prepared as described by Seraydarian et al. (1967). Density gradient centrifugation of α-actinin produced a single symmetrical peak sedimenting at 1.2 cm (Figure 26). The results of disc gel electrophoresis appear in Figure 27. Two main bands are evident in the α-actinin group with R_m values of 0.14 and 0.18.

The method of Maruyama (1965b) was used for the preparation of β-actinin. The results of density gradient centrifugation are shown in Figure 28. A single symmetrical peak was produced, which sedimented at 0.9 cm. Disc gel electrophoresis, however, showed that the β-actinin preparation consisted of at least four main bands (Figure 29) with R_m values of 0.29, 0.32, 0.39 and 0.43.

IF was prepared by the method of Hartshorne et al. (1967). Density gradient centrifugation of IF could not be carried out due to equipment failure. Disc gel electrophoresis (Figure 30) revealed that the IF group consists of three main bands with R_m values of 0.14, 0.20 and 0.32.

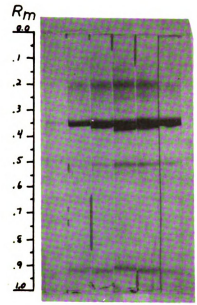


Figure 25. Serial disc gel electrophoretic analysis of the purification shown in Figure 24. From left to right, 0.5 ml samples taken at effluent volumes of 130, 150, 170, 190, 210 and 230 ml were analyzed.

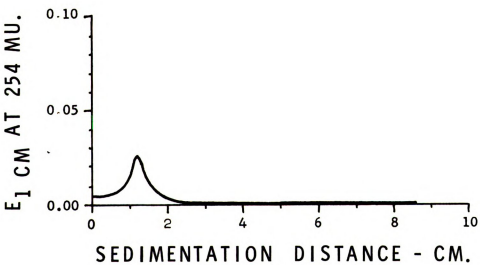


Figure 26. Density gradient centrifugation of α-actinin. Sample = 0.3 ml of α-actinin preparation containing 0.3 mg protein/ml. Sedimentation distance of α-actinin peak = 1.2 cm.

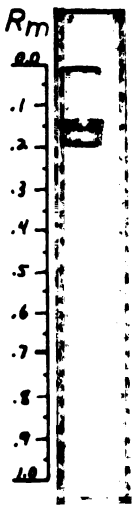


Figure 27. Disc gel electrophoresis of α-actinin. Sample = 0.06 ml of α-actinin preparation containing 0.3 mg protein/ml.

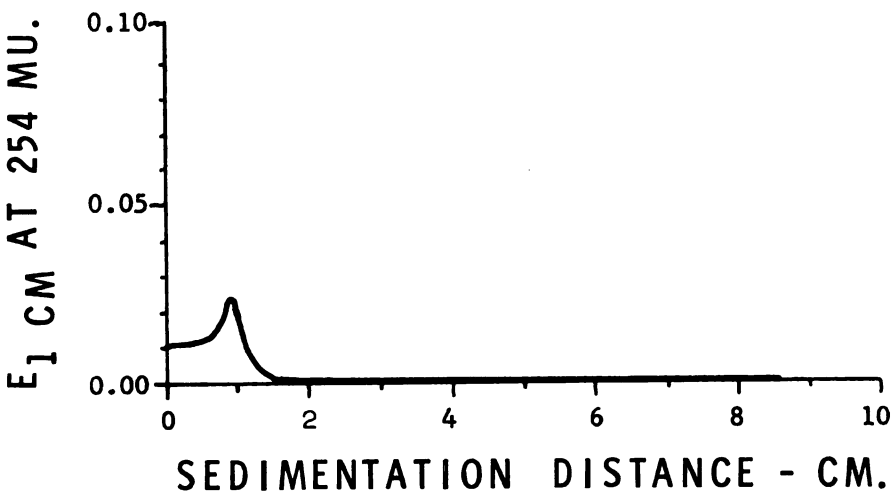


Figure 28. Density gradient centrifugation of β-actinin. Sample = 0.3 ml of β-actinin preparation containing 0.3 mg protein/ml. Sedimentation distance of β-actinin peak = 0.9 cm.

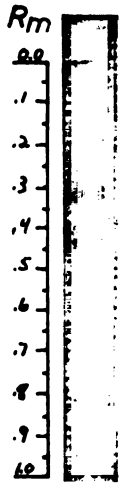


Figure 29. Disc gel electrophoresis of β-actinin. Sample = 0.1 ml of β-actinin preparation containing 0.3 mg protein/ml.

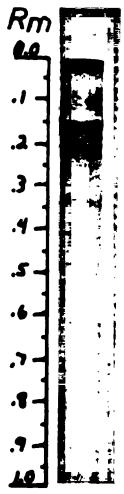


Figure 30. Disc gel electrophoresis of IF preparation. Sample = 0.05 ml of IF preparation containing 0.8 mg protein/ml.

Since preparations of ESF, α-actinin, β-actinin and IF each contained several major electrophoretic components, it is probable that present purification procedures are inadequate. Determination of their true molecular weight and elucidation of the action of urea on these proteins is necessary to properly interpret the results at hand. Thus, more work is necessary to determine whether the pure proteins exist as single bands, or if they actually break down into several species producing the patterns shown in Figures 27, 29 and 30.

Extra Protein

Extra protein was prepared and chromatographed on DEAE-cellulose as described by Perry and Zydowo (1959a). A typical pattern obtained from the chromotography of extra protein is shown in Figure 31. The peaks were identified as Fractions I, II, III and IV (Perry and Zydowo, 1959a) on the basis of the salt concentration required for their elution from the column. Perry and Zydowo (1959a) observed a small peak following Fraction I, but gave it no designation. It has been labeled as Fraction IA in Figure 31.

Serial disc gel electrophoretic analyses were performed on the eluted fractions, and are shown in Figure 32. Disc gel electrophoresis of Fraction I produced a very broad band with an R_m value of 0.14-0.21 and a more sharply defined band having an R_m value of 0.26. Fraction IA appears to contain only one electrophoretic component with an R_m value of 0.90. Fractions II and III were present only in small amounts in this preparation. Their bands, although faint, are discernible and possess R_m values of 0.03, 0.21 and 0.25. Fraction IV contained two bands, with R_m values of 0.32 and 0.51.

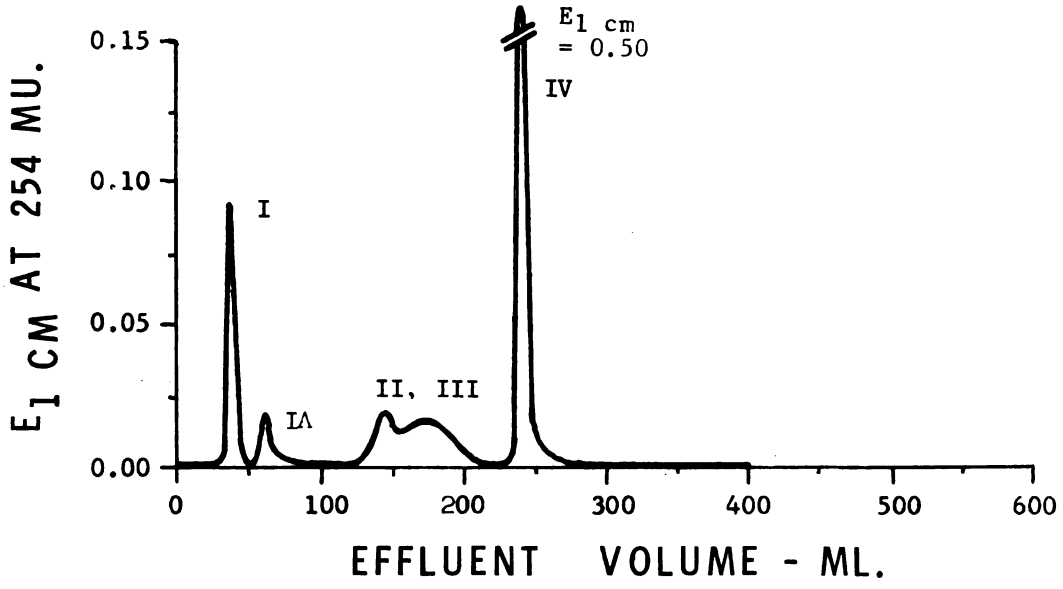


Figure 31. Chromatography of extra protein on DEAE-cellulose (Perry and Zydowo, 1959 a). Column dimensions were 2.5 x 12.0 cm. A 10 ml sample of extra protein preparation containing 1.7 mg protein/ml was applied at 0 ml effluent volume. Elution was accomplished using a linear gradient composed of 100 ml starting buffer (0.1 M KCl containing 0.02 M Tris, pH 7.6) and 100 ml of limit buffer (0.35 M KCl containing 0.02 M Tris, pH 7.6). At 200 ml effluent volume, a step was made to 2.0 M KCl containing 0.02 M Tris, pH 7.6.

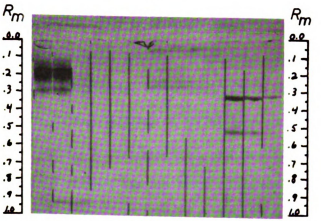


Figure 32. Serial disc gel electrophoretic analysis of the separation shown in Figure 31. From left to right, 0.12 ml samples taken at effluent volumes of 30, 40, 50, 60, 70, 80, 140, 150, 160, 170, 190, 230, 240 and 250 ml were analyzed.

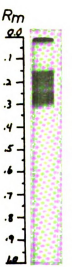


Figure 33. Disc gel electrophoresis of sarcoplasmic fraction. Sample = 0.01 ml of first extract obtained during preparation of washed muscle residue. Sample contained 17.0 mg protein/ml.

Perry and Zydowo (1959a) showed that preparations of sarcoplasmic proteins chromatographed on DEAE-cellulose behaved identically with Fraction I of the extra protein group. They then concluded that Fraction I consisted mainly of sarcoplasmic material, which had not been removed from the fibrils prior to extraction of extra protein.

In the present work, a similar test was performed in which the sarcoplasmic proteins of rabbit muscle were electrophoresed, and the resulting pattern (Figure 33) was compared with that of Fraction I. The pattern obtained from the sarcoplasmic preparation (Figure 33) appears to contain several bands with R_m values ranging from 0.15 to 0.29. These values are very similar to the R_m values of 0.14-0.23 obtained from electrophoresis of Fraction I (Figure 32). This supports the opinion of Perry and Zydowo (1959a) that Fraction I consists mainly of sarcoplasmic material.

According to Perry and Zydowo (1959a) the amounts of Fractions II and III (Figure 32) present in extra protein preparations is reduced by thoroughly washing the myofibrils prior to their extraction with KCl-phosphate solution. Therefore, the lengthy washing procedure employed during preparation of the myofibrils in the present study may well account for the relatively small amounts of Fractions II and III in the extra protein preparation.

The foregoing experiments indicate that fraction I of the extra protein group consists of sarcoplasmic components with R_m values of 0.14-0.21 and 0.26. Fraction IA has an R_m value of 0.90. Fractions II and III appear to produce the same or similar electrophoretic patterns, with bands having R_m values of 0.03, 0.21 and 0.25. Fraction IV contained two electrophoretic components with R_m values of 0.32 and 0.51.

Interrelationships of Various Proteins

Results of all experiments on individual preparations of myofibrillar proteins are summarized in Table I. The data indicate that myosin has an R_m value of 0.05-0.10, as shown by the disc gel experiments with standard proteins. Disc gel analyses also show that most myosin preparations contained reduced tropomyosin (R_m = 0.50), actin (R_m - 0.39), and two unidentified components (R_m = 0.46 and 0.58). The method of Richards et al. (1967) yielded the purest preparation of myosin with no evident contamination (Table I).

As shown in Table I, the R_m value of actin is 0.38. Actin preparations also contained reduced tropomyosin (R_m = 0.51), and other components at R_m = 0.14, 0.31 and 0.59. The band at 0.14 may correspond to α-actinin or myosin, while the band at 0.31 may be oxidized tropomyosin (Table I). The method of Hama et al. (1965) for direct isolation of F-actin from the myofibril yielded the purest actin preparation. Actin isolated by this procedure showed no other electrophoretic bands.

Tropomyosin was found to possess R_m values of 0.34 and 0.51. Based on the results of disc gel electrophoresis, tropomyosin preparations also contained varying amounts of extra protein fraction IA (R_m = 0.91) and of three unidentified components at R_m values of 0.20, 0.26 and 0.57 (Table I). Results suggest that the purest tropomyosin preparation was obtained by using the method of Bodwell (unpublished method) followed by chromatography on DEAE-cellulose (Davey and Gilbert, 1968) in the presence of 0.01M EDTA.

The lack of information about the action of urea on ESF, α-actinin, β-actinin and IF has prevented assignment of definite R_m values to these proteins. Nevertheless, identification of the major myofibrillar proteins in the disc gel system now permits one to draw some tentative conclusions.

Table 8. Summary of Behavior of Protein Preparations

Preparation	Sedimentation Distance - cm	Relative Mobility	
		Standard Protein	Contaminating Proteins
1. HM (Harris' Myosin)	1.0	0.00-0.15	0.39, 0.46, 0.50, 0.58
2. PM (Perry's Myosin)	1.0	0.05-0.10	0.39, 0.46, 0.50, 0.58
3. RM (Richard's Myosin)	1.0	0.05-0.10	
4. AA (Adelstein's Actin)	---	0.39	0.50, 0.59
5. MA (Mommaert's Actin)	4.3	0.37	0.51, 0.31
6. NFA (Natural F-actin)	---	0.38	
7. KIA (KI-extracted actin)	---	0.37	0.14
8. BAT (Bailey's Tropomyosin)	0.7	0.34, 0.51	0.26, 0.91
9. MT (Mueller's Tropomyosin)	0.7	0.34, 0.51	0.20, 0.26, 0.91
10. BOT (Bodwell's Tropomyosin)	0.6	0.34, 0.51	0.25, 0.91
11. DT (Davey's Tropomyosin)	0.6	0.34, 0.51	0.57
12. KESF (Katz' ESF)	0.6	*	0.32, 0.51
13. AESF (Azuma's ESF)	---	*	0.20, 0.25, 0.34, 0.51, 0.54, 0.92
14. α-Actinin Group	1.2	*	0.14, 0.18
15. β-Actinin Group	0.9	*	0.25, 0.29, 0.32, 0.39, 0.43, 0.51
16. Inhibitory Factor	---	*	0.14, 0.20, 0.32, 0.39, 0.60
17. Extra Protein, Fraction I	---	*	0.14, 0.21, 0.26
18. Extra Protein, Fraction IA	---	0.90	
19. Extra Protein, Fraction II	---	*	0.03, 0.21, 0.25
20. Extra Protein, Fraction III	---	*	0.03, 0.21, 0.25
21. Extra Protein, Fraction IV	---	*	0.32, 0.51

*Position of standard protein in disc gel pattern was not determined.

