ELECTROPHORETIC ANALYSIS OF PROTEINS EXTRACTED FROM BOVINE STRIATED MUSCLE

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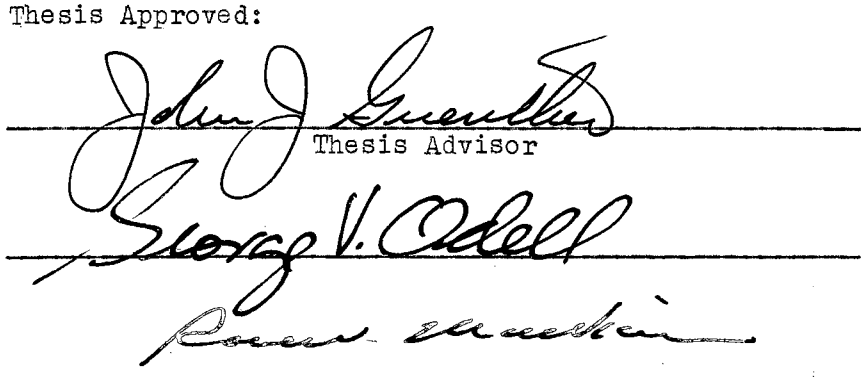

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INTRODUCTION

Protein is a major nutrient essential for human life. The "lean portion" or muscle tissue of domestic "meat" animals provides man with a concentrated source of biologically complete protein. With the exclusion of water, protein is by far the major constituent of the skeletal muscles of our meat animals. Accordingly, it could be assumed that the condition or state of muscle protein is the controlling factor or, at least, is intimately related to the factors that govern meat "quality". The study of the basic characteristics or properties of this muscle protein, however, has appealed to only a very few researchers. The reasons for this may be due, in part, to the lack of suitable analytical equipment and to the complexities involved.

Considerable work has been done with the muscle protein of rats and rabbits. Most of this research, however, was slanted towards medical or physiological problems. Although these results have been extremely useful in forming hypotheses and in drawing broad generalizations, it is firmly believed that significant progress in the production of high quality and durable meat products will be made only when the changes undergone by animal tissue protein from slaughter to consumption are known. It may well be that the answers to such phenomena as the autolytic tenderization effect of "ripening" or "aging" may evolve from a thorough understanding of the reactions of these proteins.

It has become apparent that such qualities of meat as tenderness, juiciness, flavor, etc. are complex entities, yet we are still employing many obscure, collective terms as, "soluble protein", "insoluble protein", "non-protein nitrogen", and the like, as factors with which to correlate organoleptic results. Though there may be a definite place or need for such determinations, the fact remains that no real advancement has been made in the search for an understanding of the basic nature of meat and meat products.

With this in mind, an investigation, designed to elucidate the basic nature and reactivities of the muscle tissue of beef animals, was begun in the Fall of 1960 at the OSU Meat Laboratory.

This thesis presents a portion of the initial results of this study.

REVIEW OF LITERATURE

Most investigations on the proteins of muscle, or more specifically on the proteins of the myofibril have been conducted with the muscle of rabbits and rats. Consequently, much of the present knowledge about muscle proteins of meat animals has evolved from these studies. While the fundamental information obtained from these studies may be applicable to muscle protein of meat animals, it is firmly believed that to make significant progress in the field of "meats", a basic study of the proteins of bovine striated muscle is direly needed.

The studies reviewed here will consider some of the work relative to: (1) The properties and classification of muscle protein; (2) Changes in protein during the agonal and post mortem periods; (3) Methods of fractionation and identification.

I. Properties and classification of muscle protein

The biochemistry and physiology of muscle has been intensively investigated in many laboratories. Yet, the protein constituents of the muscle cell have by no means been precisely characterized. Muscle proteins exist in extremely variable states of dispersion. Certain proteins are freely dissolved in the sarcoplasm, while others impose a rigid structure upon certain parts of the cell. Between these two extremes, there are many intermediate possibilities. While some proteins can be extracted easily with water or saline solutions without fear of modification of their properties, others can be brought into

solution only by very drastic means. Consequently, there is always the possibility of denaturation, which may greatly obscure or influence the true properties (in vitro and in situ) of the protein being investigated.

Since proteins differ broadly in their chemical and physical properties, the proper handling of proteins during fractionation and purification demands considerable knowledge of the properties of the proteins in question.

The historical classification scheme for the simple, unconjugated proteins, was based largely on their solubility behavior. Those proteins soluble in water were termed albumins; those insoluble in water but soluble in low concentration of salt were classified as globulins, histones, etc..

The first serious attempt to partition the proteins of muscle into well defined fractions and to ascertain their concentration was made by Weber and Meyer in 1933. A general type of classification for muscular proteins was suggested by Dubuisson (1954). This classification was based on: (1) specific enzymatic activity, (2) solubility and extractibility and (3) electrokinetic properties.

Springall (1954) divided the proteins of muscle into two main classes, (1) fibrous proteins (albuminoids and schlerins) and (2) globular proteins.

The proteins of the structural material of living animal systems have a fibrous structure, and it is this peculiarity which has given rise to the general class name into which they are commonly grouped. Some of the characteristic properties of the fibrous proteins were described by Springall (1954), who reported that most of these proteins are insoluble in ordinary aqueous solvents. Many of them appear to be

of an indefinite high molecular weight. The fibrous proteins are composed of long, approximately linear molecules arranged roughly parallel to the fiber axis and they are amorphous.

The globular proteins are soluble in water or in aqueous solutions of salts, acid, bases or alcohol. These molecules tend to be round in shape and to coagulate by heat.

A recent classification for muscle protein was given by Szent-Győrgyi (1960), who grouped the proteins of muscle into four major fractions. These fractions are: the sarcoplasmic proteins, the proteins of the granules, the proteins of the myofibril, and the stroma proteins. This grouping is based upon the localization of the proteins within the muscle cell, and to their particular mode of activities. The differences in the solubility of these major fractions is one of the bases of this classification.

A. The Sarcoplasmic Proteins

Frequently referred to as the "soluble proteins" of the muscle, these proteins usually exist in the free state, but may be bound by very weak forces to the proteins responsible for the rigidity of the muscle. The sarcoplasmic (myogen) fraction has been divided by Weber and Meyer (1933) into an albumin and globulin X fraction. The sarcoplasmic proteins which are soluble in dilute salt solutions (about 0.15 M, at neutral or weakly alkaline pH) were called the corpuscular proteins by Bosch (1951). Kronman et al. (1960) indicated that the water soluble group, consisted almost entirely of proteins with purely enzymatic characteristics. The myogen fraction was shown by Zender et al. (1958) to consist of myoalbumin, globulin X and various enzymes, and to be soluble in distilled water or dilute salt solutions

of 0.05 - 0.1 molarity.

Although globulin X has not been intensively studied, Dubuisson (1954) suggested that globulin X had a structural function in the living muscle.

Szent-Györgyi (1960) indicated that the sarcoplasmic proteins can be estimated after simple extraction with water or with neutral salt solutions of low ionic strength ($\frac{\Gamma}{2} < 0.02$). The solution thus obtained had a low viscosity and contained the muscle pigment, myoglobin, and enzymes. This author found that in the striated muscles of adult rabbits or chicks, about 30 percent of the total protein was of sarcoplasmic origin. This was in line with the results of Neurath et al. (1954), who concluded that the sarcoplasmic fraction comprised about 32 to 37 percent of the intracellular protein.

B. The Proteins of the Granules

Szent-Györgyi (1960) found that most of the "granular proteins" together with the sarcoplasmic proteins, could be removed from a well-homogenized muscle by solvents of low ionic strength. This author indicated that differential centrifugation was a convenient way to separate the granule protein fraction from the sarcoplasmic protein fraction, and that the important components of the granule proteins were the nuclei and the sarcomes or mitochondria.

C. The Myofibrillar Proteins

The myofibrillar proteins are frequently denoted as the "structural proteins" of muscle. Also they are often referred to as the "insoluble proteins" -to distinguish them from the "soluble" sarcoplasmic proteins. The use of these terms as a means of differentiating the two protein

classes; however, has led to no small amount of confusion. Hanson et al. (1957) reported that this fraction made up 34 percent of the total protein of the whole muscle.

Bosch (1951) suggested that myofibrillar proteins were composed of interlinked peptide chains, which could not be dissolved unless the peptide bonds were ruptured. He found that extraction with salt solutions of high concentration, e.g., 0.5 M KCl solution of pH 7.5 to 9.5 would cause rupture of the peptide chains.

Kronman et al. (1960) reported that the salt soluble fraction was composed almost entirely of the contractile proteins, actin and myosin. The solubility, electrophoretic mobility and other biochemical methods indicated the presence of a number of different proteins components in muscle extracts with buffers of high ionic strength. The solubility of some of these protein fractions may depend on whether fresh muscle or muscle in rigor was used as starting material (Szent-Györgyi, 1960).

The myofibrillar proteins are numerous and include those entities important in contraction, activities of the nucleus, and in the enzymatic reactions of the cell. In addition, some of these proteins have more than one function in the muscle; for example, the protein myosin is necessary for contraction and also as an enzyme for splitting ATP.

According to Szent-Györgyi (1960), the proteins myosin, actin, and tropomyosin account for about 80 percent of the protein of the myofibril of rabbit skeletal muscle.

Corsi et al. (1958) working with rabbit muscle demonstrated that at pH 7 to 8, and under conditions of low ionic strength, about 35 percent of the total muscle protein could be extracted from the myofibril.

For the extraction of myofibrillar proteins, Szent-Györgyi (1960) stated that neutral salt solutions of high ionic strength ($\frac{\mu}{2} > 0.5$) are required, even though, after extraction, some of them may be soluble at low ionic strength.

D. The Stroma Proteins

This fraction includes those proteins which can not be dissolved or extracted with the usual salt solutions used to extract the other protein fractions. Although proteins of this fraction could be extracted with strong urea or alkali solutions, this procedure often caused the denaturation of the proteins.

Stroma proteins comprise about 20 percent of the muscle proteins, (Hawk et al. 1954). These author's also reported that this fraction consists, in part, of the constituents of cell nuclei and connective tissue.

