INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in “sectioning” the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from “photographs” if essential to the understanding of the dissertation. Silver prints of “photographs” may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
CHENG, Chin-Sheng, 1938-
BIOCHEMICAL STUDIES OF POSTMORTEM AGING,
CALCIUM ION AND HEATING ON BOVINE
SKELETAL MUSCLE PROTEINS.

Iowa State University, Ph.D., 1976
Food Technology

Xerox University Microfilms, Ann Arbor, Michigan 48106
Biochemical studies of postmortem aging, calcium ion 
and heating on bovine skeletal muscle proteins

by

Chin-Sheng Cheng

A Dissertation Submitted to the 
Graduate Faculty in Partial Fulfillment of 
The Requirements for the Degree of 
DOCTOR OF PHILOSOPHY

Departments:  Food Technology 
Animal Science 
Major:  Meat Science

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University 
Ames, Iowa 
1976
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF SYMBOLS AND ABBREVIATIONS</td>
<td>iv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>Skeletal Muscle Tissues</td>
<td>5</td>
</tr>
<tr>
<td>Skeletal Muscle Proteins</td>
<td>9</td>
</tr>
<tr>
<td>Postmortem Muscle</td>
<td>16</td>
</tr>
<tr>
<td>Decline of ATP content and pH value</td>
<td>16</td>
</tr>
<tr>
<td>Intracellular calcium ion and properties of sarcoplasmic reticulum</td>
<td>18</td>
</tr>
<tr>
<td>Development and resolution of rigor mortis</td>
<td>24</td>
</tr>
<tr>
<td>Ca$^{2+}$-Activated Factor</td>
<td>39</td>
</tr>
<tr>
<td>Addition of Ca$^{2+}$ and EDTA to Muscle</td>
<td>42</td>
</tr>
<tr>
<td>Heating of Muscle Tissues</td>
<td>46</td>
</tr>
<tr>
<td>Structural and biochemical changes</td>
<td>46</td>
</tr>
<tr>
<td>Changes in water-holding capacity</td>
<td>55</td>
</tr>
<tr>
<td>Effects on collagen fibers</td>
<td>60</td>
</tr>
<tr>
<td>Effect on sarcoplasmic and myofibrillar proteins</td>
<td>63</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>69</td>
</tr>
<tr>
<td>Source of Muscle Tissue</td>
<td>69</td>
</tr>
<tr>
<td>Treatment of Muscle Samples for Studies of the Effects of Calcium Ion on Postmortem Biochemical Changes</td>
<td>69</td>
</tr>
<tr>
<td>Preparation of Myofibrils</td>
<td>71</td>
</tr>
<tr>
<td>Protein Extraction of Isolated Myofibrils</td>
<td>72</td>
</tr>
<tr>
<td>Heating of Muscle Tissue</td>
<td>73</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Protein Extraction of Muscle Tissue after Heating</td>
<td>74</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis</td>
<td>75</td>
</tr>
<tr>
<td>Protein Concentration and Muscle pH Measurement</td>
<td>77</td>
</tr>
<tr>
<td>RESULTS</td>
<td>78</td>
</tr>
<tr>
<td>Postmortem Biochemical Changes in Myofibrils and their Proteins</td>
<td>79</td>
</tr>
<tr>
<td>Postmortem Biochemical Changes in Muscles Heated to 45°, 50°, 55°, 60°, 70° and 80°C for 30 Minutes</td>
<td>104</td>
</tr>
<tr>
<td>Effects of Calcium Ion on the Postmortem Biochemical Changes in Muscle Proteins</td>
<td>123</td>
</tr>
<tr>
<td>Possible Accelerative Effects of Calcium Ion on Postmortem Biochemical Changes</td>
<td>165</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>201</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>222</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>227</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>230</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>251</td>
</tr>
</tbody>
</table>
**LIST OF SYMBOLS AND ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>angstroms</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>CAF</td>
<td>calcium-activated factor</td>
</tr>
<tr>
<td>CASF</td>
<td>calcium-activated sarcoplasmic factor</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane</td>
</tr>
<tr>
<td>EJ</td>
<td>expressed juice</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>r/2</td>
<td>ionic strength</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>H-S</td>
<td>Hasselbach-Schneider</td>
</tr>
<tr>
<td>in.</td>
<td>inch</td>
</tr>
<tr>
<td>K</td>
<td>potassium</td>
</tr>
<tr>
<td>Kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mamp</td>
<td>milliamperere</td>
</tr>
<tr>
<td>MCE</td>
<td>mercaptoethanol</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>Symbol</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>NaN₃</td>
<td>sodium azide</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH</td>
<td>sulfhydryl</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>Ts</td>
<td>thermal shrinkage temperature</td>
</tr>
<tr>
<td>V</td>
<td>volume</td>
</tr>
<tr>
<td>ω</td>
<td>weight</td>
</tr>
<tr>
<td>W-B</td>
<td>Warner-Bratzler</td>
</tr>
<tr>
<td>WHC</td>
<td>water-holding capacity</td>
</tr>
<tr>
<td>WRC</td>
<td>water retention capacity</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>µmole</td>
<td>micromole</td>
</tr>
</tbody>
</table>
INTRODUCTION

Many factors affect the eating qualities of meat. These factors have been characterized into three time-based groups: those determined before the birth of the animal (breed, sex, etc.), those modified by management during its life (age, composition, feed, etc.), and those affected by treatment after the slaughter of the animal (aging, cooking method, etc.) (Marsh, 1972). Meat scientists have long been interested in understanding the nature of postmortem aging and heating (cooking) processes which occur in muscle tissues. Moreover, they would like to be able to manipulate these characteristics at will for beneficial utilization of muscle tissues as a food, meat.

During the last two decades, the important role of the contractile apparatus, the myofibril, to meat quality has been recognized through fundamental biological and meat science research. The discovery of the ultrastructure of myofibrils using electron microscopy, and the molecular components of myofibrils, particularly the myofibrillar proteins and their interactions in these subcellular organelles using biochemical techniques, have established a foundation for applied meat science research. Microscopic and biochemical studies have provided evidence of a gradual degradation of Z-disk structure, the weakening of the actin-myosin interaction and proteolysis.
of certain myofibrillar proteins by a calcium activated factor during postmortem aging. Recently relationships have been established among myofibril fragmentation, calcium activated proteolysis and beef steak tenderization (Olson, 1975).

A Ca$^{2+}$-activated factor (CAF) specifically able to remove Z-disks in myofibrils has been discovered in the sarcoplasmic fraction of muscle tissue (Busch et al., 1972b). The implication of this enzyme as a causal agent in postmortem aging processes, mainly the weakening of Z-disk in myofibrils, has been demonstrated by Goll et al. (1974a) and Olson (1975). Recently, CAF has been purified and characterized (Dayton, 1975). Knowledge of the properties of CAF makes it possible to conduct further studies, such as searching for direct evidence of CAF activity in postmortem muscle and finding ways to regulate this enzyme for beneficial effects during the postmortem aging process.

It is well established that intracellular calcium ion concentration controls contraction and relaxation of muscle fibers. And this intracellular calcium ion concentration is subject to stringent controls in living muscle. Subcellular organelles, such as sarcoplasmic reticulum and mitochondria are capable of binding Ca$^{2+}$ in the presence of ATP. The Ca$^{2+}$ binding ability of these subcellular organelles gradually declines in postmortem muscle because of the depletion of ATP in the muscle tissues, decrease in intracellular pH, or the
destruction of the functionality of these organelles by some unknown factors. One of these, or a combination of these, may be responsible for loss of Ca\(^{2+}\)-binding ability. It has been suggested that the availability of Ca\(^{2+}\) in muscle fibers triggers the onset of isometric tension development and promotes CAF activity (Busch et al., 1972a, 1972b). To test whether CAF is active in postmortem muscle, Ca\(^{2+}\) chelators, such as oxalate, fluoride or EDTA could be added to tie-up calcium ion and thus inactivate the enzyme. Chelation of calcium and inactivation of CAF is based on the assumption that a certain level of Ca\(^{2+}\) concentration is essential for this enzyme to function in situ. On the other hand, it may be possible to potentiate CAF activity by raising the free calcium ion level in muscle tissue with a pre- or post-slaughter addition of some form of calcium ion.

Most postmortem biochemical changes are studied on raw muscle samples, but the subjective and objective measurements on meat palatability are usually conducted on the cooked product. The relationship between measurements made on the raw muscle samples and on cooked meat samples has been variable; however, recently a strong relationship has been demonstrated between myofibril fragmentation index of fresh tissue and cooked beef steak tenderness (Olson, 1975). Yet the nature of the thermal modification on the structure of myofibrils and on the interactions of myofibrillar proteins
is still unknown. Hence, the relationship between postmortem modifications on the myofibrils in raw samples and the thermal modifications on the myofibrils in heating require further elucidation. The understanding of the effects of heating on the myofibrillar proteins at the molecular level is essential for meaningful meat palatability research.

The objectives of this study were to 1) detect the post-mortem biochemical changes in myofibrils at the molecular level, 2) study the effects of calcium ion on postmortem biochemical changes in myofibrils, 3) use a model system to test the possible accelerative effects of calcium ion on postmortem biochemical changes, and 4) reveal the effects of heating on the nature of myofibrillar proteins.
Skeletal Muscle Tissues

Skeletal muscle fibers are held in anatomical arrangement by a series of connective tissue sheathes. The whole muscle is surrounded by a heavy connective tissue sheath called the epimysium. Perimysia anastomose from the epimysium and surround groups of muscle fibers to form muscle fiber bundles or fasciculi. And thin endomysial sheathes branch from a perimysium to encompass each individual muscle fiber (Gould, 1973). The cell membrane of the skeletal muscle fiber, the sarcolemma, is closely associated with, but separate from, the endomysium.

The skeletal muscle fiber is an unique cell because of its striated appearance. Striation of skeletal muscle fibers is caused by alternating dark and light bands that lie in register along the longitudinal axis of myofibrils. Under polarized light, the dark bands appear strongly birefringent (anisotropic), termed A bands, and the light bands are weakly birefringent (isotropic), termed I bands. Z-lines or disks bisect through the center of I bands. The region from one Z-line to the next is defined as a sarcomere and this represents the contractile unit of the myofibril.

Myofibrils are so tightly packed in the muscle fiber that the 100-200 nuclei within the cell are forced to the periphery
of the fiber next to the sarcolemma. Myofibrils are long threads of proteins lying parallel to each other along the long axis of the fiber. These myofibril threads are surrounded by a fine extensive membranous tubular network, termed the sarcotubular system. The sarcotubular system consists of the sarcoplasmic reticulum or longitudinal system (SR or L system) and transverse system (T system). Other components of the muscle cell are mitochondria, glycogen and fat droplets and these are found along the intermyofibrillar spaces. Huxley (1953) has showed the arrays of interdigitating thick and thin myofilaments in the myofibrils with electron microscopy. Thick filaments are only found in the A band whereas thin filaments attach to the Z-line and extend into the A bands. The I band contains the thin filaments and a Z-line, and the A band contains both thick and thin filaments, the H zones and the M-line.

ATP and Ca^{2+} are the two important constituents involved in muscle contraction and relaxation. In living muscle, intracellular ATP concentration is maintained at a constant level through glycolysis and a reservoir of phosphocreatine (Bate-Smith and Bendall, 1947, 1949). The enzymes for anaerobic glycolysis are in the sarcoplasmic protein fraction (Scopes, 1970) and mitochondria for aerobic glycolysis and oxidative phosphorylation are distributed nearby in the intermyofibrillar spaces (Slautterback, 1966). The substrates, glycogen and fat
droplets, are also located in the vicinity of the myofibrils. The intracellular Ca\(^{2+}\) is controlled by the sarcotubular systems. This system serves as a coupling mechanism for the nerve impulses, contraction and relaxation of myofibrils and to some extent glycolysis in muscle fibers.

There are two types of sarcotubules in the muscle fibers; one is the transverse system (T system) which is an invagination of the sarcolemma (Franzini-Armstrong and Porter, 1964; Huxley, 1964) and the other is the sarcoplasmic reticulum or longitudinal system (SR or L system). T tubules are capable of conducting nerve impulses from the sarcolemma into the myofibrils (Huxley and Taylor, 1958) and these tubules are oriented perpendicular to the long axis of the myofibril. The cisternae of the SR (or L system) membrane has a Ca\(^{2+}\)-binding ability against a concentration gradient in the presence of ATP (Constantin et al., 1965; Winegard, 1965). The L system tubular network is predominantly oriented parallel to the long axis of the muscle fibers (Peachey, 1965). Upon the depolarization of the SR membrane by an afferent nerve impulse from the T tubules, Ca\(^{2+}\) in the cisternae of the SR is released for myofibril contraction. When SR membranes are repolarized, Ca\(^{2+}\) is reaccumulated and the muscle relaxes. In addition to the Ca\(^{2+}\)-accumulating ability of the SR, the membranes of mitochondria and sarcolemma may posses a Ca\(^{2+}\)-binding ability.

A brief sketch of the protein components of the myofibril
will be presented in order to describe the general mechanism of muscle contraction and relaxation. A more detailed discussion of myofibrillar proteins will be subsequently presented in the muscle proteins section. Myofibrillar proteins are the components of the myofibrils and they are insoluble at physiological ionic condition and thus they exist as structural entities in the muscle fibers (Goll et al., 1970). By selective extraction, myosin was shown to be located in the thick filaments of A bands (Hanson and Huxley, 1955; Corsi and Perry, 1958). Recently, a new myofibrillar protein, component C, was found to form 18 bands surrounding each thick filament in the A band (Starr and Offer, 1971; Offer, 1972). M-lines consist of M proteins (Morimoto and Harrington, 1972). Each thin filament is composed of two strands of actin and tropomyosin (Huxley, 1957) and other protein attached to tropomyosin, troponin, is distributed at 40 nm intervals along the thin filaments (Endo et al., 1966). It has been shown that α-actinin is the dense, amorphous material at the Z-disk and tropomyosin may also be a protein component of the Z-filaments (Schollmeyer et al., 1973). Another protein, β-actinin, may be located at the I band (Maruyama and Ebashi, 1965).

Myofibrils serve as the contractile elements in muscle fibers and within them molecular reactions have been characterized during contraction and relaxation (Ebashi et al., 1969; Young, 1969; Taylor, 1972). They are capable of
converting the chemical energy stored as ATP into the mechan­
ical work of muscle contraction. Within the myofibril structure
there are regulatory mechanisms to respond to the changes in
the subcellular Ca^{2+} concentration. Myosin is an ATPase which
hydrolyses ATP. After the hydrolysis of bound ATP-Mg^{2+} on the
myosin molecule, myosin cross-bridges interact with actin and
the energy produced by the hydrolysis of ATP is used for
muscular contraction. The tropomyosin-troponin complex
regulates the actin-myosin interaction and myosin ATPase in
response to the intracellular Ca^{2+} concentration. When the
intracellular Ca^{2+} rises to 1 \times 10^{-6} M or higher, Ca^{2+} binds to
one of the troponin components, troponin C, and this removes
the inhibitory actions of tropomyosin and troponin I, initiates
myosin ATPase activity and actin-myosin interaction and causes
muscle contraction. Conversely, when intracellular Ca^{2+} is
1 \times 10^{-7} M or less, tropomyosin will block the actin and myosin
interaction and one of the troponin components, troponin I, will
inhibit Mg^{2+} activated actomyosin ATPase. ATP-Mg^{2+} bound on
the myosin molecule dissociates myosin from actin and as a
result muscle relaxation occurs.

Skeletal Muscle Proteins

The composition of muscle tissue ranges from 15 to 22
percent protein. This variation is due to differences in
species, age, animals, muscles, and physiological conditions. These muscle proteins can be classified into three groups (sarcoplasmic, myofibrillar and stroma proteins) based on their solubility in salt solutions of different ionic strengths. The proportion of each of these fractions to total muscle protein is 30-34 percent for sarcoplasmic proteins, 50-55 percent for myofibrillar proteins and 10-15 percent for stroma proteins (Goll et al., 1970).

Sarcoplasmic proteins are isolated by homogenizing minced muscle tissue with a low ionic strength (\(1/2 < .2\)) buffered solution and at low temperatures (near 0°C) (Helander, 1957; Goll and Robson, 1967). The supernatant of this extract contains sarcoplasmic proteins, the nuclear fraction, the mitochondria fraction and the microsomal fraction. High speed centrifugation (25,000 to 100,000 \( \times g \) for 2-3 hours) usually sediments most of the membranes and particles and as a result the supernatant contains the soluble sarcoplasmic proteins, tRNA and other soluble substances (Goll et al., 1974b). Proteins of the glycolytic enzymes comprise the major portion of the sarcoplasmic protein fraction (Scopes, 1970). The muscle pigment, myoglobin and the \(Ca^{2+}\)-activated factor (CAF) are also in this fraction.

It has been estimated that out of a total of 55 mg of sarcoplasmic proteins, approximately 40 mg are glycolytic enzymes, 5 mg is creatine kinase, 0.2 to 12 mg is myoglobin,
3 to 6 mg are extracellular proteins and 8 to 12 mg are other proteins (Scopes, 1970). Glyceraldehyde phosphate dehydrogenase (GAPDH), phosphorylase, aldolase, triose phosphate isomerase, enolase, pyruvate kinase and lactate dehydrogenase make up over half of the sarcoplasmic proteins.

The myofibrillar proteins are usually described as those proteins that are soluble in a salt solution of ionic strength of 0.4 to 1.5. But it is also known that some of the myofibrillar proteins, such as actin, tropomyosin-troponin, can be extracted with a solution of low ionic strength (Arakawa et al., 1970a, 1970b). Goll et al. (1970) suggested that myofibrillar proteins should be defined as those proteins constituting the myofibrils. There are nine myofibrillar proteins: myosin, actin, tropomyosin, troponin, α-actinin, β-actinin, component C and two M-line proteins.

The amount of each myofibrillar protein of total myofibrillar protein is as follows: myosin, 50-55 percent; actin, 15-20 percent; tropomyosin, 5-8 percent; troponin, 5-8 percent; α-actinin, 2-3 percent; β-actinin, 0.5-1 percent; component C, 2-3 percent; and M-line proteins, 3-5 percent (Goll et al., 1974a).

Myosin is the largest single protein in muscle tissue and it represents 25-30 percent of the total muscle proteins. Its quantity and properties are important to both biological function and meat quality.

The myosin molecule has a molecular weight of 460,000
daltons (Godfrey and Harrington, 1970) and it is 160 nm in length and the diameter of the rod and the head portion are 1.5 nm and 5 nm, respectively (Huxley, 1963). This large molecular weight and the large ratio of length to diameter of the molecule makes myosin unique to other muscle proteins. The natural subunits of myosin consist of two heavy chains of 200,000 daltons (Gazith et al., 1970) and four light chains of 16,000, 20,000 and 25,000 daltons (Stracher, 1969; Weeds and Lowey, 1971) as shown in SDS-polyacrylamide gel.

In view of biological function, myosin molecules are the structural components of thick filaments, have ATPase activity and have the actin binding ability for contraction. Furthermore, myosin performs both chemical and mechanical work. In respect to meat quality, myosin is directly related to water holding capacity, emulsification ability and texture of meat.

G-actin is a globular protein with a molecular weight of 42,000-44,000 daltons (Elzinga et al., 1973). The thin filament consists of two strands of G-actin molecules (Hanson and Lowy, 1963). There is one Ca^{2+} and ADP associated with each G-actin molecule in the thin filament. Actin has the ability to bind myosin and to modify the myosin Mg^{2+}-ATPase activity. The biological functions and implications of myosin to meat quality are greatly modified by the interaction of myosin with actin. Water-holding capacity, emulsification and gelation of myosin are greatly increased and stabilized by
binding actin molecules (Samejima et al., 1969), on the other hand, the contracted state of the actin-myosin interaction in cold shortened bovine muscle, or thaw rigor muscles, causes toughness (Locker, 1960; Marsh and Thompson, 1958).

The tropomyosin (TM) molecule has two subunits of 33,000 and 36,000 daltons (Cohen et al., 1972). The tropomyosin-troponin complex exerts an inhibitory effect on the actin-myosin interaction and myosin Mg$^{2+}$-ATPase (Hanson et al., 1972; Huxley, 1972).

The troponin (TN) molecule has a molecular weight of 80,000 daltons (Ebashi et al., 1971). It consists of three subunits (Greaser and Gergely, 1971; 1973); troponin T (TNT) which has a molecular weight of 37,000 - 39,000 daltons and binds to TM but not actin; troponin I (TNI) which has a molecular weight of 24,000 daltons and binds to the TNT/TNC complex and also weakly binds to TM; troponin C (TNC) which has a molecular weight of 18,500 daltons, binds to TNT but not to actin and it has four high affinity binding sites for Ca$^{2+}$. TNI strongly inhibits the Mg$^{2+}$-modified actomyosin ATPase, but this inhibition is removed when TNC binds Ca$^{2+}$.

Alpha-actinin has a molecular weight of 200,000 daltons with two identical subunits of 100,000 daltons (Robson et al., 1970; 1971). In vitro, α-actinin can exert an activating effect upon Mg$^{2+}$-modified actomyosin ATPase and superprecipitation (Ebashi et al., 1964). Singh (1974) has suggested
that α-actinin in muscle enhances the structure and stability of the Z-line.

Component C has a molecular weight of 135,000 daltons (Starr and Offer, 1971). This protein may help maintain the integrity of thick filaments during contraction (Offer, 1972).

Stroma proteins, which are insoluble in neutral aqueous solution, include collagen, elastin, reticulin and mucoproteins in the ground substance (Goll, 1965). They are the major components in the epimysial, perimysial, and endomysial connective tissue sheathes.

Collagen is the principle protein constituent (50-60 percent) of the stroma proteins. Its molecular structure consists of tropocollagen, a triple chained molecule with a molecular weight of 300,000 daltons (Veis, 1970). Collagen molecules are very resistant to extraction by neutral salt solution and enzymatic digestion because of the triple helix structures of tropocollagen peptide chains and the intermolecular covalent cross-linkages (Goll, 1965). Bailey and Lister (1968) have reported that intermolecular cross-linkages of several varieties are present in the collagen fibers. One of these may be Schiff bases which are labile to heat. These labile bonds are intermediates in the cross-linking scheme and are converted to a stable form in the maturation process. Collagen heated in a hot aqueous solution is converted to gelatin and in this form gelatin is soluble in neutral salt.
solution and susceptible to enzyme digestion. The temperature required for gelation of collagen varies from 55° to 75°C depending on the age of the animal (Goll et al., 1963; 1964b) and the extent and nature of the covalent cross-linkages in the collagen fiber (Bailey and Lister, 1968).