ESF activity has been reported in the following protein preparations; MT (Mueller, 1966), KESF (Katz, 1966), AESF (Azuma and Watanabe, 1965a, b), and extra protein fractions I, II, and III (Perry et al., 1966). As can bee seen in Table I, disc gel electrophoresis of the KESF preparation indicated that it contained only tropomyosin bands (R_m = 0.32 and 0.51). On this basis, ESF appears to be non-protein in nature, or else is simply not separated from tropomyosin by the disc gel system.

Another interpretation emerges from a comparison of the disc gel patterns of MT, AESF, and extra protein fractions I, II and III (Table I). These preparations contained common bands at R_m = 0.20, 0.26 and 0.91, suggesting that ESF might be localized in these bands. Since MT was prepared under special conditions to preserve ESF activity, it is interesting to note that the bands at R_m = 0.20, 0.26 and 0.91 are more intense in MT than in any other tropomyosin preparation. Furthermore, extra protein fractions I, II and III produce only these bands. The elusive nature of ESF has been noticed in several laboratories (Azuma and Watanabe, 1965 a, b; Perry et al., 1966; Perry, 1967a). The present study suggests that ESF is either non-protein in nature or has an R_m value of 0.20, 0.25 or 0.91 in the disc gel system.

The method of Seraydarian et al. (1967) for preparation of α-actinin appears to yield a fairly pure product. (Table I). Disc gel electrophoresis of α-actinin revealed no contamination from actin or tropomyosin. The bands produced by α-actinin (R_m = 0.14 and 0.18) resemble those produced my myosin (R_m = 0.05-0.10), and the ESF group (R_m = 0.20, 0.25 or 0.90). Thus caution must be used in interpreting the significance of bands in this area of the gel separation pattern.

β-actinin prepared as described by Maruyama (1965b) contained tropomyosin (R_m = 0.34 and 0.51), actin (R_m = 0.39), and bands at R_m = 0.25, 0.29 and 0.43 (Table I). The band at R_m = 0.25 has tentatively been identified with the ESF group. Thus, the β-actinin group is tentatively assigned R_m values of 0.29 and 0.43.

The IF preparation (Table I) appeared to contain some myosin (R_m = 0.05 - 0.15) or α-actinin (R_m = 0.14 and 0.18). In addition, the IF preparation contained two bands tentatively identified with the ESF group (R_m = 0.20 and 0.25), reduced tropomyosin (R_m = 0.32), actin (R_m = 0.39) and a component associated with the β-actinin group (R_m = 0.43). Also, a minor unidentified component was observed at R_m = 0.60. Thus IF may have an R_m value of 0.60 or it may exist as a complex of one or more of the other proteins.

As stated previously, fraction I of the extra protein group appears to consist mainly of sarcoplasmic material. Extra protein fraction IA was not of sarcoplasmic origin and was not noticeably contaminated by other myofibrillar proteins as shown by the existence of a single band at R_m = 0.90. This band may correspond to ESF (R_m = 0.20, 0.25 or 0.91). Fractions II and III appeared to consist solely of components (R_m = 0.20 and 0.25) tentatively identified with the ESF group. Electrophoretic bands produced by fraction IV suggest that tropomyosin (R_m = 0.34 and 0.51) is its main protein component.

The electrophoretic similarity between tropomyosin preparations, ESF and fraction IV of the extra protein group prompted further inquiry into the nature of these preparations. Perry and Zydowo (1959b) reported that fraction IV contains large amounts of nucleic acid complexed with a protein moiety. In the present study, absorption spectra of BOT,

KESF and fraction IV (Figure 34) indicated that BOT and KESF contain very little nucleic acid, but that fraction IV has large amounts. By using the data of Warburg and Christian (1941) and the E_{280}/E_{260} ratio of each preparation, a crude estimate of nucleic acid content can be made. The E_{280}/E_{260} ratios for BOT, KESF and fraction IV are 1.6, 1.1 and 0.5, respectively, which indicate that nucleic acids comprise about 0, 3 and over 60% of the respective preparations. The absence of nucleic acid in BOT and its presence in KESF and fraction IV were confirmed by treatment with 6% perchlorate, centrifugation and subsequent measurement of the absorption spectrum of the supernatant.

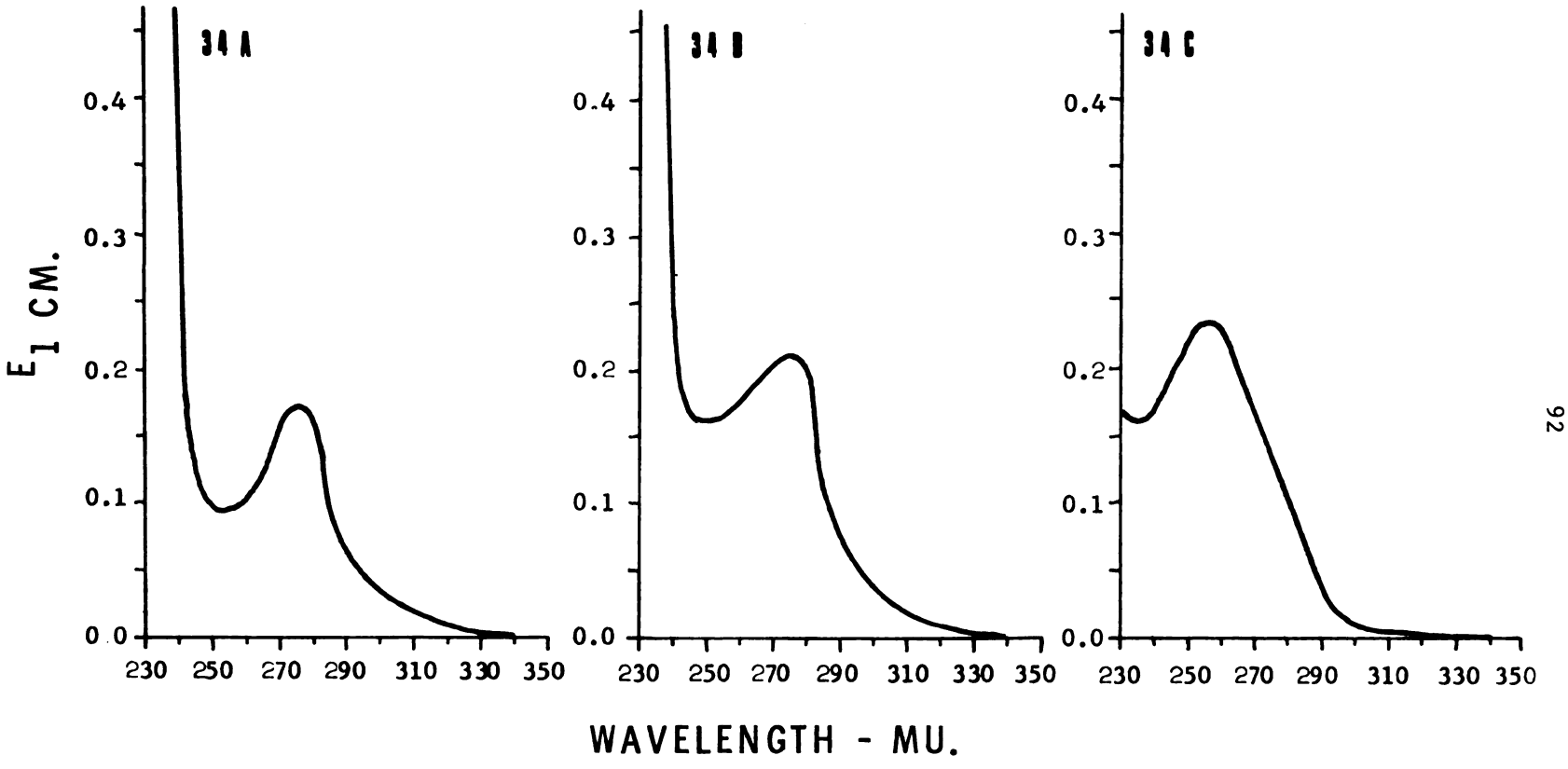


Figure 34. Ultraviolet absorption spectra of protein preparations. A, absorption spectrum of BOT preparation containing 0.2 mg protein/ml. B, absorption spectrum of KESF preparation containing 0.2 mg protein/ml. C, absorption spectrum of extra protein fraction IV containing 0.2 mg protein/ml.

Total Extractable Myofibrillar Proteins

During extraction of myofibrils with the Weber-Edsall solution, 56% of the total myofibrillar nitrogen was solublized. This value is similar to the 58% reported by Hegarty et al. (1963), but is considerably lower than the 78-87% reported by Davey and Gilbert (1968) and the 93% reported by Perry (1953). The differences in these values may well be due to variations in the extraction techniques.

Since the Weber-Edsall extract of the myofibrils is quite viscous, the finer insoluble particles are difficult to remove by centrifugation. In the present work, Weber-Edsall extracts were centrifuged 1 hour at 25,000 x g. On the other hand, Hegarty et al. (1963) utilized centrifugation for 1 hour at 1,400 x g, and Perry (1953) for 10 minutes at 10,000 x g. The more severe centrifugation treatment applied in the present research probably sedimented more colloidal material, so that the supernatant contained a lower percentage of the total protein.

Weber-Edsall extracts were prepared from six samples of washed muscle residue and from three preparations of myofibrils. Each preparation was analyzed separately using gel filtration, density gradient centrifugation and disc gel electrophoresis. Gel filtration of Weber-Edsall extracts consistently produced 4-5 peaks, but the relative heights of the peaks varied unpredictably. This variation was presumably due to the tendency of myofibrillar proteins to aggregate in solution. A typical gel filtration pattern obtained from the Weber-Edsall extract of myofibrils is shown in Figure 35. Gel filtration revealed no consistent

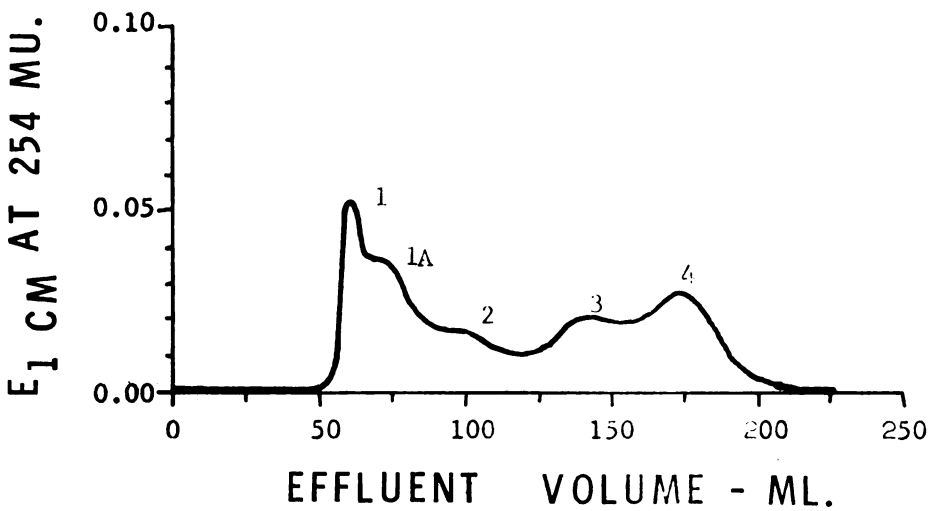


Figure 35. Gel filtration of Weber-Edsall extract. A 2 ml sample of extract containing 1.5 mg protein/ml was applied at 0 ml effluent volume. V_o of the column was 61 ml. Column dimensions were 2.5 x 37 cm. V_e/V_o values were 1.0, 1.6, 2.3 and 2.8 for peaks 1,2,3 and 4, respectively.

difference between Weber-Edsall extracts of washed muscle residue and those of prepared myofibrils.

Peak 1 in Figure 35 has a V_e/V_o value of 1.0. This peak occasionally appeared heterogeneous as illustrated by the shoulder shown in Figure 35. Assuming that separation is occurring solely on the basis of gel filtration, a component with V_e/V_o = 1.0 should have a molecular weight of 50,000,000 or larger (Bio-Rad Laboratories, 1968).

Peak 2 of Figure 35, having a V_e/V_o value of 1.6, is relatively obscure, although it was frequently more pronounced. Molecular weight-elution volume data indicated that this peak had a molecular weight of approximately 5,000,000 (Bio-Rad Laboratories, 1968).

The V_e/V_o value of component 3 (Figure 35) is 2.3, indicating a molecular weight of around 200,000. The V_e/V_o ratio of 2.8 for fraction IV (Figure 35) lies outside the range of those values listed as obtainable by gel filtration (Bio-Rad Laboratories, 1968) Thus, component IV may have been retarded by absorption effects and its molecular weight cannot be estimated by gel filtration.

Typical density gradient separation patterns from Weber-Edsall extracts of washed muscle residue and of prepared myofibrils are shown in Figures 36 A and B, respectively. Unlike gel filtration, density gradient centrifugation revealed large and consistent differences between the extracts of washed muscle residue and those of prepared myofibrils. Components 1 and 2 (Figures 36 A and B), with sedimentation distances of 1.1 cm and 2.3 cm, respectively, were present in preparations of both myofibrils and the washed muscle residue. Components 3, 4 and 5 with sedimentation distances of 4.0, 5.8, and 6.5, respectively, were present in the washed muscle residue, but were largely absent from preparations