Szent-Györgyi (1960) showed that the stroma proteins could be obtained as a residue from the prolonged extraction of a well-homogenized muscle with strong salt solutions. This residue contained material of a collagenous nature which contributed to the structure of the sarcolemma and possibly to the Z membrane.

In the following paragraphs of this section are compiled characteristic properties of the myofibrillar proteins either identified or tentatively identified.

Myosin

Myosin is quantitatively perhaps the most important protein of the myofibril. The amount of myosin in the skeletal muscle (as a whole) of the rabbit has been established to be about 8 to 10 percent according

to Szent-Györgyi (1947). Huxley et al. (1957) and Szent-Györgyi (1960) reported that myosin comprised about 50 percent of the total protein of the myofibrils.

Myosin has been known for about a century, having been discovered by Kuhne, who showed that a great amount of this protein could be extracted from muscle by a strong salt solution. Due to its role in muscle contraction, myosin has occupied the attention of many investigators. Szent-Györgyi (1948) and Springall (1954) observed that myosin consisted of thin, enlongated particles of about 2,000 to 4,000 A^{o} in length and about 25 A^{o} in diameter. Using the electron microscope, Bate-Smith (1948) observed that the diameter of the particles in a solution of myosin was approximately the same as that of the ultimate filaments of the muscle fiber, Viz, about 10 mu. Hanson et al. (1957), using comparative measurement by interference microscopy, found that at least four-fifths of the myosin in the muscle fibrils was located in the thick filaments in the A-bands. The intrinsic viscosity study of Greenstein et al. (1940) showed that myosin molecule is highly asymetric. According to the diffusion measurements of Weber et al. (1933), the molecular weight of myosin is 10^{6}. This figure is in agreement with that obtained by the ultracentrifuge measurement of Szent-Györgyi (1947) and the sedimentation rate of Fox et al. (1957). Later Szent-Györgyi (1960) reported that the light scattering measurements obtained intimated the molecular weight of myosin to be in the range of 400,000 to 500,000.

Dubuisson (1954) and Szent-Györgyi (1948) reported that the isoelectric point of myosin was at pH 5.4 and 5.3 respectively. Like

other amphoteric colloids, the myosin molecule particle has at the alkaline side of its isoelectric point a negative charge due to the dissociation of its acidic groups.

Myosin is a hydrophilic colloid, and it freely dissolves in water giving a clear solution. Szent-Györgyi (1947) suggested that although myosin behaves like a globulin, in that it precipitates with $(NH_4)_2SO_4$ as it passes half saturation, it can not be classed as a globulin because it is soluble in water.

Myosin is completely denatured by strong acids, organic solvents, freeze drying or by heat. Myosin has a unique property. Though hydrophilic, it is quantitatively precipitated from its aqueous solution by very small concentrations of neutral salts, such as, KCl. Szent-Györgyi (1947) observed that a very low salt concentration (0.001 M KCl) was sufficient to cause turbidity, and that 0.025 M salt caused complete precipitation. Myosin is very sensitive to the action of heavy metals and it strongly binds and accumulates them, undergoing denaturation in the process. This is an important consideration in working with myosin. Metal-free water, distilled from glass vessels through glass condenser must be used, also the salts used for the extraction of myosin in high concentration should be of high purity. Potassium chloride has been preferred as the neutral salt in this work, but its action is not specific.

To bring myosin from striated muscle into solution, Szent-Györgyi (1947) indicated that KCl must be present in at least 0.5 M concentration. In later work, he (Szent-Györgyi, 1960) reported that myosin is readily soluble in salt solutions of ionic strength higher than 0.3. Once the myosin is dissolved and the myosin polymer broken into smaller units,

it is soluble even in 0.1 M KCl. To precipitate myosin from its solution, the salt must be diluted to an ionic strength below 0.2 (Szent-Györgyi, 1960).

In the crystallization of myosin, myosin is first dissolved in a strong salt solution and then precipitated by a process of dilution. Thus any excess of salt during extraction involves additional dilution and consequently there will be more loss of myosin during the process of crystallization.

Some of the proteolytic enzymes change the properties of myosin in a rather unique fashion. Szent-Györgyi (1953) found, that the addition of trypsin, very quickly lowered the viscosity of myosin solution without reducing its ATPase activity. This lowering of viscosity indicated that the myosin molecules were divided into smaller particles. Also, Fox et al. (1957) reported that urea apparently had both an unfolding and a disaggregating influence on myosin molecule.

Szent-Györgyi (1953) found that the myosin molecule was composed of six small Meromyosin molecules. Two of these subunit molecules were thicker, 30 A^{o} wide, 435 A^{o} long and sedimented faster, with a molecular weight of 230,000. These were called "heavy" or "H" meromyosin. Others were slender, 17 A^{o} wide, 550 A^{o} long, had a molecular weight of 96,000, and were called "light" or "L" meromyosin. The same author in 1955 showed that the "H" meromyosin had the full ATPase activity of the whole myosin molecule and interacted with actin, while the "L" meromyosin seemed to be primarily involved in shortening the "myosin - actin complex".

Dubuisson (1954) found that myosin could exist in at least three states of folding: (1) α myosin - the half folded state in the resting

muscle, (2) B-myosin - the fully extended state in the stretched muscle, and (3) the supercontracted or intermediate state. Also, he reported that the composition of the myosin from different species of animals (rabbit, dog, fish, lobster) seemed to be the same.

Actin

Actin is the second major structural protein of the muscle. Straub (1942), was the first to isolate and characterize this protein. Huxley (1960) wrote that actin was located in the thin filaments of both the A and I bands of the fibril. Its quantity was estimated to be 20 to 25 percent of the fibrillar protein.

Actin is a globular colloid, is readily denatured by heat and its isoelectric point, according to Szent-Györgyi (1947), is at pH 4.7. It is characterized by its ability to combine with myosin to give a colloidal system whose state is profoundly affected by ATP, and to exist in globular (G-actin) as well as in fibrous (F-actin) form. G-actin can be readily polymerized to F-actin by addition of $MgCl_2$.

G-actin is freely soluble in water. Its molecular weight as reported by Szent-Györgyi (1947), ranges between 35,000 to 70,000. However, Straub (1942) and Fox et al. (1957), both reported the molecular weight of actin to be 75,000.

Springall (1954), Fox et al. (1957) demonstrated that actin, in a salt free water solution, consisted of approximately spherical molecules of G-actin. On addition of a salt, the G-actin molecules polymerized to form very long, fibrous, giant molecules of F-actin. These fibrous structures showed striations at 54 A° internals - corresponding to the original G-actin monomers.

Though present in resting muscle in the fibrous form, actin can be extracted only in its globular form, and the method of extraction involves the change from the fibrous into the globular state.

Szent-Györgyi (1947, 1948), demonstrated that, to extract actin from striated muscle myosin must be eliminated, its unextracted residue denaturated, and the actin depolymerized. The myosin is first extracted with a strong salt solution. Its residue is then denatured by adding acetone which at the same time, may break the hydrogen bonds linking actin to the structure. This same author also showed that the polymerization of G-actin into F-actin is connected with a sharp rise in viscosity, thus making it possible to follow this process by means of a viscosimeter. Any salt that does not denature actin will cause its polymerization.

The rate of the G-actin - F-actin transformation increases greatly with the actin concentration and is also catalyzed by myosin. In muscle, where the concentration of actin is high and myosin is present, polymerization may be exceedingly fast.

Actomyosin

It is well known that actin and myosin form a complex (actomyosin). The name was first given by Szent-Györgyi.

With the electron microscope, Szent-Györgyi (1953) observed that actomyosin was composed of very long filaments of about 150 A^{o} in diameter. Low temperature favors dissolution and dissociation of the complex, indicating that actomyosin formation is slightly endothermic.

Szent-Györgyi (1947) found that when an aqueous solution of myosin and F-actin was mixed, they united to form the highly viscous actomyosin, also no change in the pH of the solution was noted.

Actomyosin is not a stoichiometric compound. Myosin and actin will unite to form actomyosin in all proportions, but the viscosity as well as other physical properties of these compounds will differ, depending on the relative concentration of the two proteins. In an experiment designed to find the ratio of myosin and actin that gave maximum actomyosin formation, Johnson et al. (1951), using the ultracentrifuge, reported that a myosin-actin ratio of 3:1 resulted in maximum formation of the complex. However, in a similar experiment, when a viscosimeter was used to follow actomyosin formation, these same workers reported a 2:1 ratio of myosin-actin to be best. Szent-Györgyi (1960) stated that the stoichiometry of the combination is around 4 or 5:1. Like F-actin, G-actin also unites with myosin to form G-actomyosin, but the union of these two substances is not accompanied by a rise in viscosity. The viscosity of G-actomyosin is equal to that of myosin.

Actomyosin and ATP

The concentration of ATP in the normal muscle, was estimated to be 0.005 M, (Szent-Györgyi, 1947). Dubuisson (1954) reported that myosin and actin were unable to combine with each other to form actomyosin as long as ATP was present in the solution. Therefore the disappearance of actomyosin in the normal muscle could be explained as a result of the presence of ATP in the muscle.

The effect of ATP in reducing the viscosity of actomyosin solution had been explained by Szent-Györgyi (1947), as a result of actomyosin dissociation in the solution.

Many investigators have used different techniques such as sedimentation rate, birefringence of flow, light scattering and other biophysical methods to explain this phenomenon. Their results have

supported those of Szent-Györgyi.

Weber (1956) found that the high intrinsic viscosity of actomyosin solution was reduced to a value close to that for "L" myosin when ATP was added. He explained his results by assuming that ATP caused the dissociation of the actomyosin into L-myosin and actin. Also he estimated that L-myosin represented 2/3 to 3/4 of the total protein in the solution that he employed.