Collagen has a very unusual amino acid composition; 33 percent is glycine, 13 percent is proline, 10 percent is hydroxyproline, and it has a small quantity of hydroxylysine (Ramachandran, 1963). The unique amount of hydroxyproline in collagen is usually used in assays for quantitative estimation of the collagen content in muscle tissues (Goll et al., 1963).

Elastin also has a unique amino acid composition that is similar to collagen. Elastin is a minor component of most muscles (Bendall, 1967). In muscles containing a large amount of elastin, most of the elastin exists in the epimysium or perimysium.

Reticulin is similar to collagen in morphology, shrinkage temperature and amino acid composition (Kono and Colowick, 1961). Some authors considered it to be in the same class of proteins as those of collagen fibers (Seifter and Gallop, 1966).
Postmortem Muscle

Decline of ATP content and pH value

After exsanguination of the animal, the muscle tissues in the carcass are not under the control of the nerve, circulatory and hormonal systems regulating biochemical reactions in a living organism (Lawrie, 1966). The replenishment of oxygen and nutrients and other substances, and the removal of the products of metabolism, e.g., lactic acid, cease in those muscle fibers following the cessation of circulation. The relationships among glycogen levels, post-mortem ATP degradation, ultimate muscle pH and postmortem rigor mortis have been extensively studied by many workers (Bate-Smith and Bendall, 1947, 1949; Cassens and Newbold, 1966, 1967a, 1967b; Newbold, 1966). The postmortem metabolism of skeletal muscle tissues has been intensively studied and reviewed (Kastenschmidt, 1970; Kastenschmidt et al., 1968). The high energy phosphorous compounds, phosphocreatine and ATP are used to maintain the cell's integrity and the biochemical processes in the organisms. They are rephosphorylated by aerobic and anaerobic glycolysis as long as enzymatic systems are functioning and substrates are available. Phosphocreatine and hence ATP concentration remains constant in the muscle fibers when the rate of regeneration and consumption of the high energy compounds are in balance. Due to
the limited availability of oxygen in postmortem muscle fibers, anaerobic glycolysis, which is a rather inefficient system compared with aerobic glycolysis, becomes the predominant pathway for regeneration of the high energy phosphate compounds. Consequently, concentration of phosphocreatine falls because of its conversion to ATP. At the same time, lactic acid, the product of anaerobic glycolysis, begins to accumulate in the cells and the muscle pH starts to decline. ATP remains unchanged until much of the phosphocreatine disappears. ATP concentration will finally reach a level that is about 20 percent of its initial level and the ultimate pH of muscle may drop to 5.5 depending on the amount of glycogen residue in the muscle at death and the integrity of glycolytic enzymes in postmortem muscle. These biochemical reactions are completed in six hours at 37°C, or 24 hours at 1°C, in bovine sternomandibularis muscle (Newbold, 1966). The depletion of ATP and the accumulation of lactic acid in muscle is a most pronounced process during the first 12 to 24 hours.

Hydrolysis of ATP provides energy for muscle contraction and shortening in the presence of an adequate level of Ca\(^2+\) in the muscle fibers. Excised pre-rigor muscle will shorten in storage whereas excised post-rigor muscle will not (Marsh et al., 1968). This cold shortening phenomena which has profound effects on meat tenderness (Locker, 1960; Marsh and Leet, 1966) is closely related to the ATP level in the muscle
(Newbold and Harris, 1972).

On the other hand, relaxation of muscle and the maintenance of a relaxed, resting state depends on the suppression of the myofibrillar ATPase activity and the plasticizing effect of ATP (Weber and Portzehl, 1954). In living muscle, only 20 percent of the cross-bridges on the thick filaments were actually attached to the thin filaments at any one time during muscle contraction (Huxley and Brown, 1967). However, when ATP was depleted, 100 percent of the cross-bridges on thick filaments were locked on the thin filaments causing inextensibility of the muscle (Huxley, 1968).

**Intracellular calcium ion and properties of sarcoplasmic reticulum**

The concentration of calcium ion in muscle tissue increases during postmortem storage (Arnold et al., 1956; Nakamura, 1973a, 1973b; Jergenson, 1975). Nakamura (1973b) found that water extractable calcium from one gram of chicken pectoralis major muscle increased from 0.031 μmoles at-death to 0.08 μmoles in 8 hours postmortem and reached a maximum of 0.13 μmoles at 24 hours of storage in drained crushed ice. Jergenson (1975) observed that the calcium ion in the supernatant from a high speed centrifugation of procine semitendinosus muscle increased rapidly during the first day of storage and the maximum values were reached at 2 days of storage at
5°C. The increase in extractable calcium ion in postmortem muscle could result from both intracellular calcium ion and extracellular calcium ion and hence these results only provide some comparative information.

Other investigators have studied the properties of isolated sarcoplasmic reticulum in vitro from postmortem muscles for the purpose of relating the changes in intracellular calcium ion concentration to the biochemical and physical changes in postmortem muscle (Greaser et al., 1967, 1969a; G. R. Schmidt et al., 1970; Eason, 1969). Greaser et al. (1967) demonstrated that the calcium-accumulating activity of the microsome fraction, isolated by centrifugation between 8,000 to 30,000 x g, from porcine longissimus muscle, was reduced by 40 percent within the first three hours postmortem and then further decreased to 10 percent after 24 hours of postmortem storage. A large decline in pH and the development of rigor shortening were observed concomitantly during this period. This led these authors to suggest that postmortem shortening was triggered by the availability of intracellular calcium ion due to the inactivation of the calcium ion pump in the sarcoplasmic reticulum. In subsequent studies (Greaser et al., 1969a) a relationship between the rate of reduction in Ca\(^{2+}\) binding ability of the isolated SR and the rate of pH decline and rigor development in porcine longissimus muscle was shown.

Eason (1969) studied the Ca\(^{2+}\)-binding ability of the
isolated sarcoplasmic reticulum from bovine *semitendinosus* muscle during isometric tension development at 2°C. The Ca\(^{2+}\)-binding ability of the isolated SR from muscle at the onset of tension development was only 35 percent of the Ca\(^{2+}\) binding ability of the isolated SR from at-death muscle, but the Ca\(^{2+}\)-binding ability of the isolated SR from the muscle at maximum tension was reduced to 15 percent of that from at-death muscle samples. These observations demonstrated that sarcoplasmic reticulum in situ loses its Ca\(^{2+}\)-binding ability during tension or rigor development. However, G. R. Schmidt et al. (1970), suggested that the release of Ca\(^{2+}\) from the SR triggers rigor development in postmortem muscle, yet rigor mortis can develop under conditions where there is little loss in calcium binding ability of the sarcoplasmic reticulum per se. This observation is supported by the studies of Hay et al. (1973c) who showed that the Ca\(^{2+}\)-accumulating ability of the isolated fragmented sarcoplasmic reticulum from chicken breast muscle increased with postmortem storage at 2°C. This discrepancy in the Ca\(^{2+}\)-binding ability of chicken fragmented SR from large domestic animal SR may be due to the rapid cooling of the chicken carcass because low pH and high temperature is critical to the integrity of SR (Hay et al., 1973c; Greaser et al., 1969b).

Many reports have suggested mechanisms or causes for the release of calcium ion from the SR in postmortem muscle
Bendall (1960), in a review paper, proposed that cold shortening and thaw rigor in the pre-rigor bovine muscle was caused by the release of calcium ion from sarcoplasmic reticulum when muscle tissue is exposed to a temperature near the freezing point. An extensive salt "flux" occurs in the muscle tissues with a release of calcium ion and a temporary inactivation of the sarcoplasmic reticulum. When the temperature is raised, the cold-shortened muscle will lengthen again if the muscle contains enough ATP (Marsh, 1966). A thin, frozen, fresh muscle strip will not show thaw rigor contraction if the strip is allowed to thaw rapidly (Bendall, 1960).

Greaser et al., (1969b) demonstrated that incubation of the SR, isolated from at-death muscle, in a medium of low pH (5.5) and at a high temperature (25 or 37°C) impaired the Ca\(^{2+}\)-binding ability of the isolated SR. The conditions of low pH (5.5) and low temperature (0°C) or high pH (7.2) and high temperature (37°C) are relatively ineffective in reducing the Ca\(^{2+}\)-binding ability of isolated SR. Nakamura (1973b) has shown that a maximum amount of water extractable calcium from chicken breast muscle was obtained from an at-death bird injected with epinephrine before slaughter. Furthermore he found a relationship between the depletion of ATP and the
amount of water extractable calcium ion from chicken breast muscle during postmortem storage. Since hydrolysis of ATP is essential to the active transport of calcium ion in the SR, the depletion of ATP could result in the release of calcium ion from the SR. 

Hay et al., (1973c) assayed the Ca$^{2+}$-binding activity of the isolated SR from chicken breast muscle at various combinations of pH and temperature. The Ca$^{2+}$ uptake by the isolated SR assayed at pH 5.6 and 2°C was only 25 percent of the control which was assayed at pH 7.2 and 25°C. In an assay at pH 5.6 and 25°C, the Ca$^{2+}$ uptake by the isolated SR was 50 percent of the control. This showed that the Ca$^{2+}$ uptake by SR was greatly retarded by the condition of low pH (5.6) and even greater by the combination of low pH (5.6) and low temperature (2°C). They (Hay et al., 1973c) suggested that lowering the carcass temperature to 2°C may either block the Ca$^{2+}$-binding sites in the SR or that the ATPase activated pump becomes less efficient allowing a reduction in uptake of Ca$^{2+}$.

Goll et al., (1971, 1974a) proposed that a limited proteolysis of sarcoplasmic reticulum membrane accounts for the observations of postmortem loss of Ca$^{2+}$-binding ability, although no experimental evidence was provided. However, it has been shown that brief tryptic digestion of isolated SR would markedly reduce Ca$^{2+}$-binding ability and slightly activate ATPase activity (Ikemoto et al., 1968; Inesi and
Asi, 1968). Neelin and Ecobichon (1966) have also suggested that postmortem destruction of the sarcotubular membranes may involve hydrolytic enzymes (lipases, phospholipases, proteases) released after death from lysosomes.

In summary, the release of calcium ion from the SR in postmortem muscle may be caused by: 1) low temperatures, such as 0-5°C, blockage of the Ca\(^{2+}\)-binding sites in the SR and/or inactivation of the ATPase pump even in the presence of ATP and intact SR membranes; 2) ATP depletion; 3) low pH and high temperature and, 4) a limited proteolysis within the muscle fiber.

The measurement of the changes in the concentration of intracellular calcium ion is rather difficult. Nauss and Davies (1966) have shown however, that postmortem tension development is accompanied by an increased rate of calcium ion efflux even in the presence of ATP. Kushmerick and Davies (1968) have studied thaw contraction and development of thaw rigor in frog muscle and found that thaw rigor was characterized by a release of calcium ion and rapid utilization of ATP. Because postmortem tension development is observed in most cases (Busch et al., 1972a) it is clear that the increased concentration of intracellular calcium ion in postmortem muscle results from a loss of the mechanism controlling calcium ion in the muscle fibers. The rate and extent of this elevation in the concentration of intracellular calcium ion in
postmortem muscle should correspond approximately to the rate and extent of postmortem tension development. No information in regard to the exact sources for this increase in the concentration of intracellular calcium ion is available. The release of calcium ion from the SR in postmortem muscle fibers is the most likely major source for the increase in the intracellular Ca$^{2+}$, although the increased calcium ion may come from calcium formerly bound by other subcellular membranes and from extracellular calcium ion.

**Development and resolution of rigor mortis**

It is well known that pre-rigor muscle is quite tender, rigor muscle is very tough and that rigor muscle after storage will gradually become tender (Paul et al., 1952; Marsh, 1964; Goll et al., 1964a). In a parallel observation on the postmortem changes in muscle texture it has been reported that muscles on the carcass gradually develop rigidity and then soften after storage (Bendall, 1960). The nature of the development of rigor or toughness and the resolution of rigor or tenderness has been studied intensively and elucidated in terms of the ultrastructural and molecular level of muscle proteins. In addition, there has been a tremendous amount of research carried out to determine the causal events or agents leading to postmortem changes. As a result some profound
and enlightening information has been reported during the last two decades on rigor mortis (Bendall, 1960, 1972; Locker, 1960; Locker and Hagyard, 1963; Marsh, 1972; Goll, 1968; Goll et al., 1970, 1971, 1974a; Busch et al., 1972a, 1972b).

Postmortem tension development and decline phases of bovine muscle, corresponding to the development and resolution of rigor, have been reported by several workers (Busch et al., 1967, 1972a; Goll, 1968; Goll et al., 1970, 1971, 1974a). To determine the various phases of rigor, an isometer was designed to detect the amount of isometric tension developed by a muscle strip at constant length during postmortem storage (Jungk et al., 1967; Busch et al., 1967). The isometric tension development was a manifestation of postmortem muscle shortening. It was also observed that the isometric tension development paralleled the postmortem changes in tenderness of excised bovine muscle, i.e., rigor shortened muscle was tough and resolved muscle was tender (Goll et al., 1964a). This postmortem isometric tension measurement was the first to relate the development and resolution of rigor mortis to meat tenderness changes.

In subsequent studies on isometric tension, Busch et al. (1972a) studied the effects of temperature on the development of isometric tension in postmortem bovine, porcine and rabbit muscle using more refined and sophisticated instrumentation, an E&M physiograph equipped with isometric transducers. It
was shown that postmortem isometric tension development and decline occurred in bovine, porcine, and rabbit muscle at all temperatures studied (2°, 16°, 25° and 37°C). For bovine semitendinosus and psoas muscle, isometric tension development was maximal at 2°C, minimal at 16°C, and at 37°C isometric tension was approximately one-half that developed at 2°C. Porcine and rabbit longissimus muscle developed the most isometric tension at 37°C and the least at 2-16°C (Busch et al., 1972a). These effects of temperature on isometric tension development were exactly the same as the effects of temperature on shortening of pre-rigor muscle (Locker and Hagyard, 1963).

The differences between postmortem muscle shortening at 2° and 37°C was that shortening at 2°C occurred in the presence of 5-6 mM of ATP and started at a pH value above 6.0, and shortening at 37°C began at pH values below 6.0, (Busch et al., 1967). It is clear that attempted shortening of postmortem muscle causes the rigor development that is measured as isometric tension.

ATP concentration gradually declines in postmortem muscle and finally reaches such a low level that cross-bridges on the thick filaments interact with the thin filament. Postmortem muscle then becomes inextensible and fixed at its existing stage of contraction when ATP is depleted (Newbold and Harris, 1972). At low temperature (2°C), rigor shortening develops more quickly than extensibility is lost, but at
higher temperatures (37°C), the onset of rigor and the loss of extensibility closely coincide (Goll, 1968). The effects of temperature on sarcomere length corresponded to shortening and postmortem isometric tension pattern (Locker and Hagyard, 1963; Busch et al., 1972a; Stromer et al., 1967a). It was suggested that the changes in banding pattern of postmortem muscle occurred much the same as Huxley and Hanson's sliding filament model of muscle contraction (Stromer and Goll, 1967a, 1967b; Stromer et al., 1967a). Partial lengthening of contracted sarcomeres in rigor muscle was also demonstrated ultrastructurally in postmortem muscles (Stromer et al., 1967a; Gothard et al., 1966; Takahashi et al., 1967). This lengthening of contracted sarcomeres of post-rigor muscle provides a partial explanation of the resolution of rigor as defined as a decline of postmortem isometric tension.

The resolution of rigor has been proposed to be from: 1) weakening of cross-linkage between thick and thin filaments or actin-myosin interaction, and 2) degradation of Z-disk structure or weakening of I-Z bonds (Stromer and Goll, 1967a, 1967b; Stromer et al., 1967a; Goll, 1968; Goll et al., 1970, 1971, 1974a; Fukazawa et al., 1970; Davey and Gilbert, 1967, 1968a, 1968b; Takahashi et al., 1967).

Postmortem rigor shortening has been observed to have great effects on meat quality. Locker (1959) found that "various muscles of ox enter rigor mortis in differing states
of contraction" and the same author (Locker, 1960) further observed that there was a relation between the degree of muscle contraction and meat tenderness. The term "actomyosin toughness" was coined to distinguish it from that contributed by connective tissue (background toughness) in meat (Locker, 1960). Moreover, Marsh (1972) stated that if excised muscle is allowed to shorten to one-third of its initial excised length, the resulting degree of toughness will considerably exceed that due to any of the usually recognized toughening factors - breed, age, lack of postmortem aging, etc.

Since Locker's findings on contraction state and cold shortening (Locker, 1960; Locker and Hagyard, 1963) the factors affecting muscle shortening and the relation between shortening and tenderness have been extensively and carefully studied (Locker and Hagyard, 1963; Goll et al., 1964a; Marsh and Leet, 1966; Herring et al., 1964, 1965a, 1965b, 1967a; Busch et al., 1967).

Meat toughening, however, is not directly proportional to the extent of shortening or stretching (Marsh and Leet, 1966; Herring et al., 1967a). In shortening of up to 20 percent, or over 60 percent, very little toughness developed, but in 35 percent to 60 percent shortening, extensive toughness developed (Marsh and Leet, 1966). Herring et al. (1967a) studied the relationship between tenderness and the degree of shortening and stretching in bovine muscle. They found that shear value
and panel tenderness were linearly related to fiber diameter and that shear force and sarcomere length appeared to have a curvilinear regression relationship. Stretching muscle by 12 percent only slightly improved tenderness and further stretching did not have any further beneficial effect. Consequently, it was concluded that prevention of rigor shortening (to less than 25 percent) was more important to tenderness than stretching muscle (Herring et al., 1967a; Marsh and Carse, 1974).

A simple packing density of muscle fibers does not seem to be enough to explain the relationship between muscle shortening and meat tenderness (Marsh and Carse, 1974). The extent of cross-linkage formation between thick and thin filaments or the "actin-myosin interaction" in rigor muscle accounts for another important part of the variation in meat tenderness (Fujimaki et al., 1965a, 1965b). However, many workers do not favor this view (Marsh, 1954; Hay et al., 1972). Careful study of the data in the literature (Marsh and Leet, 1966; Herring et al., 1967a; Souton et al., 1973) shows that shortening of more than 25 percent corresponds to sarcomere lengths of less than 1.6 μm or 1.7 μm. At this point the I band has completely disappeared, and it is a critical point for inducing toughness by rigor shortening. It seems that the presence of the I band in postmortem myofibrils is essential for acceptable meat tenderness. This
will be further discussed in the subsequent review of the effect of heating on meat.

A large number of studies have been done on the nature and causes of the resolution of rigor in terms of the improvement of postmortem tenderness or the decline of postmortem isometric tension. The rate and extent of improvement in postmortem tenderness depend on storage temperature and the degree of restraint imposed on the muscle (Locker and Hagyard, 1963; Marsh and Thompson, 1958; Busch et al., 1967, 1972a; Goll et al., 1964a; Parrish et al., 1973b).

Goll et al. (1964a) made a comparison of the postmortem tenderness changes of bovine semitendinosus muscles between excised muscle and the companion one attached to the carcass. They found that excised muscle was least tender at 6-12 hours postmortem and thereafter it gradually improved in tenderness. The muscle left in the carcass was least tender right after death and gradually reached its level of tenderness within 24-48 hours postmortem. However, even after aging for 13 days the excised sample was still less tender than the one left attached on the carcass.

Herring et al. (1967a) demonstrated that tenderness of bovine semitendinosus increased for all samples with various amounts of shortening (12 percent to 48 percent) and stretching (12 percent to 48 percent) in both A and E maturity animals as assessed by both taste panel scores and shear force values.
However, tenderness of contracted muscles (shortened 24 percent or more) did not reach acceptable levels even after 10 days of aging. Davey et al. (1967) showed that postmortem aging had some effect on improved tenderness when 20 percent muscle shortening occurred, but there was no improvement in tenderness when shortening of 40 percent or more occurred with aging.

Elevated storage temperatures (such as 15°-20°C) greatly accelerate the rate of postmortem tenderness improvement (Sleeth et al., 1957, 1958; Busch et al., 1967; Parrish et al., 1969, 1973b; Martin et al., 1971). Busch et al. (1967) demonstrated that steaks from excised muscle stored at 16°C for 2 days were more tender than the steaks stored at 2°C for 13 days. Parrish et al. (1973b) found that rib steaks from carcass stored at 16°C for one day were as tender as steaks from the control side stored at 2°C for 7 days postmortem. This increase in tenderness obtained by aging carcasses at higher temperature was attributed to greater fragmentation of myofibrils during postmortem storage.

In postmortem muscle, the thick filaments interact with the thin filaments to form actomyosin when ATP in muscle tissue is exhausted. This actin-myosin interaction results in muscle inextensibility and thick and thin filaments are locked in that particular state of contraction. It is believed that rigor toughness is due to the shortening of sarcomere length resulting from the actin-myosin interaction (Locker, 1960; Locker and
Hagyard, 1963; Goll et al., 1964a; Marsh and Leet, 1966; Marsh and Carse, 1974).

Many reports have shown that the actin-myosin interaction in rigor muscle is modified during postmortem storage (Stromer and Goll, 1967a, 1967b; Stromer et al., 1967a; Goll et al., 1970, 1971, 1974a; Gothard et al., 1966; Takahashi et al., 1967; Fukazawa and Yasui, 1967). One of these modifications is a reversal of shortening. That contracted sarcomeres lengthened at a low level of ATP in postmortem muscle indicated the weakening of the actin-myosin interaction (Goll, 1968). Greater postmortem lengthening of sarcomeres was also observed in the muscles attached to the skeleton than the muscle excised immediately after death (Fukazawa and Yasui, 1967). Other evidence to suggest weakening of the actin-myosin interaction in postmortem muscle were the changes in myofibrillar ATPase activity (Fujimaki et al., 1965a; Goll and Robson, 1967; Robson et al., 1967) and in ATP dissociability of the actin-myosin complex (Fujimaki et al., 1965b). Incubation of isolated myosin B at low pH results in an increase of myofibrillar ATPase activity similar to that observed in postmortem muscle (Okitani et al., 1967). Recently, Suzuki and Goll (1974) demonstrated that a Ca\(^{2+}\) activated enzyme isolated from rabbit longissimus muscle could activate Mg\(^{2+}\)-modified myofibrillar ATPase activity. Another
factor which may be responsible for part of the postmortem modification of actin-myosin interaction is the alteration of sulfhydryl groups in myosin or actin, or both (Strandberg et al., 1973).