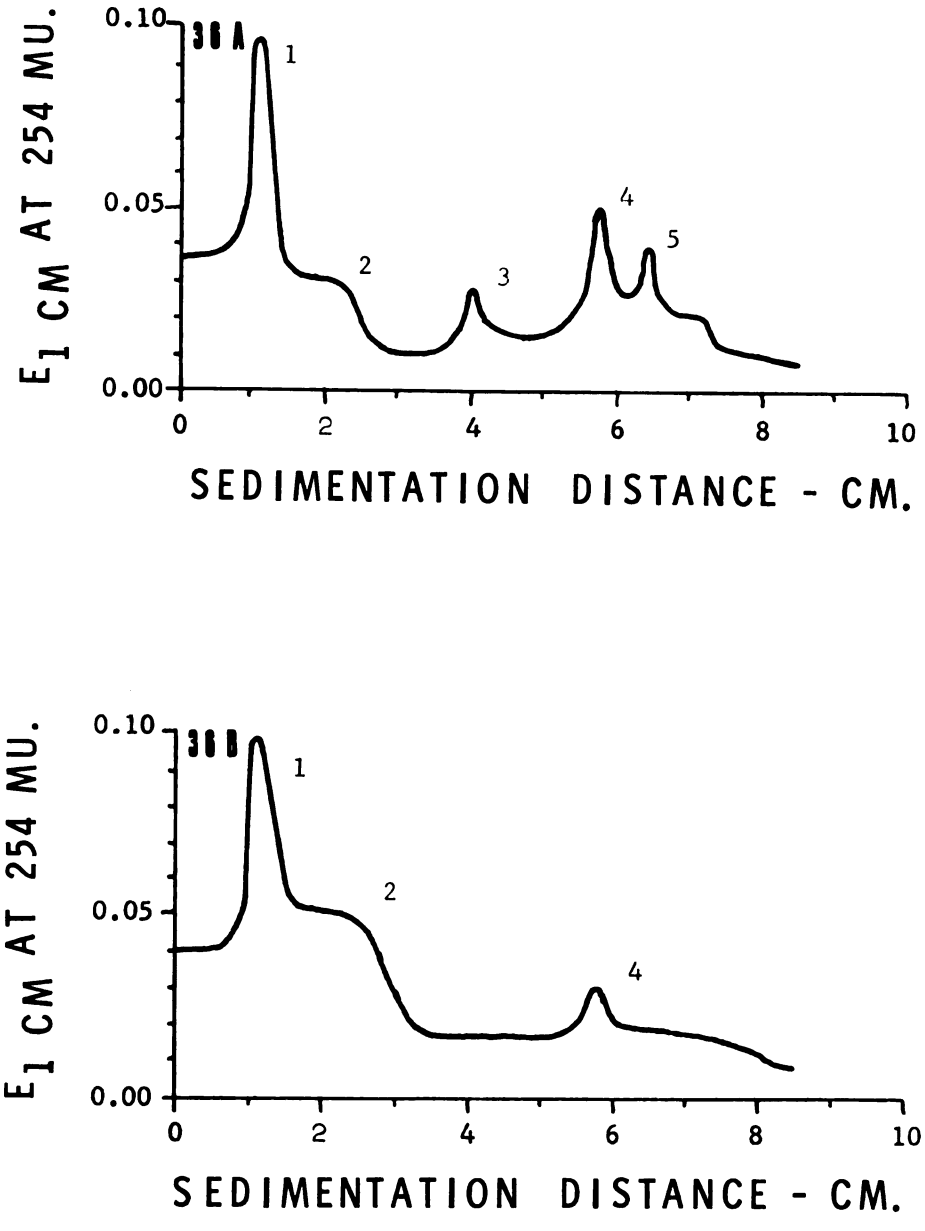


Figure 36. Density gradient centrifugation of Weber-Edsall extract. A, sample = 0.6 ml of Weber-Edsall extract from washed muscle residue. B, sample = 0.6 ml of Weber-Edsall extract from prepared myofibrils. Samples contained 1.5 mg protein/ml. Sedimentation distances in Figure 36A were 1.1, 2.3, 4.0, 5.8, and 6.5 cm for peaks 1, 2, 3, 4 and 5, respectively. Sedimentation distances in Figure 36B were 1.1, 2.3 and 5,8 cm for peaks 1, 2 and 4, respectively.

of myofibrils. Essentially complete removal of components 3, 4 and 5 was accomplished by extended washing procedures during preparation of the myofibrils.

Whether components 3, 4 and 5 originate in the sarcoplasm, or whether they are directly associated with the myofibrils and are leached out during washing procedures is not known. As stated previously, the majority of the protein (96%) was found to reside in fractions I and II. In addition, only fractions I and II demonstrated Mg^{++}- or Ca^{++}-activated ATPase activity. Thus, it appears that the bulk of the myofibrillar protein resides in components I and II.

A typical disc gel electrophoretic separation of the Weber-Edsall extract is shown in Figure 37A. Weber-Edsall extracts produced some bands during disc gel electrophoresis. However, disc gel electrophoresis revealed no difference between Weber-Edsall extracts of washed muscle residue and those of prepared myofibrils.

As previously indicated, the degree of centrifugation utilized in preparing the Weber-Edsall extract can greatly influence the amount of myofibrillar protein remaining in the supernatant. Dilution with Weber-Edsall solution prior to centrifugation reduced the amount of gelatinous material collecting in the lower half of the centrifuge tube. In order to ascertain if the same proteins were present in the supernatant extract and the gelatinous material, the gelatinous material was re-extracted with additional Weber-Edsall solution followed by electrophoretic separation of the extract. Electrophoretic results are presented in Figure 37B. The Weber-Edsall extract of the gel component contained more myosin-like material than the original extract. Therefore, it appears that any change in extracting conditions which influences the amount of undissolved gel component will alter the nature of the Weber-Edsall extract.

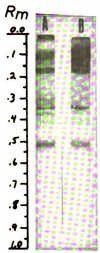


Figure 37. Disc gel electrophoresis of Weber-Edsall extract. A, sample = 0.05 ml of extract of prepared myofibrils. B, sample = 0.05 ml of extract of the gel component. Samples contained 1.5 mg protein/ml.

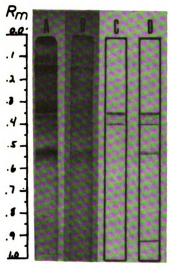


Figure 38. Disc gel electrophoresis of Weber-Edsall extract of myofibrils. Sample in each case was 0.05 ml of extract containing 2.2 mg protein/ml. A, stained with Amido Black. B, stained with Coomassie Blue. C, drawing of disc gel, which had been stained with Acridine Orange. D, drawing of disc gel, which had been stained with Methylene Blue.

Further insight into the nature of the Weber-Edsall extract was gained by additional staining of the disc gels. Figure 38 illustrates several identical disc gel separations of the Weber-Edsall extract. The gel in Figure 38A was stained with Amido Black, and in 38B with Coomassie Blue, a general protein stain. Coomassie Blue developed visible bands in the same positions as did Amido Black, although the Coomassie Blue bands were much less intense.

According to Perry and Zydowo (1959b), several of the myofibrillar proteins, especially myosin and tropomyosin, are often isolated in complex with nucleic acids. Therefore, gels were stained with Acridine Orange (Figure 38C) and Methylene Blue (Figure 38D), both of which are specific for nucleic acids (Richards et al., 1965; Peacock and Dingman, 1967). The appearance of bands at R_m values of 0.34-0.40, 0.51 and 0.92 indicates that bound nucleic acids were present in oxidized tropomyosin (R_m = 0.34), actin (R_m - 0.39), reduced tropomyosin (R_m = 0.51) and extra protein fraction IA (R_m = 0.91).

Tropomyosin has frequently been isolated in combination with nucleic acids (Poglazov, 1966). In the present work it has already been shown that tropomyosin in combination with nucleic acids may actually make up extra protein fraction IV. The presence of nucleic acids in fraction IA of the extra protein is confirmed by the absorption spectrum of fraction IA shown in Figure 39. The E_{280}/E_{260} ratio of fraction IA is 1.0. indicating that it contains about 3-4% nucleic acid (Warburg and Christian, 1941). A similar confirmation of nucleic acid in actin preparations could not be done, since the bound nucleotide necessary for stability of actin prevented accurate measurement of the absorption spectrum.

The presence of -SH groups in the major myofibrillar proteins and the possibility that -SH groups are involved in muscle contraction is

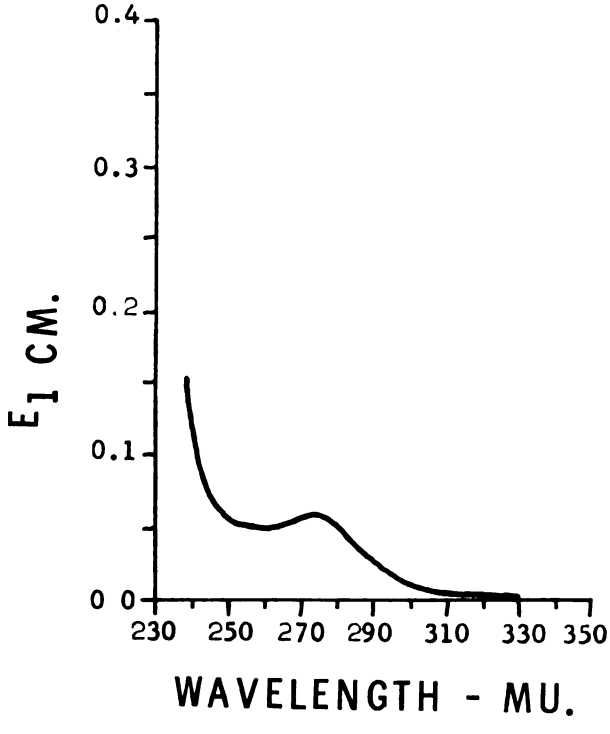


Figure 39. Absorption spectrum of extra protein fraction IA. Sample = column eluate collected at 60 ml effluent volume during the chromatography of extra protein (Figure 31).

well recognized (Poglazov, 1966; Perry, 1967a). A qualitative view of the thiol content of the proteins contained in the Weber-Edsall extract was obtained by the use of the DDD (2,2'-dihydroxy-6,6'-dinaphthyldisulfide) stain. This stain is specific for sulfhydryls and interference from non-sulfhydryl compounds is negligible (Pearse, 1960). Two identical disc gel patterns of the Weber-Edsall extract are shown in Figure 40 of the present study. Gel A was stained with Amido Black and gel B with DDD. It is obvious that -SH groups generally are detectable in most myofibrillar components.

The foregoing experiments indicate that the Weber-Edsall extract contains at least three sizes of aggregates with molecular weights of about 50,000,000; 5,000,000 and 200,000. Weber-Edsall extracts of washed muscle residue contained several rapidly sedimenting non-proteinaceous fractions, which were not present in extracts of prepared myofibrils. However, disc gel electrophoresis indicated that the amounts and kinds of proteins extracted are the same for the washed muscle residue and the prepared myofibrils. The gel component of the Weber-Edsall extract contains more myosin-like material than the supernatant solution after centrifugation.

The nucleic acid extracted by the Weber-Edsall solution seems to be mainly associated with oxidized and reduced tropomyosin, extra protein fraction IA, and possibly actin. Free -SH groups are detectable in virtually every electrophoretic component present in the Weber-Edsall extract.

Studies on Actomyosin

Composition of Actomyosin

Actomyosin prepared from the Weber-Edsall extract after the method of Morita and Tonomura (1960) was analyzed by disc gel electrophoresis. Typical disc gel patterns from the Weber-Edsall extract and the subsequent actomyosin preparation are shown in Figure 41A and B, respectively. Comparison of the two disc gel patterns indicates that purification of actomyosin removed most of the material between R_m = 0.15 and R_m = 0.30. Further, the intensity of the oxidized tropomyosin band (R_m = 0.34) was greatly reduced, and the bands with R_m values of 0.47 and 0.59 became more intense.

As can be seen, the actomyosin complex contained myosin (R_m = 0.05-0.15), possibly α-actinin (R_m = 0.14 or 0.18), oxidized tropomyosin (R_m = 0.34), actin (R_m = 0.38), reduced tropomyosin (R_m = 0.51), and extra protein fraction IA (R_m - 0.91). In addition, several unidentified components are present at R_m = 0.47 and 0.59.

The presence of separate actin (R_m = 0.38) and myosin (R_m - 0.05-0.10) bands in the actomyosin preparation suggests the 7 M urea present in the gels may have dissociated the actomyosin complex (Figure 41B). This observation agrees with the conclusion of Snellman and Erdös (1948) and of Bárany et al. (1960), who have previously reported that urea inhibits formation of the actomyosin complex. In contrast, the finding of Roettcher and Straub (1963) that intact myofibrils may retain their contractility

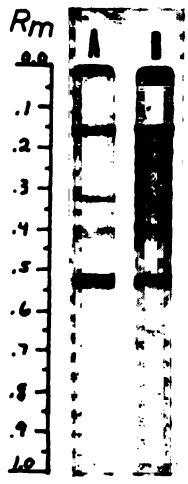


Figure 40. Disc gel electrophoresis of Weber-Edsall extract. Sample in each case was 0.05 ml of extract containing 1.5 mg protein/ml. A, stained with Amido Black. B, stained with DDD.

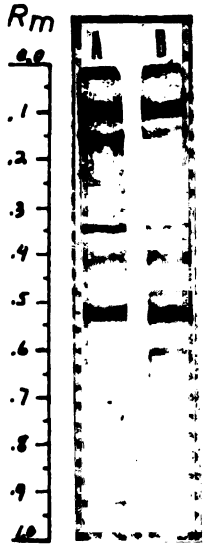


Figure 41. Disc gel electrophoretic comparison of Weber-Edsall extract and actomyosin. A, sample = 0.05 ml of Weber-Edsall extract containing 1.5 mg protein/ml. B, sample = 0.05 ml of actomyosin preparation containing 1.8 mg protein/ml.

in the presence of urea suggests that urea may not actually inhibit actomyosin formation. It is possible that the dissociation properties of actin and myosin may be different for purified proteins and in myofibrils. Further investigations will be necessary to establish the dissociation properties under such conditions.

Gel filtration of actomyosin produced the chromatogram shown in Figure 42. Assuming that separation occurred solely on the basis of gel filtration, peak 1 of Figure 42 consisted of material with a molecular weight in the range of 50,000,000 or larger; whereas peak 2 had a molecular weight of about 6,000,000 (Bio-Rad Laboratories, 1968). Obviously, the two fractions separated by gel filtration are aggregates of several proteins and would not be expected to have molecular weights in agreement with values for the various purified components.

The nature of peaks 1 and 2 was further investigated by disc gel electrophoresis. Serial disc gel separations performed on the gel filtration fractions (Figure 42) are presented in Figure 43. The characteristic darkening of the myosin bands by the gel filtration process has been observed and discussed previously herein. Peak 1, eluted at an effluent volume of 50 to 70 ml, appeared to consist largely of non-migrating material (R_m = 0.00), myosin (R_m = 0.05-0.15) and actin (R_m = 0.39). However, peak 2, which was eluted at effluent volumes of 80, 90 and 100 ml, contained no aggregated material at R_m = 0.00. Thus, peak 2 contained myosin (R_m = 0.05-0.15), actin (R_m = 0.39), reduced tropomyosin (R_m = 0.51) and an unidentified component at R_m = 0.59. The latter component appears to be identical to the band at R_m - 0.59 $\pm$ 0.01, which has been observed earlier herein to occur in preparations of HM, PM, AA and IF (Table I). Attempts to identify this band were unsuccessful in the present study.

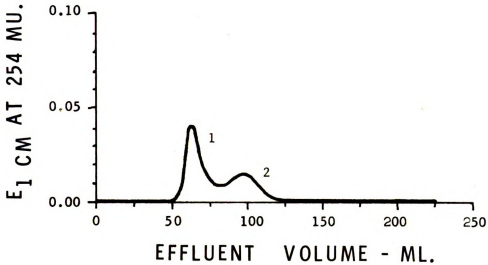


Figure 42. Gel filtration of actomyosin. A 2 ml sample of actomyosin preparation containing 2.2 mg protein/ml was applied at 0 ml effluent volume. Column dimensions were 2.5 x 38 cm. V_o of the column was 63 ml. V_e/V_o ratios for peaks 1 and 2 were 1.0 and 1.5, respectively.