The concentration of certain salts in the actomyosin solution has been reported to effect the dissociation phenomenon. Using an actomyosin solution which contained 0.1% ATP, Szent-Györgyi (1957) found that if the concentration of KCl was less than 0.02 M or greater than 0.16 M, the actomyosin complex dissociated, while actomyosin precipitated when the concentration of KCl was less than 0.16 M or stronger than 0.02 M. The same author noted that there were differences between F-actomyosin and G-actomyosin dissociation phenomenon. The F-actomyosin was dissociated by ATP at high or low salt concentrations, while G-actomyosin dissociated at any slat concentration.

Tropomyosin

The fibrous character protein "Tropomyosin" was first isolated from the fibrillar fraction of skeletal and cardiac muscle by Bailey (1946). The quantity of tropomyosin in muscle is rather small as compared to that of actin or myosin. Huxley et al. (1960) reported that tropomyosin made up about 11 percent of the muscle fibrils, while Szent-Györgyi (1960) lowered this figure to 5 percent.

The function of this protein is yet unknown, but since tropomyosin is not present in the sarcoplasm, it would appear to be associated with the fibril itself, where it is firmly bound either to the myosin or to

some other structural component (Bailey, 1946). Tropomyosin has many properties in common with myosin. This similarity in properties lead Neurath and Bailey (1954) to state that tropomyosin could be a prototype of myosin.

Two types of tropomyosin, the water soluble and the water insoluble have been recognized by Huxley and Hanson (1960). These authors reported that the water soluble tropomyosin represented a small percentage of the total protein in the muscle. In rabbit skeletal muscle it accounts for about 8 percent of the total protein. The water insoluble tropomyosin, however, was found to account for about one-third of the total muscle protein.

Bailey (1948), indicated that tropomyosin had a molecular weight of 90,000 (in the dimer form) and 53,000 (in the monomer form), at pH 6.3 and 3 respectively. This change in the molecular weight was explained as a result of a depolymerization effect due to the change of pH.

Working with a salt solution extract of fish muscle, Hamoir (1951) isolated two proteins which showed the solubility properties of tropomyosin. Although the two compounds were indistinguishable by electrophoresis, they crystallized in different forms and behaved very differently in the ultracentrifuge. One of these proteins was identical to the tropomyosin isolated by Bailey. The second protein, found to contain pentosenucleic acid, lead Hamoir (1951) to name it (nucleotropomyosin).

Delta Protein (Δ protein)

Using electrophoretic separation as a tool for studying muscle protein, Amberson *et al.* (1957), purportedly encountered a previously

unreported fibrous protein. The protein had an electrophoretic mobility greater than that of myosin and was termed "Delta" protein by these researchers.

The viscosity, flow-birefringence, sedimentation rate and electrophoretic studies conducted by White et al. (1957) showed that delta protein and tropomyosin were very similar in their properties. The two proteins had almost identical electrophoretic mobilities and isoelectric points. Also both were very soluble in distilled water and in salt solutions of low ionic strength. However, delta protein differed from tropomyosin in its ultraviolet absorption spectra. These results lead the same authors to suggested that delta protein was a polymerized form of tropomyosin.

Further work in this field by Amberson et al. (1957) indicated that delta protein formed a complex with myosin, which was designated by the authors as delta-myosin.

Gamma Myosin

The protein gamma myosin or "contraction" was first suggested by Dubuisson (1948). This author found that an apparently new protein with a very low electrophoretic mobility appeared when fatigued muscle or muscle in rigor was extracted with salt solution.

The presence of many other myofibrillar proteins (Metamyosin, X protein, D protein, etc.) have been reported by various investigators (Perry, 1953; Raeber et al. 1955). However, very little basic information about these proteins has been established.

II. Protein alterations during the agonal and post mortem period

Rigor mortis or the period of contraction and "stiffening" of muscle tissue after the death of the animal has long been known to occur. Also,

an increase in meat tenderness after the post rigor stage has been observed. Yet, neither the mechanism of rigor mortis, nor the physical, physiological and biochemical factors involved with this increased tenderness is well understood.

It is clear that different types of enzymes are involved in this post rigor tenderization process. While it is recognized that the glycolytic enzymes which catabolize glycogen into lactic acid in the absence of oxygen are responsible for part of the contraction phase of rigor (Zender et al. 1958), the mechanism by which the autolytic enzymes cause tenderization is not understood (Weinberg et al. 1960). Undoubtedly, these enzymes are effecting a degree of proteolysis, but whether or not this breakdown of muscle protein is completely responsible for increased tenderization is not known.

Protein changes associated with increasing tenderness have been investigated by many researchers. Husaini et al. (1950) used twenty-eight cattle in a study where tenderness was determined at 3 and 15 days post mortem. It was found that muscle hemoglobin had no relation to tenderness at 3 days, but a very significant correlation was observed with tenderness at 15 days, and with the increased tenderness during the 12 days interval. These observations prompted the authors to conclude that muscle plasma played an important role in meat tenderization during the post rigor period. Other researchers (Szent-Györgyi, 1947; Feeney, 1960; Zender et al. 1958) found that after the death of the animal, ATP was gradually decomposed and actomyosin formed accordingly. Consequently, at the end of this period, muscle lost the potential energy necessary for contraction and behaved as a rigid tissue. The disappearance of rigor mortis was suggested to be due to

the release of actin from the protein structure in the muscle (Kronman et al. 1960). Szent-Györgyi (1952) believed that the tenderization process involved the disintegration of the actin filaments. The relation of tenderness to protein alteration during post mortem aging has been investigated by Wierbicki et al. (1954), and from the data presented there seemed to be no doubt that muscle plasma was intimately related to tenderness in meat. However, the authors suggested that the increase in tenderness with post mortem aging could also be related to (a) the dissociation of actomyosin or some similar protein changes which would increase protein extractability and (b) redistribution of ions within the muscle, thus causing an increased hydration and tenderness. This was in agreement with the work of Feeney (1960) and Szent-Györgyi (1947) which showed that the relative concentration of various ions in the muscle plasma was affecting tenderness. Later, Wierbicki et al. (1956) working with beef muscle in an effort to find a more significant relation between tenderness and post mortem changes, suggested that tenderness might be related to the degree of hydration of meat proteins and thus post mortem tenderization might be due to certain ion - protein or protein - protein interactions rather than the classical proteolysis or dissociation of actomyosin.

Using pre- and post rigor muscle extracts for the determination of protein, Weinberg et al. (1960) concluded that the increase in actomyosin and decrease in free myosin in post rigor as compared to pre-rigor extracts was due to the increase in the amount of actin extracted. Since actin contributes to the structure of myofibrils by associating either with itself or with some other protein to form filaments, the increase in post rigor extraction of actin (due to a

dissociation of actin from the myofibrils) might render the muscle more tender by weaking the muscle structure (fibers).

While it is generally accepted that connective tissue has some affect on tenderness, research conducted in recent years indicates that post mortem tenderization involves greater changes in the muscle protein than in the connective tissue.

III. Electrophoretic methods of protein fractionation and identification

Electrophoresis had been defined as "the migration of charged particles of a colloidal solution in an electrical field" (Weinberg et al. 1960). The colloidal molecules of the protein contain electrically charged groups which are considered to be distributed over the surface of the entire molecule. It is these charged molecules in a colloidal solution that migrate toward the poles in an electrical field.

Dubuisson (1954) pointed out that the electrophoresis method was first applied to the separation of blood protein by Hardy in 1899. However, it was not until after Tiselius's work in the 1930's that this method received any substantial attention. According to Block et al. (1958), the first report on the use of paper electrophoresis was that of Konig, which appeared in Portuguese in 1937. The method was modified by Martin and Synge in 1945. Also, they used the terms "ionphoresis" to define processes concerned with the movement in an electrical field of relatively small ions, "electrophoresis" for movement of large molecules and particles and "electrodialysis" for the removal of smaller ions from larger molecules and particles.

It is a well known fact that different ions migrate at different velocities in an electrical field of given strength. This mobility depends on certain factors which Block et al. (1958) and Fox et al. (1957)

have grouped as follows:

A. Those characteristics related to the ion itself; namely, the magnitude of the net charge on the protein ion, size, shape, tendency to dissociate, and amphoteric behavior.

B. Environmental: electrolyte concentration, ionic strength, dielectric properties, chemical properties, pH, temperature, viscosity, and the presence of non-polar molecules which might influence viscosity and dielectric properties of the electrolyte or which may interact to form charged complexes.

C. Character of the applied field, its intensity, purity (presence of alternating-current components), and distribution along the migration path.

The experimental devices which have been utilized in studying the electrophoretic behavior of protein have been categorized by Fox et al. (1957).

A. Direct observation: This method is applicable to particles which can be seen with the aid of a microscope; that is, gross suspensions. Migration is carried out in a suitable cell mounted on the stage of a microscope and the velocity of the particles is determined by direct timing.

B. Zone electrophoresis: This is a process of migration in an electric field in solutions that are stabilized from convection by some supporting medium. Because of the freedom from convection, ions of different mobilities travel as separate

zones (Cowgill et al. 1957).

C. Moving boundary electrophoresis (Paper Electrophoresis): The migration of ions in solution in an electrical field at a rate and direction which are primarily a function of the ionic charge (Cowgill et al. 1957).

Paper electrophoresis has occupied a position of primary importance in the study of proteins. This important analytical technique can provide both a qualitative and quantitative analysis of the composition of protein mixtures. In addition, electrophoresis is a sorting process; for it enables the separation of closely related proteins which are not readily separable by the more conventional fractional precipitation techniques. Bailey (1942) and Neuberger (1958) reported that the electrophoretic method of investigation of the homogeneity of a protein, with respect to its electrical charge, was often more sensitive than the ultracentrifugal method of measuring the homogeneity, with respect to particle weight. This method was also used to ascertain the purity of protein preparations (Bonner, 1950) and to determine a proteins isoelectric point (Dubuisson, 1954). The interaction of proteins with each other and with smaller ions was detected by this method (Block et al. 1958; Fox et al. 1957).

Electrophoresis of Muscle Protein

Due to the fact that the area and electrophoretic mobility of each protein component are dependent upon the species of the animal, kind of muscle, condition of muscle, extraction and electrophoresis conditions (pH, ionic strength, buffer -- etc.), most of the protein components of a mixture have not been precisely identified as to exact location on a paper strip. Consequently, workers in this field have used letters or

numbers to identify particular protein components.