A second major alteration observed in the myofibril isolated from postmortem muscle is the gradual degradation of Z-disk structure. Stromer et al. (1967a) showed the disruption of Z-lines in bovine muscles stored at 2° or 16°C for 13 days. Takahashi et al. (1967) reported that loss of the zigzag configuration in the Z-line was noticeable in chicken muscle stored at 5°C for 24 hours. Henderson et al. (1970) observed that postmortem Z-disk breakdown occurred more rapidly in muscles postmortem aged at 25° or 37°C than at 2° or 16°C. Hay et al. (1973a) also observed the rupture of Z-lines in chicken breast muscle aged at 2°C for 2 days and the highly diffuse Z-line in samples aged for 7 days.

Degradation of Z-disk structure in the myofibril was shown in the increase of postmortem myofibril fragmentation when muscle samples were subjected to moderate homogenization. The number of sarcomeres in each myofibril decreased in the myofibrils prepared from postmortem muscle in comparison to the isolated myofibrils prepared from the sample immediately after death (Fukazawa and Briskey, 1969; Davey and Gilbert, 1967, 1969; Davey and Dickson, 1970; Henderson et al., 1970; Parrish et al., 1973b; Takahashi et al., 1967). The breakage of myofibrils
was usually found at the Z-disk or I-Z juncture suggesting that the Z-disk is the weak point in the structure of the myofibril (Stromer and Goll, 1967a; Takahashi et al., 1967; Fukazawa and Briskey, 1969).

Biochemical studies have also shown the weakening of bonds holding actin filaments to Z-lines in postmortem muscle. Davey and Gilbert (1968a) reported that myofibrillar protein from the isolated myofibrils increased from 54 percent for one-day postmortem muscle to 75 percent for 17-day postmortem muscle using an extraction solution containing 0.6 M KCl, 0.1 M phosphate, 1 mM Mg\textsuperscript{2+}, 10 mM pyrophosphate, pH 6.4 (Hasselbach-Schneider solution). These extracted proteins were further characterized with a moving-boundary electrophoresis and they showed that only myosin was extracted from at-death myofibrils and that additional proteins, actin and other water soluble proteins, were extracted in increasing amounts from postmortem myofibrils. Hasselbach and Schneider (1951) showed that strong KCl solution with 10 mM pyrophosphate dissociated the actin and myosin interaction to selectively extract myosin from washed myofibrils. The increased quantity of actin present in the extract from postmortem muscle indicate that either the F-actin filament depolymerization occurred or the bonds holding actin filaments to Z-disks weakened during postmortem storage of muscle. Haga et al. (1966a) demonstrated that the breakage of thin filaments from Z-disks
preceded the extraction of actin. Hence, the increased total protein and actin present in the protein extracts of postmortem muscle led Davey and Gilbert (1968b) to propose that meat progressively lost the tensile strength of the myofibrillar component of muscle during aging because of the weakening and final dissolution of the Z-disk structure. Results by Penny (1968) confirm these observations.

Chaudhry et al. (1969) observed that extractability of myofibrillar proteins increased markedly during aging at 2°, 16° and 25°C with 0.5 M KCl, 0.1 M potassium phosphate, pH 7.4. And this increased extractability of 0.5 M KCl began to appear 16-24 hours postmortem for rabbit and bovine muscle at 2°C, about 12 hours postmortem for bovine muscle at 16°C, and about 3-6 hours postmortem for rabbit muscle at 25°C. In a subsequent study (Chaudhry, 1969), it was found that rupture of the bonds between the I-Z filaments was the rate-limiting step in actomyosin extraction. Brief digestion of myofibrils with trypsin or papain caused the removal of Z-lines and the treated myofibrils were rapidly solubilized by high KCl solution.

Fukazawa et al. (1970) used a solution of 0.3 mM NaHCO₃ to extract well washed myofibrils at room temperature for 4 hours and they then fractionated the protein extracts into two fractions by the method of Ebashi et al. (1964); one was equivalent to α-actinin and the other was tropomyosin-
troponin. Proteins in both fractions increased from chicken pectoral muscles postmortem stored at 3°C for 48 hours. Further characterization of these fractions showed that fraction 1, equivalent to α-actinin fraction from post-rigor muscle, had noticeably higher accelerating effects on the superprecipitation of trypsin treated myosin B than fraction 1 from the pre-rigor muscle sample. The EGTA-sensitizing activity, which is a measure of the integrity of tropomyosin troponin complex in myofibrils, declines in fraction 2 from the 48 hour postmortem samples in spite of the increase in quantity of proteins extracted. It was proposed that the increased amount of extracted protein represented the release of α-actinin activity and the destruction and final dissolution of the Z-line structure during postmortem storage of chicken pectoral muscle.

Results of the histological and biochemical studies in the preceding discussions provide strong evidences that the rupture of Z-line structure and perhaps the weakening of I-Z bonds take place in the postmortem muscles.

Several researchers have investigated the possible causal agents or factors involved in the postmortem degradation of Z-disk structure (Fukazawa and Briskey, 1969; Davey and Gilbert, 1969; Busch et al., 1972a, 1972b; Goll et al., 1971, 1974a).

Fukazawa and Briskey (1969) found that the amount of
residues of sarcoplasmic protein fraction retained in the myofibril fraction caused a greater myofibrillar fragmentation. However, addition of fresh isolated sarcoplasmic protein to fresh prepared myofibrils did not show any effect.

Davey and Gilbert (1969) demonstrated that some factor in the sarcoplasmic protein fraction was responsible for the degradation or weakening of Z-disks and that this factor in sarcoplasmic protein fraction was inhibited by addition of 5 mM EDTA and 50 mM phosphate. These results implied that the factor in the sarcoplasmic protein fraction was calcium ion dependent.

Biochemical studies have provided direct evidence that calcium ion is involved in the degradation of the Z-line. It was noted that addition of calcium ion to the extract in a high concentration of KCl solution caused the release of F-actin from myofibrils and actomyosin was extracted (Haga et al., 1965, 1966b; Chaudhry, 1969). If phosphate buffer was used instead of Tris buffer in the high concentration of KCl extraction of minced muscle tissues only myosin was extracted. Chaudhry (1969) pointed out that phosphate was a strong calcium ion chelator and that this would retard the rupture of Z-disk structure.

In the study of postmortem isometric tension development and decline, Busch et al. (1972a) observed that muscle strips incubated in a solution containing 1 mM EDTA or 1 mM Ca$^{2+}$
showed no difference in isometric tension development. However, marked difference was noted in the isometric tension decline; 1 mM calcium ion accelerated the isometric tension decline whereas 1 mM of EDTA retarded the isometric tension decline (Busch et al., 1972a).

Phase microscopic examination showed that at-death myofibrils retained their Z-lines and rest length banding pattern and that similar structures and patterns were observed in the myofibrils from muscle strips incubated in 1 mM EDTA or 1 mM EGTA at 37°C for 24 hours. But incubation of the muscle strips with 1 mM Ca$^{2+}$ and 5 mM Mg$^{2+}$ at 37°C for 24 hours caused loss of Z-lines and resulted in extensive fragmentation of myofibrils into 2-4 sarcomere segments during myofibril preparation (Busch et al., 1972b). With electron micrographs it was further shown that thin filaments and M lines were intact in the myofibrils from muscle strips incubated in 1 mM Ca$^{2+}$ and 5 mM Mg$^{2+}$. This indicated that Ca$^{2+}$ had a very specific effect on Z-line structures (Busch et al., 1972b). In this same study a sarcoplastic protein fraction was isolated which was capable of removing Z-lines in the presence of 10 mM Ca$^{2+}$. This crude protein fraction was named Ca$^{2+}$-activated sarcoplasmic factor (CASF). It was suggested that the Ca$^{2+}$-activated factor in muscle tissue was responsible for Z-line degradation in postmortem muscle.
Ca$^{2+}$-Activated Factor

Goll and co-workers (Busch et al., 1972a, 1972b; Suzuki and Goll, 1974; Goll et al., 1974a; Parrish et al., 1975) have shown that Ca$^{2+}$-activated factor (CAF) was the causal agent related to postmortem modification of the actin-myosin interaction and the degradation of Z-disks. It has been suggested that CAF is the most important single factor in postmortem muscle changes (Goll et al., 1974a).

Recently, Dayton et al. (1974a, 1974b) successfully purified and characterized this enzyme. CAF was located in the sarcoplasmic protein fraction and required 1 mM Ca$^{2+}$ for maximum proteolytic activity, but 0.1 mM Ca$^{2+}$ gave detectable activity. CAF has an optimal pH of 7.5 and the enzyme is relatively inactive below pH 6.0. It was most active at 37°C, but some activity was detected even at 0°C. However, at 37°C, rapid autolysis occurred and in a prolonged incubation for 12 hours more soluble peptide materials were released from casein at 0°C than at 25°C. It appears that the enzyme is more stable at low temperatures near 0°C. The enzyme was inhibited by iodoacetate and heavy metal ions such as Fe$^{2+}$. Furthermore, purified CAF was very potent in the removal of Z-lines from isolated myofibrils in the presence of Ca$^{2+}$. Alpha-actinin is also released in an intact form from myofibrils by CAF. CAF has no effect on purified myosin, actin, α-actinin and
troponin C, but it degrades purified tropomyosin, troponin T, troponin I and component C. The rate of degradation of tropomyosin and troponin T decreased slightly when CAF was reacted with intact myofibrils. Degradation of troponin T, which has a molecular weight of 38,000 daltons, by CAF produces fragments with molecular weights of approximately 10,000 and 13,000 daltons via a series of transient intermediates of approximately 30,000 daltons.

Hay et al. (1973b) observed a 30,000 dalton band from postmortem myofibrils using SDS-polycrylamide gel electrophoresis. They suggested that this 30,000 dalton band was derived from one of the troponin subunits by a limited proteolysis during postmortem storage. However, they argued the importance of proteolysis in the aging process was inconsequential for chicken leg muscle because this 30,000 dalton band only appeared in myofibrils after 48 hours of aging when most changes associated with tenderness had already occurred. It was suggested that the proteolysis-tenderness relationship may only apply to certain muscle types.

Olson (1975) found that postmortem tenderness changes coincided with the change in the myofibril fragmentation index and these changes in postmortem storage corresponded to an increase of a 30,000 dalton component and the decrease of troponin T in the isolated myofibrils. It was shown that the total crude CAF activity assayed in vitro from bovine muscles
was related to the observed changes in tenderness, myofibril fragmentation index and degradation of troponin T in post-mortem muscle fibers of longissimus and semitendinosus muscles. High temperature aging accelerated these events. The demonstration of CAF activity in postmortem improvements in tenderness was a major achievement in the molecular explanation of beef tenderness. Jergenson (1975) showed that the postmortem degradation of troponin T was slightly more pronounced in porcine dark semitendinosus than the light portion. However, it was shown again that the appearance of 30,000 dalton component in the isolated myofibrils from postmortem muscle samples became apparent after 2 days storage at 2°C when large postmortem changes in tenderness had already occurred.

Penny et al. (1974) demonstrated a direct effect of CAF on the tenderness of freeze dried steaks from bovine semitendinosus. The freeze dried steaks rehydrated with a CAF containing solution were considerably more tender than the control steaks. More extensive degradation of Z-lines was noted in the sample reconstituted with CAF. This demonstrated a direct and close relationship between Z-line degradation and improved tenderness.
Addition of Ca$^{2+}$ and EDTA to Muscle

The role of calcium ion in living muscle and postmortem muscle has received extensive study because calcium ion is the chemical agent involved in triggering muscle contraction and postmortem rigor shortening. It is also able to accelerate glycolysis and rigor development (loss of extensibility) in postmortem muscle. Because postmortem rigor shortening and postmortem glycolysis have profound effects on meat quality, several studies on postmortem muscle have been undertaken to investigate the effect of addition or chelation of calcium ion (Weiner and Pearson, 1969; Pearson et al., 1973; Khan and Kim, 1975).

Pearson et al. (1973) showed that grinding bovine longissimus and biceps femoris muscles immediately after slaughter accelerated postmortem glycolysis. The ultimate pH of 5.6 was obtained about 6 hours postmortem in comparison to 16 hours for the intact control muscle sample stored at room temperature. Addition of calcium ion to the ground meat sample only had a small effect on accelerating postmortem glycolysis. It was possible that grinding ruptured subcellular membranes and released calcium ion. Injection of calcium ion into excised pre-rigor ovine muscle induced pronounced shortening ranging from 31 percent to 36 percent after storage at 15°C for 24 hours. Shear force increased markedly in those
muscle samples injected with calcium ion. These results demonstrated that calcium ion accelerated rigor development and triggered rigor shortening and toughening of meat despite aging at 15°C, a storage temperature where little shortening takes place in the control sample.

Khan and Kim (1975) added micro-injections of calcium ion into chicken breast muscle pre-rigor and post-rigor and observed that treatment of intact muscle post-rigor significantly lowered the shear force of meat compared with treatment immediately after slaughter (pre-rigor muscle). However, an aging period of more than 24 hours was required to obtain marked differences. Addition of calcium ion to minced pre-rigor muscle accelerated both postmortem glycolysis and adenosine nucleotide breakdown. Chelation of calcium ion by EDTA slowed down the breakdown of adenosine nucleotides as well as glycolysis.

Antemortem injection of either calcium ion chelator, EDTA or EGTA, inhibited muscle shortening, but they did not have an effect on ATP depletion and ultimate pH value (Weiner and Pearson, 1969). The shear values for muscle treated with EDTA were lower than those of untreated samples from rabbits and pigs. The cooking loss was highly correlated to initial muscle pH and ATP level and were also highly related to ultimate pH value of the samples. It was suggested that acceleration of postmortem glycolysis increased cooking loss.
Howard and Lawrie (1956a) carried out experiments to study the effect of pre-slaughter injection of calcium ion on beef quality. Blood calcium level was raised from 10 mg/100 ml to 30 mg/100 ml by injection of 20 percent calcium borogluconate. A level between 20 and 30 mg/100 ml was maintained for the subsequent 30 minutes before the animal was slaughtered. The chemical and physical postmortem changes were measured on the muscle samples stored under moist nitrogen at 37°C. The treated samples had shorter delay time prior to rigor mortis and more rapid decline in creatine phosphate, ATP and muscle pH values compared with the control samples. These effects were more noticeable in psoas muscle than in longissimus muscle.

In an extension of their original study Howard and Lawrie (1956b) showed that the loss of weight during freezing and storage of side of carcass from the treated animals was not significantly different from the control. Also the butcher's and laboratory drip were similar for both treated and control samples. The palatability of the treated samples showed no consistent effect in respect to any of the criteria of quality.

Injection of calcium ion into excised pre-rigor muscle caused shortening and toughening in muscles (Weiner and Pearson, 1969; Pearson et al., 1973; Khan and Kim, 1975), however, these shortening and toughening effects were not observed in muscles left attached to the carcass (Howard and Lawrie, 1956b). It
seems probable that muscles restrained on the carcass prevented the shortening and toughening effects caused by calcium ion.

In living muscle nerve impulses cause depolarization in subcellular membranes and as a consequence release calcium ion from the SR. The same principle was used by Carse (1973) to study the effect of electrical stimulation on postmortem changes in lamb muscles. Carse (1973) reported that electrical stimulation of freshly slaughtered lamb carcasses by 250v pulses for 30 minutes accelerated postmortem glycolysis and hastened postmortem rigor development. Meat from stimulated carcasses put into a blast freezer after 5 hours postmortem at 18°C were as tender as untreated carcasses held for 16 hours at 18°C before freezing. Although it was not investigated, electrical stimulation may have possibly caused the release of calcium ion from subcellular membranes of muscle fibers and accelerated postmortem reactions favorable to tenderness.
Structural and biochemical changes

Effects of heating on muscle tissues and their components in relation to meat palatability have been reviewed extensively (Hamm, 1960, 1966; Paul, 1963; Draudt, 1972; Laakkonen, 1973). Heating of muscle tissues causes the shrinkage and solubilization of connective tissues, specifically the collagen fibers, and induces shrinkage and coagulation of muscle fibers (Ritchey et al., 1963; Machlik and Draudt, 1963; Hostetler and Landmann, 1968). In heating, the reduction in volume and weight of meat is accompanied by a loss of muscle fluids (Hamm, 1966; Irmiter et al., 1967; Ritchey, 1965). The heating processes modify the structure and chemical properties of muscle tissues in such a manner as to have direct effects on the cooking yields and palatability of cooked meats.

Lowe (1955) reported that meat heated to an internal temperature of 90°C lost 34.6 percent in weight and 16.6 percent in volume. Giles (1969) observed that whole meat strips only showed slight shrinkage at 60°C, but shortened about 20 percent after heating at 70°C for 100 minutes. Sarcomere lengths shortened about 15 percent from strips heated at 70°C for 20 minutes and shortened to 20 percent after 100 minutes of heating at 70°C. Both the A band and the I band showed the same relative degree of shortening as the sarcomere.
Also the amount of shortening in sarcomere length was the same as that for whole muscle strips. Shortening of sarcomeres by heating was caused by coagulation of myofibrillar proteins and shrinkage of myofibrils (Hamm and Deatherage, 1960; Schmidt and Parrish, 1971). This is in contrast to rigor shortening involving a sliding of interdigitating thick and thin filaments in which only the width of I bands changes (Huxley, 1953; Stromer et al., 1967a). In rigor shortening, the percentage shortening or sarcomere length decrease represents the degree of overlapping of thick and thin filaments, i.e. the degree of muscle contraction (Marsh and Leet, 1966; Herring et al., 1967a). The extent of shrinkage in sarcomere length of heated muscle also depends on the initial unheated sarcomere length (Hegarty and Allen, 1972, 1975; Bouton et al., 1974). Hegarty and Allen (1975) showed that the greatest shortening due to heating occurred in the longest sarcomeres and that the degree of shortening was dependent on the degree of restraint imposed on the meat during heating.

Hostetler and Landmann (1968) microscopically observed the effect of heating on the isolated muscle fiber. Reduction of the width of fibers began at 45°C and it continued to decline until 60°C internal temperature. Decrease in length of fibers started at 55°C and continued to decrease to 80°C. The change in birefringence of heated samples showed that a drastic decline in birefringence occurred at 50°C and that
muscle fibers became isotropic by the time they reached 58° to 80°C. Hostetler and Landmann (1968) pointed out that the greatest shortening in fiber length occurred between 61° and 68°C. The greatest weight loss per minute of broiling time occurred at this temperature range (Ritchey, 1965).

Machlik and Draudt (1963) studied the effect of time and temperature on the shear patterns of small cylinders of Choice grade beef semitendinosus muscle heated for several hours between 50° and 90°C at 1°C intervals. A marked decrease in shear force was observed at 58°C after heating for 11 minutes, minimum shear values were obtained in the range of 60-64°C after heating for 30-60 minutes and shear force values increased at 68°C or higher. It was suggested that the drastic decline of shear values in muscle heated to 58°C was related to connective tissue shrinkage. And that in the range of 60-64°C, the collagen shrinkage reaction was completed quickly, whereas the hardening of myofibrillar proteins associated with higher temperatures, such as at 68°C or higher, was avoided. A point to be noted in this study, however, was the large sample variation. Similar results of the effect of heating on shear values have been reported by several researchers (Cover et al., 1962; Ritchey and Hostetler, 1964; J. G. Schmidt et al., 1970; Paul et al., 1973; Bouton and Harris, 1972a; Purchas, 1973; Bouton et al., 1974; Parrish et al., 1973b).
J. G. Schmidt et al. (1970) observed that minimum shear values for veal longissimus muscle was an internal temperature of 50°C whereas that for A and D maturity beef was 60°C. Significantly higher shear values were noted in the steaks from D maturity animals compared with veal and A maturity beef between 50 to 90°C. Increase in internal temperature above 60°C caused a slight increase in shear values in A and D maturity steaks and accompanying this was a slight decline in expressible juice ratio and increase in cooking loss.

Bouton and Harris (1972a) reported that there were important changes at three temperature zones, i.e., (a) room temperature (raw) to 50°C; (b) between 50 and 60°C; and (c) between 60 and 75°C. Temperatures up to 50°C had a toughening effect and this appeared to be related to the changes in water-holding capacity. Heating in the range of 50°C to 60°C was related to connective tissue softening changes and the temperatures between 60°C and 75°C resulted in increased cooking loss, fiber shortening and toughness.

Purchas (1973) studied the effects of heating from 40°C to 80°C on the contracted (cold shortened) and noncontracted (excised muscle stored at 16-20°C for 24 hours) beef semitendinosus. He found that the shear values for the contracted muscle increased from 40°C to 80°C with the greatest increase occurring between 50°C and 60°C, and that for the noncontracted
muscle sample a marked decline was noted between 50° and 60°C and then a slight increase occurred at 70° and 80°C. The curve of shear values for contracted muscle samples cooked from 48° to 80°C paralleled the curve of cooking loss. Other workers have confirmed this observation of cooking loss with increased temperature (J. G. Schmidt et al., 1970; Bouton and Harris, 1972a; Parrish et al., 1973a).

Bouton et al. (1974) studied the effects of heating on myofibrils and connective tissues in muscle samples (aged for 2 days) of different contraction states. They reported that adhesion values, which is a measure of connective tissue strength, of veal muscle samples decreased markedly at 50° to 60°C regardless of myofibrillar contraction state. Warner Bratzler (WB) shear values obtained for samples of calf muscle cooked to 50° or 60°C, however, was dependent on myofibrillar contraction state. This is in agreement with the work of Purchas (1973). WB shear values increased significantly as cooking temperature was increased from 60° to 90°C in the contracted muscle. Prolonged heating of contracted muscle at 90°C only slightly decreased WB shear values, even though there was a further large decrease in adhesion values. It seems that toughness and cooking loss of muscle heated from 50° to 90°C from more mature muscle are mainly caused by coagulation of myofibrillar proteins, especially those in the A band region.
As discussed previously, the toughness effect of rigor shortening became obvious only when rigor shortening was greater than 23 percent (Marsh and Leet, 1966; Marsh, 1972) or when the sarcomere length of raw meat was less than 1.6 μm or 1.7 μm (Herring et al., 1967a; Marsh and Carse, 1974). Marsh and Carse (1974) have made calculations to show that 24 percent of shortening in muscle corresponds to 1.6 μm of sarcomere length only shows the A bands and Z-lines based on the values that the A band and Z-line have widths of 1.5 μm and 0.1 μm, respectively (Bendall and Voyle, 1967). Hence, based on the relationship between degree of rigor shortening and tenderness (Marsh and Leet, 1966; Marsh and Carse, 1974) and on the increase of WB shear values in heating contracted muscle from 50° to 90°C (Purchas, 1973; Bouton et al., 1974), it seems that only a minimum of I bands in the myofibrils is essential to obtain a palatable meat after cooking.