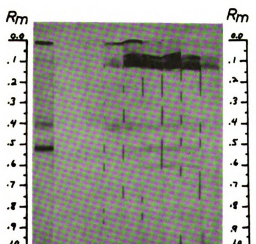


Figure 43. Serial disc gel electrophoretic analyses of the gel filtration pattern from actomyosin shown in Figure 42. At far left, a 0.05 ml actomyosin sample containing 2.2 mg protein/ml was analyzed. Then, proceeding from left to right, 0.10 ml samples taken at 50, 60, 70, 80, 90 and 100 ml effluent volume were analyzed.

The presence of reduced tropomyosin in peak 2 of Figure 42 provided direct evidence that tropomyosin is an integral part of the large aggregates contained in this fraction. Tropomyosin, applied by itself to the column, was found to have a Ve/Vo ratio of 2.2 for the oxidized species and 2.7 for the reduced species; whereas, the Ve/Vo ratio of peak 2 is 1.5. Thus, tropomyosin appears to be an integral part of the purified actomyosin complex, apparently as a large aggregate. This was shown to be true since neither of the gel filtration peaks corresponded to the Ve/Vo ratio of tropomyosin, yet tropomyosin was present in the disc gel pattern of peak 2. Similar determinations could not be reliably made for actin and myosin, apparently due to spontaneous aggregation of these purified proteins on the gel column.

Sucrose density gradient centrifugation of actomyosin usually produced two peaks as shown in Figure 44A. Peaks 1 and 2 sedimented at 1.1 and 2.0 cm, respectively. Johnson and Rowe (1964) have speculated on the nature of the two peaks produced by actomyosin in the ultracentrifuge. They suggested that the two peaks may result from two different types of actin participating in the actomyosin complex.

In the present study, the nature of peaks 1 and 2 from ultracentrifugation of purified actomyosin was investigated by disc gel electrophoresis. Serial disc gel analyses of the separation achieved by density gradient centrifugation are shown in Figure 45A. The ultracentrifuge pattern from 0.0 to 1.0 cm consisted mostly of oxidized tropomyosin (R_m = 0.34 $\pm$ 0.02). The sharp rise in E_{245} at 1.1 cm (peak 1, Figure 44A) was accompanied by the appearance in the disc gel pattern of myosin (R_m = 0.05-0.15), actin (R_m = 0.39), reduced tropomyosin (R_m = 0.51), an unidentified band at R_m = 0.60 $\pm$ and extra protein fraction IA. Thus,

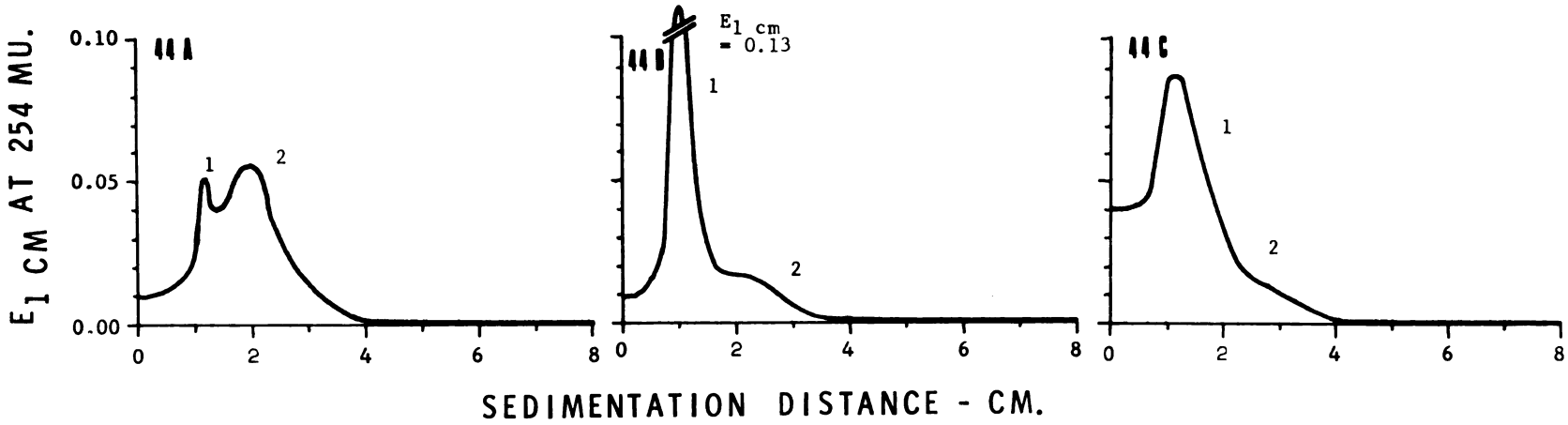
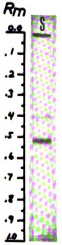


Figure 44. Density gradient centrifugation of actomyosin. In each case, a 0.6 ml actomyosin sample containing 2.6 mg protein/ml was analyzed. A, control separation with sample and gradient containing no added pyrophosphate or EDTA. B, sample and gradient contained 1 mM sodium pyrophosphate (pH 7.6) and 1 mM $MgCl_2$. C, sample and gradient contained 1 mM EDTA (pH 7.6).



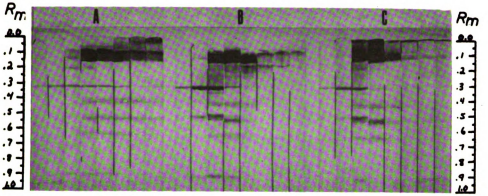


Figure 45. Serial disc gel electrophoretic analyses of the density gradient separations of actomyosin shown in Figure 44. S, analysis of 0.05 ml of original actomyosin sample before density gradient centrifugation. A, analysis of separation pattern shown in Figure 44A. From left to right, the separation pattern (Figure 44A) was analyzed at depths of 0.0 - 0.3, 0.3 - 0.7, 0.7 - 1.0, 1.0 - 1.3, 1.3 - 1.7, 1.7 - 2.0, 2.0 - 2.3 and 2.3 - 2.7 cm. B, analysis of separation pattern shown in Figure 44B. Distribution of samples is same as in Figure 45A. C, analysis of separation pattern shown in Figure 44C. Distribution of samples is same as in Figure 45A.

peak 1 consisted of myosin, oxidized tropomyosin, actin, reduced tropomyosin and an unidentified band at R_m = 0.60.

Disc gel analyses showed that oxidized tropomyosin, the unidentified band having an R_m value of 0.60 ($\pm$ 0.02) and extra protein fraction IA were present in peak 2 of Figure 44A. However, myosin (R_m = 0.05-0.15), actin (R_m 0.39) and reduced tropomyosin (R_m = 0.51) were present throughout peak 2 (1.67-2.67 cm). Thus, it appears that peak 1 from ultracentrifugation of actomyosin consisted of myosin, oxidized tropomyosin, actin, reduced tropomyosin, the unidentified component with an R_m value of 0.60 and extra protein fraction IA; whereas, peak 2 consisted mainly of myosin, actin and reduced tropomyosin. This information suggests that the two ultracentrifugal peaks of actomyosin arise from differences in the combination of proteins participating in the complex, rather than from two different forms of actin as was suggested by Johnson and Rowe (1964).

In the density gradient centrifugation pattern of actomyosin (Figure 44A), myosin was detected at 1.0-2.7 cm, oxidized tropomyosin at 0.3-2.0 cm, actin at 1.0-2.7 cm and reduced tropomyosin at 1.0-2.7 cm. Experiments with purified proteins have previously indicated that myosin and tropomyosin produce relatively sharp peaks sedimenting at 1.0 and 0.7 cm, respectively (Figures 5 and 14). Ultracentrifugation of actin produced a broad peak at 4.3 cm (Figure 12A). The unnatural sedimentation behavior of these proteins in Figure 44A indicates that their molecular size, configuration or relative specific density had changed, possibly due to their participation in the actomyosin complex.

The original actomyosin sample (Figure 45 S) contained only the reduced species of tropomyosin, yet after density gradient centrifugation

(Figure 44A), the separation pattern contained oxidized tropomyosin (Figure 45A). These results indicate that the oxidized tropomyosin was formed during density gradient centrifugation and was not present in the original actomyosin preparation. This may explain the reason that oxidized tropomyosin is spread over such a large range in the density gradient pattern.

The foregoing experiments suggested that actomyosin is dissociated by 7 M urea. Disc gel electrophoresis of purified actomyosin revealed the presence of myosin, actin, reduced tropomyosin, extra protein fraction IA and unidentified components at R_m = 0.35, 0.47, and 0.59. Disc gel patterns also indicated the possible presence of α-actinin and varying amounts of oxidized tropomyosin.

Gel filtration separated purified actomyosin into two fractions. One fraction had a molecular weight in the range of 50,000,000 and consisted mainly of myosin and actin. The other fraction was a smaller aggregate, having a molecular weight of about 6,000,000, and consisted of myosin, actin, reduced tropomyosin and an unidentified component at R_m = 0.59.

Density gradient centrifugation also separated actomyosin into two components. The slower sedimenting component consisted of myosin, oxidized tropomyosin, actin, reduced tropomyosin and an unidentified band at R_m = 0.60. The faster sedimenting species consisted of myosin, actin and reduced tropomyosin. Gel filtration behavior and sedimentation in the ultracentrifuge indicated that myosin, actin and tropomyosin all play direct roles in the make-up of the actomyosin complex.

Composition of Natural Actomyosin (NAM)

According to Schaub et al.(1967), "natural actomyosin" prepared by repeated precipitation at an ionic strength of 0.04 exhibits ESF activity. On the other hand, purification of actomyosin by conventional methods often yields a preparation with little or no ESF activity (A. Weber and Winicur, 1961; Ebashi, 1966). This information suggests that "natural actomyosin" contains ESF, while conventional actomyosin preparations often contain little or none. Therefore, in the present study, preparations of actomyosin and "natural actomyosin" were analyzed by disc gel electrophoresis to determine if differences in protein composition might account for differences in their enzymatic properties. Results are shown in Figure 46.

The major noticeable difference between actomyosin and "natural actomyosin" (Figure 46) was the higher content of extra protein fraction IA (R_m = 0.91) in "natural actomyosin". This suggests that the higher ESF activity noted in "natural actomyosin" preparations (Schaub et al., 1967) may reside in the extra protein fraction IA, which has an R_m value of 0.91.

Effect of Pyrophosphate on Actomyosin

Pyrophosphate in the presence of trace Mg^{++} ions has been used by many workers as a chemical analog to study the action of ATP on myofibrillar proteins (Azzone and Dobrilla, 1964). In the present work, density gradient centrifugation in the absence of pyrophosphate produced the separation shown in Figure 44A. An identical separation (Figure 44B) was performed on the same actomyosin preparation in the presence of 1mM sodium pyrophosphate (pH 7.6) and 1mM $MgCl_2$ (Tonomura and Sekiya, 1961; Levy and Fleisher, 1965). The protein sample was first brought to 1mM

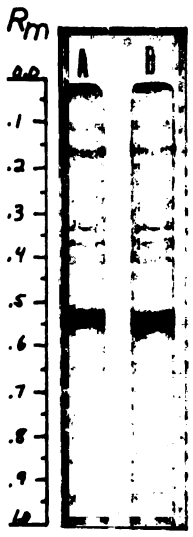


Figure 46. Disc gel electrophoretic comparison of actomyosin and natural actomyosin. A, sample = 0.03 ml of actomyosin preparation containing 3.0 mg protein/ml. B, sample = 0.03 ml of "natural actomyosin" preparation containing 3.5 mg protein/ml.

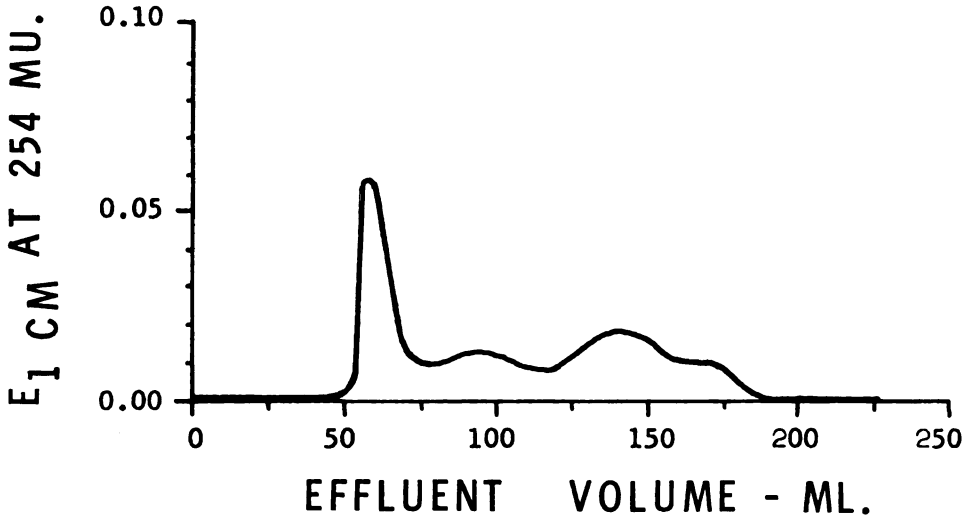


Figure 47. Typical gel filtration pattern obtained from Weber-Edsall extract of muscle samples in all microbiological experiments. A 2 ml sample of extract was applied at 0 ml effluent volume. V_o of the column was 60 ml. Column dimensions were 2.5 x 37 cm.

pyrophosphate and 1mM $MgCl_2$ by appropriate addition of the solid reagents, allowed to equilibrate 1 hour in the cold, then subjected to density gradient centrifugation. Results are shown in Figure 44B.

Pyrophosphate (Figure 44B) effected a large increase in the height of peak 1 (sedimentation distance = 1.1 cm) and a concurrent decrease in peak 2 (sedimentation distance = 2.0 cm). The majority of workers have interpreted the decrease in rapidly sedimenting material and the simultaneous increase in slowly sedimenting materials to mean that ATP and its chemical analogs dissociate actomyosin (Johnson and Rowe, 1964). However, some workers (Blum and Morales, 1963; Morales et al.,1955: von Hippel et al., 1957; Johnson and Rowe, 1964) have found evidence that actomyosin undergoes certain changes, but does not dissociate in the presence of ATP.

In the present study, the ultracentrifugal separation shown in Figure 44B was analyzed by disc gel electrophoresis. The results, which are shown in Figure 45B, indicate that the material sedimenting at less than 1 cm consisted mainly of oxidized tropomyosin (R_m = 0.34), reduced tropomyosin (R_m = 0.51) and extra protein fraction IA (R_m = 0.91). The sharp peak, which sedimented at 1.1 cm, contained myosin (R_m = 0.05-0.15), some unidentified components between R_m = 0.15 and R_m = 0.30, oxidized tropomyosin (R_m = 0.34), actin (0.38), reduced tropomyosin (0.51), an unidentified band (0.60) and extra protein fraction IA (0.91). At 2.0 cm from the top of the gradient (Figure 44B) a small amount of peak 2 still remained, but apparently was not further dissociated by the addition of pyrophosphate. Disc gel electrophoretic analysis (Figure 45B) revealed that the undissociated remainder of peak 2 contained mostly myosin (R_m = 0.05-0.15).