Bate-Smith (1940) used 0.05 M phosphate buffer pH 7.0 for the extraction of water soluble proteins of rabbit muscle. Two distinct boundaries, with electrophoretic mobilities of 5.3 and 1.6 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} appeared. It was noticed that these respective fractions corresponded, property-wise, to myoalbumin and myogen. It was also found that the second boundary was definitely not homogeneous.

The dark and light skeletal muscles of rabbits were used in electrophoretic studies by Amberson et al. (1949). Protein was extracted with 0.1 M potassium phosphate buffer ($\frac{\Gamma}{2}$ = .28 at pH 7.6). Four electrophoretic components, A, B, C and D were observed in the electrophoretic patterns of the protein extracted from the dark muscles, while three components, A, B and D were obtained from the light muscle extractions. These components were identified by the author's as:

Component D: myoalbumin-

average mobility of 5.64 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} (4.51 - 6.12)

Component C: myosin-

average mobility of 3.66 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} (3.33 - 3.86)

Component B: myoglobin-

average mobility of 2.80 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} (2.59 - 3.10)

Component A: unidentified-

average mobility of 1.92 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} (1.75 - 2.26)

In later work, using the same pH, ionic strength and buffer, White et al. (1957) found that the average values for 72 determinations which were electrophoresed for the same period of time (6 hrs.) were 4.6 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} for myoalbumin and 3.1 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} for myosin. However, when electrophoretic analysis time was extended to

12-48 hours, component D split into two or occasionally three peaks. These workers believed that one of these peaks was probably tropomyosin, while the other was delta protein.

The electrophoretic study of myosin isolated from rabbit muscle by Ziff and Moore (1944) showed that myosin consisted of only one component. Also, they found that the mobilities of myosin were 2.60, 2.79, 2.94 and 3.10 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, when phosphate buffers at pH 6.2 and borate buffers at pH 7.4, 7.8 and 8.6 respectively, were used for the electrophoretic separations. This was in agreement with the work of Spicer et al. (1951), who observed that myosin was electrophoretically homogeneous, while actin consisted of several components.

The protein solution obtained by extraction of myofibrils isolated from fresh muscle with low ionic strength buffer (0.25 M KCl, 0.05 M sodium phosphate - pH 6.8) was analyzed by Perry et al. (1958). Electrophoresis was carried out in 0.25 M KCl, containing 0.05 M phosphate buffer at pH 6.8. Three components A, B and C, in order of decreasing mobility appeared on the electrogram. Component A, with an average mobility of 3.8 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, appeared as a symmetrical peak and contained about 10-12 percent of the total nitrogen of the myofibril. This component was established to be tropomyosin. Component B, with an average mobility of 3.1 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} represented about 15-20 percent of the total myofibril protein. This component was thought to be a type of irreversibly inactivated actin in the depolymerized form. The C component was not identified, but had an average mobility of 1.2 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} and was estimated to represent about 5 percent of the total soluble protein fraction.

Jacob (1947) grouped the seven electrophoretic patterns obtained from rabbit muscle extracted with a NaH_2PO_4 — Na_2HPO_4 — 0.05 M NaCl buffer of ionic strength 0.15 and pH 7.6 into three groups. Group I, accounted on the average for 72 percent of the refractive increment of the extracts and consisted of those proteins with the highest isoelectric points. The isoelectric points ranged from pH 6.0 to 6.75. Group II, included 16 percent of the refractive increment and had isoelectric points in the region of pH 5.0 to 5.5. Group III, represented 5 to 8.6 percent of the refractive increment and had considerable anodic mobility. Bosch (1951), working with rabbit muscle, reported that a KCl - phosphate solution of pH 7.5 and ionic strength of 0.13 had the most adequate properties for extraction of the muscle proteins of the myogen-globulin-X group. He observed nine electrophoretic patterns, of which eight occurred in concentrations sufficiently high to permit their numerical evaluation from the diagrams.

Working with the water soluble proteins of bovine skeletal muscle, Kronman et al. (1960) obtained patterns similar to those reported by Jacob (1947). Also, he observed great variation in the relative amounts of these components from muscle to muscle and from animal to animal. Finally, no systematic variation was observed.

Systematic electrophoresis analysis were carried out by Zender et al. (1958) on the proteins extracted from rabbit and lamb muscle by 1 M glycine buffer solution at pH 8.6. Muscle samples were stored for 100 days at 25° C. and 38° C. and tested at certain intervals. The electrograms thus obtained showed great differences. One protein constituent was noticed to have a very high electrophoretic mobility. The workers suggested that during the early days of storage, the

protein split into subunits of low molecular weight and later in storage, to amino acids. In a subsequent experiment, Longissimus dorsi muscle from 18 month old choice steer calves to study post mortem and freezing changes, Kronman et al. (1960) found that the protein concentration in their extracted solutions were 1.3, 1.2 and 1.1 gram/100 ml in fresh, aged and frozen muscle respectively. The electrophoretic analysis showed no consistent change in the relative amounts of each protein component after aging and freezing. Proteins having mobilities in excess of 50 x 10^{-6} were found. Aging or freezing resulted in a loss of protein components having mobilities in excess of 65 x 10^{-6}. This effect was particularly pronounced in the case of the frozen samples, where components having mobilities in excess of 70 x 10^{-6} were completely absent. These losses were accompanied by an increase in the amount of material of lower mobility. The researches concluded that a maximum of 30 percent of the soluble protein might have been converted to amino acids and low molecular weight peptides upon freezing and aging.

EXPERIMENTAL PROCEDURE

Material

The experimental material used in this study was obtained from the Longissimus dorsi muscle of the beef carcass (1st through 4th lumbar vertebrae section). The relative ease and shortness of time required to dissect this muscle from the stunned animal, together with the fact that the Longissimus dorsi is purported to be the major "quality" muscle of the carcass were the major reasons why this particular sample was selected as the basic material for this study.

Sample Preparation and Extraction

All preparatory and extraction operations were performed in the cold room (Ca. 2° C.). Samples were trimmed of external fat and epimysial connective tissue, diced and transferred to a Waring blender, which had been previously cleaned and thoroughly washed in glass distilled water. Muscle tissue was then blended for 4 minutes into a "homogeneous paste". Sub-samples of this homogenate were then transferred to an Omni-mixer and extracted with the desired buffer for 5 minutes at 14,000 R.P.M.. The material was then transferred to 50 ml graduated tubes and centrifuged for 30 minutes at 2200 x g. The resulting supernatants were then filtered through Whatman No. 1 filter paper, collected in test tubes and stored at 1° C. until electrophoresis could be accomplished.

Electrophoretic Analysis

Samples were electrophoresed in a Durrum Ridgepole Electrophoresis cell (Spinco Model R apparatus), in a cold room at 4° C.. The electrophoresis procedures were essentially as outlined in the instruction manual supplied with this apparatus. Prior experimentation, revealed that approximately 16 hours were required for best protein separation on the paper strips. The instrument's power supply controls were monitored at certain intervals throughout the electrophoresis period. The initial and final voltage and current potentials were recorded. The initial current was adjusted to 10 MA, where possible. However, since electrophoresis buffer pH ranged from 5 to 9, such a milliampere reading could not always be attained.

The paper strips were processed and developed according to the procedures outlined in table I.

Immediately prior to scanning, the paper strips were passed over amonium hydroxide vapor to develop maximum color. After developing, the paper strips were individually transferred to the Spinco Model RB analytrol, the primary purpose of which was to evaluate the stained material separated electrophoretically along the strips. In achieving this, the analytrol combined two function, that of a calibrated recording densitometer and that of an automatic integrator. The densitometer measured the amount of light absorbed by the material distributed along the paper strips and simultaneously recorded this information as a curve on a chart. At the same time, the integrator automatically produced saw-tooth patterns which represented the area under the curve immediately above. One saw-tooth, (that is, one unit of integration) was equal to 0.1 sq. cm..

TABLE I

PROCEDURE FOR PROCESSING AND DEVELOPING THE PAPER ELECTROPHORESIS STRIPS

Strips from electrophoresis cell

First drying	:	Oven 110-120° C.	30-60 minutes
Pre-rinse	:	95% Ethanol	6 minutes
Stain	:	0.1% Bromphenol Blue in Ethanol	20 minutes
First rinse	:	5% Glacial Acetic Acid	6 minutes
Second rinse	:	5% Glacial Acetic Acid	6 minutes
Third rinse	:	5% Glacial Acetic Acid + 5% Sodium Acetate	4 minutes
Final drying	:	Oven 110-120° C.	30 minutes

Store for scanning

From the integrated curves so produced, the percent and mobility of the particular protein fraction separated were calculated. The percent of each protein fraction was calculated as a portion of the total protein material separated. The electrophoretic mobility, "u", of each fraction was determined as the velocity, V, measured under standard conditions; that is, per unit field, X, expressed as volts per cm.. The mobility of each protein fraction was calculated independent of the other fractions separated on the particular strip.

Other Determinations

The protein content of the muscle extracts was determined by means of the macro-Kjeldahl technique. Experimentation revealed that 1:4 muscle to buffer ratio provided adequate amounts of protein in the extracts for suitable electrophoretic separation.

Effect of pH of Extraction Buffer

To study the effect of pH of extraction buffer, and in order to obtain the best extraction pH, 0.1 M potassium phosphate buffer solutions were used. The different buffer pH's used were: pH 2, 3, 4, 5, 6, 6.5, 7, 7.5 and 8.6. In addition 0.1 M potassium phosphate and glycine buffers at pH 7.5 were tested as extraction buffers.

Effect of Type and pH of Electrophoresis Buffer

The buffers used in this portion of the study consisted of solutions of diethyl barbituric acid, sodium-diethyl barbiturate, potassium phosphate and glycine. Finally, the effect of 0.1 M glycine buffer were studied at pH's of: 5, 6, 6.5, 7, 7.5, 8, 8.6 and 9.