Bouton et al. (1975a) reported that there were significant postmortem aging x temperature interactions for both initial yield force and peak force value measurements with the Warner-Bratzler shear device. Initial yield force values, which were related to myofibril strength, increased with cooking temperature but these values were reduced by postmortem aging. Calf longissimus muscle aged at 0-1°C for 3 weeks, showed a marked decrease in initial yield force and peak force and an increase in cooking loss at 60° and 90°C compared with
unaged samples. Both mechanical measurement values were less for aged than for unaged muscle samples. These results showed that weakening of myofibrillar structure by aging compensated for the toughening effect of coagulated myofibrillar proteins heated to high temperature. Similar results were also reported by Purchas (1973).

The effect of degree of marbling and internal temperature of doneness on beef rib steaks has been studied (Parrish et al., 1973a; Parrish, 1974). Marbling, the amount of intramuscular fat of beef longissimus muscle, is one of the major components used in the Federal beef quality grading system. For example, a steak from a U.S. Prime carcass is usually considered to have superior palatability attributes to one from U.S. Choice or Good grade beef, because of higher amount of intramuscular fat content. However, Parrish et al. (1973a) showed that increased quantities of marbling did not compensate for the reduction in palatability attributes of rib steaks heated to higher internal temperatures. A sensory panel found that degree of marbling had no significant effect on palatability attributes, but that degree of doneness was an important effector of juiciness, flavor, tenderness and overall acceptability. Furthermore, there was no interaction between degree of marbling and doneness. This work further substantiated that collagenic and myofibrillar proteins are the constituents of muscle tissue mainly involved with tenderness
Morphological changes in connective tissues and muscle fibers have been observed with light and electron microscopy (Paul, 1965; Schmidt and Parrish, 1971; Giles, 1969; Schaller and Powrie, 1972; Cheng and Parrish, 1975).

Paul (1965) studied the heat induced changes in connective tissue using light microscopy and reported that ribbons or bands of fibers were in the perimysial before heating. After heating a banding or clumping was initially noted in collagen fibers and upon further heating conversion of the fibrous clumping to granulation was subsequently observed. The endomysial sheath remained unchanged in heated muscle tissues.

Giles (1969) observed the fine structure of perimysial and endomysial collagen fibrils and found that they exhibited a 640 Å periodicity banding pattern when heated at 60°C from 10 to 140 minutes. At 70°C the collagen fibrils were swollen and denatured and no longer exhibited their characteristic fine striation structure and 640 Å periodicity.

Schmidt and Parrish (1971) made observations with light and electron microscopy on the changes in connective tissues and myofibrils in broiled steaks heated to 50°, 60°, 70°, 80° and 90°C internal temperatures. Endomysial connective tissue shrinkage was initiated at 50°C and completed at approximately 70°C. Perimysial connective tissue shrinkage required internal temperature of 70°C and higher before any significant fiber
changes were observed. Granulated materials were shown in the clefts between endomysial sheath and muscle fibers in the samples heated to 70°C or higher internal temperature. The endomysial sheath remained intact in samples heated to 90°C. With electron and phase contrast microscopy it was shown that myofibrillar proteins were compressed and sarcomeres shortened at 50°C. Heating to 60°C caused initiation of disintegration and coagulation of thin and thick filaments and further myofibrillar protein shrinkage. Heating to 70 and 80°C caused more disintegration of thin filaments and coagulation of thick filaments. At 90°C, an amorphous structure resulted, but regardless of the internal temperature the principal banding features of the sarcomere remained identifiable.

Schaller and Powrie (1972) observed bovine longissimus muscle, chicken semitendinosus and trout dorsal muscle heated to 60°C and 97°C in a water bath for an unspecified period of time with scanning electron microscopy. Raw muscle samples were cryofractured in liquid nitrogen and the fragmented samples were air dried at room temperature under a stream of nitrogen gas after dehydration with gradient ethanol. For muscles heated to 97°C, the structural integrity of myofibrils was lost near or at the transverse elements and granular materials beneath the sarcolemma of fibers from beef and chicken muscle were observed. Further investigation indicated that isolated sarcoplasm heated to 97°C formed
granular materials. They suggested that the granular materials observed in heated beef and chicken muscles could be due in part to precipitated sarcoplasmic proteins. Changes in muscle myofibrils from beef and chicken heated to 60°C were small although extensive damage was apparent in muscle from rainbow trout.

Changes in water-holding capacity

Because water-holding capacity (WHC) of meat has profound effects on meat palatability and processing (curing and cooking) yield, it has been studied very extensively (Hamm, 1960; Hamm and Deatherage, 1960; Cover et al., 1962; Ritchey and Hostetler, 1964; Sanderson and Vail, 1963; Bouton and Harris, 1972a; Bouton et al., 1973, 1975b). Hamm (1960) defined the WHC of meat as the ability of meat to hold fast to its own or added water during application of forces (pressing, heating or centrifugation, etc.). According to Hamm (1960), only 4-5 percent of the total water present in meat is bound tightly as "true hydration water". This type of water is hardly influenced by the shape and electrical charge of the muscle proteins. Another portion of water in muscle tissue is termed the physical-chemically "free" water which is immobilized within the microstructure of the myofibrils in muscle fibers (Hamm, 1960, 1970). The free water immobilized within muscle tissues can be liberated by application of a force and the
amount of water released, termed "loose water" depends on the methods used for its determination. The water retained by the tissue after application of a force is termed "bound" water.

The myofibrillar proteins, especially myosin and actin, are the major muscle proteins responsible for binding hydration water and for immobilization of "free" water in meat (Hamm, 1960, 1970).

Heating of muscle tissues causes denaturation and coagulation of myofibrillar proteins, shrinkage of myofilaments and tightening of the microstructure of myofibrils. All of these affects bring about an increase in the amount of "loose water" in muscle tissues. This increase in "loose water" by increased temperature or longer periods of heating muscle tissue is evidenced by greater cooking loss. The loss of WHC of muscle during heating is also evident in that a greater quantity of immobilized water is readily forced out of muscle tissue by a relatively low force. The amount of water forced out of muscle tissues is often termed expressed juice (EJ).

Hamm and Deatherage (1960) studied the WHC of muscle by adding 60 percent water to heated muscle and compared the percent bound water after pressing heated and unheated muscle tissue. They found that percent bound water drastically declined from 70 percent in muscle heated to 30°C and to less than 20 percent in muscle heated to 70°C.
In the same study, they (Hamm and Deatherage, 1960) also observed that the rigidity of heated muscle samples markedly increased from the 30°C to 50°C and then it leveled off. Although the changes in rigidity did not correspond to the changes in WHC of samples heated to temperatures greater than 60°C, their observations on WHC did demonstrate the continuous denaturation of myofibrillar proteins heated to higher temperatures.

Cover et al. (1962) studied the heating effects on juiciness and two components of tenderness: softness to tongue and cheek and softness to tooth pressure. They reported that meat became drier and harder when heated from 61° to 80°C internal temperature. This occurred more markedly in biceps femoris than in longissimus dorsi. Juiciness was not closely associated with any of the six components of tenderness but they found that moisture losses, as a percentage of that in raw, increased from 61° to 80°C and again from 80° to 100°C, which was the inverse of panel juiciness scores.

Weir (1960) separated juiciness into two effects: the impression of wetness during the first chews produced by the rapid release of meat fluids, and a sustained juiciness due to slow release of serum and to the stimulating effect of fat on salivary flow. Cover et al. (1962) suggested that panel juiciness scores were largely due to the first effect since it was usually scored during the first chews.
Sanderson and Vail (1963) reported mean press fluid values of 54.0, 45.2, and 36.4 percent for the steaks cooked to internal temperatures of 60°, 70° and 80°C, respectively. Bound water (not released by pressing but removed by vacuum oven drying) varied little when related to dry matter in the meat at each stage of cooking. It was further shown that shear values and panel tenderness scores indicated little change with degree of cooking in the *longissimus* muscle, but a considerable decrease occurred for *seminembranosus* and *semitendinosus* muscle.

Bouton and Harris (1972a) heated beef deep pectoral muscle from veal and 2-3 year old steers to 40°, 50°, 60°, 70°, 80° or 90°C for one hour. The percentage cooking loss increased drastically from 50°C to 80°C and the percent expressed juice (centrifugation at 100,000 x g for 60 minutes) was maximum at 50°C and then decreased inversely with cooking loss. As discussed previously, the changes in cooking loss corresponded to changes in shear values observed in contracted muscle samples heated from 40° to 80°C, but they were not parallel with the changes in shear values for the noncontracted muscle samples heated from 40° to 80°C (Purchas, 1973; Bouton et al., 1974).

In another study, Bouton et al. (1973) found that water retention capacity (WRC, a measurement of bound water) and expressed juice (EJ) both decrease with increasing temperature.
WRC values increased markedly with increasing pH, but the increase in EJ values was small and not always significantly related to pH. Taste panel scores for juiciness were not significantly related to pH. Consequently, juiciness was more closely related to EJ values, which changed little with increasing pH, than to WRC values when there is wide variation in pH values. A similar suggestion has been made by Cover et al. (1962).

Further study by Bouton et al. (1975b) indicated that EJ measurements would seem to have greater applicability than cooking loss measurements in assessing juiciness in cooked meat, but under many circumstances cooking loss measurements would be more convenient to use.

On the basis of reported results the effects of heating on WHC can be summarized as follows: 1) percent expressed juice (EJ) is closely related to the panel juiciness scores, particularly the juicy feeling of the first few chews; 2) percent cooking loss is directly related to cooking yields and the dryness feeling of cooked meat; 3) moisture loss in heated muscle contributes to increased toughness, however, this effect is complexed with other components related to tenderness, such as connective tissues and myofibrillar contraction state. It is clear, however, that tenderness and juiciness are closely related.
Effects on collagen fibers

Collagen fibers suddenly contract to one-third of their original length upon heating to certain temperatures. This temperature dependent characteristic is termed the thermal shrinkage temperature, $T_s$ (Goll et al., 1964b). Collagen shrinkage is caused by a rupture of the interchain cross linkages of collagen (Gustavson, 1955). Goll et al. (1964b, 1964c) showed that soluble substances (hydroxyproline and ninhydrin-positive materials) were released from bovine muscle collagen fibers during thermal shrinkage. These released substances are readily solubilized with neutral salt solution (Goll et al., 1964b,c), by Ringer's solution (Hill, 1966; Herring et al., 1967b), or by warm water (Paul et al., 1973; McCrae and Paul, 1974). Hydroxyproline can be readily assayed and used to determine collagen solubilized or the degree of thermal modification of collagen fiber molecules.

Goll et al. (1964b) studied the heating ($25^\circ$, $45^\circ$, $55^\circ$, $60^\circ$, $65^\circ$ and $70^\circ$C) effects on isolated loose connective tissue of bovine biceps femoris from various age groups. They found that solubilized collagen expressed as amount of hydroxyproline released increased with higher heating temperatures for all age groups. The rate and extent of collagen solubilization was age dependent. They (Goll et al., 1964b) also reported that the thermal shrinkage temperature, defined as a sudden release of soluble
hydroxyproline, increased with advancing age, i.e., near 55°C for Group I (veal), 55-60°C for Group II (steers), 60-65°C for Group III (cow) and over 70°C for Group IV (aged cow). The total hydroxyproline content, however, was not significantly different among the four age groups. WB shear values have shown, however, that tenderness decreases with age (Goll et al., 1963; J. G. Schmidt et al., 1970). This decrease in tenderness is more closely related to the nature of collagen rather than the amount. Collagen from more mature animals may possess more frequent or stronger cross linkages (Goll et al., 1964b).

Herring et al. (1967b) studied the collagen solubility of longissimus and semimembranosus from A, B and E maturities by heating the muscle samples in Ringer's solution at 77°C for 10 minutes. They reported that collagen solubility decreased significantly with each advancing maturity group in both longissimus and semimembranosus and that collagen solubility was higher for longissimus than semimembranosus muscle. A high correlation was found between collagen solubility and panel tenderness scores for different maturity groups, but this was not observed within maturity groups.

Machlik and Draudt (1963) found a marked decrease in shear occurred rapidly between 58° and 60°C and another decrease occurred between 71° and 75°C, this decrease becoming more pronounced with increasing temperature. They
concluded that the marked decline in shear at 58°C was due to collagen shrinkage and between 71° and 75°C it was due to the degradation of collagen fibers.

Penfield and Meyer (1975) oven roasted bovine semitendinosus muscle cores to four end points (40°, 50°, 60° and 70°C) and at two heating rates (93°C and at 143°C). They found that slower heating and higher end points produced more tender cores and resulted in greater solubilization of hydroxyproline containing materials. They reported a significant relationship between percentage of solubilized hydroxyproline and shear values during heating cores from 50°-60°C, however, a greater increase in percentage of solubilized hydroxyproline from 60° to 70°C was accompanied by only a small decrease in shear values. Similar results have been reported by Paul et al. (1973).

McCrae and Paul (1974) compared the collagen solubility of bovine semitendinosus muscle heated to internal temperature of 70°C by oven broiling, roasting, braising and microwave oven for 30 minutes. They found that microwave cooking solubilized more collagen from meat samples than did conventional cooking methods. Furthermore there were no significant differences in collagen solubility among different heating rates using the three conventional methods.

Bouton and co-workers (Bouton and Harris, 1972a, 1972b; Bouton et al., 1974, 1975a) measured adhesion values of
muscles heated to different internal temperatures with an Instron Universal Testing Machine. Adhesion value was defined as a measurement of connective tissue strength in muscle tissue. They showed that adhesion values decreased with higher temperatures and longer heating time with the greatest decrease occurring between 50° and 60°C for samples from veal and steers. Adhesion values were animal age and muscle type dependent, but postmortem aging and muscle pH had no effect on adhesion values. It seems that measurement of adhesion values by an Instron is a more sensitive method than by measurement of WB shear value for determining the changes in the strength of connective tissue in muscle tissue as affected by heating.

**Effect on sarcoplasmic and myofibrillar proteins**

Heating of muscle causes denaturation and coagulation of muscle proteins, resulting in hardening of muscle fibers and expulsion of muscle fluids (Hamm, 1960).

Hamm and Deatherage (1960) compared the effect of heating on hydration and rigidity to changes in the solubility of muscle proteins with increasing temperature. Solubility of the sarcoplasmic protein fraction changed little between 20° and 40°C, but the greatest decrease was observed between 40° to 60°C. Only small amounts of myofibrillar proteins were extracted from muscle heated between 60° to 80°C. Based on
the changes in hydration, rigidity and protein solubility, it was concluded that the greatest denaturation of the muscle proteins occurred between 40° and 80°C (Hamm and Deatherage, 1960). They pointed out that the decrease in myofibrillar protein solubility was more pronounced than the drop in hydration at 40°C but the small change in protein solubility was not comparable with the marked decline in the hydration between 60° and 80°C.

Heating of muscle also causes an increase in pH and a decrease in carboxyl groups in muscle proteins. A delay phase in the changes in hydration, rigidity and pH was noted between 50° and 55°C. Based on the decrease in carboxyl groups between 50° and 55°C, it was proposed that two different kinds of carboxyl groups exist each differing in their sensitivity to heat (Hamm and Deatherage, 1960). Two kinds of carboxyl groups may be located in separate structural proteins (such as myosin, actin, tropomyosin). It is possible that different myofibrillar proteins are denatured by heat in different ways. Although, they measured the viscosity and ATP sensitivity of protein solutions, no conclusive evidence was obtained to support their proposal (Hamm and Deatherage, 1960).

Paul et al. (1966) found that with increasing temperature, or increasing time at a given temperature, the nitrogen containing compounds in the sarcoplasmic and myofibrillar fractions decreased, while the denatured proteins (soluble
in 0.1N NaOH) increased, and the stroma fraction remained relatively constant. These results coupled with shear values showed that the increase in shear values between 65° and 75°C was due to denatured muscle proteins.

Randall and MacRae (1967) observed 3 cathodic bands and 13 anodic bands with starch gel electrophoresis of the water soluble fraction of bovine skeletal muscle. Similar results were observed by Laakkonen et al. (1970) with polyacrylamide gel electrophoresis. The changes in the nature of the water soluble proteins and juices of bovine muscle during low-temperature heating were also followed. It was found that the slowest moving anodic proteins coagulated first and that myoglobin and myoalbumins were altered significantly only by prolonged holding of meat at 60°C. Lee et al. (1974) used SDS-polyacrylamide gel electrophoresis to characterize the nature of water-soluble proteins from bovine muscle heated to 65°, 70°, 75°, 80°, 85° and 90°C. They found that there were five major bands at 65°C and that even at 90°C there was a fair amount of residual undenatured myoglobin.

Locker (1956) heated purified myosin and found that at 53°C, 82 percent to 92 percent of pure myosin was coagulated and at least four subunits of the myosin molecule were split off in a soluble state. Bourdillon (1956) reported the isolation of a heat stable crystalline protein after heating of muscle at 90°C. Since the heat stable protein has a large
length to diameter ratio it could be tropomyosin.

Tsai et al. (1972) demonstrated that myosin was the essential myofibrillar proteins in the stabilization of emulsions and in the formation of stable gel after heating at 70°C for 5 minutes. The emulsifying capacities of myosin, actin, tropomyosin-troponin and sarcoplasmic proteins were equally effective at a protein concentration of 12 mg/ml.

Samejima et al. (1969) demonstrated that myosin B forms a harder gel with more water content than myosin A when heated at 60°C for 30 minutes. F-actin alone exerts little influence on heat-gelling properties, but addition of F-actin to myosin A greatly improves the gel strength. It was also shown that the whole myosin molecule is necessary for gel formation function. Heavy meromyosin or light meromyosin, the proteolytic subunits of myosin by trypsin digestion, formed a gel with less gel strength than the gel from the parent protein, myosin.

Many studies have tried to investigate the mechanism of heat denaturation of proteins. The initial changes in heating of muscle tissues are the unfolding of peptide chains and the formation of new electrostatic and/or hydrogen cross-linkages. Further heating caused aggregations of peptide chains (Hamm and Deatherage, 1960; Tombs, 1970). And it is the aggregation of peptide chains which are responsible for the observed phenomena of gelation and hardening (Tombs, 1970).
Hamm and Hofmann (1965) have shown that heating of myofibrils to 70°C resulted in an increased number of sulfhydryl groups reacting with NEM (N-ethyl-maleimide). Heat denaturation causes an unfolding of the peptide chains and a release of reactive sulfhydryl groups hidden within the folded structure of the nature proteins.

Total sulfhydryl groups were relatively constant up to 70°C, and then they were reduced during further heating to higher temperatures. The reduction in total sulfhydryl groups resulted in the formation of disulphide bonds but these were reversed with reducing agents such as NaBH₄ (Hamm and Hofmann, 1965; Dubé et al., 1972).

Tombs (1970) described two kinds of protein-protein interaction in the heat processing of protein substances; covalent kinds of which the only probable kind is the disulphide bond, and noncovalent kinds involving electrostatic hydrogen bonds and hydrophobic interactions. Samejima et al. (1969), in the study of heat gelling properties of myofibrillar proteins, showed that neither a reducing reagent such as thioglycol or Na₂S₀₃, nor a SH-blocking reagent such as p-chloromercuribenzoate prevented the solution from coagulating, but rather accelerated the reaction up to 60°C. They (Samejima et al., 1969) suggested that heat coagulation of myofibrillar proteins was not due to the oxidation of
SH-groups, but to the intermolecular association of other side groups on the molecules.
MATERIALS AND METHODS

Source of Muscle Tissue

Muscle samples were obtained from the *M. longissimus* of 400-500 Kg, 12-18 months of age, about Choice grade, bovine animals fed similar rations at the Iowa State University Beef Nutrition Farm. Animals were slaughtered at the ISU Meat Laboratory and at-death muscle samples were obtained from the short loin region of the *M. longissimus* within one hour after exsanguination. Muscle samples were taken to the Food Research Laboratory and used immediately for myofibril preparation, for studies of the effects of calcium ion on postmortem biochemical changes and for studies of heat-induced biochemical changes in muscle proteins. Muscle was removed from the companion side of the carcass after aging for one day at 2°C. Subsequent aging was carried out by storing the excised one-day postmortem muscles in the Food Research Laboratory cold room at 2°C.

Treatment of Muscle Samples for Studies of the Effects of Calcium Ion on Postmortem Biochemical Changes

At-death bovine *M. longissimus* was excised from the carcass within one hour after exsanguination. The muscle samples were diced to less than 1 cubic centimeter after the samples
were trimmed free of connective and fat tissues. To control the concentration of calcium ion in the at-death muscle samples, the diced muscle samples were treated in the following ways: 1) 100 g of sample was mixed with 5 ml of 20 mM NaN_3; 2) 100 g of sample was mixed with 5 ml of 20 mM NaN_3, 20 mM CaCl_2; and 3) 100 g of sample was mixed with 5 ml of 20 mM NaN_3, 200 mM potassium oxalate. After thoroughly mixing the samples with the treatment solutions, the samples were ground once to facilitate the rapid penetration of added chemicals into muscle fibers. The samples so treated were referred to as the ground muscle control, the 1 mM calcium ion sample and the 10 mM oxalate sample. A sample treated with NaN_3 without grinding was referred to as the intact muscle control. The samples were stored in beakers and covered with Saran Wrap in the cold room (2°C) or on a laboratory bench (room temperature ~23°C).