In the presence of pyrophosphate (Figure 44B), the myosin moiety of actomyosin sedimented near the true value for purified myosin, 1.1 cm. Likewise, the oxidized tropomyosin moiety behaved much like purified tropomyosin, sedimenting around 0.6 cm. The fact that myosin and oxidized tropomyosin sedimented at or near the true values for these purified proteins indicates that they are probably not part of a large protein complex, but instead are apparently dissociated from actomyosin by pyrophosphate.

On the other hand, actin and reduced tropomyosin are both detected at about 1 cm from the top of the gradient (Figure 44B). Purified preparations of actin and tropomyosin sediment at 4.3 and 0.6 cm, respectively (Table I). Thus, the addition of pyrophosphate caused the actin and tropomyosin moieties to sediment more slowly, however, the sedimentation rate was not the same as that of the purified proteins. Therefore, it seems probable that actin and tropomyosin are not completely dissociated from actomyosin by the presence of pyrophosphate.

The foregoing experiments indicate that pyrophosphate decreases the sedimentation rate of the protein moieties of actomyosin, apparently causing a general dissociation of actomyosin. The behavior of actomyosin in the ultracentrifuge suggests that pyrophosphate completely dissociates myosin from the actomyosin complex, yet leaves actin and reduced tropomyosin in a partially dissociated or an otherwise unnatural state. Pyrophosphate decreased the sedimentation distance of the unidentified component (R_m = 0.60) and of extra protein fraction IA (R_m = 0.91), apparently dissociating them from the actomyosin complex. Since sedimentation distances for the purified counterparts of the unidentified band at R_m = 0.60 and of extra protein fraction IA were not determined in the present work

(Table I), it is difficult to say whether they are completely dissociated, or remain partially complexed in the presence of pyrophosphate.

Effect of EDTA on Actomyosin

Density gradient centrifugation of actomyosin produced the pattern shown in Figure 44A. An identical separation, performed on the same actomyosin preparation in the presence of 0.001M EDTA (pH 7.6) is shown in Figure 44C. Sufficient EDTA was added to the protein sample to adjust it to a concentration of 1mM. The solution was readjusted to pH 7.6, allowed to equilibrate for 1 hour in the cold and then subjected to density gradient centrifugation.

EDTA caused an increase in peak 1 and a decrease in peak 2 (Figure 44C), which was somewhat like the effect of ATP (Figure 44B). In the case of EDTA, however, the change was not so complete, and peak 1 did not have the sharp separation so apparent with ATP.

The contents of peaks 1 and 2 (Figure 44C) were analyzed by disc gel electrophoresis. The results (Figure 45C) showed that myosin, actin and tropomyosin sedimented much like they did in the presence of ATP (Figure 44B). This suggests that EDTA dissociated myosin from the actomyosin complex, while leaving actin and tropomyosin bound in some manner. However, the effect of EDTA on actomyosin is not identical with that of ATP. Comparison of Figures 45B and 45C shows that the ultracentrifugal pattern of actomyosin in the presence of EDTA produced much less material at R_m = 0.15-0.30. Furthermore, EDTA appears to have drastically reduced the amount of extra protein fraction IA. This is an interesting occurrence, since experiments described herein have already suggested that extra protein fraction IA is identical to or contains the elusive ESF. Perry (1967a) speculated that the presence or absence of minute amounts of Ca^{++} will

govern the properties of ESF and its ability to function as a regulatory protein during muscular contraction. More work is needed to establish whether the band at R_m = 0.91 is actually ESF and to elucidate the effects of EDTA upon this component.

Bacterial Action on Myofibrillar Proteins

A series of 12 experiments were conducted as previously described with Achromobacter liquefaciens, Clostridium perfrigens, Micrococcus luteus, Pediococcus cerevisiae, Pseudomonas fluorescens, Streptococcus faecalis and natural flora obtained from commercial hamburger. Two of the experiments, one with Clostridium perfringens and the other with Pseudomonas fluorescens, were discontinued after plate counts disclosed that the inoculum was not viable. The remaining eight experiments are listed in Table II.

Much difficulty was encountered in getting the micro-organisms to grow. Good growth was achieved only with Micrococcus luteus and the natural culture from commercial meat. The failure of the microorganisms to multiply is difficult to explain, since stock cultures were subcultured twice to bring them to an active state before inoculation into the ground muscle.

Salt-soluble proteins from control and inoculated muscle samples were prepared after 0, 8 and 20 days incubation time. The samples were then analyzed by gel filtration, density gradient centrifugation and disc gel electrophoresis as previously outlined. Gel filtration of extracts from control and inoculated samples from all microbiological experiments (Table II) yielded the typical separation pattern shown in Figure 47, and revealed no differences due to aging or bacterial growth.

Density gradient centrifugation patterns from microbiological Experiments No. 1 through 6 (Table II) are given in Figures 48A through I.

Density gradient separations from the fresh control (Figure 48A) and from the incubated control (Figure 48B) suggest that the aging process caused a decrease in the components sedimenting at 4.0, 5.8 and 6.5 cm. The decrease due to aging is tabulated in Table II for each microbiological experiment. As can be seen (Table II), incubation at 10°C caused the components sedimenting at 4.0, 5.8 and 6.5 cm to decrease more than did incubation at 3°C.

Typical density gradient centrifugation patterns from fresh control and fresh inoculated samples are shown in Figures 48A and C, respectively. No difference was found between patterns for the fresh control and those from fresh inoculated samples in any of the microbiological experiments. However, centrifugation patterns from incubated control samples (Figure 48B) and incubated inoculated samples (Figures 48D, E, F, G, H and I) indicated that bacterial growth may cause a decrease in the heights of the peaks sedimenting at 4.0, 5.8 and 6.5 cm. The decrease due to bacterial growth is listed in Table II for each microbiological experiment.

As shown in Table II, Experiments No. 1, 2, 5 and 6 (inoculations performed with Streptococcus faecalis, Pediococcus cerevisiae, Pediococcus cerevisiae and Pseudomonas fluorescens, respectively) exhibited no noticeable increase in viable bacteria during incubation, and accordingly revealed little or no decrease in the components sedimenting at 4.0, 5.8 and 6.5 cm. In contrast, Experiments No. 3 and 4 (inoculation performed with mixed culture in each case) showed large increases in the number of viable bacteria during incubation, and exhibited large decreases in the components sedimenting at 4.0, 5.8 and 6.0 cm.

As previously described herein, the ultracentrifugal peaks sedimenting at 4.0, 5.8 and 6.5 cm did not represent myofibrillar proteins. The exact

Table II. Microbiological Experiments

No.	Animal	Inoculation Organism	Storage Temp	Incubation Time	Bacterial Count		Percent of Peak Height* Remaining After	
					Control	Inoculated	Aging (20 days)	Bacterial Growth (20 days)
1	Pig	Streptococcus faecalis	3°C	0 days	0	2.2×10^4		
				8	4	5.0×10^3		
				20	1.0×10^3	1.0×10^4	56%	50%
2	Pig	Pediococcus cerevisiae	3°C	0	0	1.6×10^6		
				8	0	5.6×10^4		
				20	0	1.6×10^6	50	43
3	Pig	Mixed culture	3°C	0	1.6×10^2	5.0×10^3		
				8	0	1.3×10^8		
				20	0	6.0×10^9	43	14
4	Pig	Mixed culture	3°C	0	2.0×10^2	3.1×10^3		
				8	0	1.0×10^6		
				20	0	2.0×10^8	43	28
5	Pig	Pediococcus cerevisiae	3°C	0	0	1.3×10^5		
				8	0	1.3×10^5		
				20	0	4.0×10^4	60	60
			10°C	0	0	1.3×10^5		
				8	0	9.0×10^4		
				20	0	4.7×10^5	50	50
6	Pig	Pseudomonas fluorescens	3°C	0	0	2.1×10^3		
				8	0	0		
				20	0	0	50	50
			10°C	0	0	2.1×10^3		
				8	0	2.4×10^2		
				20	mold	mold	40	40

Table II (con't)

No.	Animal	Inoculation Organism	Storage Temp	Incubation Time	Bacterial Count		Percent of Peak Height* Remaining After	
					Control	Inoculated	Aging (20 days)	Bacterial Growth (20 days)
7	Rabbit	Micrococcus luteus	10°C	0	0	3.3×10^4		
				8	0	1.1×10^4		
				20	0	mold	**	**
		Achromobacter liquefaciens	10°C	0	0	1.5×10^5		
				8	0	3.4×10^7		
				20	0	5.9×10^9	**	**
8	Pig	Micrococcus luteus	10°C	0	5	1.2×10^4		
				8	0	2.0×10^3		
				20	mold	mold	**	**
		Achromobacter liquefaciens	10°C	0	5	7.9×10^4		
				8	0	7.2×10^6		
				20	mold	4.8×10^8	**	**

*Percent of initial average height of peaks sedimenting at 4.0, 5.8 and 6.5 cm in Figures 48A through I. Percentages were determined from the relationships:

$$\text{percent remaining after aging} = \frac{\text{average height observed in 20-day control sample}}{\text{average height observed in 0-day control sample}}$$

$$\text{percent remaining after bacterial growth} = \frac{\text{average height observed in 20-day inoculated sample}}{\text{average height observed in 0-day control sample}}$$

**Ultracentrifuge was inoperative during Experiments No. 7 and 8.

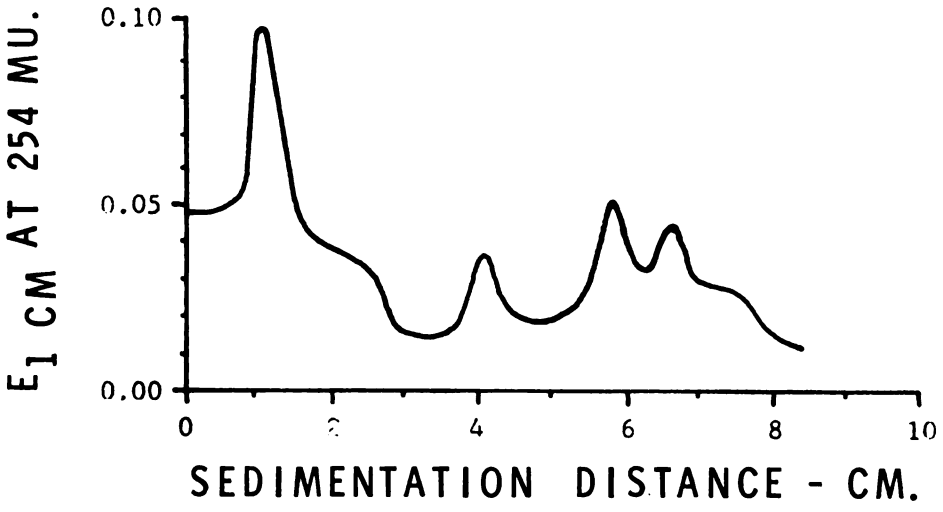


Figure 48A. Typical density gradient centrifugation pattern obtained from Weber-Edsall extract of fresh control muscle samples. Sample = 0.6 ml of Weber-Edsall extract.

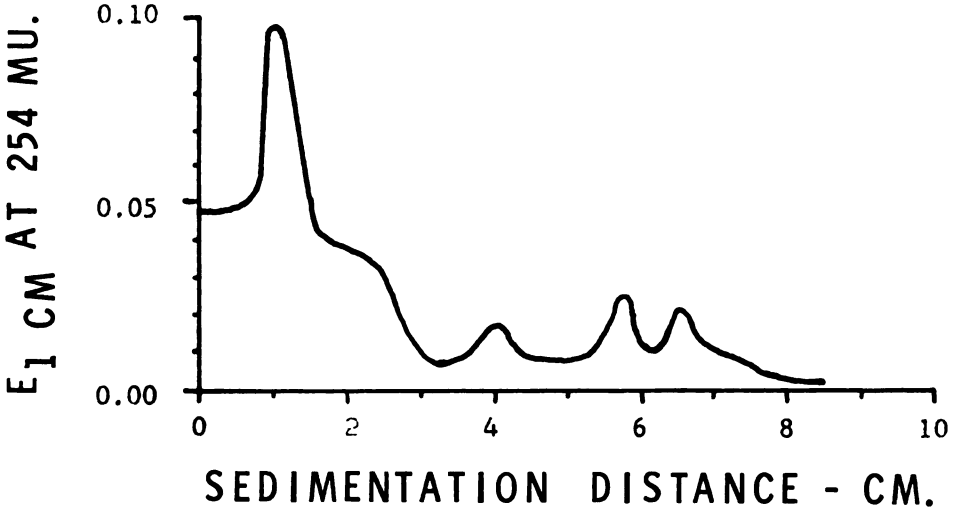


Figure 48B. Typical density gradient centrifugation pattern obtained from Weber-Edsall extract of control muscle samples after 20 days incubation at either 3 or 10°C. Sample = 0.6 ml of Weber-Edsall extract.

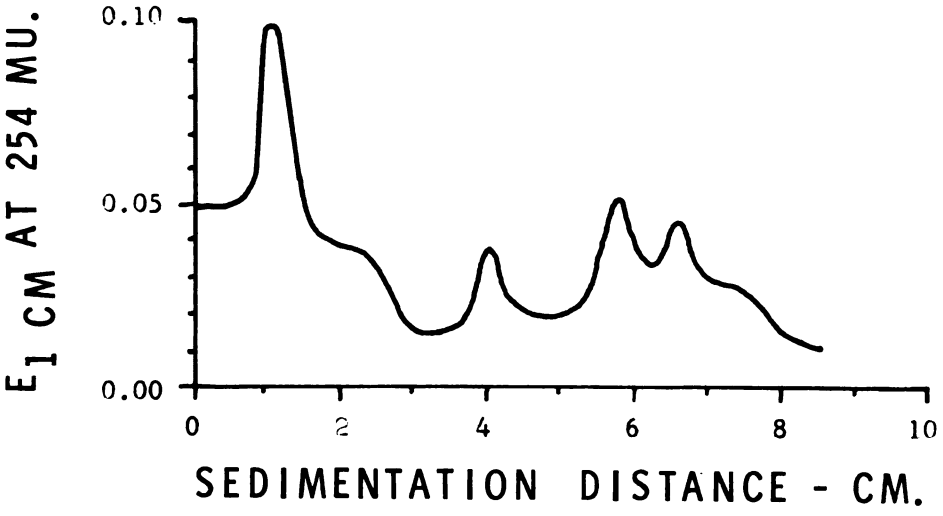


Figure 48C. Typical density gradient centrifugation pattern obtained from Weber-Edsall extract of fresh inoculated muscle samples. Sample = 0.6 ml of Weber-Edsall extract.