Post Mortem Changes in Muscle Protein

The experimental material used in this phase of the experiment was obtained from the Longissimus dorsi muscle of a 5 year old, Commercial grade, Hereford cow. The cow was stunned, bled and the Longissimus dorsi muscle from the left side was dissected, immediately chilled in a crushed ice bath and removed to the cold room. The operation required approximately 4 minutes.

A 100 gram sample was removed from the muscle and prepared and homogenized as stated above. A 40 gram sample of the homogeneous paste thus obtained was extracted with a high ionic strength dibasic potassium phosphate buffer, (pH 7.0, in 0.5 M potassium chloride, total ionic strength 0.55). This extract was further treated exactly as indicated in table II.

Then, a second 40 gram sample of the original homogeneous paste was extracted with a low ionic strength dibasic potassium phosphate buffer, (pH 7.0, ionic strength 0.10). The extract was further treated as indicated in table III.

The remaining portion of the Longissimus dorsi muscle was then stored in the aging room (34° F.) and tested as outlined above after 2 and 14 days aging.

The pH of the fresh, 2 and 14 days aged muscle was also determined.

Finally, electrophoretic patterns were obtained on solutions of commercially purified collagen, elastin, myoglobin and alpha globulin.

TABLE II

METHOD FOR THE PREPARATION OF HIGH IONIC STRENGTH EXTRACTS OF BOVINE LONGISSIMUS DORSI MUSCLE

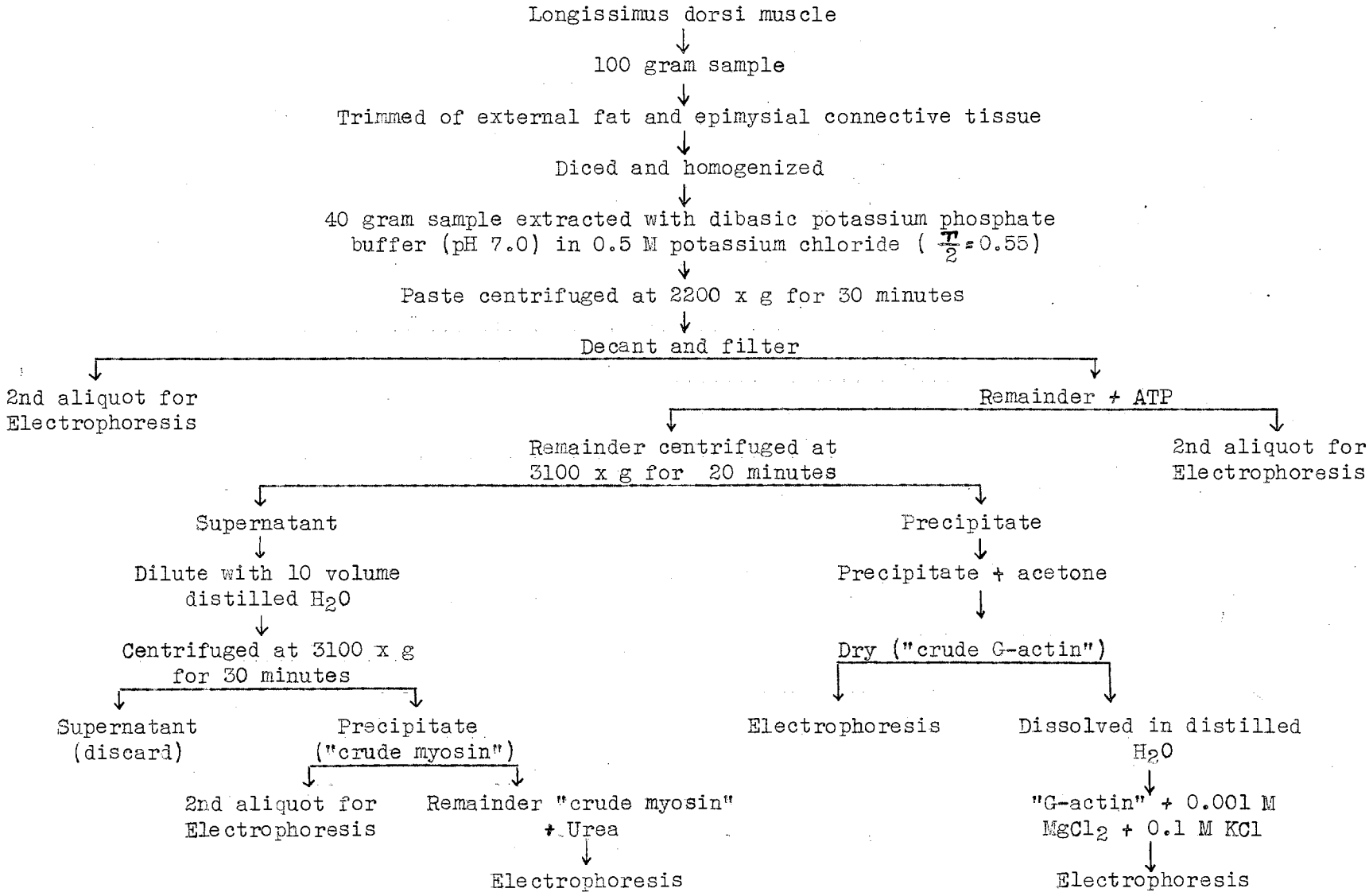


TABLE III

METHOD FOR THE PREPARATION OF LOW IONIC STRENGTH EXTRACTS OF BOVINE LONGISSIMUS DORSI MUSCLE

Longissimus dorsi muscle

↓

100 gram sample

↓

Trimmed of external fat and epimysial connective tissue

↓

Diced and homogenized

↓

40 gram sample extracted with dibasic potassium phosphate buffer (pH 7.0) at 0.1 ionic strength

↓

Paste centrifuged at 2200 x g for 30 minutes

↓

Decant and filter

↓

Electrophoresis

RESULTS AND DISCUSSION

The results and discussion section are presented in four parts as follows:

I. The effect of pH of extraction buffer on muscle proteins extracted and their resulting electrophoretic separations.

II. The effect of type and pH of electrophoresis buffer on protein separation.

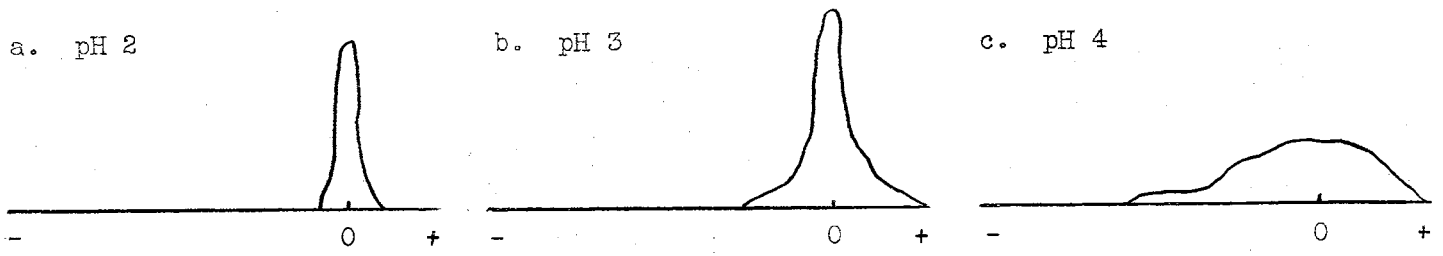
III. Post mortem changes in muscle proteins extracted with high and low ionic strength buffers.

IV. Identification of some electrophoretically separated proteins.

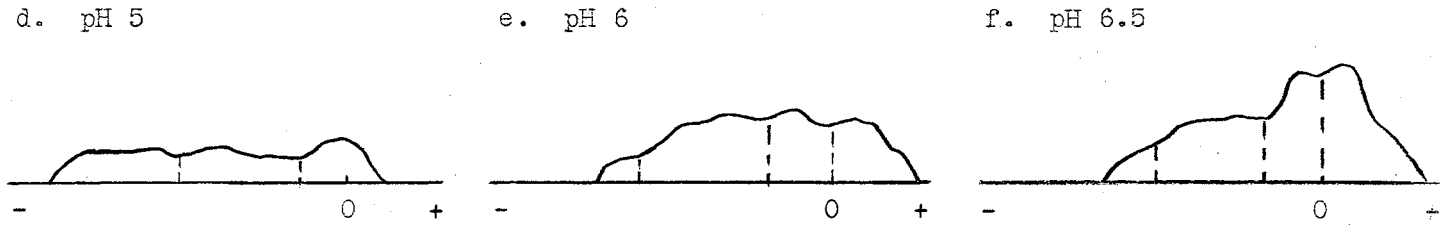
I. The Effect of Buffer pH on the Extraction of Muscle Proteins

The electrophoretic patterns of the soluble proteins extracted from Longissimus dorsi muscle with 0.1 M potassium phosphate buffer of various hydrogen ion concentrations are shown in figures 1 a, b, c, d, e, f, g, h, and i. These extracts were electrophoresed for approximately 16 hours against a 1 M glycine buffer, pH 8.6.

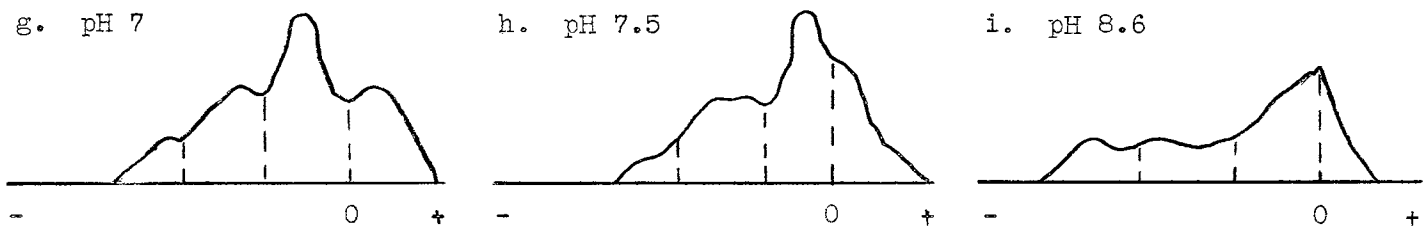
A single, non-mobile component was obtained with the pH 2 extraction buffer (figure 1 a). Similar protein separations were obtained with extraction buffers of pH 3 and 4 respectively (figures 1 b and c). There was however, a trailing out of this single component, both to the negative and positive electrodes. The curve for the pH 3 extracted



non-mobile non-mobile



Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$								
	4.73	2.80		3.76	3.03	.97	1.45	3.40	2.67	.85	1.82
Relative area %	31.60	36.80	31.60	5.70	42.80	24.30	27.20	5.80	32.00	30.00	32.20



Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$												
	3.76	2.67	1.33	1.45	3.40	2.42	1.09	1.7	4.49	3.03	1.33	.85
Relative area %	6.60	27.10	39.10	27.20	4.90	33.20	35.80	26.10	18.70	21.90	43.7	15.6

Figure 1 - Typical electrophoretic pattern obtained from extracts of Longissimus dorsi muscle when electrophoresed for approximately 16 hours with 1 M glycine pH 8.6.