For studies of the possible accelerative effect of calcium ion on postmortem biochemical changes, the treated at-death muscle samples were incubated in a water bath at 37°C for 1 to 2 1/2 hours. Both the temperatures of the water bath and meat were monitored with thermometers. The come-up time for a 70 g - 80 g muscle sample in a 300 ml beaker to reach 37°C was about five minutes.
Preparation of Myofibrils

Muscle samples were homogenized three times of 15 seconds duration each with an interval of 5 seconds between each duration in 10 volumes (V/W) of a 2°C isolating medium containing 0.25M sucrose, 0.05M Tris-acetate, 1 mM EDTA, 1 mM NaN₃, pH 7.6 with a Waring Blendor. The homogenate was sedimented at 1,000 x g for 15 minutes. Then the supernatant was passed through glasswool to remove fatty materials and used as a sample of sarcoplasmic proteins. The residue was resuspended in 5 volumes (V/W) of the previously mentioned isolating medium with a stir rod, sedimented again at 1,000 x g for 10 minutes and the supernatant decanted. The sediment was resuspended in 10 volumes (V/W) of the solution containing 100 mM KCl, 20 mM K phosphate (pH 6.8), 1 mM EDTA, 1 mM NaN₃ and passed through a polyethylene strainer to remove connective tissue and debris. The supernatant was decanted and the sediments were washed two more times by suspending in 5 volumes (V/W) of the previously mentioned buffered 100 mM KCl solution and sedimented at 1,000 x g for 10 minutes. Finally the sediments were resuspended in 5 volumes (V/W) of 100 mM KCl, 20 mM K phosphate (pH 6.8), 1 mM EDTA and 1 mM NaN₃.
Protein Extraction of Isolated Myofibrils

Isolated myofibrils were extracted with three types of solutions: 1) Hasselbach-Schneider (H-S) solution containing 0.6M KCl, 0.1M K phosphate, 1 mM MgCl₂, 10 mM sodium pyrophosphate, pH 6.4; 2) 1 mM Tris, pH 8.5 solution and 3) a high ionic strength solution containing 0.6M KCl, 0.1M K phosphate, pH 7.4. Not all three types of solutions were used in each experiment. The protein concentration of myofibrils used in extraction was 3 percent (i.e., 3g of myofibrils in 100 ml solution) for the extraction with H-S solution and high ionic strength solution containing 0.6M KCl, 0.1M K phosphate, pH 7.4 and 2 percent (i.e., 2g of myofibrils in 100 ml solution) for the extraction with 1 mM Tris, pH 8.5 solution. The concentration of myofibrils in the extraction suspension was so chosen so that the determination of protein concentration with the biuret method was optimal and the residue chemicals were reduced to minimal amounts.

Solutions were precooled to 2°C and all operations were conducted at 2°C.

Myofibril suspension (in 100 mM KCl, 20 mM K phosphate, pH 6.8, 1 mM EDTA and 1 mM NaN₃) was sedimented at 15,000 x g for 15 minutes and the supernatant decanted. The residues were then suspended in H-S solution or high ionic strength solution containing 0.6M KCl, 0.1M K phosphate, pH 7.4. For
the extraction with 1mM Tris, pH 8.5 solution, the sedimented myofibrils were resuspended in a solution containing 100 mM KCl, 10 mM Tris-HCl, pH 8.5, sedimented at 15,000 x g for 15 minutes and the supernatant decanted. The sediments were resuspended with 1 mM Tris solution (fresh made solution, ~pH 9.3). The desired pH values of myofibrils suspensions were adjusted after the myofibrils were completely suspended in the extraction solution.

Complete suspension of myofibrils into the extraction solution was taken as zero minutes of extraction. The suspension was then further extracted in the cold room (2°C) by gentle magnetic bar stirring for certain periods of time as indicated in the results section. At each time, 10 ml of suspension was removed and centrifuged at 15,000 x g for 15 minutes. The volume and the protein concentration of the supernatant were measured. A duplicate of 1 ml of myofibril suspension after extraction for 40 minutes to 60 minutes was removed and diluted to 5 ml with 2.5M KOH solution. The total protein concentration was determined for this sample by the biuret procedure.

Heating of Muscle Tissue

2.5g of diced muscle sample was placed in a glass test tube (25 ml) and heated in a water bath to the desired
temperature and held for a total of 30 minutes. Temperatures of muscle sample and water bath were simultaneously monitored with thermometers. The come-up time was around 3-5 minutes. After heating at the designated temperature test tubes were cooled in ice.

Protein Extraction of Muscle Tissue after Heating

After heating, the 2.5g of muscle sample was homogenized with a Waring Blendor for three 20 second periods with 5 second intervals between in 30 ml of solution containing 0.25M sucrose, 0.05M Tris, 1 mM EDTA, 1 mM NaN₃, pH 7.6 and sedimented at 15,000 x g for 15 minutes. The sediments were re-suspended in 20 ml of the same sucrose solution and extracted in the cold room for 1 hour. Then the suspension was sedimented at 15,000 x g for 15 minutes. The residues were extracted with 25 ml of a solution containing 0.6M KCl, 0.1M K phosphate, pH 7.4 in the cold room for 1 hour and then they were centrifuged at 15,000 x g for 15 minutes. The extraction was repeated twice more, one for an hour and the other for 12 hours. Before centrifugation, 1mM MgCl₂ and 1mM sodium pyrophosphate were added to the protein suspension to facilitate the separation of supernatant and residues in the raw muscle samples and the samples heated to 45°, 50° and 55°C. The volume and protein concentration of the supernatant were measured for each
extraction. The results were reported as mg protein per g of fresh muscle tissue.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

Myofibrils and protein extracts were run on SDS polyacrylamide gel electrophoresis according to the method of Weber and Osborn (1969) to determine alterations of isolated myofibrils and the composition of protein extract of postmortem muscle and heated samples.

Both 7 1/2 percent acrylamide and bis-acrylamide in a 75:1 weight ratio and 10 percent acrylamide and bis-acrylamide in a 37:1 weight ratio were used in gels which were run in 5 mm (inside diameter) x 120 mm tubes.

Myofibrils, in preparation for SDS-polyacrylamide gel electrophoresis, were dissolved in 2.5 percent SDS, 1 percent 2-MCE solution to a final concentration of 1 mg protein per ml and incubated at room temperature for 3-6 hours.

Samples of dissolved myofibrils and protein extracts were mixed with SDS-tracking dye in a ratio of 2:1 (V/V) and were boiled for 10 minutes before loading samples onto the gels. The SDS-tracking dye solution was composed of 5.2 percent SDS, 1.5M (or 32 percent) 2-MCE, 0.03 percent bromophenol blue and 20 percent glycerol in 60 mM Na phosphate buffer, pH 7.0. The loads applied to the gels were 10 μg to 30 μg for 7 1/2 percent
gels and 10 µg to 15 µg for 10 percent gels. The protein loads are indicated in the gel figures in the results section. The gels and the upper and lower reservoirs contained 100 mM Na phosphate (pH 7.1) and 0.1 percent SDS. Electrophoresis was performed at 20°-25°C in gels 8 cm long and at a constant current of 6 mamp per gel until the tracking dye had moved to approximately 1 cm to 1.5 cm from the bottom of the tube. The gels were stained in a solution of 0.1 percent Coomassie Brilliant Blue, 50 percent methanol and 7 percent glacial acetic acid for 12-16 hours and they were destained twice in a quick gel destainer (Canalco Co.) for 20 minutes in a solution of 7.5 percent (V/V) acetic acid and 5 percent (V/V) methanol. After destaining, the gels were stored in the dark in destaining solution until the background gel stain had been completely removed. The gels were photographed with a Nikon single-lens reflex camera on Kodak Panatomic X film. Some gels were photographed with a Polaroid camera on Kodak Ektapan film (4 x 5 in., No. 4162, thick). The identification of myofibrillar proteins was made by running the standard known proteins with SDS-polyacrylamide gel electrophoresis at the same time. No dialysis was carried out to remove excess KCl in the protein extracts with H-S solution or 0.6M KCl solution. After dilution, the concentration of KCl in protein solution applied to gels was usually less than 150 mM and no
precipitation of potassium dodecyl sulfate was observed in the protein solution.

The procedures used for electrophoresis of protein extracted from heated muscle samples were essentially the same as the method used for the raw muscle sample studies with the exception that 8 1/2 percent acrylamide and bis-acrylamide in a 45:1 weight ratio was used.

Protein Concentration and Muscle pH Measurement

The protein concentration of isolated myofibrils and protein solutions were determined by the biuret procedure of Gornall et al. (1949) or the method of Robson et al. (1967).

One-two g of muscle sample was homogenized with 15 ml of neutralized 2 mM iodoacetate solution in a Waring Blender for 10 seconds and pH values were measured with Radiometer pH Meter 26.
RESULTS

The first phase of this study was to determine the postmortem biochemical alterations in the integrity of isolated myofibrils and their proteins from at-death and postmortem muscle with SDS-polyacrylamide gel electrophoresis. The biochemical changes in postmortem muscle fibers were studied by selectively extracting myofibrillar proteins from myofibrils with Hasselbach-Schneider (H-S) solution and a low ionic strength salt solution (1 mM Tris, pH 8.5). The postmortem biochemical changes were also studied in at-death muscle and 1-day and 7-day postmortem muscle samples by heating samples in a water bath to 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes. The results obtained with protein extraction and electrophoresis were used to interpret the nature of the postmortem modification in the interaction and the integrity of myofibrils and their proteins.

Another phase of this study was to determine the effect of addition and chelation of calcium ion to a muscle mince on myofibrillar proteins during postmortem storage. The purpose of this was to test the possible direct relationship between availability of calcium ion and postmortem biochemical changes in muscle fibers. Furthermore, the possible accelerative effect of calcium ion on postmortem biochemical changes were studied by incubating at-death muscle of different
treatments (ground muscle control, 1 mM Ca\(^{2+}\) sample, 10 mM oxalate sample) at 37°C for 1 to 2 1/2 hours. The alterations in the integrity of myofibrillar proteins and the extractability of \(\alpha\)-actinin by 1 mM Tris, pH 8.5 solution were followed in the samples after incubation and in the subsequent post-mortem storage.

The following are the results of the studies of post-mortem aging, calcium treatment and heating on muscle proteins.

**Postmortem Biochemical Changes in Myofibrils and their Proteins**

In this study, myofibrils were prepared from at-death muscle from four animals and the samples were stored at 2°C for 1, 2 and 10 days. The changes in the composition of isolated myofibrils and the changes in the integrity of myofibrillar proteins were detected by using SDS-polyacrylamide gel electrophoresis. The 7 1/2 percent and 10 percent SDS polyacrylamide gels of isolated myofibrils from at-death and postmortem muscle samples at 2°C for 1, 2 and 10 days are shown in Fig. 1. No changes were observed in the major myofibrillar proteins, myosin, actin, tropomyosin and \(\alpha\)-actinin of isolated myofibrils from postmortem muscles. The only postmortem alteration observed was the gradual disappearance of the troponin T band, a 38,000 dalton protein, and a corresponding increase in the intensity of a 30,000
Fig. 1. SDS - 7 1/2 percent and 10 percent polyacrylamide gels of myofibrils prepared from bovine longissimus muscle at-death and at different times of postmortem storage at 2°C.

Note the gradual decrease of the troponin T (TnT) band and the gradual increase of the 30,000 dalton band from 0 to 10 days postmortem. No other major changes of other band are noted.

Protein load: 28 μg for 7 1/2 percent gels; 14 μg for 10 percent gels.
dalton band. This was very clear in the isolated myofibrils from 2-day postmortem muscle samples. Troponin T had completely disappeared from myofibrils isolated from 10-day postmortem muscle.

The composition of the major sarcoplasmic proteins extracted with 0.25M sucrose solution from at-death and postmortem stored muscle samples at 2°C for 1 and 10 days was also determined with SDS-gel electrophoresis (Fig. 2). The minor band (arrow) just below phosphorylase (~94,000 daltons) disappeared in the postmortem muscle samples stored at 2°C for 1 and 10 days. No other postmortem alteration in the sarcoplasmic proteins was observed.

To further study the postmortem alterations in the integrity and in the interaction of myofibrils and their proteins, isolated myofibrils were extracted with H-S solution (0.6M KCl, 0.1M phosphate, 1 mM MgCl₂, 10 mM sodium pyrophosphate, pH 6.4) and a low ionic strength solution (1 mM Tris, pH 8.5).

Percentage extractability of proteins with H-S solution increased markedly in all isolated myofibrils from postmortem muscle samples compared with at-death muscle samples (Fig. 3). Using a 60 minute extraction period with H-S solution and calculating percentage extractability to total myofibrillar protein content the following results were obtained: 65 percent for the 1 day postmortem sample, nearly 75 percent for
Fig. 2. SDS - 10 percent polyacrylamide gels of sarcoplasmic proteins prepared from bovine \textit{longissimus} muscle at-death and at different times of post-mortem storage at 2°C. 

Note the disappearance of the minor band (arrow) just below phosphorylase in 1-day and 10-day postmortem muscles.

Protein load: 14 \textmu{g}.
DAYS POSTMORTEM

0  1  10

PHOSPHORYLASE

DEHYDROGENASE

MYOGLOBIN
Fig. 3. The time-course of extraction with H-S solution of isolated myofibrils from bovine longissimus muscle at-death (DAY-0) and at different times (DAY-1, DAY-2 and DAY-10) of postmortem storage at 2°C.
the 2-day postmortem sample, and 70 percent for the 10-day postmortem sample. In contrast, only 37 percent was extracted for the at-death sample. With prolonged extraction (for 420 minutes) of isolated myofibrils with H-S solution, the percentage extractability was only 43 percent for at-death muscle sample, and nearly 75 percent for all postmortem muscle samples (Fig. 3). Hence, myofibrillar proteins from postmortem muscle are more soluble by H-S solution than from at-death muscle; however, there is a certain percentage (about 25 percent) that are not soluble by H-S solution even under the conditions of postmortem aging or prolonged extraction times.

The amount of protein extracted from isolated myofibrils from postmortem muscle by 1 mM Tris, pH 8.5 is shown in Fig. 4. The percentage extractability was less from 1-day postmortem samples than it was from at-death samples and then it progressively increased from 2-day and 10-day postmortem samples. A 40 minute extraction period with 1 mM Tris, pH 8.5, resulted in 4.5 percent for at-death, 3.5 percent for 1-day, 6.5 percent for 2-day and nearly 7.8 percent extractability for 10-day postmortem muscle samples. Prolonged extraction (420 minutes) of isolated myofibrils with 1 mM Tris, pH 8.5 solution caused a noticeable change for at-death (7.8 percent) and 1-day postmortem samples (5 percent), but little change occurred for 2- and 10-day postmortem samples (Fig. 4).
Fig. 4. The time-course of extraction with 1 mM Tris, pH 8.5 solution of isolated myofibrils from bovine longissimus muscle at-death (DAY-0) and at different times (DAY-1, DAY-2 and DAY-10) of postmortem storage at 2°C.
SDS-polyacrylamide gels of the proteins extracted with H-S solution are shown in Figs. 5 and 6. The SDS-polyacrylamide gels showed that myosin was the predominant protein extracted from at-death and postmortem muscle samples stored at 2°C for 1, 2 and 10 days. All subunits of the myosin molecule were shown, the heavy chain of 200,000 daltons and the light chains of 24,000 and 19,000 daltons. The proteins of the thin filaments, actin (44,000 daltons), tropomyosin (35,000 daltons) and troponin T (38,000 daltons) were extracted with H-S solution in small quantities from at-death and 1-day postmortem muscle samples. With prolonged extraction (420 minutes) the intensity of the proteins associated with the thin filaments increased slightly. In particular, the amount of actin increased slightly from 2- and 10-day postmortem muscle samples. The disappearance of troponin T (38,000 daltons) and the appearance of a 30,000 dalton band occurred in the protein extracts with H-S solution in the 2- and 10-day postmortem muscle samples.

The results of SDS-polyacrylamide gels showed that H-S solution was very specific for extraction of the thick filament protein, myosin, and it was able to extract proteins of the thin filaments, actin, tropomyosin, and troponin only in limited quantities. The increase in percentage extractability of myofibrillar proteins from postmortem muscle with H-S solution appeared to be mainly due to the increase in the
Fig. 5. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (H-S solution) from isolated myofibrils of bovine longissimus muscle at-death (DAY-0) and 1-day postmortem storage at 2°C. Troponin T (TNT), Tropomyosin (TM).

Protein load: 30 μg.
Fig. 6. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (H-S solution) from isolated myofibrils of bovine longissimus muscle 2-day and 10-day postmortem storage at 2°C. Troponin T (TNT), Tropomyosin (TM).

Protein load: 30 μg.
POSTMORTEM TIME
EXTRACTION TIME (hr) 0 1/2 7

MYOSIN HEAVY CHAIN

DAYS

DAY 2

DAY 10

ACTIN

TNT

30,000 DALTONS

MYOSIN LIGHT CHAINS
amount of myosin extracted.

Figs. 7 and 8 illustrate the results (SDS-polyacrylamide gels) of the protein extracted with 1 mM Tris, pH 8.5 solution. It is clear that actin, tropomyosin and troponin of thin filaments, component C and myosin light chains of thick filaments were extracted from at-death myofibrils by low ionic strength solution at pH 8.5 (Fig. 7). However, a protein of the Z-disk, α-actinin, was only extracted from at-death muscle in trace amounts even with prolonged extraction periods (420 minutes) but the amount of α-actinin increased very noticeably in postmortem muscle samples even with short extraction times (Fig. 8). For all postmortem samples, actin was the major protein extracted, component C appeared to increase slightly in the protein extracted from one day and gradually decreased in 2- and 10-day postmortem muscle samples, and tropomyosin extractability declined markedly in 1-day postmortem muscle samples. The decline in tropomyosin extraction is a likely explanation for the reduction in total percentage extractability in 1-day samples (Fig. 4). Also, a trace of the myosin heavy chain (200,000 daltons) was observed for all postmortem muscle samples.

Figs. 9 and 10 show the proteins extracted from isolated myofibrils by 0.6M KCl, 0.1M K phosphate, pH 7.4 solution for at-death and postmortem muscle samples. All myofibrillar
Fig. 7. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution) from isolated myofibrils of bovine longissimus muscle at-death (DAY-0) and 1-day postmortem stored at 2°C. Troponin T (TNT), Tropomyosin (TM). Protein load: 10 µg.
<table>
<thead>
<tr>
<th>COMPONENT-C</th>
<th>α-ACTININ</th>
<th>ACTIN</th>
<th>TNT</th>
<th>TM</th>
<th>MYOSIN LIGHT CHAIN</th>
<th>TNI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>POSTMORTEM TIME</th>
<th>EXTRACTION TIME (hr)</th>
<th>DAY 0</th>
<th>DAY 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1/2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1/2</td>
<td>7</td>
</tr>
</tbody>
</table>
Fig. 8. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution) from isolated myofibrils of bovine longissimus muscle 2-day and 10-day postmortem stored at 2°C. Tropomyosin (TM), Troponin I (TNI).

Protein load: 10 μg.
POSTMORTEM TIME
EXTRACTION TIME (hr)

DAYS

| 0 | 1/2 | 7
|---|-----|---

DAYS

| 0 | 1/2 | 1 | 40
|---|-----|---|---

MYOSIN
HEAVY CHAIN

COMPONENT

α-ACTININ

ACTIN

TM

TNI
Fig. 9. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (0.6M KCl, 0.1M K phosphate, pH 7.4) from isolated myofibrils of bovine *longissimus* muscle at-death (DAY-0) and 1-day postmortem stored at 2°C. Tropomyosin (TM), Troponin T (TNT), Troponin I (TNI).

Protein load: 30 µg.
<table>
<thead>
<tr>
<th>POSTMORTEM TIME</th>
<th>DAY 0</th>
<th>DAY 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXTRATION TIME (hr)</td>
<td>0 1 7</td>
<td>0 1 7</td>
</tr>
<tr>
<td>MYOSIN HEAVY CHAIN</td>
<td><img src="image1.png" alt="Blots" /></td>
<td><img src="image2.png" alt="Blots" /></td>
</tr>
<tr>
<td>( \alpha )-ACTININ</td>
<td><img src="image3.png" alt="Blots" /></td>
<td><img src="image4.png" alt="Blots" /></td>
</tr>
<tr>
<td>ACTIN</td>
<td><img src="image5.png" alt="Blots" /></td>
<td><img src="image6.png" alt="Blots" /></td>
</tr>
<tr>
<td>TNT</td>
<td><img src="image7.png" alt="Blots" /></td>
<td><img src="image8.png" alt="Blots" /></td>
</tr>
<tr>
<td>TM</td>
<td><img src="image9.png" alt="Blots" /></td>
<td><img src="image10.png" alt="Blots" /></td>
</tr>
<tr>
<td>TNI</td>
<td><img src="image11.png" alt="Blots" /></td>
<td><img src="image12.png" alt="Blots" /></td>
</tr>
</tbody>
</table>
Fig. 10. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (0.6M KCl, 0.1M K phosphate, pH 7.4) from isolated myofibrils of bovine longissimus muscle 2-day and 10-day postmortem stored at 2°C. Tropomyosin (TM), Troponin I (TNI).
Protein load: 30 μg.
POSTMORTEM TIME
EXTRACTION TIME (hr)

DAYS 0 1 7

MYOSIN HEAVY CHAIN
α-ACTININ

DAYS 0 1 7

ACTIN
TM

MYOSIN LIGHT CHAIN
TNI
proteins were extracted by high ionic strength solution, but the amount of actin and α-actinin extracted were relatively small from at-death muscle samples (Fig. 9), but they gradually increased from postmortem muscle samples (Fig. 10). The disappearance of troponin T (38,000 daltons) and the appearance of a 30,000 dalton band were also observed in the protein extracts from postmortem muscle samples. This was most noticeable from 2- and 10-day postmortem muscle samples (Fig. 10). Obviously, 0.6M KCl, 0.1M K phosphate, pH 7.4 solution was not as selective in the extraction of myofibrillar proteins as H-S solution was (Figs. 5 and 6) because H-S solution predominantly extracted myosin whereas 0.6M KCl, 0.1M K phosphate, pH 7.4 solution extracted noticeable quantities of actin and α-actinin.

Postmortem Biochemical Changes in Muscles Heated to 45°, 50°, 55°, 60°, 70° and 80°C for 30 Minutes

Most postmortem biochemical changes are studied on raw muscle samples, but most subjective and objective measurements on meat palatability are usually conducted with cooked muscle samples. A recent study has shown a strong relationship between the postmortem biochemical changes of the raw sample and the palatability of cooked meat (Olson, 1975; Parrish et al., 1975). It was found that correlation coefficients
between myofibril fragmentation index and W-B shear force ranged from -0.65 to -0.97 and between myofibril fragmentation index and sensory tenderness ranged from 0.63 to 0.94 for steaks from M. longissimus of veal, A maturity and C maturity carcasses. Other workers have shown that postmortem aging compensated for the increase in the toughening effect caused by higher internal temperature of cooked meat (Bouton et al., 1975a; Purchas, 1973). It was the purpose of this part of the study to use biochemical techniques to detect postmortem changes in sarcoplasmic and myofibrillar proteins of muscles heated to 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes.

The amounts of sarcoplasmic proteins in the first extraction of 0.25M sucrose buffered solution are graphically illustrated in Fig. 11. Two main observations can be cited about the effect of heating on sarcoplasmic protein extractability. First the most drastic reduction in extractability occurred in samples heated to 50°C (day-0 sample) or to 55°C (postmortem samples). Extractability continued to gradually decline at 60°C and then it leveled off at 70° and 80°C. Secondly, sarcoplasmic proteins extractability were greater from at-death muscle than it was from the 1- and 7-day postmortem muscle samples when heated to 45°, 55° and 60°C.