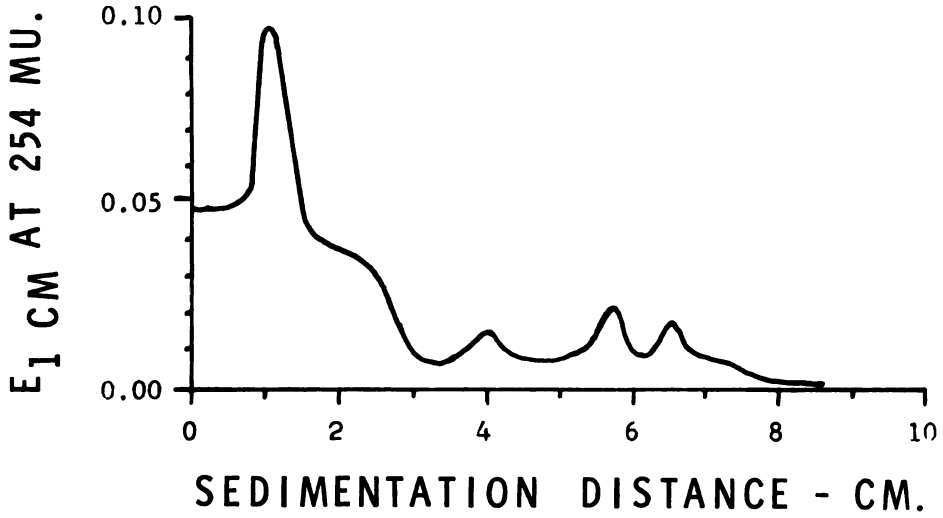


Figure 48D. Density gradient centrifugation pattern obtained from inoculated sample in Experiment No. 1 (inoculation performed with Streptococcus faecalis) after 20 days incubation at 3°C. Sample = 0.6 ml of Weber-Edsall extract.

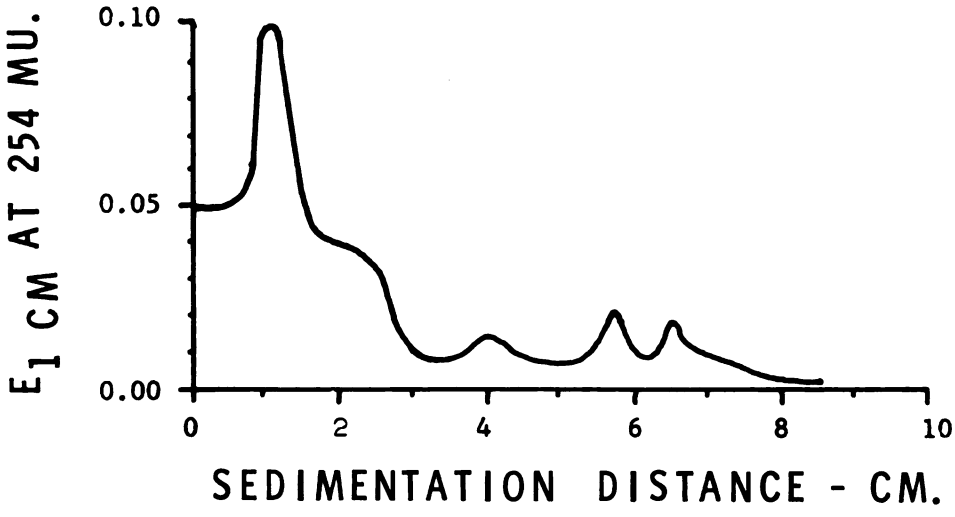


Figure 48E. Density gradient centrifugation pattern obtained from inoculated sample in Experiment No. 2 (inoculation performed with Pediococcus cerevisiae) after 20 days at 3°C. Sample = 0.6 ml of Weber-Edsall extract.

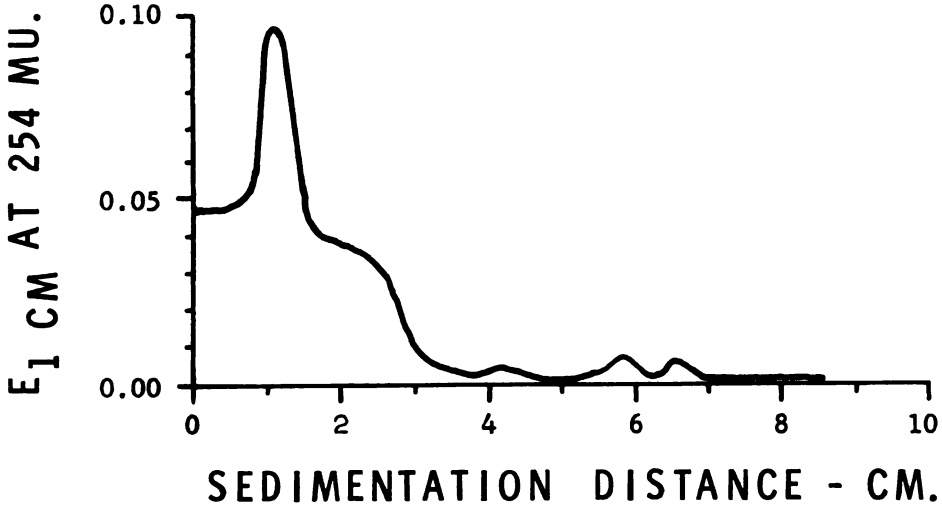


Figure 48F. Density gradient centrifugation pattern obtained from inoculated sample in Experiment No. 3 (inoculation performed with mixed culture from commercial meat) after 20 days of incubation at 3°C. Sample = 0.6 ml of Weber-Edsall extract.

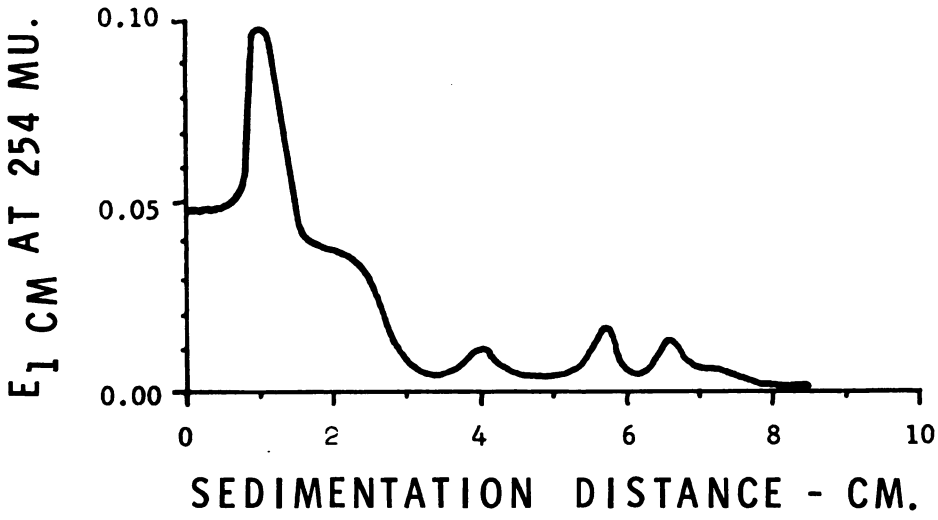


Figure 48G. Density gradient centrifugation pattern obtained from inoculated sample in Experiment No. 4 (inoculation performed with mixed culture from commercial meat) after 20 days of incubation at 3°C. Sample = 0.6 ml of Weber-Edsall extract.

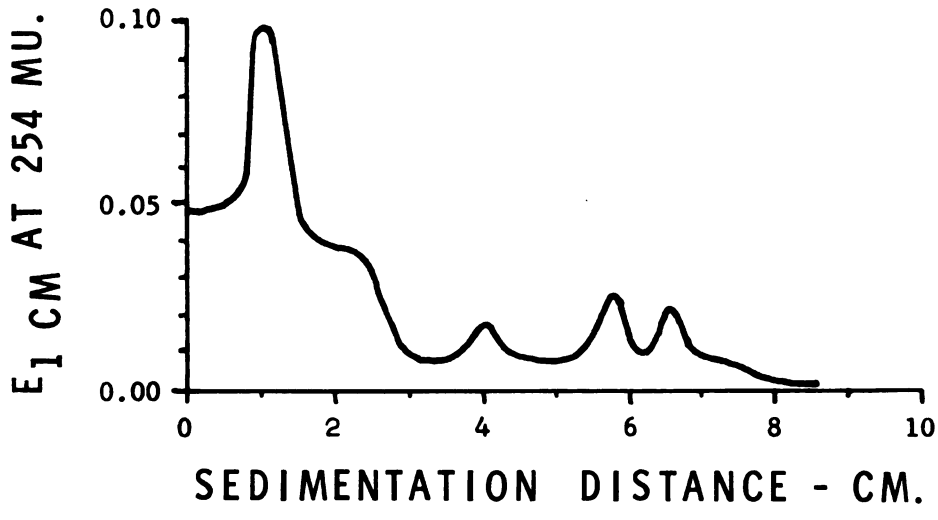


Figure 48H. Density gradient centrifugation pattern obtained from inoculated sample in Experiment No. 5 (inoculation performed with Pediococcus cerevisiae) after 20 days at either 3 or 10°C. Sample = 0.6 ml of Weber-Edsall extract.

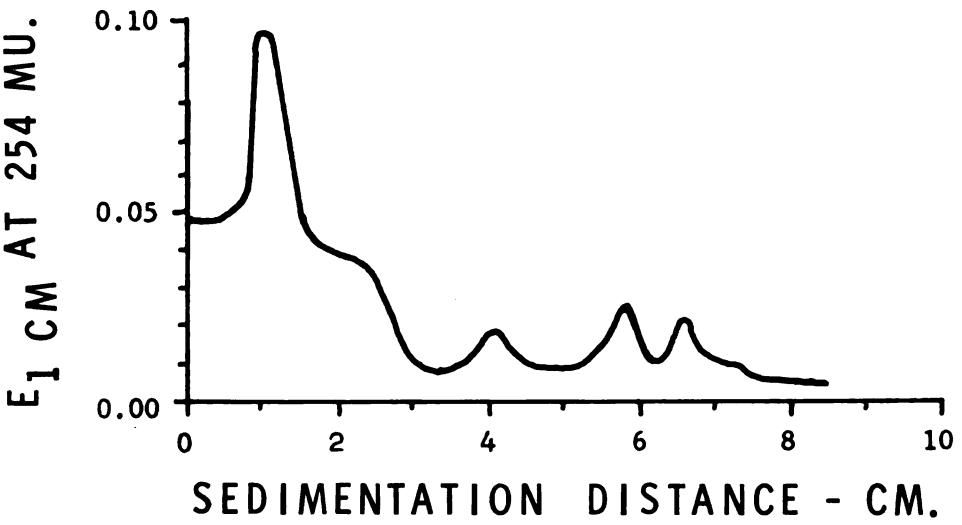


Figure 48I. Density gradient centrifugation pattern obtained from inoculated sample in Experiment No. 6 (inoculation performed with Pseudomonas fluorescens) after 20 days of incubation at either 3 or 10°C. Sample = 0.6 ml of Weber-Edsall extract.

nature of these peaks was not ascertained in the present work. The decrease in the height of these peaks would suggest that they were actually being used as nutrients by the bacteria. However, the possibility that the bacteria merely altered these fractions, thereby making them insoluble, was not ruled out in this study. In this regard, it is interesting to note the work of Jay and Kontou (1967) and Lerke et al. (1967), who presented evidence showing that the common spoilage bacteria of meat do not use existing proteins for food, but instead prefer small non-protein nitrogenous molecules.

In the present work, disc gel electrophoresis was used to study the effects of bacterial growth on salt soluble proteins during Experiments No. 5, 6, 7 and 8 (Table II). Typical disc gel patterns obtained during these experiments are shown in Figure 49. Results indicated no noticeable changes in the salt-soluble proteins during bacterial growth.

The present microbiological experiments also permit examination of species differences between salt-soluble proteins from the rabbit and the pig. Neither gel filtration nor density gradient centrifugation showed any consistent differences between the salt-soluble proteins of the rabbit and those of the pig. Disc gel electrophoresis (Figure 49) indicated that the salt-soluble proteins from pork had the same or similar electrophoretic properties as those from the rabbit. However, Weber-Edsall extracts prepared from pig muscle consistently produced a darker band at $R_m = 0.59$ than those prepared from rabbit muscle. This band appears to be identical with the unidentified component at $R_m = 0.59$, which has appeared in protein preparations throughout the course of the present study (Table I).

The fact that Weber-Edsall extracts of pig muscle produce a darker band at $R_m = 0.59$ does not necessarily mean that this component is present in greater amounts in pig muscle. It could be more easily extracted from

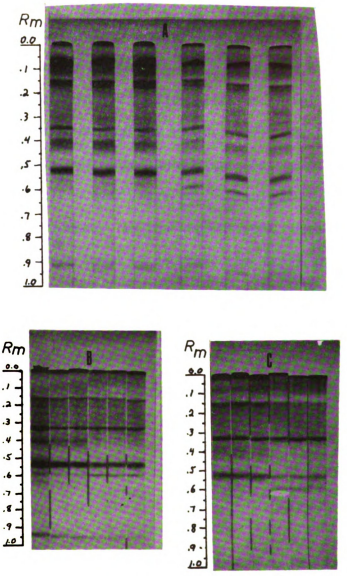


Figure 49. Disc gel electrophoresis of Weber-Edsall extracts obtained during microbiological experiments No. 7 and 8 (Table II). A, extracts were prepared on the day of inoculation. From left to right, extracts were obtained from Experiment No. 7 (rabbit) control, No. 7 (rabbit) inoculated with Achromobacter liquifaciens, No. 7 (rabbit) inoculated with Micrococcus luteus, No. 8 (pig) control, No. 8 (pig) inoculated with Achromobacter liquifaciens and No. 8 (pig) inoculated with Micrococcus luteus. B, extracts were prepared after 8 days incubation at 10°C. Distribution of samples is the same as in Figure 49A. C, extracts were prepared after 20 days incubation at 10°C. Distribution of samples is same as in Figure 49A. In each case, sample = 0.05 ml of Weber-Edsall extract, except in the case of the 8-day control from Experiment No. 7 (gel on far left in Figure 47B), where 0.10 ml of extract was applied.

pig muscle, or it may have a greater affinity for the Amido Black stain. Any one of these possibilities could produce a darker band at $R_m = 0.59$.

The foregoing experiments (Table II) indicated no detectable changes in the salt-soluble proteins of muscle during bacterial spoilage. This is in agreement with the work of Jay (1966, 1967), Jay and Kontou (1967) and Lerke et al. (1967), which indicated that the common meat spoilage organisms do not usually attack large protein molecules, but instead utilize non-protein nitrogen sources. In the present work, both the aging process and bacterial growth were found to decrease the amount of certain non-protein, ultraviolet-absorbing components, which were detectable in the ultracentrifuge, but did not appear to be derived from the myofibrils. Results of disc gel electrophoresis indicated that the Weber-Edsall extract of pig muscle produced a more intense band at $R_m = 0.59$ than that from rabbit muscle.