Extraction buffer used: 0.1 M potassium phosphate

protein showed extreme positive kurtosis, while that for the pH 4 extraction exhibited a negative kurtose curve.

When the muscle sample was extracted with pH 5 phosphate buffer, a somewhat different separation was observed (figure 1 d). While only one non-mobile peak appeared with pH 2, 3 and 4 buffers, three components were obtained with the pH 5 buffer. The non-mobile component occupied 31.60% of the total area and the two other mobile fractions accounted for 36.80% and 31.60% of the total area.

Figures 1 e and 1 f, respectively, show the electrograms obtained when the same buffer at pH 6 and 6.5 was utilized for extraction. Here, an additional component, or a total of four protein fractions were observed. Three of the four components moved toward the negative pole, while the fourth component moved toward the positive pole. The pattern obtained with the pH 6.5 buffer showed essentially the same four components, however, a much clearer partition was observed with this buffer than with the pH 6 extraction buffer. However, the relative magnitudes and mobilities of these fractions were approximately the same.

The most definite and clearest separation of the extracted proteins (under experimental conditions employed) were observed when the pH of the extraction buffer was adjusted to 7 (figure 1 g).

The electrograms of the proteins extracted with the 7.5 and 8.6 buffers were similar in that four components were obtained (figures 1 h and i), however, a less distinct separation was noted between the negatively charged component and the slowest moving component of the positively charged groups. The precise bounderies between the four peaks obtained at pH 8.6 were even less specific than those at pH 7.5.

Similar electrophoretic patterns were obtained when 0.1 M glycine buffer of pH 7 was used for extraction as were obtained with the 0.1 M

dibasic potassium phosphate buffer of pH 7, when other conditions of extraction and electrophoresis were held constant.

These results show that the pH of extraction buffer had a profound effect on extraction of muscle proteins.

At pH s of 5 and below the muscle proteins were quite insoluble in the extraction buffer. Also, it can be seen that the proteins extracted were, for the most part, non-mobile and were probably denatured during the extraction process.

As the buffer pH was increased to 6 and above, the muscle proteins apparently were more soluble. Hence, more proteins were extracted and their respective separations on the paper strips were much more distinct.

Patterns for extractions above pH 7.5 also showed a tendency towards decreased solubility and lack of response to electrophoretic separation.

II. The Effect of Type and pH of Electrophoresis Buffer on Protein Separation

Preliminary investigation indicated that the type or nature of electrophoresis buffer employed might have very significant effects on the separation, mobilities, etc. of the muscle proteins applied to the paper electrophoresis strips. In order to verify this, and to select the type of buffer that would give optimum separation of the soluble muscle proteins, a sample of the Longissimus dorsi muscle was extracted with 0.1 M dibasic potassium phosphate buffer (pH 7.0), and electrophoresed against buffer solutions of, glycine, "B^2" (mixture of diethyl barbituric acid and sodium diethyl barbiturate), monobasic potassium phosphate and a mixture of: sodium, 5,5-diethyl barbiturate, sodium acetate, KCl and acetic acid.

The results of these determinations are shown in figure 2 a, b, c and d. These electrograms indicated that the best separation of the

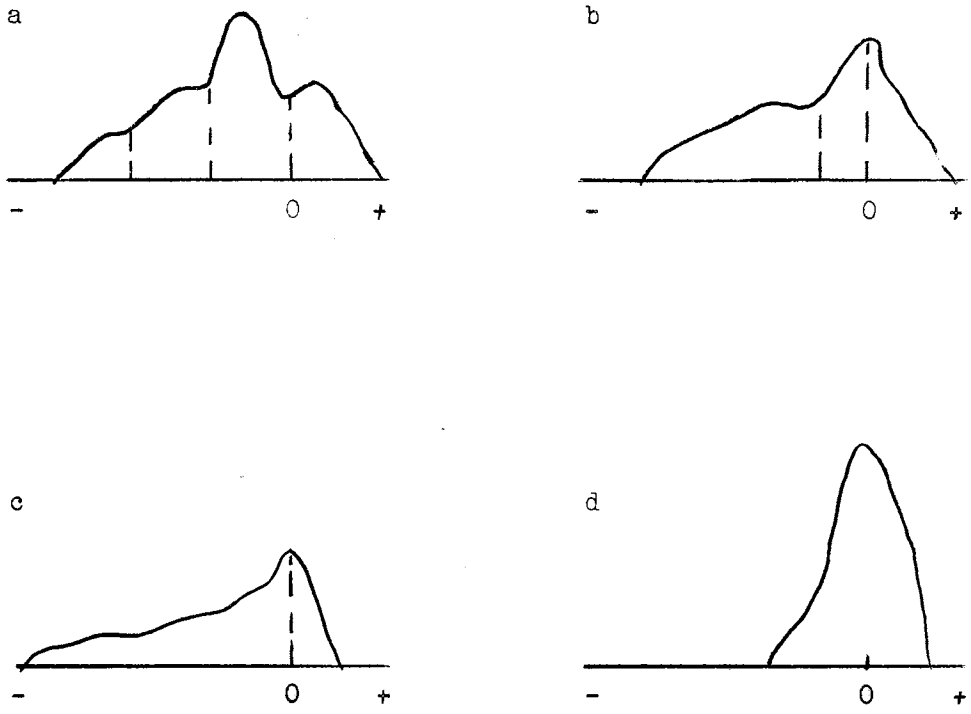


Figure 2 - Typical electrophoretic pattern secured from extracts of Longissimus dorsi muscle with 0.1 M dibasic potassium phosphate buffer pH 7.0 for approximately 16 hours.

Electrophoresis buffer:

a. 1 M glycine pH 8.6

b. $\frac{\mu}{2}$ = 0.075 "B_2" (mixture of diethyl barbituric acid and sodium diethyl barbiturate pH 8.6

c. .025 M monobasic potassium phosphate pH 5

d. 0.5 M (mixture of sodium, 5,5-diethyl barbiturate, sodium acetate, KCl and acetic acid) pH 7.4

soluble proteins was obtained when the extractions were electrophoresed against glycine buffer. This was probably due to the low electric conductivity of the glycine buffer, which permitted the use of the necessary field strength for proper separation of the protein material, thus eliminating excessive heat production and possible protein denaturation.

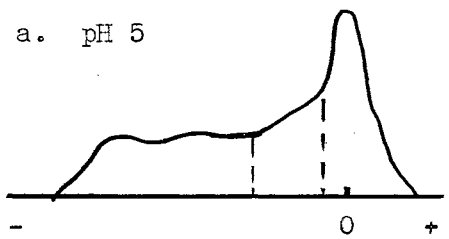
The glycine buffer, therefore, was used as the electrophoresing buffer in all subsequent tests.

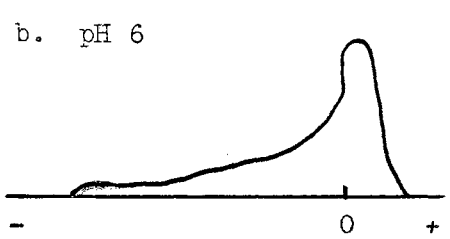
In figure 3 a, b, c, d, e, f, g and h, are presented typical separations obtained from muscle extracts electrophoresed against 1 M glycine buffer at hydrogen ion concentrations of 5, 6, 6.5, 7, 7.5, 8, 8.6 and 9 respectively. These patterns illustrate the effect of pH on the number, relative area, definement and mobility of the protein fractions separated.

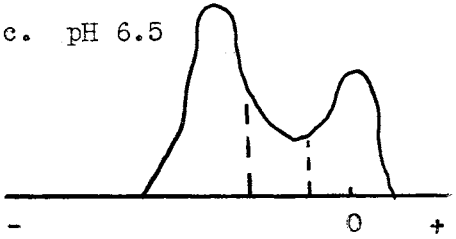
In figure 3 a, b, c and d, the patterns obtained were quite variable with respect to number of components, percentage of each component and mobility. There was however, a rather consistent non-mobile or denatured fraction occurring in each of these patterns. The number and definition of the mobile components or fractions showed no consistent trend with buffer hydrogen ion concentration.

However, as the pH was increased from 7.5 to 8.6 their appeared to be a fairly consistent trend in the number of fractions separated (figure 3 e, f and g). The 8.6 buffer gave the best separation as evidenced by peak definition, both on the paper strips and curves.

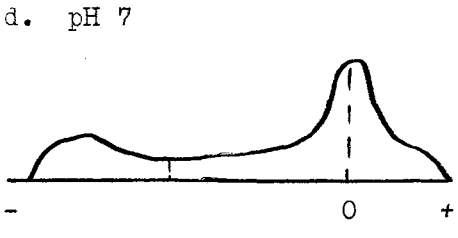
When the extracted material was electrophoresed at pH 9, there was an apparent loss of one of the positively charged fractions and also a decided loss in mobility.