The changes in extractability of myofibrillar proteins by the first extraction with a buffered 0.6M KCl solution are
Fig. 11. Effect of heating on the extractability (0.25 M sucrose, 0.05 M Tris, 1 mM EDTA and 1 mM NaN3, pH 7.6) of sarcoplasmic proteins from at-death and postmortem stored at 2°C bovine longissimus muscle.
Fig. 12. Effect of heating on the extractability (0.6M KCl, 0.1M K phosphate, pH 7.4) of myofibrillar proteins from at-death and postmortem stored at 2°C bovine longissimus muscle.
graphically depicted in Fig. 12. Also a very noticeable large decline in myofibrillar proteins occurred in those postmortem muscle samples heated to 55°C. Heating at-death muscle samples to 50°C resulted in a precipitous decline in the amount of myofibrillar proteins extracted, compared with 1- and 7-day postmortem muscle samples.

The SDS-polyacrylamide gels of proteins extracted with 0.25M sucrose from at-death, 1- and 7-day postmortem muscle samples are shown in Figs. 13, 14 and 15. Phosphorylase (~94,000 daltons) was completely denatured in all muscle samples heated to 50°C. There were four bands of sarcoplasmic proteins denatured at lower heating temperatures for postmortem muscles compared with at-death muscle samples. This difference may be due to the difference in muscle pH between at-death and postmortem muscle samples. All sarcoplasmic proteins were denatured at 70°C with the exception of myoglobin.

The SDS-polyacrylamide gels of myofibrillar proteins extracted with 0.6M KCl solution from at-death and 1- and 7-day postmortem muscle samples are shown in Figs. 16, 17 and 18. No myosin heavy chain was observed in any of the three muscle samples heated to 55°C; however, samples heated to 55°C or 60°C clearly show that the thin filament proteins, actin, tropomyosin and troponin, are more heat tolerant than those of the thick filament. The quantity of the myosin
Fig. 13. SDS - 8 1/2 percent polyacrylamide gels of sarcoplasmic proteins extracted with sucrose solution (0.25M sucrose, 0.05M Tris, 1 mM EDTA, 1 mM NaN₃, pH 7.6) from at-death bovine longissimus muscle heated at 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes.

Protein load: 20 µg.
112

HEATING TEMPERATURE (°C)

RAW 45 50 55 60 70 80

PHOSPHORYLASE

44,000 DALTONS

DEHYDROGENASE

MYOGLOBIN
Fig. 14. SDS – 8 1/2 percent polyacrylamide gels of sarcoplasmic proteins extracted with sucrose solution (0.25M sucrose, 0.05M Tris, 1 mM EDTA, 1 mM NaCl, pH 7.6) from 1-day postmortem stored at 2°C bovine longissimus muscle heated at 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes. Protein load: 20 μg.
HEATING TEMPERATURE (°C)

RAW 45 50 55 60 70 80

- PHOSPHORYLASE
- 44,000 DALTONS
- DEHYDROGENASE
- MYOGLOBIN

°C
Fig. 15. SDS - 8 1/2 percent polyacrylamide gels of sarcoplasmic proteins extracted with sucrose solution (0.25M sucrose, 0.05M Tris, 1 mM EDTA, 1 mM NaN3, pH 7.6) from 7-day postmortem stored at 2°C bovine longissimus muscle heated at 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes. Protein load: 20 μg.
<table>
<thead>
<tr>
<th>HEATING TEMPERATURE (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHOSPHORYLASE</td>
</tr>
<tr>
<td>44,000 DALTONS</td>
</tr>
<tr>
<td>DEHYDROGENASE</td>
</tr>
<tr>
<td>MYOGLOBIN</td>
</tr>
</tbody>
</table>
Fig. 16. SDS - 8 1/2 percent polyacrylamide gels of myofibrillar proteins extracted with high ionic strength solution (0.6M KCl, 0.1M K phosphate, pH 7.4) from at-death bovine longissimus muscle, heated at 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes. Troponin T (TNT), Troponin I (TNI), Tropomyosin (TM).

Protein load: 20 µg.
HEATING TEMPERATURE (°C)

RAW  45  50  55  60  70  80

MYOSIN HEAVY CHAIN

α-ACTININ

ACTIN

TNT

MYOSIN LIGHT CHAINS
Fig. 17. SDS - 8 1/2 percent polyacrylamide gels of myofibrillar proteins extracted with high ionic strength solution (0.6M KCl, 0.1M K phosphate, pH 7.4) from 1-day postmortem stored at 2°C bovine longissimus muscle heated at 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes. Troponin T (TNT), Troponin I (TNI), Tropomyosin (TM).
Protein load: 20 µg.
<table>
<thead>
<tr>
<th>HEATING TEMPERATURE (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW 45 50 55 60 70 80</td>
</tr>
</tbody>
</table>

**MYOSIN**

- Heavy Chain
- \(\alpha\)-Actinin

**ACTIN**

- TNT
- TM

**MYOSIN LIGHT CHAIN**

- TNI
Fig. 18. SDS - 8 1/2 percent polyacrylamide gels of myofibrillar proteins extracted with high ionic strength solution (0.6M KCl, 0.1M K phosphate, pH 7.4) from 7-day postmortem stored at 2°C bovine longissimus muscle heated at 45°, 50°, 55°, 60°, 70° and 80°C for 30 minutes. Tropomyosin (TM), Troponin I (TNI).

Protein load: 20 μg.
heavy chain was highest in the 7-day postmortem muscle sample heated to 50°C for 30 minutes. Higher intensity of actin band in the SDS-polyacrylamide gels of raw samples and the samples heated to 70°C was observed in postmortem muscle samples than in at-death samples. The amount of tropomyosin was reduced markedly in all three muscle samples heated to 70°C, however, traces of actin and tropomyosin were still observed in samples heated to 80°C. The effect of heating on α-actinin was not clearly illustrated with high KCl solution extraction. However, it is possible that α-actinin is completely denatured at 50°C because it has been denatured in porcine longissimus muscle at this temperature (personal observation).

Effects of Calcium Ion on the Postmortem Biochemical Changes in Muscle Proteins

The postmortem biochemical changes in muscle fibers observed in the first phase of this study resemble the results obtained from brief digestion of isolated at-death myofibrils with Ca$^{2+}$-activated factor (CAF). CAF hydrolyzes troponin T (38,000 daltons) into a 30,000 dalton component in isolated myofibrils (Dayton et al., 1974a, 1974b; Olson, 1975) and CAF is very potent in releasing α-actinin from the Z-lines of isolated myofibrils (Busch et al., 1972b; Dayton et al., 1974a, 1974b; Suzuki and Goll, 1974; Olson, 1975)
and hence weakens I-Z bonds (Goll et al., 1970). Dayton et al. (1974a, 1974b) reported that a minimum of 0.1 mM calcium ion was necessary for CAF activity. It would seem possible that CAF activity could be detected in postmortem muscle fibers by controlling the subcellular concentration of calcium ion in the muscle. By addition of calcium ion or a calcium ion chelator, such as oxalate, fluoride or EDTA to an at-death muscle sample, CAF activity should be activated or retarded. To determine the effects of calcium ion on the postmortem biochemical changes in muscle fibers, a second phase of this study was carried out by using minced at-death muscle samples with addition of 1 mM calcium ion or 10 mM oxalate and storage at 2° and 23°C. Three animals were used in this part of the study. The results presented are considered typical or characteristic of the observations.

Myofibrils and sarcoplasmic proteins were isolated from treated muscle samples postmortem stored at 2°C or room temperature (23°C) for 1, 2 and 10 days. The treatment of muscle samples included the control ground muscle, the 1 mM calcium ion sample and the 10 mM oxalate sample.

Both 7 1/2 percent and 10 percent SDS-polyacrylamide gels of isolated myofibrils are shown in Figs. 19, 20 and 21. The gels of the at-death muscle sample clearly show troponin T and no 30,000 dalton band (Fig. 19). After storage at 2°C for 1 day, the 30,000 dalton component begins to show up and
Fig. 19. SDS - 7 1/2 percent and 10 percent polyacrylamide gels of myofibrils prepared from at-death and 1-day postmortem bovine longissimus muscle.

At-death muscle sample, Io; 1-day postmortem muscle samples, I (ground muscle control); II (1 mM Ca^{2+} sample); III (10 mM oxalate sample). a (postmortem stored at 2°C); b (postmortem stored at 23°C). Troponin T (TNT), Troponin I (TNI).

Protein load: 28 µg for 7 1/2 percent gels; 14 µg for 10 percent gels.
troponin T still persists in the ground muscle control and the 1 mM calcium ion sample. After storage at room temperature for 1 day, however, the 30,000 dalton component was very clear in both the ground muscle control and the 1 mM calcium ion sample whereas troponin T was completely absent. However, no change was noted in the 10 mM oxalate sample stored at 2°C or room temperature; i.e., troponin T remained unchanged and no 30,000 dalton component appeared. These results were shown more clearly in 10 percent gels. Other changes shown in the 7 1/2 percent gels were the extra minor bands in the M-protein region and the reduced intensity of component C in the ground muscle control and the 1 mM calcium ion sample stored at room temperature (Fig. 19).

The postmortem biochemical changes were more noticeable in 2-day postmortem muscles in both the ground muscle control and the 1 mM calcium ion sample (Fig. 20). The disappearance of troponin T and the appearance of the 30,000 dalton component were also observed in the 10 mM oxalate sample stored for 2 days at room temperature, but they were not observed in samples stored at 2°C. The intensity of troponin I was reduced in the ground muscle control and the 1 mM calcium ion sample stored for 2 days at room temperature (Fig. 20).

Storage of muscle samples at room temperature for 10 days resulted in the disappearance of troponin T with the exception of the 10 mM oxalate sample stored at 2°C in which
Fig. 2J. SDS - 7 1/2 percent and 10 percent polyacrylamide gels of myofibrils prepared from 2-day postmortem bovine longissimus muscle.
I (ground muscle control); II (1 mM Ca$^{2+}$ sample); III (10 mM oxalate sample). a (postmortem stored at 2°C); b (postmortem stored at 23°C). Troponin T (TNT), Troponin I (TNI).
Protein load: 28 µg for 7 1/2 percent gels; 14 µg for 10 percent gels.
Fig. 21. SDS - 7 1/2 percent and 10 percent polyacrylamide gels of myofibrils prepared from 10-day postmortem bovine longissimus muscle.

I (ground muscle control); II (1 mM Ca$^{2+}$ sample); III (10 mM oxalate sample). a (postmortem stored at 2°C); b (postmortem stored at 23°C). Troponin I (TNI).

Protein load: 28 µg for 7 1/2 percent gels; 14 µg for 10 percent gels.
a trace of troponin T could still be observed (Fig. 21). The 30,000 dalton component was observed in all samples stored at 2°C for 10 days, but it was less intense in the 10 mM oxalate sample. Furthermore, the 30,000 dalton band was evidently degraded because it was absent in the gels from the ground muscle control and the 1 mM calcium ion sample stored at room temperature for 10 days. Only a trace of the 30,000 dalton band was still observed in the 10 mM oxalate sample stored at room temperature for 10 days. The troponin I band appeared to be altered in all samples stored at room temperature for 10 days. Extra minor bands in the M-protein region increased in intensity for all samples stored at room temperature for 10 days.

The sarcoplasmic proteins extracted from at-death muscle samples and the three treatment samples stored at 2°C and room temperature for 1, 2 and 10 days are shown in Figs. 22 and 23. At least 7 major bands and 6 minor bands were found in gels for the sarcoplasmic proteins isolated from at-death muscle samples (Fig. 22). The minor band (arrow) just below phosphorylase was not seen in all postmortem samples stored at 2°C or room temperature for 1, 2 and 10 days with the exception of the 10 mM oxalate sample stored at 2°C. The intensity of the band around 44,000 daltons gradually decreased in the control sample and the 1 mM calcium ion sample stored at room temperature for 1, 2 and 10 days. It
Fig. 22. SDS - 10 percent polyacrylamide gels of sarcoplasmic proteins prepared from at-death and 1-day postmortem bovine longissimus muscle.

At-death muscle sample, I; day-1 postmortem muscle samples, I (ground muscle control); II (1 mM Ca^{2+} sample); III (10 mM oxalate sample). a (postmortem stored at 2°C); b (postmortem stored at 23°C).

Protein load: 14 μg.
PHOSPHORYLASE

44,000 DALTONS
DEHYDROGENASE

MYOGLOBIN
Fig. 23. SDS - 10 percent polyacrylamide gels of sarcoplasmic proteins prepared from 2-day and 10-day postmortem bovine longissimus muscle.

I (ground muscle control); II (1 mM Ca^{2+} sample); III (10 mM oxalate sample). a (postmortem stored at 2°C); b (postmortem stored at 23°C).

Protein load: 14 μg.
POSTMORTEM TIME  DAY 2  DAY 10
Ia  Ib  IIA  IIB  IIIa  IIIb  Ia  Ib  IIA  IIB  IIIa  IIIb

PHOSPHORYLASE

44,000 DALTONS
DEHYDROGENASE

MYOGLOBIN
also appeared to be slightly reduced in the 10 mM oxalate sample stored at room temperature for 10 days (Fig. 23). The sarcoplasmic proteins from the 10 mM oxalate sample stored at 2°C for 1, 2 and 10 days were similar in composition to those sarcoplasmic proteins from the at-death muscle sample.

The results obtained with SDS-polyacrylamide gel electrophoresis indicated that chelating the calcium ion in muscle tissue inhibited or retarded the postmortem changes which were observed in isolated myofibrils and sarcoplasmic proteins from postmortem muscles. No apparent differences were noted between the myofibrils and sarcoplasmic protein isolated from the ground muscle control and the 1 mM calcium ion sample, but differences were evident with the 10 mM oxalate sample. Storage of muscle samples at room temperature hastened the postmortem changes in the isolated myofibrils and sarcoplasmic proteins.

The isolated myofibrils from at-death and postmortem muscle samples of different treatments (the ground muscle control, the 1 mM calcium ion sample and the 10 mM oxalate sample) stored at 2°C for 1 and 2 days were extracted with H-S solution. The amount of protein extracted with H-S solution increased for all postmortem muscle samples, but more was extracted from the ground muscle control and the 1 mM calcium ion sample than from the 10 mM oxalate sample.
Fig. 24. Effects of Ca$^{2+}$ on postmortem changes in the percentage extractability with H-S solution of isolated myofibrils from at-death and postmortem stored at 2°C bovine longissimus muscle.

- o: at-death intact muscle, •: ground muscle control, Δ: 1 mM Ca$^{2+}$ sample, ▲: 10 mM oxalate sample, ————, 1-day postmortem muscle; ————, 2-day postmortem muscle. The muscle pH values are shown in parentheses.
(Fig. 24). The increase in percentage extractability appeared to be independent of muscle pH. The 10 mM oxalate sample had a relatively high pH value of 5.95 at 1-day postmortem; however, it had a much lower percentage extractability than the ground control or the 1 mM calcium ion sample which had muscle pH values of 5.36 and 5.20 at 1-day postmortem. Hence, the postmortem changes in percentage extractability with H-S solution may be related more to the availability of calcium ion in muscle tissues than to the pH value of muscle tissue.

Protein extractability of isolated myofibrils from at-death muscle sample and the postmortem samples stored at 2°C and room temperature (23°C) for 1 and 2 days using 1 mM Tris, pH 8.5 solution were also studied. The results are graphically shown in Figs. 25 and 26. For those muscle samples stored at 2°C, the largest amount of protein extracted on a percentage of total myofibrillar protein basis was the sample containing 1 mM calcium ion, next was the ground muscle sample control and the least was the 10 mM oxalate sample (Fig. 25). These differences were more noticeable in the 2-day postmortem samples than in the 1-day postmortem samples. However, the protein extractability increased markedly from 1-day to 2-day in all three postmortem stored samples. Again, the postmortem changes in the protein extractability with 1 mM Tris, pH 8.5 solution was related
Fig. 25. Effects of Ca\textsuperscript{2+} on postmortem changes in the percentage extractability with 1 mM Tris, pH 8.5 solution of isolated myofibrils from at-death and postmortem stored at 2°C bovine longissimus muscle.

○: at-death intact muscle, ●: ground muscle control, △: 1 mM Ca\textsuperscript{2+} Sample, ▲: 10 mM oxalate sample, --- ---, 1-day postmortem muscle; ---- --, 2-day postmortem muscle. The muscle pH values are shown in parentheses.
Fig. 26. Effects of Ca$^{2+}$ on postmortem changes in the percentage extractability with 1 mM Tris, pH 8.5 solution of isolated myofibrils from at death and postmortem stored at 23°C bovine longissimus muscle.

○: at-death intact muscle, ●: ground muscle control, Δ: 1 mM Ca$^{2+}$ sample, ▲: 10 mM oxalate sample, -------, 1-day postmortem muscle; ---------, 2-day postmortem muscle. The muscle pH values are shown in parentheses.
more to the availability of calcium ion in muscle than to the pH values of muscle (Fig. 25). The protein extracted with 1 mM Tris, pH 8.5 solution of isolated myofibrils from all three postmortem samples stored at room temperature increased very noticeably (Fig. 26). The rate of protein extraction was greatly accelerated by storage of muscle samples at room temperature. Even the addition of 10 mM oxalate to at-death muscle stored at room temperature was unable to retard the decline in muscle pH value and the increase in protein extractability during postmortem storage (Fig. 26). The differences between the samples in the presence and absence (chelated) of calcium ion, however, were still clearly evident.

Protein extracts (H-S and 1 mM Tris solutions) were further characterized by SDS-polyacrylamide gel electrophoresis. H-S protein extracts are shown in Figs. 27, 28, 29 and 30 and 1 mM Tris solution protein extracts are shown in Figs. 31, 32 and 33.

Fig. 27 shows the nature of the H-S extracted proteins from at-death muscle for 0, 1/2, 1, 7 and 24 hours. It again shows that H-S solution was specific for the extraction of proteins of the thick filaments (myosin, both heavy and light chains, M-proteins and component C). Only very small quantities of the thin filament proteins, actin, tropomyosin, troponin and α-actinin were extracted. The amount of thin
Fig. 27. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (H-S solution) from isolated myofibrils of at-death bovine longissimus muscle. Troponin T (TNT), Troponin I (TNI), Tropomyosin (TM).
Protein load: 30 µg.
HOURS EXTRACTION
0 1/2 1 7 24

MYOSIN HEAVY CHAIN

ACTIN
TNT
TM

MYOSIN LIGHT CHAINS
TNT
Fig. 28. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (H-S solution) from isolated myofibrils of 1-day postmortem stored at 2°C bovine longissimus muscles (ground muscle control, 1 mM Ca$^{2+}$ sample, 10 mM oxalate sample). Numbers indicate hours extraction. Identification of individual myofibrillar protein is the same as indicated in Fig. 27. Protein load: 30 µg.
Fig. 29. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (U-S solution) from isolated myofibrils of 2-day postmortem stored at 2°C bovine longissimus muscles (ground muscle control, 1 mM Ca²⁺ sample, 10 mM oxalate sample). Numbers indicate hours extraction. Identification of individual myofibrillar protein is the same as indicated in Fig. 27. Protein load: 30 μg.
filament proteins extracted was only slightly increased after a 24 hour extraction period; however, \( \alpha \)-actinin remained very resistant to extraction with H-S solution. These observations are consistent with the results reported in the first part of this thesis on studies of postmortem biochemical changes.

Fig. 28 shows the SDS-polyacrylamide gels of the H-S extracted proteins from three samples (ground muscle control, 1 mM calcium ion and 10 mM oxalate samples) stored at 2°C for 1 day. It is very noticeable in the 1 mM calcium ion sample that the amount of \( \alpha \)-actinin and actin increased. These results were more distinguishable in the 2-day postmortem sample (Fig. 29). In addition, trace amounts of the 30,000 dalton band were observed in the H-S protein extracts of isolated myofibrils from the control sample and the 1 mM calcium ion sample, but it was not seen in the 10 mM oxalate sample stored at 2°C for 2 days. Troponin T was present in the H-S protein extracts from all three muscle samples. Tropomyosin was extracted by H-S solution in larger quantities from the control sample and the 1 mM calcium ion sample stored at 2°C for 2 days than at-death and 1-day postmortem muscle samples.

The SDS-polyacrylamide gels of the protein extracted with H-S solution from isolated myofibrils of the three muscle treatments stored at 2°C for 10 days are shown in
Fig. 30. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (H-S solution) from isolated myofibrils of 10-day postmortem stored at 2°C bovine longissimus muscle (ground muscle control, 1 mM Ca²⁺ sample, 10 mM oxalate sample). Numbers indicate hours extraction. Identification of individual myofibrillar protein is the same as indicated in Fig. 27.

Protein load: 30 µg.
Fig. 30. Alpha-actinin was found in both the control sample and 1 mM calcium ion sample, but it was not found in the 10 mM oxalate sample. Troponin T completely disappeared and the 30,000 dalton band was present in both the control sample and the 1 mM calcium ion sample. Also the intensity of the 30,000 dalton band was greater in the 1 mM calcium ion sample than the control sample. Troponin T was present and only a trace amount of 30,000 dalton band was found in the protein extracts from the 10 mM oxalate sample stored at 2°C for 10 days. The largest amount of extracted tropomyosin and actin was in the 1 mM calcium ion sample and the smallest was in the 10 mM oxalate sample. It was also shown that the intensity of actin, troponin T, tropomyosin and 30,000 dalton band increased simultaneously with prolonged extraction (with H-S solution) indicating that these bands might be all from thin filaments (Fig. 30).

The SDS-polyacrylamide gels of the protein extracts from at-death muscle myofibrils showed that only component C and actin were extracted with 1 mM Tris, pH 8.5 solution for 0 minute through 7 hours (Fig. 31). After samples were stored at 2°C for 1 day, α-actinin was extracted in larger quantities and the amount of tropomyosin extracted also increased in both the control sample and the 1 mM calcium ion sample (Fig. 32). These changes were more demonstrable after storage at 2°C for 2 days (Fig. 33). The SDS-polyacrylamide
Fig. 31. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution) from isolated myofibrils of at-death bovine longissimus muscle.

Protein load: 10 µg.
Fig. 32. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution) from isolated myofibrils of 1-day postmortem stored at 2°C bovine longissimus muscle (ground muscle control, 1 mM Ca²⁺ sample, 10 mM oxalate sample). Numbers indicate hours extraction. Troponin T (TNT), Tropomyosin (TM).