SUMMARY

The salt soluble proteins from skeletal muscle were separated and characterized by gel filtration, density gradient centrifugation, ion exchange chromatography and disc gel electrophoresis. Gel filtration and density gradient centrifugation afforded only crude fractionation, so that supplemental methods for separation were necessary. Ion exchange chromatography separated the Weber-Edsall extract of skeletal muscle into 8-11 fractions, but the method was too slow and cumbersome for routine analyses. Disc gel electrophoresis in the presence of 7 M urea was the most useful tool for fractionation and analyses of myofibrillar proteins, separating the Weber-Edsall extract into 8-11 principal bands.

Each of the known myofibrillar proteins was isolated and purified by several methods. The purified proteins were then subjected to gel filtration, density gradient centrifugation and disc gel electrophoresis for further purification and characterization. Results showed that many of the so-called "pure preparations" of individual myofibrillar proteins contained significant amounts of contaminants that could only be removed by special techniques.

A comparison of four different procedures for preparing myosin indicated that chromatography on DEAE-Sephadex A-50 gave the purest preparation. Disc gel electrophoresis in 8 M urea showed several bands at R_m values between 0.00 and 0.15. The material remaining at the origin probably consisted of myosin monomers and myosin aggregates. The bands

at R_m = 0.05-0.15 were tentatively identified as being myosin polypeptide sub-units.

Actin isolated directly from the myofibrils resulted in the purest actin preparation on comparing with three other isolation procedures. This preparation produced a single diffuse band at R_m = 0.39 by disc gel electrophoresis.

Tropomyosin was prepared by four different methods. The purest preparation was obtained by ammonium sulfate fractionation and isoelectric precipitation followed by chromatography on DEAE-cellulose in the presence of 0.01 M EDTA. Two bands on disc gel patterns with R_m values of 0.34 and 0.50 were identified as tropomyosin. The slower moving component was apparently due to oxidation of the -SH groups, whereas, the faster moving component was reduced tropomyosin.

Troponin (ESF) was prepared by two procedures. Disc gel bands were found in both preparations at R_m = 0.34 and 0.50, which corresponded to the oxidized and reduced forms of tropomyosin. This suggests that troponin was not separated from tropomyosin or else was non-protein in nature.

Preparations of α-actinin, β-actinin, inhibitory factor and the extra protein group were made using only one method for each. Neither α-actinin, β-actinin nor inhibitory factor were sufficiently pure to permit identification.

DEAE-cellulose chromatography of the extra protein group separated it into four main fractions, which have been previously identified as Fractions I, II, III and IV on the basis of salt concentration required for elution. A small peak following Fraction I was designated as Fraction IA. Fraction I was identified as consisting mainly of sarcoplasmic

proteins. Fractions II and III were not identified, while Fraction IV contained considerable amounts of tropomyosin. Examination of the behavior of Fraction IA indicates that it may be identical to troponin.

Weber-Edsall extracts of washed muscle residue and of prepared myofibrils were compared. Although extracts of washed muscle residue contained non-proteinaceous components absorbing in the ultraviolet region, the amount and types of proteins appeared to be the same. Weber-Edsall extracts contained myosin, actin, oxidized and reduced tropomyosin, extra protein Fraction IA and probably α-actinin. Specific staining of electrophoretic gels of Weber-Edsall extracts indicated that the nucleic acids are complexed with tropomyosin, extra protein Fraction IA and possibly actin. In addition, Weber-Edsall extracts contained several unidentified components on disc gel electrophoresis, having relative mobilities of 0.15-0.30, 0.36 and 0.59.

Actomyosin preparations contained myosin, actin, reduced tropomyosin, varying amounts of extra protein Fraction IA and probably α-actinin. Several unidentified components were also present at relative mobilities of 0.36, 0.47 and 0.59 on disc gels. Actomyosin preparations contained two fractions detectable by gel filtration and density gradient centrifugation. Gel filtration yielded two peaks with apparent molecular weights of 50,000,000 and 6,000,000. The former peak consisted mainly of actin and myosin, apparently aggregated together, while the second contained mainly actin, myosin, reduced tropomyosin and an unidentified component at $R_m = 0.60$.

Density gradient centrifugation of actomyosin produced one fast- and one slow-sedimenting peak. The slow-sedimenting peak consisted of

myosin, oxidized tropomyosin, actin, reduced tropomyosin and an unknown component at R_m - 0.60. The fast-sedimenting peak consisted mainly of myosin, actin and reduced tropomyosin. Oxidized tropomyosin was not present in the initial actomyosin sample and appeared to have been formed during density gradient centrifugation. Results of gel filtration and density gradient centrifugation indicated that myosin, actin and tropomyosin are all involved in the actomyosin complex.

Pyrophosphate was found to have a general dissociating action on actomyosin, decreasing the sedimentation rate of all detectable protein moieties. Sedimentation behavior indicated that pyrophosphate caused an actual dissociation of myosin from the actomyosin complex, yet left actin and reduced tropomyosin in a partially dissociated or otherwise unnatural state. Pyrophosphate also decreased the sedimentation rate of an unknown component (disc gel electrophoretic R_m = 0.50) and of extra protein Fraction IA. However, the extent of dissociation from the actomyosin complex was not determined in the present study.

The effect of EDTA on actomyosin was similar to the action of pyrophosphate. In general, EDTA appeared to dissociate myosin and partially dissociate actin and reduced tropomyosin. Nevertheless, the action of EDTA and pyrophosphate was not entirely the same. EDTA markedly decreased the amount of extra protein fraction IA detectable by disc gel electrophoresis.

In the microbiological experiments, bacterial growth had no detectable effect on the extraction and fractionation of myofibrillar proteins. However, both the aging process and bacterial growth decreased the amount of certain ultraviolet-absorbing components, which were detectable by density gradient centrifugation. Preliminary experiments indicated that these ultraviolet-absorbing components were not proteins and that they did not

come from the myofibrils. Disc gel electrophoretic patterns showed that Weber-Edsall extracts from pig longissimus dorsi muscle produced a darker band at R_m = 0.60 than similar extracts from the rabbit.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adelstein, R.S., J. E. Godfrey and W. W. Kielley. 1963. G-actin: Preparation by gel filtration and evidence for a double stranded structure. Biochem. Biophys. Res. Comm. 12:34.

American Instrument Co. 1961. The determination of nitrogen by the Kjeldahl procedure including digestion, distillation, and titration. Reprint No. 104.

Azuma, N. and S. Watanabe. 1965a. The major component of metin from rabbit skeletal and bovine cardiac muscle. J. Biol. Chem. 240: 3847.

Azuma, N. and S. Watanabe. 1965b. The minor component of metin from rabbit skeletal muscle, J. Biol. Chem. 240:3852.

Azzone, G. F. and G. Dobrilla. 1964. The mechanism of relaxation of muscle fibers--The relaxing effect of ATP and pyrophosphate on isolated myofibrils. In Biochemistry of Muscle Contraction. Ed. J. Gergely, p. 255. Little, Brown and Company, Boston, Mass.

Bailey, K. 1946. Tropomyosin: A new asymmetric protein component of muscle. Nature 157:368.

Bailey, K. 1948. Tropomyosin: A new asymmetric component of the muscle fibril. Biochem. J. 43:271.

Bailey, K. 1951. End-group assay in some proteins of the keratin-myosin group. Biochem. J. 49:23.

Banga, I. and A. Szent-Györgyi. 1942. Preparation and properties of myosin A and B. Studies Inst. Med. Chem., Univ. Szeged. 1:5.

Bárany, M., K. Bárany and W. Trautwein. 1960. Die Hemmung der Aktin-L-Myosin Interaktion in lebeden und extrahierten Muskeln durch Urea. Biochim. Biophys. Acta 45:317.

Bárany, M. and F. Jaisle. 1960. Kontraktionszklus und interaction zwischen aktin und L-myosin under der werkung spezifischer interaktions-inhibitoren. Biochim. Biophys. Acta 41:192.

Baril, E. F., D.S. Love and H. Hermann. 1966. Investigation of myosin heterogeneity observed during chromatography on DEAE-cellulose. J. Biol. Chem. 241:822.

Beaton, G.H., A.E. Selby and Audrey M. Wright. 1961. Starch gel electrophoresis of rat serum proteins. I. Procedure and designation of components. J. Biol. Chem. 236:2001.

Bendall, J.R. 1964. Meat Proteins. In Symposium on Foods: Proteins and Their Reactions. Eds. H.W. Schultz and A.F. Anglemier. p. 225. AVI Publishing Company, Inc., Westport, Conn.

Bio-Rad Laboratories. 1968. Price List T. Bio-Rad Laboratories, Richmond, Calif.

Bloom, W. and D.W. Fawcett. 1968. A Textbook of Histology. W. B. Saunders Co., Philadelphia, Pa.

Blum, J.J., and M.F. Morales. 1953. The interaction of myosin with adenosinetriphosphate. Arch. Biochem. Biophys. 43:208.

Borton, R.J. 1966. Emulsifying and other properties of porcine muscle tissue as related to microbial contamination. M.S. Thesis. Michigan State Univ., East Lansing, Mich.

Briskey, E.J., 1967. Myofibrillar proteins of skeletal muscle. In Proceedings of the Meat Industry Research Conference. p. 1 American Meat Institute Foundation, Chicago, Ill.

Briskey, E.J., K. Seraydarian, and W.F.H.M. Mommaerts. 1967a. The modification of actomyosin by α-actinin. II. The effect of α-actinin upon contractility. Biochim, Biophys. Acta 133:412.

Briskey, E.J., K. Seraydarian, and W.F.H.M. Mommaerts. 1967b. The modification of actomyosin by α-actinin. III. The interaction between α-actinin and actin. Biochim. Biophys. Acta 133:424.

Carsten, M.E., 1963. Actin, its amino acid composition and its reaction with iodoacetate. Biochemistry 2:32.

Carsten, M.E. 1968. Tropomyosin from smooth muscle of the uterus. Biochemistry 7:960.

Cohen, L.B., 1966. Viscosity of G-ADP and G-ATP actin. Arch. Biochem. Biophys. 117:289.

Crambach, A., R.A. Reisfeld, M. Wyckoff and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem. 20:150.

Czok, R., and Th. Bücher, 1960. Crystallized enzymes from the myogen of rabbit skeletal muscle. In Advances in Protein Chemistry. Vol. 15. Eds. C.B. Anfinsen, Jr., M.L. Anson, K. Bailey, and J. T. Edsall. Academic Press, New York, N. Y.

Davey, C.L., and K.V. Gilbert, 1968. Studies on meat tenderness. 6. The nature of myofibrillar proteins extracted from meat during aging. J. Food Sci. 33:343.

Davis, B. J. 1964. Disc Electrophoresis--II. Method and application to human serum proteins. Ann. New York Acad. Sci. 121:404.

Dreizen, P., L.C. Gershman, P.P. Trotta and A. Stracher. 1967. Myosin subunits and their interaction. In The Contractile Process. p. 85. Little, Brown and Company, Boston, Mass.

Ebashi, S., 1963. Third component participating in the superprecipitation of "Natural Actomyosin." Nature 200:1010.

Ebashi, S. 1966. Structural proteins controlling the interaction between actin and myosin. In Progressive Muskeldystrophie·Myotonie· Myasthenie. Ed. Erich Kuhn. p. 506. Springer-Verlag, Berlin· Heidelberg.

Ebashi, S. and F. Ebashi. 1964. A new protein component participating in the superprecipitation of myosin B. J. Biochem. 55:604.

Ebashi, S. and F. Ebashi. 1965. α-Actinin, a new structural protein fron striated muscle. I. Preparation and action on actomyosin-ATP interaction. J. Biochem. 58:7.

Ebashi, S. and A. Kodama. 1965. A new protein factor promoting aggregation of tropomyosin. J. Biochem. 58:107.

Ebashi, S. and A. Kodama. 1966. Interaction of tropqnin with F-actin in the presence of tropomyosin. J. Biochem. 59:425.

Ebashi, S., and K. Maruyama. 1965. Preparation and some properties of α-actinin-free actin. J. Biochem. 58:20.

Endo, M., Y. Nonomura, T. Masaki, I. Oshtuki, and S. Ebashi. 1966. Localization of native tropomyosin in relation to striation patterns. J. Biochem. 60:605.

Evans, J.B. and C.F. Niven, Jr. 1960. Microbiology of Meat. Bacteriology. In The Science of Meat and Meat Products. Ed. American Meat Institute Foundation. p. 151. W. H. Freeman and Company, San Francisco, Calif.

Frazier, W.C. 1958. Food Microbiology. McGraw-Hill Book Company, Inc., New York, N.Y.

Fujimaki, M., A. Okitani, and N. Arakawa. 1965. The changes of "myosin B" during storage of rabbit muscle. Part I. Physico-chemical studies on "myosin B." Agr. Biol. Chem. 29:581.

Gifford, G.T., and L. Yuknis. 1965. Protein patterns in human parotid saliva. J. Chromatography 20:150.

Goll, D., W.F.H.M. Mommaerts, and K. Seraydarian. 1967. Is α-actinin a constituent of the Z-band of the muscle fibril? Fed. Proc. 26:499.

Goll, D.E., and R.M. Robson, 1967. Molecular properties of post-mortem muscle. I. Myofibrillar nucleosidetriphosphatase activity of bovine muscle. J. Food Sci. 32:323.

Guba, F. and F. B. Straub. 1943. On the specificity of the ATP effect. Studies Inst. Med. Chem., Univ. Szeged 3:49.

Haga, T., K. Maruyama and H. Noda, 1965. Formation of actomyosin during the extraction of muscle mince with Weber-Edsall solution. Biochim. Biophys. Acta 94:226.

Hagdahl, L., 1964. On the use of coupled columns in chromatography. Science Tools 1:21.

Hama, H., K. Maruyama, and H. Noda, 1965. Natural F-actin. I. Direct isolation of F-actin from myofibrils and its physico-chemical properties. Biochim. Biophys. Acta 102:249.

Hamoir, G. 1951. Fish tropomyosin and fish nucleotropomyosin. Biochem. J. 48:129.

Hamoir, G., and L. Laszt. 1962. La tropomyosine B de carotides de bovide. Biochim. Biophys. Acta 59:365.

Hanson, J., and H.E. Huxley, 1953. Structural basis of the cross-striations in muscle. Nature 172:530.

Hanson, J., and H.E. Huxley. 1955. The structural basis of contraction in striated muscle. Sympos. Soc. Exptl. Biol. 9:228.

Hanson, J., and H.E. Huxley. 1957. Quantitative studies on the structure of cross-striated myofibrils. II. Investigations by biochemical techniques. Biochim. Biophys. Acta 23:250.

Hanson, J., and J. Lowy, 1963. Structure of F-actin and of actin filaments isolated from muscle. J. Mol. Biol. 6:46.

Hanson, J., and J. Lowy. 1964. The structure of actin. In Biochemistry of Muscle Contraction. Ed. J. Gergely. p. 141. Little, Brown and Company, Boston, Mass.