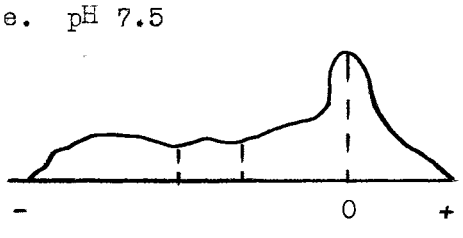


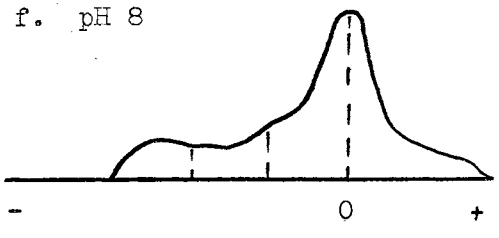




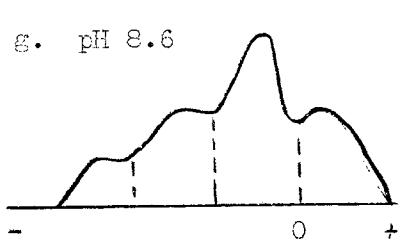
Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	3.41	1.16	.80	
Relative area %	40.40	25.00	34.60	
Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	2.15			
Relative area %	100.0			
Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	1.87	.93		
Relative area %	48.78	19.51	31.71	

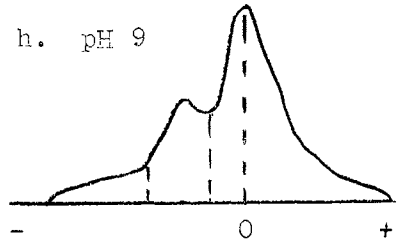






Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	3.90	2.50	1.30		
Relative area %	30.0	37.5	32.5		
Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	3.91	2.0	1.27	1.36	
Relative area %	29.29	11.63	31.81	27.27	
Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	2.50	1.66	.83	1.58	
Relative area %	11.20	14.40	33.40	41.00	





Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	3.76	2.67	1.33	1.45	2.85	1.37	.45	2.28
Relative area %	6.60	27.10	39.10	27.20	8.80	23.60	23.50	44.10

Figure 3 - Typical electrophoretic pattern secured from extracts of Longissimus dorsi muscle with 0.1 M dibasic potassium phosphate buffer pH 7.0 for approximately 16 hours.

Electrophoresis buffer: 1 M glycine.

It was evident from these data that the pH of the electrophoretic buffer exerted considerable influence on both the separation of protein fractions and their respective velocities. No doubt, this was due to the magnitude of the net charge on the protein, their tendency to dissociate differently in the various electrical fields and their amphoteric behavior.

III. Post Mortem Changes in Proteins from the Bovine Longissimus dorsi Muscle

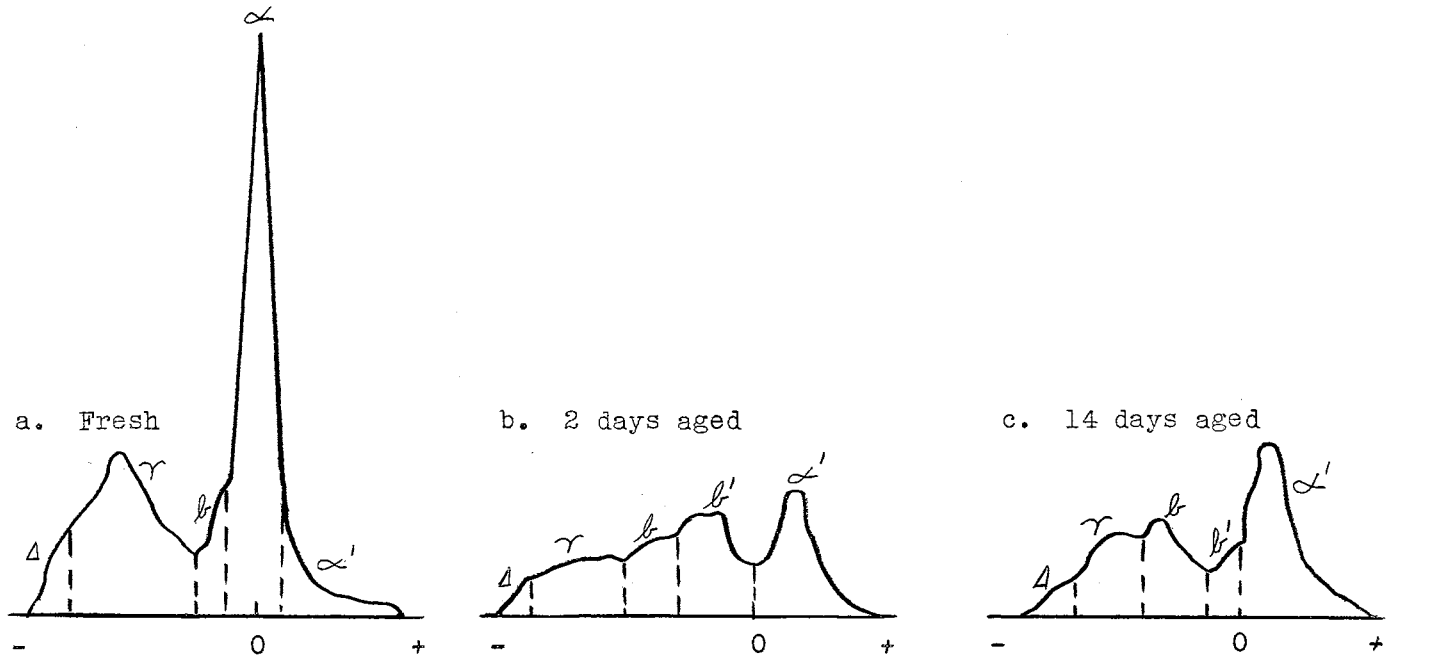
A. High Ionic Strength Extracts

In figures 4 a, b, and c are presented the electrophoretic analyses of proteins extracted from freshly excised, 2 and 14 days aged muscle. Figures 5 a, b, and c present the results obtained when adenosine triphosphate (ATP) was added to the extracts prior to electrophoresis. For ease of reference, the protein peaks were individually labeled.

Although five definite fractions were obtained in each of these electrograms, very significant differences were observed between the "fresh" and "aged" samples.

The α' fraction, which migrated towards the positive electrode increased significantly as the muscle was aged. This component accounted for 7.70%, 32.60% and 38.46% of the total protein area of the freshly excised, and the 2 and 14 days aged muscle, respectively (figure 4 a, b and c).

The non-mobile fraction, α, which composed 41.00% of the fresh sample was conspicuously absent from the curves of the aged muscle. It can be seen that in the absence of this component, in the aged samples, there was formed, in addition to the build up of the α' fraction, a new positively charged protein, which was labeled ℓ'.

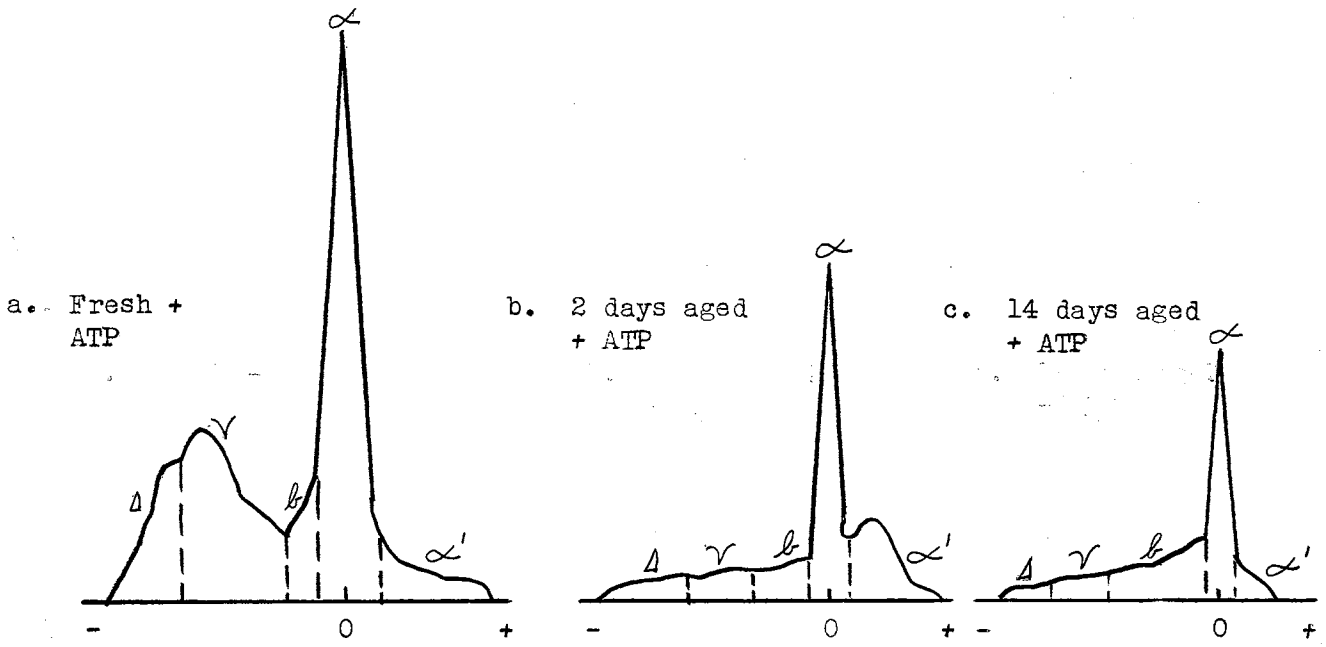


Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	1.88	1.48	.56		1.25	3.17	2.71	1.54	.99	1.63	2.82	2.07	1.22	0.47	1.69
Relative area %	6.41	32.09	12.80	41.00	7.70	2.40	23.80	15.20	26.00	32.60	2.56	23.09	25.4	10.25	38.46

Figure 4 - Typical electrophoretic pattern of proteins extracted from Longissimus dorsi muscle with dibasic potassium phosphate buffer (pH 7.0) in 0.5 M potassium chloride ($\frac{\pi}{2} = 0.55$).