Protein load: 10 µg.
Fig. 33. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution) from isolated myofibrils of 2-day post-mortem stored at 2°C bovine longissimus muscle (ground muscle control, 1 mM Ca²⁺ sample, 10 mM oxalate sample). Numbers indicate hours extraction. Troponin T (TNT), Tropomyosin (TM).

Protein load: 10 μg.
gels also showed that α-actinin was still resistant to extraction by 1 mM Tris, pH 8.5 solution in isolated myofibrils from the 10 mM oxalate samples stored at 2°C for 1 and 2 days (Fig. 32 and Fig. 33).

After muscle samples were stored at room temperature for 1 or 2 days, the extraction of α-actinin from isolated myofibrils by 1 mM Tris solution was greatly accelerated in the control sample and the 10 mM oxalate sample (Fig. 34). Alpha-actinin extraction was greater for 1 mM calcium ion sample stored at 2°C than the sample stored at room temperature for 1 or 2 days. Addition of 10 mM oxalate to the at-death muscle sample, however, was ineffective in retarding postmortem changes of the sample stored at room temperature for 2 days. Also to be noted was that more α-actinin was extracted whereas the amount of troponin T disappeared in the isolated myofibrils. It is clearly evident that the intensity of the α-actinin band is inversely proportional to the intensity of the component C and troponin T bands in the protein extracted with 1 mM Tris solution.

Postmortem biochemical changes were studied to illustrate the effect of calcium ion on postmortem changes. These postmortem biochemical changes include; 1) the changes in the integrity of myofibrillar and sarcoplasmic proteins; 2) the postmortem increase in percentage extractability of protein from isolated myofibrils by H-S solution and 1 mM Tris
Fig. 34. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of bovine longissimus muscle at-death (Io) and with different treatments (I, ground muscle control; II, 1 mm Ca\textsuperscript{2+} sample; III, 10 mM oxalate sample) postmortem stored at 2\textdegree C (a) or at 23\textdegree C (b). Troponin T (TNT), Tropomyosin (TM).

Protein load: 10 \( \mu \text{g} \).
**POSTMORTEM DAYS**

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>DAY 0 AND DAY 1</th>
<th>DAY 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>I₀</td>
<td>Iₐ</td>
<td>I₋</td>
</tr>
<tr>
<td>COMPONENT C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ACTININ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**COMPONENT C**

**α-ACTININ**

**ACTIN**

**TNT**

**TM**
solution; and 3) the changes in the extraction of α-actinin by H-S solution and 1 mM Tris solution. The results showed that the addition of 1 mM calcium ion to at-death muscle samples activated the postmortem biochemical changes and the presence of 10 mM oxalate, in most cases, retarded the postmortem biochemical changes. Higher postmortem temperature activated the postmortem biochemical changes.

Possible Accelerative Effects of Calcium Ion on Postmortem Biochemical Changes

The results in the preceding studies on the effect of calcium ion on postmortem changes suggested that calcium ion was involved with CAF activity. It is very possible that CAF activity starts some time after the initiation of tension development in postmortem muscle (Busch, 1969). It was shown that the initiation of tension development in bovine semitendinosus was 3 to 6 hours postmortem at 2°, 16°, 25° and 37°C storage temperature (Busch et al., 1972a). This is about the same time the SR looses its Ca^{2+}-binding ability (Greaser et al., 1969a; Eason, 1969). Hence, it is possible that postmortem changes involving calcium ion and CAF activity start about 3 to 6 hours postmortem. If the concentration of calcium ion is a major factor in controlling CAF activity in muscle, addition of calcium ion to at-death
muscle should activate CAF activity at least 3 to 6 hours earlier than that normally occurring in postmortem muscle.

Carcass temperature remains above 30°C or higher after exanguination of animal for several hours under conventional operations (Watt and Herring, 1974). This elevated temperature in a carcass should stimulate CAF activity if an optimal concentration of calcium ion is present. Consequently, a model system was developed in the third phase of this study for the purpose of determining the possible accelerative effects of calcium ion on postmortem biochemical changes. This was accomplished by incubating at-death muscle samples (consisting of an intact muscle control, a ground muscle control, and ground muscle in the presence of 1 mM calcium ion or 10 mM oxalate) for 1 to 2 1/2 hours at 37°C. A comparison was made of differences in the biochemical changes.

The changes in protein extractability, using 1 mM Tris from muscle samples of different treatments incubated at 37°C for 75 minutes are shown in Fig. 35. Protein extractability increased for all treatments with the exception of the 10 mM oxalate sample. Large increases in protein extractability were noted in the intact sample and the 1 mM calcium ion sample after incubation at 37°C. The extractability of myofibrillar proteins from the 10 mM oxalate sample increased only slightly (Fig. 38), or even declined slightly (Fig. 35)
Fig. 35. Effects of Ca$^{2+}$ on the percentage extractability (1 mM Tris, pH 8.5 solution, 1 hour extraction) of proteins from isolated myofibrils of at-death bovine longissimus muscle (a, intact muscle control; b, ground muscle control; c, 1 mM Ca$^{2+}$ sample; d, 10 mM oxalate sample) upon incubation at 37°C for 75 minutes. The dashed line indicates extractability of intact at-death muscle without incubation. Muscle pH values are shown in parentheses.
Fig. 36. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of at-death bovine longissimus muscle (a, intact muscle control; b, ground muscle control; c, 1 mM Ca^{2+} sample; d, 10 mM oxalate sample) after incubation at 37°C for 75 minutes. Intact at-death muscle without incubation, Ao.

Note that the greatest extraction of α-actinin was from the 1 mM Ca^{2+} sample after incubation. Troponin T (TNT), Troponin I (TNI), Tropomyosin (TM).

Protein load: 10 μg.
COMPONENT C

α-ACTININ

ACTIN
TNT
TM

MYOSIN LIGHT CHAIN
TNI
after incubation.

The protein constituents of the 1 mM Tris extract were further characterized with SDS-polyacrylamide gel electrophoresis. Again, it was shown that α-actinin was very resistant to extraction by 1 mM Tris solution from isolated myofibrils of at-death controls before incubation. After incubation the extraction of α-actinin from isolated myofibrils by 1 mM Tris solution increased. The increase in the extraction of α-actinin was most noticeable in the 1 mM calcium ion sample and the least in the 10 mM oxalate sample and the intact muscle control after incubation (Figs. 36 and 40). Differences in the intensity of the troponin T band between the samples before and after incubation and among the samples after incubation were also observed. The largest reduction in the troponin T band was noted in the 1 mM calcium ion sample. The 10 mM oxalate sample was not different from the at-death control sample without incubation. These results were consistently observed in all five animals used in this part of the experiment. The extent of increase in the extraction of α-actinin in 1 mM calcium ion sample after incubation as shown in the SDS-polyacrylamide gels (Figs. 36 and 40), was approximately equal to that of the muscle sample aged at 2°C for 24 hours or less (Fig. 7).

The integrity of myofibrillar and sarcoplasmic proteins from muscle samples of different treatments incubated at 37°C
for 1 to 2 1/2 hours was also studied. The SDS-polyacrylamide gels of isolated myofibrils showed that a 30,000 dalton band was observed only in the 1 mM calcium ion sample after incubation and it was not seen in any other muscle samples (the ground muscle, the 10 mM oxalate sample after incubation) (Fig. 39). However, this was found for three of the five animals used in this experiment. Troponin T band was present in all muscle samples after incubation.

Fig. 37 showed that two bands were altered in the sarcoplasmic proteins from the sample with 1 mM calcium ion after incubation, one minor band (arrow) just below phosphorylase, and the other around 44,000 dalton, possibly creatine kinase (Scopes and Penny, 1971). These alterations in sarcoplasmic proteins were consistently observed in the sample with 1 mM calcium ion after incubation.

These increases in α-actinin solubility and the appearance of a 30,000 dalton band in the isolated myofibrils suggest that the incubation at 37°C and the addition of 1 mM calcium ion to at-death muscle tissue does accelerate the reactions associated with CAF activity.

Dayton (1975) observed that purified CAF was more active at 37°C than at 25°C and was least active at 2°C. However, incubation at 37°C caused rapid autolysis of CAF in the presence of 1 mM calcium ion. Hence, the total CAF activity during prolonged incubation was higher at 2°C than at 25°C,
Fig. 37. SDS - 10 percent polyacrylamide gels of sarcoplasmic proteins from at-death bovine longissimus muscle of different treatments (a, intact muscle control; b, ground muscle control; c, 1 mM Ca^{2+} sample; d, 10 mM oxalate sample) after incubation at 37°C for 75 minutes. Intact at-death muscle without incubation, Ao.

Note the disappearance of the minor band (arrow) just below phosphorylase and the 44,000 dalton band in the 1 mM Ca^{2+} sample after incubation.

Protein load: 14 µg.
which in turn was higher than at 37°C. The incubation at 37°C of at-death muscle in the presence of 1 mM calcium ion for 1 to 2 1/2 hours may result in the autolysis of CAF activity and the impairment of further postmortem aging phenomena. To answer this question, the incubated and nonincubated at-death muscle samples with different treatments were stored and the differences in postmortem changes were compared.

The postmortem changes in protein extractability with 1 mM Tris solution (1 hour extraction) for incubated and nonincubated muscle samples are shown in Fig. 38. After incubation of the treated muscle samples at 37°C for 75 minutes, the protein extracted with 1 mM Tris solution increased for all samples. The percentage of protein extracted with 1 mM Tris solution (1 hour extraction) was only 3.8 percent for the at-death muscle sample before incubation, but after incubation, it was increased to 6.8 percent in the 1 mM calcium ion sample, to 5.8 percent in the ground muscle control and to 4.2 percent in the 10 mM oxalate sample.

The treated at-death muscle samples (ground muscle control, 1 mM calcium ion and 10 mM oxalate samples) incubated at 37°C for 75 minutes and without incubation were further stored at 2°C for 1, 2 and 9 days. The differences in the percentage extractability of proteins with 1 mM Tris
Fig. 38. Postmortem changes in the percentage extractability (1 mM Tris, pH 8.5 solution, 1 hour extraction) of proteins from isolated myofibrils of bovine longissimus muscle (○, ground muscle control; △, 1 mM Ca$^{2+}$ sample; □, 10 mM oxalate sample). Open signs are nonincubated and treated muscle samples; closed signs are the treated at-death samples incubated at 37°C for 75 minutes.
Fig. 39. SDS - 10 percent polyacrylamide gels of isolated myofibrils from at-death bovine longissimus muscle (b, ground muscle control; c, 1 mM Ca\(^{2+}\) sample; d, 10 mM oxalate sample) after incubation at 37°C for 75 minutes. Intact at-death muscle without incubation, Ao. Troponin T (TNT), Tropomyosin (TM).

Note the 30,000 dalton band in the 1 mM Ca\(^{2+}\) sample after incubation.

Protein load: 14 μg.
solution (1 hour extraction) between incubated and nonincubated samples were compared at each postmortem storage period. The percentage extractability increased in the nonincubated muscle samples postmortem stored at 2°C whereas it declined in the incubated muscle samples (Fig. 38). The percentage extractability with 1 mM Tris solution (1 hour extraction) was consistently lower for the 10 mM oxalate sample either incubated or nonincubated postmortem stored at 2°C.

The SDS-polyacrylamide gels of proteins extracted with 1 mM Tris solution are shown in Figs. 40, 41, 42 and 43. Alpha-actinin was observed in the 1 mM calcium sample and only trace amounts were found in the ground muscle control after incubation at 37°C for 75 minutes (Fig. 40). There was essentially no difference between the samples incubated and nonincubated when stored at 2°C (Figs. 41, 42 and 43). However, the differences among treatments were very apparent. Addition of 10 mM oxalate to at-death muscle sample retarded the postmortem changes observed in the control sample and the 1 mM calcium ion sample stored at 2°C for 2 days (Figs. 41 and 42). The difference in sample treatments diminished after storage at 2°C for 9 days (Fig. 43). The same muscle pH of 6.0 was observed in the 10 mM oxalate samples postmortem stored at 2°C for 1, 2 and 9 days (Fig. 46); however, the extractability of α-actinin with 1 mM Tris solution was
Fig. 40. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of at-death bovine longissimus muscle (b, ground muscle control; c, 1 mM Ca²⁺ sample; d, 10 mM oxalate sample) after incubation at 37°C for 75 minutes. Intact at-death muscle without incubation, Ao. Troponin T (TNT), Tropomyosin (TM).

Note that the greatest extraction of α-actinin was from 1 mM Ca²⁺ sample after incubation.

Protein load: 10 μg.
COMPONENT C

α-ACTININ

ACTIN
TNT
TM
Fig. 41. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of nonincubated and incubated bovine longissimus muscle (b, ground muscle control; c, 1 mM Ca$^{2+}$ sample; d, 10 mM oxalate sample) postmortem stored at 2°C for 1 day. Troponin T (TNT), Tropomyosin (TM).

Protein load: 10 μg.
Fig. 42. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of nonincubated and incubated bovine longissimus muscle (b, ground muscle control; c, 1 mM Ca\(^{2+}\) sample; d, 10 mM oxalate) postmortem stored at 2°C for 2 days. Troponin T (TNT), Tropomyosin (TM).

Protein load: 10 μg.
Fig. 43. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of nonincubated and incubated bovine longissimus muscle (b, ground muscle control; c, 1 mM Ca²⁺ sample; d, 10 mM oxalate sample) postmortem stored at 2°C for 9 days. Tropomyosin (TM).

Protein load: 10 μg.
NONINCUBATED

b c d

COMPONENT-C

α-ACTININ

ACTIN

TM

30,000 DALTONS

INCUBATED

b c d
quite different between the 2-day and the 9-day postmortem 10 mM oxalate samples (Fig. 42, d vs. Fig. 43, d). This seems to indicate that the change in extractability of α-actinin was not related to the muscle pH. The 30,000 dalton band was very clear in all muscle samples (the ground muscle, 1 mM calcium ion and 10 mM oxalate samples) after storage at 2°C for 9 days (Fig. 43).

The amount of α-actinin extracted after incubating muscle samples for 2 1/2 hours increased very markedly (Fig. 44). In this experiment, 10 mM floride was used in place of 10 mM oxalate to chelate calcium ion. Floride appears to be a stronger chelator than does oxalate because the extractability of α-actinin increased markedly and the troponin T band disappeared in all muscle samples, except the sample with 10 mM floride after storage of incubated muscle samples for 7 days (Fig. 45, e).

The postmortem decline of at-death muscle pH was accelerated by incubation at 37°C in the presence of 1 mM calcium ion. The typical ultimate muscle pH of 5.5 was reached in the 1 mM calcium ion sample after incubating at 37°C within 30 minutes (Fig. 47) whereas the control ground required from 75 minutes to 2 hours to reach pH 5.5 (Figs.
Fig. 44. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of at-death bovine longissimus muscle (a, intact muscle control; b, 10 mM Mg\(^{2+}\) sample; c, 10 mM Mg\(^{2+}\), 1 mM Ca\(^{2+}\) sample; d, 1 mM Ca\(^{2+}\) sample; e, 10 mM F\(^{-}\) sample) after incubation at 37°C for 2 1/2 hours. At-death intact muscle without incubation, Ao. Troponin T (TNT), Tropomyosin (TM).

Protein load: 10 µg.
Fig. 45. SDS - 7 1/2 percent polyacrylamide gels of protein extracts (1 mM Tris, pH 8.5 solution, 1 hour extraction) from isolated myofibrils of bovine longissimus muscle (a, intact muscle control; b, 10 mM Mg$^{2+}$ sample; c, 10 mM Mg$^{2+}$, 1 mM Ca$^{2+}$ sample; d, 1 mM Ca$^{2+}$ sample; e, 10 mM F$^{-}$ sample) after incubation at 37°C for 2 1/2 hours and postmortem stored at 2°C for 7 days. Troponin T (TNT), Tropomyosin (TM).

Protein load: 10 µg.
Fig. 46. Effects of addition of $1 \text{ mM Ca}^{2+}$ or $10 \text{ mM oxalate}$ on rate of pH fall in bovine longissimus muscle after incubation at $37^\circ \text{C}$ for 75 minutes and subsequent postmortem storage at $2^\circ \text{C}$ for 1, 2 and 9 days.

•, ground muscle control; ▲, $1 \text{ mM Ca}^{2+}$ sample; ■, $10 \text{ mM oxalate}$ sample.
INCUBATION TIME (MINUTES)  POSTMORTEM TIME (DAYS)

MUSCLE pH VALUE

- CONTROL
- OXALATE

\[ \text{INCUBATION TIME (MINUTES)} \]
\[ \text{POSTMORTEM TIME (DAYS)} \]

\[ \text{MUSCLE pH VALUE} \]

1. 2 9

15 45 75
Fig. 47. Effects of addition of 1 mM Ca$^{2+}$, or 10 mM Mg$^{2+}$, or 10 mM F$^{-}$ on rate of pH fall in bovine longissimus muscle after incubation at 37°C for 2 1/2 hour and subsequent postmortem storage at 2°C for 7 days. ●, ground muscle control; ○, 10 mM Mg$^{2+}$ sample; ▲, 1 mM Ca$^{2+}$ sample; ■, 10 mM F$^{-}$ sample.
Addition of 10 mM oxalate or 10 mM fluoride retarded or inhibited the postmortem muscle pH decline (Figs. 46 and 47). Addition of 10 mM EDTA had no effect on the ultimate muscle pH during postmortem storage (Fig. 48).
Fig. 48. Effects of addition of 1 mM Ca$^{2+}$ or 10 mM EDTA on rate of pH fall in bovine longissimus muscle after incubation at 37°C for 75 minutes and subsequent postmortem storage at 2°C for 1, 2 and 9 days.

•, ground muscle control; ▲, 1 mM Ca$^{2+}$ sample; ■, 10 mM EDTA sample.
DISCUSSION

Many of the changes that occur in myofibrillar proteins during the postmortem aging of skeletal muscle are very discrete ones. Hence, very sensitive techniques must be employed to detect these changes. SDS-polyacrylamide gel electrophoresis is a technique which meets these requirements. Sodium dodecyl sulfate (SDS) is an anionic detergent which can be used to solubilize the whole isolated myofibril and dissolve the soluble proteins in the presence of 2-MCE. SDS-polyacrylamide gel electrophoresis with its high sensitivity and precision readily lends itself to the qualitative and quantitative study of the composition and the integrity of isolated myofibrils and their protein extracts. Hence, by designing and executing experiments which seek fundamental explanations to postmortem muscle phenomena, more extensive and reliable information will be available for translating these phenomena into more meaningful and applicable terms to the use of muscle as a food.

A significant postmortem alteration in the integrity of isolated myofibrils from postmortem stored muscles has been shown to occur in the degradation of troponin T, a subunit component of troponin found in the thin filament, to a 30,000 dalton component using SDS-polyacrylamide gel electrophoresis (Hay et al., 1973b; Olson, 1975; Jergenson,
1975). Somewhat surprisingly, however, no changes in the major myofibrillar proteins (myosin, actin, tropomyosin, and α-actinin) were observed in isolated myofibrils from postmortem muscle. Hay et al. (1973b), based on SDS polyacrylamide gels observations that postmortem degradation of troponin T into a 30,000 dalton component occurred in myofibrils, concluded that a very limited and specific proteolysis took place in myofibrils of postmortem muscles. Unfortunately, the mechanism of the postmortem degradation of the troponin subunit was not clear to them and the relationship between the postmortem degradation of troponin subunit and the postmortem changes in tenderness was not explained by these workers. Recently, however, Olson (1975) was able to clearly demonstrate the cause for and relationship of molecular changes in postmortem muscle. He (Olson, 1975) found that higher myofibril fragmentation index and tenderness characteristics (measured by W-B shear-force and sensory panel evaluation of steaks from longissimus muscles from veal, A maturity and C maturity carcasses) corresponded to greater troponin T degradation and the more intense appearance of a 30,000 dalton component. These results suggested that postmortem events associated with tenderness changes and postmortem degradation of the troponin subunit were highly related. Moreover, this limited and specific proteolysis of troponin T was determined to be caused by
CAF activity in postmortem muscle.

CAF enzyme has been shown to be a very potent agent in removing Z-lines from isolated myofibrils in vitro (Busch et al., 1972b; Dayton et al., 1974a, 1974b; Suzuki and Goll, 1974; Olson, 1975). Furthermore, works by Busch et al. (1972b) and Dayton et al. (1974a, 1974b) demonstrated that CAF was calcium dependent. Ca\(^{2+}\) was found to be necessary for removal of Z-disk because isolated myofibrils incubated with CAF in the absence of Ca\(^{2+}\) or without CAF in the presence of Ca\(^{2+}\) had intact Z-disks; however, when isolated myofibrils were incubated with both CAF and Ca\(^{2+}\), Z-disks were removed. CAF in vitro requires 0.1 mM Ca\(^{2+}\) for detectable proteolysis of isolated myofibrils (Dayton et al., 1974a, 1974b; Suzuki and Goll, 1974). Olson's recent study demonstrated the significance of myofibril fragmentation, degradation of troponin T, the appearance of 30,000 dalton component and CAF to postmortem tenderization of bovine muscle. These studies taken together clearly illustrate the importance of CAF and calcium ion to postmortem meat quality. These could even be more important than we can perceive at the moment because this limited and specific proteolysis may be the basis for producing both high quality fresh and cured meat. That is, calcium ion and CAF may be associated with the eating characteristics (tenderness, juiciness, etc.) and processing characteristics (water-binding ability,
emulsification, cutting and cooking loss, etc.) of meat. Obviously, beneficial regulation or modification of these characteristics would have gigantic economic, palatability and nutritional implications to the meat industry.

The experiments reported in this thesis have attempted to elucidate the role of myofibrillar proteins and the effect calcium ion has on these proteins during postmortem storage of bovine muscle.

In the first part of this study the observation reported by previous workers was substantiated in that myofibrils isolated from postmortem muscle had a loss of troponin T and an increase in the 30,000 dalton component. Furthermore, no changes were noted in the major myofibrillar proteins of isolated myofibrils, myosin, actin, tropomyosin and α-actinin during postmortem storage. To further investigate the possibility of postmortem modifications in some of the major myofibrillar proteins, experiments to selectively extract myosin with H-S solution and α-actinin with 1 mM Tris solution from isolated myofibrils of postmortem stored bovine muscles were designed and carried out. SDS-polyacrylamide gel electrophoresis was used to monitor and identify these changes. It was found that there were postmortem alterations in the thick filaments and the Z-disks. The postmortem alteration in the thick filament was evidenced by the
increased extraction of myosin with H-S solution and in the weakening and degradation of Z-disks in myofibrils by increased extraction of α-actinin with 1 mM Tris solution.