Harris, M., and C.H. Suelter, 1967. A simple chromatographic procedure for the preparation of rabbit-muscle myosin A free from AMP deaminase. Biochim. Biophys. Acta 133:393.

Hartshorne, D.J., S.V. Perry, and V. Davies. 1966. A factor inhibiting the adenosine triphosphatase activity and the superprecipitation of actomyosin. Nature 209:1352.

Hartshorne, D.J., S.V. Perry, and M.C. Schaub. 1967. A protein factor inhibiting the magnesium-activated adenosine triphosphatase of desensitized actomyosin. Biochem. J. 104:907.

Hasselbach, W., 1953. Elektronmikroskopische Untersuchungen an Muskelfibrillen bei totaler und partieller Extraktion des L-Myosins. Z. Naturforsch. 8B:449.

Hasselbach, W., and G. Schneider, 1951. L-Myosin and actin contents of rabbit muscle. Biochem. Z. 321:462.

Hayashi, T., 1967. Reactivities of actin as a contractile protein. In The Contractile Process. p. 119. Little, Brown and Company, Boston, Mass.

Hegarty, G.R., L.J. Bratzler and A.M. Pearson. 1963. The relationship of some intracellular protein characteristics to beef muscle tenderness. J. Food Sci. 28:525.

Holtzer, A., R. Clark and S. Lowey. 1965. The conformation of native and denatured tropomyosin. Biochem. 4:2401.

Huxley, H.E., 1953. X-ray analysis and the problem of muscle. Proc. Roy. Soc. London. Ser. B. 141:59.

Huxley, H.E. 1957. The double array of filaments in cross-striated muscle. J. Biophys. Biochem. Cytol. 3:631.

Huxley, H.E. 1960. Muscle cells. In The Cell. Eds. J. Bracket and A. E. Mirsky. Vol. 4, p. 365. Academic Press, New York, N.Y.

Huxley, H.E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J. Mol. Biol. 7:281.

Huxley, H.E. 1965. Structural evidence concerning the mechanism of contraction in striated muscle. In Muscle. Eds. W.M. Paul, E.E. Daniel, C.M. Kay, and G. Monckton. p. 29. Pergamon Press Ltd., Oxford, England.

Huxley, H.E. 1967. Recent X-ray diffraction and electron microscope studies of striated muscle. In The Contractile Process. p. 71. Little, Brown and Company, Boston, Mass.

Huxley, H.E. and J. Hanson. 1957. Quantitative studies on the structure of cross-striated myofibrils. I. Investigations by interference microscopy. Biochim. Biophys. Acta 23:229.

Jay, J.M. 1966. Influence of postmortem conditions on muscle microbiology. In The Physiology and Biochemistry of Muscle as a Food. Eds E.J. Briskey, R.G. Cassens, and J.C. Trautman. p. 387. The University of Wisconsin Press, Madison, Wis.

Jay, J.M. 1967. Nature, characteristics and proteolytic properties of beef spoilage bacteria at low and high temperatures. Appl. Microbiol. 15:943.

Jay, J.M., and K. Kontou. 1967. Fate of free amino acids and nucleotides in spoiling beef. Appl. Microbiol. 15:759.

Johnson, P., and A.J. Rowe. 1964. An ultracentrifuge study of the actin-myosin interaction. In Biochemistry of Muscle Contraction. Ed. J. Gergely. p. 279. Little, Brown and Company, Boston, Mass.

Jolley, W. B., H.W. Allen and O.M. Griffith. 1967. Ultracentrifugation using acrylamide gel. Anal. Biochem. 21:454.

Kasai, M., E. Nakano, and F. Oosawa. 1965. Polymerization of actin free from nucleotides and divalent cations. Biochim. Biophys. Acta 94:494.

Katz, A.M. 1966. Purification and properties of a tropomyosin-containing protein fraction that sensitizes reconstituted actomyosin to calcium-binding agents. J. Biol. Chem. 241:1522.

Knappeis, G.G., and F. Carlson. 1962. Ultra-structure of the Z-disc in skeletal muscle. J. Biophys. and Biochem. Cytol. 13:323.

Kominz, D.R., A. Hough, P. Symonds and K. Laki. 1954. The amino-acid composition of actin, myosin, tropomyosin and the meromyosins. Arch. Biochem. Biophys. 50:148.

Laki, K, and J. Standaert. 1960. The minimal molecular weight of actin estimated with the use of carboxypeptidase A. Arch. Biochem. Biophys. 86:16.

Largier, J.F., and A. Polson. 1964. Estimation of molecular weight of β-glucuronidase from Patella barbara by gel diffusion filtration. Biochim. Biophys. Acta 79:626.

Lawrie, R.A. 1966. Meat Science. Pergamon Press, London, England.

Lerke, P., L. Farber and R. Adams. 1967. Bacteriology of spoilage of fish muscle. IV. Role of protein. Applied Microbiology 15:770.

Levy, H., and M. Fleisher. 1965. Studies on the superprecipitation of actomyosin suspensions as measured by the change in turbidity. I. Effects of adenosine triphosphate concentration and temperature. Biochim. Biophys Acta 100:471.

Lewis, M.S., K. Maruyama, W.R. Carroll, D.R. Kominz and K. Laki. Physical properties and polymerization reactions of native and inactivated G-actin. Biochemistry 2:34.

Maruyama, K.. 1965a. A new protein-factor hindering network formation of F-actin in solution. Biochim. Biophys. Acta 94:208.

Maruyama, K. 1965b. Some physico-chemical properties of β-actinin, "actin-factor," isolated from striated muscle. Biochim. Biophys. Acta 102:542.

Maruyama, K., and S. Ebashi. 1965. ∝-Actinin, a new structural protein from striated muscle. II. Action on actin. J. Biochem. 58:13.

Maruyama, K., H. Hama, and Y. Ishikawa. 1965. Some physico-chemical properties of KI-extracted F-actin. Biochim. Biophys. Acta 94:200.

Mihalyi, E., and A.J. Rowe. 1966. Studies on the extraction of actomyosin from rabbit muscle. Biochem. Z. 345:267.

Mommaerts, W.F.H.M., 1952. The molecular transformations of actin. J. Biol. Chem. 198:445.

Mommaerts, W.F.H.M. 1966. Molecular Alterations in myofibrillar proteins. In Physiology and Biochemistry of Muscle as a Food. Eds. E.J. Briskey, R.G. Cassens and J.C. Trautman. p. 277. Univ. of Wisconsin Press, Madison, Wis.

Morales, M.F., J. Botts, J.J. Blum, and T.L. Hill. 1955. Elementary processes in muscle action: An examination of current concepts. Physiol. Rev. 35:475.

Morita, F., and Y. Tonomura. 1960. Kinetic analysis of the pyrophosphate-myosin B system by the use of the light-scattering method. J. Am. Chem. Soc. 82:5172.

Mueller, H. 1966. EGTA-sensitizing activity and molecular properties of tropomyosin prepared in presence of a sulfhydryl protecting agent. Biochem. Z. 345:300.

Needham, D.M., and J.M. Williams. 1963. Proteins of the uterine contractile mechanism. Biochem. J. 89:552.

Oppenheimer, H., K. Barany, G. Hamoir, and J. Fenton. 1967. Succinylation of myosin. Arch. Biochem. Biophys. 120:108.

Peachey, L.D. 1968. Muscle. Ann. Rev. Physiol. 30:401.

Peacock, A.C., and C.W. Dingman. 1967. Resolution of multiple ribonucleic acid species by polyacrylamide gel electrophoresis. Biochemistry 6:1818.

Pearse, A.G.E. 1960. Histochemistry. Little, Brown and Company, Boston, Mass.

Pepe, F.A. 1966. Some aspects of the structural organization of the myofibril as revealed by antibody-staining methods. J. Cell Biol. 28:505.

Peterson, E.A., and H.A. Sober. 1962. Column chromatography of proteins: Substituted celluloses. In Methods in Enzymology. Eds., S.P. Colowick and N.O. Kaplan. Vol. V, p.3. Academic Press, New York, N.Y.

Perry, S.V. 1951. The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle. Biochem. J. 48:257.

Perry, S.V. 1953. The protein components of the isolated myofibril. Biochem. J. 55:114.

Perry, S.V. 1955. Myosin adenosinetriphosphatase. In Methods in Enzymology. Eds. S.P. Colowick and N.O. Kaplan. Vol. II, p. 582. Academic Press Inc., New York, N.Y.

Perry, S.V. 1965. Muscle proteins in contraction. In Muscle. Eds. W.M. Paul, E.E. Daniel, C.M. Kay, and G. Monckton. p. 29. Pergamon Press Ltd., Oxford, England.

Perry, S.V. 1967a. The structure and interactions of myosin. In Progress in Biophysics and Molecular Biology. Vol. 17, p. 325. Pergamon Press Ltd., Oxford, England.

Perry, S.V. 1967b. Introduction. In The Contractile Process. p. 63. Little, Brown and Company, Boston, Mass.

Perry, S.V. and A. Corsi. 1958. Extraction of proteins other than myosin from the isolated rabbit myofibril. Biochem. J. 68:5.

Perry, S.V., V. Davies and D. Hayter. 1966. "Natural" tropomyosin and the factor sensitizing actomyosin adenosine triphosphatase to ethlenedioxybis (ethyleneamino) tetraacetic acid. Biochem. J. 99:1c.

Perry, S.V. and M. Zydowo. 1959a. The nature of the extra protein fraction from myofibrils of striated muscle. Biochem. J. 71:220.

Perry, S.V. and M. Zydowo. 1959b. A ribonucleoprotein of skeletal muscle and its relation to the myofibril. Biochem. J. 72:682.

Poglazov, B.F. 1966. Structure and Function of Contractile Proteins. Academic Press, New York, N.Y.

Porter, K.R. 1961. The sarcoplasmic reticulum: Its recent history and present status. J. Biophys. Biochem. Cyt. 10:Suppl. p. 219.

Richards, E.G., C.S. Chuny, D.B. Menzel and H.S. Olcott. 1967. Chromatography of myosin on diethylaminoethyl-Sephadex A-50. Biochemistry 6:528.

Richards, E.G., J.A. Coll and W.B. Gratzer. 1965. Disc electrophoresis of ribonucleic acid in polyacrylamide gels. Anal. Biochem. 12:452.

Robertson, J.D. 1957. The cell membrane concept. J. Physiol. 140:58P.

Roettcher, M. and R.W. Straub. 1963. Zur Hemmung der Muskelkontraktion durch Urea. Pfluegers Arch. 277:207.

Rozsa, G., A. Szent-Györgyi and R.W.G. Wyckoff. 1950. The fine structure of myofibrils. Exptl. Cell Res. 1:194.

Schaub, M.C., D.J. Hartshorne, and S.V. Perry. 1967. The adenosine-triphosphatase activity of desensitized actomyosin. Biochem. J. 104:263.

Seifter, S. and P.M. Gallop. 1966. The structure proteins. X. The structural proteins of the myofibril. In The Proteins. Ed. H. Neurath. p. 372. Academic Press Inc., New York, N.Y.

Seraydarian, K., E.J. Briskey and W.F.H.M. Mommaerts. 1967. The modification of actomyosin by α-actinin. I. A survey of experimental conditions. Biochim. Biophys. Acta 133:399.

Small, P.A., W.F. Harrington and W.W. Kielley. 1961. The electrophoretic homogeneity of the myosin subunits. Biochim. Biophys. Acta 49:462.

Snellman, O. and T. Erdös. 1948. Ultracentrifugal analysis of crystallized myosin. Biochim. Biophys. Acta 2:650.

Straub, F.B. 1942. Actin. Studies Inst. Med. Chem., Univ. Szeged. 2:3.

Szent-Györgyi, A. and P. Kaminer. 1963. Metin and Metactomyosin. Proc. Nat. Acad. Sci. U.S. 50:1033.

Szent-Györgyi, A.G. 1951a. The reversible depolymerization of actin by potassium iodide. Arch. Biochem. Biophys. 31:97.

Szent-Györgyi, A.G. 1951b. A new method for the preparation of G-actin. J. Biol. Chem. 192:361.

Szent-Györgyi, A.G., D. Mazia and A. Szent-Györgyi. 1955. On the nature of the cross-striation on body muscle. Biochim. Biophys. Acta 16:339.

Szentkiralyi, E.M. 1961. The binding of H-meromyosin on cross-striated myofibrils. Exptl. Cell Res. 22:18.

Takashina, H. and M. Kasuya. 1967. Inactivation of myosin B by low concentrations of urea. Arch. Biochem. Biophys. 118:475.

Tonomura, Y. and K. Sekiya. 1961. Viscometric study on myosin B solution. Arch. Biochem. Biophys. 94:512.

Tsao, T.C. 1953. Fragmentation of the myosin molecule. Biochim. Biophys. Acta 11:368.

Tsao, T.C., K. Bailey and G.S. Adair. 1951. The size, shape, and aggregation of tropomyosin particles. Biochem. J. 49:27.

von Hippel, P.H., M. Gellert and M.F. Morales. 1957. Some physical and enzymatic phenomena in solutions of myosin B. Conference on the Chemistry of Muscular Contraction. p. 1. Igaku Shoin, Tokyo, Japan.

Warburg, O. and W. Christian, 1941. Isolierung und Kristallisation des Gärungs-ferments Enolase. Biochem. Z. 310:384.

Watanabe, S. and W. Sleator, Jr., 1957. EDTA relaxation of glycerol-treated muscle fibers, and the effects of magnesium, calcium, and manganese ions. Arch. Biochem. Biophys. 68:81.

Watanabe, S. and I. Staphrans, 1966. Purification of the relaxing protein of rabbit skeletal muscle. Proc. Nat. Acad. Sci. U.S. 56:572.

Weber, A. 1956. The ultracentrifugal separation of L-myosin and actin in an actomyosin sol under the influence of ATP. Biochim. Biophys. Acta 19:345.

Weber, A. and S. Winicur, 1961. The role of calcium in the superprecipitation of actomyosin. J. Biol. Chem. 236:3198.

Weber, H.H. 1964. Introduction. In Biochemistry of Muscle Contraction. Ed. J. Gergely. p. 193. Little, Brown and Company, Boston, Mass.

Weiner, P.D. and A.M. Pearson, 1966. Inhibition of rigor mortis by ethlenediamine tetraacetic acid. Proc. Soc. Exp. Biol. Med. 123:185.

Wetlaufer, D.B. and J.T. Edsall, 1960. Sedimentation of myosin in urea solutions. Biochim. Biophys. Acta 43:132.

Woods, E.F. 1965. Peptide chains of tropomyosin. Nature 207:82.

Woods, E.F. 1967. Molecular weight and subunit structure of tropomyosin B. J. Biol. Chem. 242:2859.

Zwann, J. 1965. Detection of protein-bound sulfhydryl groups after agar gel electrophoresis. Anal. Biochem. 15:369.