Electrophoresis buffer: 1 M glycine pH 8.6.

a. Fresh muscle: protein extracted within 11 minutes after the death of the animal
b. Aged muscle: protein extracted within 2 days
c. Aged muscle: protein extracted within 14 days



Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	2.01	1.38	.55		1.25	2.71	1.72	0.90		1.26	2.82	2.07	.75		.56
Relative area %	12.10	28.07	8.70	42.60	8.53	4.34	4.44	8.60	56.52	26.10	5.55	16.60	33.33	33.42	11.10

Figure 5 - Typical electrophoretic pattern of protein extracted from Longissimus dorsi muscle with dibasic potassium phosphate buffer (pH 7.0) in 0.5 M potassium chloride $\frac{\pi}{2} = 0.55$ after the addition of ATP.

Electrophoresis buffer: 1 M glycine pH 8.6.

a. Fresh muscle: protein extracted within 11 minutes after the death of the animal
b. Aged muscle: protein extracted within 2 days
c. Aged muscle: protein extracted within 14 days

Since the freshly excised muscle contained ATP, and since the same α fraction appeared when ATP was added to the extract (figure 4 a and figure 5 a, b, and c), it seemed reasonable to assume that this fraction, α, consisted of an ATP - protein complex.

It was further observed that when the ATP was known to have been depleted (note - figure 4 b and c; muscle pH 5.6), the α' component did not appear in any great amount. It appeared that the presence of ATP, in some way, prevented the formation of the α' fraction. Since it is known that ATP will prevent the formation of, or cause dissociation of the protein actomyosin, the complex formed by the fusion of the fibrillar proteins actin and myosin, it was believed that fraction α' might very well be actomyosin. It is pointed out that this protein has not been identified electrophoretically, heretofore. To further test this observation, ATP was added to the 2 and 14 days aged muscle extracts (figure 5 b and c). Results showed that the α' fraction failed to appear in any great amount. If this tentative identification is correct, then the actual build up of actomyosin, at least until 14 days aging as indicated in figure 4 b and c, is of interest; for in previous literature it has been suggested that part of the "increased tenderness" encountered during meat aging was due to a dissociation of actomyosin. The results obtained in this study would indicate that such is not the case, for actomyosin is, in fact, increased during aging. Consequently, the conclusion that the increased tenderness of aged meat is due, in part, to the dissociation of actomyosin may not be correct.

Further justification of the conclusion that component α was either an ATP - protein complex or at least was ATP dependent is offered by the electrograms appearing in figures 4 a and 5 a, b, and c. These

curves represented the separations obtained when ATP was either known to be present, as in the fresh muscle extraction of figure 4 a or when ATP was added to the extracts, as in figures 5 a, b, and c.

Experimental data also showed that the γ fraction, which represented a considerable portion of the total protein of freshly excised muscle, 32.09% and 28.07% respectively of figure 4 a and 5 a, decreased as the muscle was aged (figure 4 b, c, and 5 b, c). In the center of this component, a faint brown band appeared on the paper strips prior to staining. While separating commercially purified proteins, it was noticed that the chromoprotein, myoglobin, always produced this phenomena on the strip. The average mobility of this component, in the fresh muscle patterns, was 1.48×10^{-5} cm^2 $volt^{-1}$ sec^{-1}. This was in close agreement to that of the "pure" myoglobin (1.45×10^{-5} cm^2 $volt^{-1}$ sec^{-1}). Though myoglobin was heterogeneous in its electrophoretic behavior, it was entirely possible that portion of the γ fraction could have been present in the myoglobin.

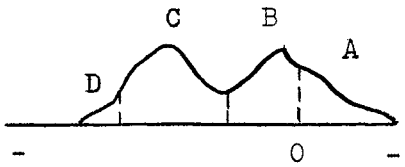
Finally, in comparing the Δ fractions of the fresh and aged muscle, it was found that this fraction accounted for only about one-third as much of the total area in the aged muscle as it did in the fresh sample. The same relationship was true when ATP was added to the extracts.

B. Proteins Extracted with Low Ionic Strength Buffers

Electrophoresis of the protein solutions obtained by extracting Longissimus dorsi muscle with low ionic strength phosphate buffer revealed four components (figures 6 a, b, and c).

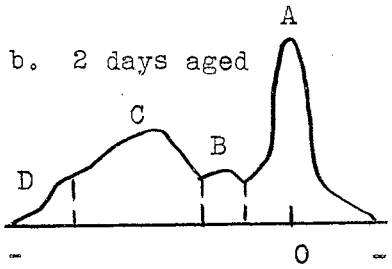
There seemed to be a rather inconsistent change in the mobility of fraction A of the fresh and aged muscle extracts. Whereas this component had a mobility 0.98×10^{-5} cm^2 $volt^{-1}$ sec^{-1} and accounted

a. Fresh



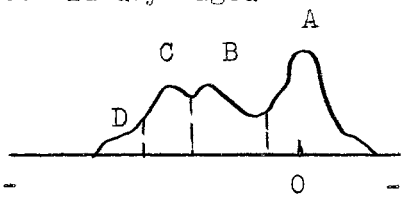
Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	1.88	1.50	.56	.98
Relative area %	4.78	47.61	33.33	14.28

b. 2 days aged



Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	2.22	1.80	.72	.66
Relative area %	8.58	40.00	8.58	42.84

c. 14 days aged



Mobility $cm^2\ v^{-1}\ sec^{-1} \times 10^{-5}$	1.62	1.26	.84	.60
Relative area %	4.18	24.0	29.16	41.66

Figure 6 - Typical electrophoretic pattern of proteins extracted from Longissimus dorsi muscle with dibasic potassium phosphate buffer (pH 7.0) and $\frac{\Gamma}{2} = 0.1$.

Electrophoresis buffer: 1 M glycine pH 8.6.

a. Fresh muscle: protein extracted within 11 minutes after the death of the animal
b. Aged muscle: protein extracted within 2 days
c. Aged muscle: protein extracted within 14 days

for 14.28% of the total protein of the freshly excised muscle, it apparently became non-mobile and represented 42.84% and 41.66% of the total protein in the 2 and 14 day aged muscle respectively.

The B fraction showed an increase in mobility as the muscle was aged. This fraction decreased in concentration upon aging.

The percent area of fraction C decreased significantly as the muscle was aged. Its mobility however, decreased only after 14 days aging.

Finally, neither the concentration nor the mobility of component D showed a particular trend with aging.

IV. Protein Identification

Identification of protein fractions from literature values of their electrophoretic mobilities and relative concentration are complicated by the fact that no standard procedure exists for electrophoretic analysis. A variety of buffer systems, electrophoretic equipment etc., has been utilized. The problem is further confused by the fact that many of the protein components have never been isolated, purified or characterized and are only known to be present on the basis of their enzymatic activity.

A number of "commercially purified" proteins were electrophoresed, under test conditions utilized above, and their electrophoretic characteristics are given in table IV.

In addition, some "crudely purified" fibrillar proteins were analyzed electrophoretically and their characteristics mobilities are shown in table V. It is pointed out, however that these proteins were not exhaustively purified.

TABLE IV

ELECTROPHORETIC MOBILITY OF SOME
"COMMERCIALLY PURIFIED" PROTEINS

	Mobility x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}
Collagen	*+ 0.67
Elastin	+ 0.53
Albumin	+ 6.91
Alphaglobulin	+ 3.22
Myoglobin	*- 1.45

Electrophoresis condition: 1 M glycine, pH 8.6 for approximately 16 hours

*+ moving toward the positive electrode
*- moving toward the negative electrode

TABLE V

ELECTROPHORETIC MOBILITY OF SOME PROTEIN FRACTIONS, ISOLATED FROM THE LONGISSIMUS DORSI MUSCLE AT DIFFERENT INTERVALS FROM THE DEATH OF THE ANIMAL

	Mobility x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}		
	Freshly Excised Muscle	Aged 2 days	Aged 14 days
Actin (G)	*-1.25	-0.44	-0.56
Actin + 0.1 M KCl + Traces of $MgCl_2$ (F-actin)	-0.62	-0.43	-0.47
Myosin	-0.62	-0.45	-0.56
Myosin + Urea	-0.43	-0.45	-0.56

Electrophoresis condition: 1 M glycine buffer, pH 8.6

*- moving toward the negative electrode

SUMMARY

In this study, paper electrophoresis technique were used to study the protein composition of bovine striated muscle.

Experimental results indicated that the hydrogen ion concentration of extraction buffer had a profound effect on both the number of protein fractions extracted and the subsequent partition of these fractions on the paper strips. The most satisfactory results were obtained when 0.1 M dibasic potassium phosphate buffers of pH 7.0 were used.

Results also showed that the type and pH of electrophoresis buffer significantly influenced the separation and mobility of the muscle proteins applied to the electrophoresis strips. In this regard, it was found that 1 M glycine buffer adjucted to pH 8.6 showed the best electrophoretic separation of the extracted protein.

In studying the post mortem changes of muscle proteins, dibasic potassium phosphate of high ($\frac{\Gamma}{2} = 0.55$) and low ($\frac{\Gamma}{2} = 0.10$) ionic strength were used. While 5 different fractions were obtained with the high ionic buffer, only 4 protein fractions appeared when the low ionic buffer was used. Apparently one of the fibrillar proteins failed to be extracted by the latter buffer.

One of the protein fractions obtained with the high ionic strength buffer was tentatively identified as actomyosin. This fraction increased significantly as the muscle was aged, accounting for 7.70%, 32.00% and 38.46% of the total protein of the fresh, 2 and 14 days aged

muscle, respectively. It was further observed that ATP prevented the formation of this fraction. These results appear to contradict the present hypothesis that actomyosin content of muscle is decreased upon aging. Consequently, the previous conclusion that increased tenderness of aged meat is due to a dissociation of actomyosin may not be true.

In comparing the electrophoretic patterns of the muscle extracts with some commercially purified proteins, it was found that one of the positively charged fractions of the protein extracts had electrophoretic characteristics quite similar to those of the chromo protein "myoglobin". This fraction accounted for about 32% of the total protein of the freshly excised muscle and had a mobility of 1.48×10^{-5} cm^2 $volt^{-1}$ sec^{-1}.

Finally, the electrophoretic characteristics of some commercially purified proteins and some "crudely purified" fibrillar proteins were determined.

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