The marked increase in amount of extractable proteins from isolated myofibrils of postmortem muscle with H-S solution was mainly due to increased extractability of myosin and only partially due to increased extractability of actin and other myofibrillar proteins. A major reason for this is because 10 mM sodium pyrophosphate in H-S solution is capable of dissipating the actin-myosin interaction, thus aiding in the selective extraction of myosin. These results suggest that postmortem alterations occur in the structural architecture of thick filaments. The slight increase in amount of actin in the H-S solution extract is interpreted as a postmortem weakening of Z-disks, or I-Z bonds, in the myofibrils. However, the increase in actin in the protein extract with H-S solution became moderately noticeable only at 10 days postmortem (2°C). Davey and Gilbert (1968b) reported that the amount of actin extracted from isolated myofibrils of 21-day postmortem muscle samples with H-S solution was not sufficient to form actomyosin with all the extracted myosin. They suggested that inactive actin may also be extracted. They concluded, however, that postmortem weakening of Z-lines in myofibrils caused the postmortem increase in the percentage extractability of isolated myofibrils with H-S solution. The marked postmortem increase in
the percentage of proteins extracted as myosin with H-S solution reported in this thesis, however, indicate that the main structural alterations occurred in the thick filaments during postmortem storage of muscle.

Since myosin is the most abundant single protein component in myofibrils (Goll et al., 1974a) and since it plays an important role in water-holding capacity and texture of meat (Hamm, 1970), any postmortem alteration in the structure of thick filaments should significantly contribute to the postmortem modification of myofibrils. Hence, factors that may affect the postmortem change in the percentage extractability of proteins, especially myosin, are important. Three possibilities are readily apparent; one is the forces involved in the aggregation of myosin molecules into thick filaments, especially the rod portion of myosin molecules; another may be the postmortem alteration in component C which involves the packing of myosin molecules (Starr and Offer, 1971; Offer, 1972); and the third possible region that may have an effect on the structure of thick filaments is the M-line (Knappeis and Carlsen, 1968).

The rods of myosin molecules make up the backbone of thick filaments (Harrison et al., 1971) and are composed of a high portion of negatively charged sidechains (Lowey et al., 1969). The shift in muscle pH will certainly affect the electrostatic forces of these charged sidechain groups in the myosin molecules. However, Davey and Gilbert (1968b) showed that the ultimate muscle pH determined the rate and
maximum limit of percentage extractability with H-S solution. The postmortem increase in extractability with H-S solution was lowest in the muscle with the ultimate muscle pH of 5.4 or 5.5. The postmortem increase in extractability with H-S solution increased concomitantly with the increase in muscle ultimate pH to 6.3. Hence, it is clear that the low muscle pH of 5.5 per se in the postmortem muscle fibers was not the instrumental factor causing the postmortem increase in percentage extractability with H-S solution.

Component C is located in nine stripes 43 nm apart on either side of the M-line in the thick filament (Offer, 1972). The amount of component C extracted with 1 mM Tris solution increased in the 1-day postmortem muscle sample (Fig. 7) and decreased in 2- and 10-day postmortem samples (Fig. 8). Also the 1 mM Tris protein extracts of isolated myofibrils from postmortem muscle sample showed that the extractability of \( \alpha \)-actinin was inversely proportional to the amount of troponin T and component C extracted (Figs. 44, 45). These results suggest that postmortem degradations of troponin T and component C occur simultaneously. It is likely that postmortem alterations in component C are the major factors causing the postmortem increase in myosin extractability with H-S solution. Unfortunately no histological studies have been carried out to determine the possibility of postmortem modification of myosin molecule packing in the thick filaments.
to corroborate these biochemical observations. It is interesting to note that CAP degrades component C (Dayton et al., 1974a, 1974b) and it seems probable that the post-mortem increase in myosin extractability with H-S solution is related to the CAF activity in the degradation of component C of postmortem muscle fibers.

Knappeis and Carlsen (1968) suggested that the main function of the M-line is to maintain thick filaments in proper longitudinal and lateral register. Any alteration in M-line structure or its proteins may affect the packing of thick filaments in the myofibrils and hence the extractability of proteins at thick filaments. Many properties of the M-line resemble that of the Z-line in myofibrils; such as its removal by extraction with low ionic solution (Stromer et al., 1967b) and its susceptibility to brief trypsin digestion (Stromer et al., 1967a). Postmortem degradation of M-line has been observed in bovine, porcine and rabbit muscle (Henderson et al., 1970) and in chicken breast muscle (Hay et al., 1973a). It is likely that post-mortem degradation of M-line is one of the causes for the postmortem increase in the percentage extractability with H-S solution. However, in this study a protein of 160,000 daltons, suggested as a protein component of the M-line (Landon and Oriol, 1975; Masaki and Takaiti, 1974), was not extracted by 1 mM Tris, pH 8.5 solution from isolated
myofibrils of at-death or postmortem muscle samples (Figs. 7 and 8). Hence, no conclusion can be made to show the relationship between the postmortem degradation of M-line and postmortem increase in myosin extractability by H-S solution. Busch et al. (1972b) showed that isolated at-death myofibrils incubated in the presence of both 10 mM Ca\(^{2+}\) and Ca\(^{2+}\)-activated sarcoplasmic factor (CASF) resulted in total removal of Z-lines without evident degradation of the A band or the M-line as detected with electron microscopy.

Postmortem alterations in the Z-disk of myofibrils were very evident in this study. The SDS-polyacrylamide gels showed that \(\alpha\)-actinin, a protein of Z-lines, in the at-death myofibrils was very resistant to extraction with low or high ionic strength solutions, but that after postmortem aging it became readily extractable. The protein extracted with the solution containing 0.6M KCl, 0.1M K phosphate, pH 7.0 showed that \(\alpha\)-actinin was not extracted from at-death myofibrils, but was extracted from myofibrils of postmortem muscle samples and that the extraction of actin increased in the postmortem muscle in comparison with at-death sample. This increase in extractability of actin in postmortem muscle was also shown in heated muscle at 70° for 30 minutes as well as in the raw samples (Fig. 16 vs. Figs. 17 and 18). Haga et al. (1966a) showed that the breakage of thin filaments from Z-disks in the myofibrils preceded the extraction of
actin by high ionic solution. Both the postmortem increase in the extraction of α-actinin and actin in the protein extractions with the solution containing 0.6M KCl, 0.1M K phosphate, pH 7.0 indicate that weakening and degradation of I-Z bonds take place in postmortem muscle fibers.

That postmortem alterations occurred in Z-disks were even more conclusively shown in the extraction of myofibrils with 1 mM Tris, pH 8.5 solution. Alpha-actinin was not extracted from at-death myofibrils with 1 mM Tris, pH 8.5 even with prolonged extraction times (48 hr). In the 1-day postmortem sample, however, the amount of α-actinin increased markedly. And the extractability of α-actinin from isolated myofibrils continued to increase upon further postmortem aging. This marked alteration in extractability of α-actinin from isolated myofibrils indicated that the postmortem weakening of Z-disk structure or I-Z bonds occurred progressively in postmortem aging.

Fukazawa et al. (1970) reported that the protein extracted with 0.3 mM NaHCO₃ solution increased in postmortem muscle and this extract showed α-actinin activity. These observations were shown along with the destruction and final dissolution of the Z-line structure during postmortem storage of chicken pectoral muscle at 2°C for 48 hours. The results reported in this thesis using 1 mM Tris solution to extract isolated myofibrils, and using SDS-polyacrylamide gel
electrophoresis to identify the extracted proteins, were more conclusive and sensitive in showing the postmortem weakening of Z-disks or I-Z bonds.

Thus, it appears that the procedure used in this study to extract and detect α-actinin is by far the most sensitive and reproducible procedure showing postmortem weakening of Z-lines. The postmortem decline of muscle pH to 5.5 is an unlikely explanation for the postmortem increase in extractability of α-actinin by 1 mM Tris, pH 8.5 solution. It is known that I-Z bonds are labile to alkali pH (Arakawa et al., 1970c). This is further supported by the results in this thesis that the same muscle pH of 6.0 was observed in the 10 mM oxalate sample at 2- and 10-day postmortem storage at 2°C (Fig. 46) and the extractability of α-actinin increased from 2- to 10-day postmortem muscle samples (Figs. 42, d vs. 43, d).

CAF is very potent in removing Z-lines from isolated myofibrils in the presence of 1 mM Ca^{2+} (Busch et al., 1972b; Suzuki and Goll, 1974; Dayton et al., 1974a, 1974b). Incubation of at-death muscle strips with 1 mM Ca^{2+} has been shown to have an effect on the integrity of Z-disks in myofibrils (Busch et al., 1972b). It seems probable that the postmortem increase in the extractability of α-actinin with 1 mM Tris solution is the result of a limited and specific proteolysis associated with CAF activity in postmortem muscle
fibers. And this limited and specific alteration could hardly be expected to be detected by histological observations (such as electron microscopy), especially at 24 to 48 hours postmortem stored muscle at 2°C, when the most important postmortem changes were taking place.

It was reported in the first part of the results section of this thesis that postmortem changes caused by limited and specific proteolysis in myofibrils was manifested by the postmortem alterations in thick filaments and Z-disk structure. That postmortem degradation of myofibrillar proteins was only observed in troponin T and possibly in component C without affecting any major myofibrillar proteins supports the suggestion by Goll et al. (1971) that fission of only a limited number of peptide bonds would be sufficient to produce postmortem alterations in muscle. The results also suggest that this limited and specific proteolysis is probably caused by CAF activity, a proteolytic enzyme endogenous to postmortem muscle fibers. The effects of CAF activity in situ in postmortem muscle fibers were further tested by adding or chelating calcium ion in at-death muscle because, as discussed previously, a minimum of 0.1 mM of calcium ion was required for CAF activity (Dayton et al., 1974a, 1974b; Suzuki and Goll, 1974). The results obtained in the second phase of this study showed that addition or chelation of calcium ion did potentiate or retard all the
postmortem biochemical changes.

To test the effects of calcium ion on the postmortem biochemical changes associated with CAF activity, minced at-death muscle samples were mixed with 1 mM Ca\textsuperscript{2+} or 10 mM oxalate and ground once to ensure the penetration of added chemicals. Oxalate instead of EDTA was used to chelate calcium ion in muscle fibers because oxalate penetrates muscle fibers rapidly (Diculescu et al., 1971) and because the sarcolemma of muscle fibers is apparently impermeable to added EDTA at least during first 5-6 hours postmortem at 37°C (Busch et al., 1972a).

Postmortem biochemical changes observed in the first part of this study were repeatedly observed in the ground muscle control and 1 mM Ca\textsuperscript{2+} sample. These included: 1) the gradual loss of troponin T and the concurrent appearance of a 30,000 dalton component in isolated myofibrils; 2) postmortem increase in extraction of myosin from isolated myofibrils with H-S solution; and 3) postmortem increase in extraction of α-actinin from isolated myofibrils with 1 mM Tris, pH 8.5 solution. Addition of 1 mM Ca\textsuperscript{2+} to at-death muscle potentiated all postmortem biochemical changes in comparison with the ground control sample, as evidence by the more rapid occurrence of the 30,000 dalton component at 2°C and the greatest postmortem increase in protein extractability with both H-S solution and 1 mM Tris, pH 8.5 solution from the 1 mM Ca\textsuperscript{2+}
sample during postmortem storage. On the other hand, addition of 10 mM oxalate to at-death muscle sample retarded all postmortem biochemical changes. The results indicated that all postmortem biochemical changes reported in this thesis were related to the availability of calcium ion in the postmortem muscle fibers and were characteristic of CAF activity (Dayton et al., 1974a; 1974b). It is therefore evident that postmortem biochemical changes are caused by CAF activity in postmortem muscle fibers and that availability of Ca\(^{2+}\) is a major factor controlling CAF activity in postmortem muscle. The rate and extent of postmortem biochemical changes were greatly activated by higher postmortem storage temperature at 23°C suggesting that these changes were enzymatic in nature.

As mentioned previously, calcium ion concentration increase in postmortem muscle fibers occurs about 3-6 hours postmortem and that this is the same time the SR of muscle looses its Ca\(^{2+}\)-binding ability (Greaser et al., 1969a; Busch, 1969). Based on the evidence that availability of calcium ion is a major factor in controlling CAF activity in the muscle fiber, pre-slaughter or immediate post-slaughter administration of calcium ion may be advantageous in activating the CAF activity earlier after slaughter during the time the temperature of a carcass is still high. The results in the third part of this thesis showed that the postmortem biochemical
changes associated with CAF activity were activated by incubation of at-death muscle at 37°C for 75 minutes in the presence of 1 mM calcium ion. These changes were evident in that the 30,000 dalton component appeared more rapidly in isolated myofibrils and α-actinin was extracted with 1 mM Tris solution in greater quantity in the 1 mM Ca$^{2+}$ sample. Incubation of at-death muscle at 37°C for 75 minutes also accelerated postmortem glycolysis in the presence of 1 mM Ca$^{2+}$. However, no detrimental effects on CAF activity during subsequent postmortem aging was noted in the incubated at-death muscle in comparison with the nonincubated at-death muscle, since no differences in the postmortem biochemical changes were noted between incubated and nonincubated from subsequent postmortem stored samples at 2°C.

It has been shown that postmortem isometric tension decline was related to calcium ion in muscle fibers (Eason, 1969; Goll et al. 1971; Busch et al., 1972b). It is very likely that these calcium ion dependent postmortem biochemical changes reported in this thesis are the molecular basis of postmortem isometric tension decline (resolution of rigor mortis). The postmortem isometric tension decline probably is the result of postmortem modification of thick filaments and of Z-disks in the myofibrils by CAF activity triggered by elevated calcium ion concentration in postmortem muscle fibers. Hence, the results reported in this thesis add
further evidence to support the proposals by Goll and co-workers that resolution of rigor mortis and postmortem tenderization of skeletal muscle are the results of postmortem weakening of Z-disks (myofibril fragmentation) and that CAF activity is responsible for these postmortem modifications (Goll, 1968; Goll et al., 1970, 1971, 1974a; Busch et al., 1972b; Parrish et al., 1975). Postmortem modification of thick filaments in myofibrils may also contribute a significant role in the resolution of rigor mortis, but more intensive investigations must be carried out before definite conclusions can be made.

CAF activity in postmortem muscle has been suggested as one of the major factors causing increased meat tenderness during postmortem aging (Goll et al., 1974a). Results in this study demonstrated that the major postmortem biochemical changes were caused by CAF activity in muscle and that the availability of calcium ion in muscle appeared to be a major factor controlling the CAF activity. It was also shown that incubation of at-death ground muscle with 1 mM calcium ion at 37°C for 1 to 2 1/2 hours accelerated CAF activity and there was no detrimental effects on CAF activity in the subsequent postmortem aging. It is very likely that postmortem tenderness changes in muscle could be accelerated by pre- or post-slaughter administration of a calcium salt and that CAF activity could be activated right after the death
of animals in taking advantage of high carcass temperature. The activation of CAF activity is valuable in terms of energy and time savings under conventional postmortem aging processes. However, several considerations should be evaluated before practical operation is initiated. Some of these should include: 1) the optimal concentration of $\text{Ca}^{2+}$; 2) the techniques to elevate the concentration of calcium ion in muscle fibers; 3) the increased chances for bacteria contamination; 4) the possible effects of shortening induced by the elevated calcium ion level in muscle fibers; and 5) the effects of low pH and high carcass temperature on the properties of myofibrillar proteins.

The results obtained in this study also clarify some of the nature of changes in protein solubility. During postmortem storage, rapid decline of muscle pH to 5.5 when muscle temperature was still high (37°C) caused the severe denaturation of myofibrillar proteins and the reduction of protein extractability during subsequent postmortem storage (Sayre and Briskey, 1963; Scopes, 1964). The postmortem increase in protein extractability of myofibrillar proteins has also been well documented (Davey and Gilbert, 1968a; Chaudhry et al., 1969; Penny, 1968). As demonstrated in this study, these postmortem increases in the extractability of myofibrillar proteins were related to the availability of calcium ion in the muscle fibers via activation of CAF activity.
This increased activity altered the structure of myofibrils and the interactions of myofibrillar proteins. Another consideration is that the ultimate muscle pH may have effects on CAF activity in postmortem muscle and on the denaturation of myofibrillar proteins and as a consequence affect changes in protein extractability. Studies of postmortem changes in protein extractability should therefore take into simultaneous consideration of both aspects of myofibrillar protein denaturation and CAF activity. In addition, the nature of the postmortem changes in protein extractability of individual myofibrillar proteins, as shown in the SDS-polyacrylamide gels, should be included for the purpose of providing more meaningful information and for more accurate interpretation of results.

The effect of heating on small samples of meat using a controlled temperature water bath to simulate cookery conditions was determined by examining heat-induced changes in myofibrillar proteins by SDS-polyacrylamide gel electrophoresis. The major findings were the differences in the response of major myofibrillar proteins to heating temperatures. The protein of thick filaments, myosin, was completely denatured at 55°C; the protein of the Z-disks, α-actinin was completely denatured at 50°C; and the proteins of thin filaments, actin, tropomyosin, troponin, were completely denatured between 70° and 80°C. These distinct
heat-induced biochemical differences present a much more lucid explanation of the molecular changes that occur in different parts of the myofibril, since, ultrastructurally, A bands and I bands and Z-disks are regularly distributed along the entire length of the myofibril and each of these regions responds differently to heat. Thus, it is very likely that the effects of thermal alterations on the structure of myofibrils and in the interactions of myofibrillar proteins are even greater than those of postmortem aging.

As shown in SDS-polyacrylamide gels, actin and tropomyosin appeared to be completely extracted by the solution containing 0.6M KCl, 0.1M K phosphate, pH 7.4 when muscle was heated to 55° or 60°C. Haga et al. (1966a) showed that the detachment of thin filaments from Z-disk preceded the extraction of actin by the solution of 0.6M KCl, 0.1M K phosphate, pH 7.4. If this fact holds for heated muscle, then the dissociation of thin filaments from Z-disks, or from thick filaments, should occur in the muscle samples heated to 55° or 60°C because at these temperatures, proteins of thick filaments and Z-disk are all denatured and the proteins of thin filaments remain in their native state.

The drastic decline in shear values in muscle samples heated from 55°C to 60°C has been suggested as the result of thermal shrinkage of collagen fibers (Machlik and Draudt, 1963; J. G. Schmidt et al., 1970; Bouton and Harris, 1972a;
Paul et al., 1973). However, the extent of thermal shrinkage in collagen fibers is not proportional to the extent of changes in shear values, especially for mature animals, at the temperature of 55°C and 60°C. Goll et al. (1964b) reported that the thermal shrinkage temperature of connective tissue increased with advancing age; i.e. near 55°C for veal, 55-60°C for steers, 60-65°C for cow, and over 70°C for aged cow. The minimum shear values are for steaks from bovine longissimus cooked to an internal temperature of 60°C for A and D maturity animals (J. G. Schmidt et al., 1970; Bouton and Harris, 1972a). Furthermore, it is clear, as discussed previously, that shear values mainly measure the muscle fiber strength and that adhesion values represent the strength of connective tissues (Bouton and Harris, 1972a, 1972b; Bouton et al., 1974, 1975a). It is very likely that the drastic decline in the shear values in the samples heated from 55° to 60°C is mainly related to the thermal modifications in the I-Z bonds and in actin-myosin interaction in the myofibrils.

Bramblett et al. (1959) showed that the length of time meat was held at an internal temperature of 57° to 60°C appeared to be a decisive factor and closely related to an increase in tenderness of bovine muscle. The thermal modifications in the interaction of myofibrillar proteins may provide an explanation for this tenderness effect by holding meat at 57° to 60°C during cooking. The weakening
of I-Z bonds and the alterations in the thick filaments in postmortem aging may have additive effects to the thermal modifications on the I-Z bonds and on actin-myosin interaction. This theory is partially supported by the observation in SDS-polyacrylamide gels that larger quantities of thin filament proteins, actin and tropomyosin, were extracted from 1- and 7-day postmortem samples than from at-death samples, either raw or heated to 70°C.
SUMMARY

Several experiments were carried out to determine the biochemical changes in myofibrils and their proteins during postmortem storage and their relationship to availability of calcium ion and to CAF activity. SDS-polyacrylamide gel electrophoresis was the principal technique used to detect these changes because it offers several distinct advantages over other systems. One is that it has the sensitivity to detect subtle changes that occur in proteins during postmortem storage that usually otherwise would go undetected, and another is that the bands of myofibrillar proteins can be visually seen and identified. Hence SDS-polyacrylamide gel electrophoresis proved to be of invaluable assistance in interpreting postmortem muscle changes and relating these changes to the effects of calcium ion and CAF activity.

In the first phase of experiments on postmortem storage at 2°C, myofibrils were isolated from at-death and 1-, 2- and 10-day postmortem bovine longissimus muscle. SDS-polyacrylamide gels showed that during postmortem storage, the degradation of troponin T and the concurrent appearance of a 30,000 dalton component occurred without affecting any of the major myofibrillar proteins, myosin, actin, α-actinin and tropomyosin, of isolated myofibrils. These results extended and confirmed our earlier results with postmortem muscle and
consequently added validity and substance for subsequent experiments in this study.

To further study molecular changes occurring in post-mortem muscle, myofibrils were isolated and extracted with Hasselbach-Schneider (H-S) solution and 1 mM Tris, pH 8.5 solution. The percentage extractability of proteins from isolated myofibrils with H-S solution increased markedly in the muscles postmortem stored at 2°C for 1 and 2 days. SDS-polyacrylamide gels showed that H-S solution was very specific for the extraction of the thick filament protein, myosin, M proteins and component C, and was capable of extracting proteins of the thin filament, actin, tropomyosin and troponin, in limited quantities from either at-death or postmortem muscle samples. The postmortem increase in the percentage extractability of proteins from isolated myofibrils with H-S solution was mainly an increase in myosin. These results, therefore, suggest that the post-mortem increase in myosin extractability may occur because of modifications in component C.

Marked postmortem increase in the percentage extractability of proteins from isolated myofibrils with 1 mM Tris, pH 8.5 solution was also observed. SDS-polyacrylamide gels showed that 1 mM Tris, pH 8.5 solution was able to extract the thin filament proteins, actin, tropomyosin, troponin, and the thick filament proteins, component C and myosin light
chains. Alpha-actinin, a protein of the Z-disk was very resistant to 1 mM Tris, pH 8.5 solution extraction from isolated myofibrils of at-death muscle, but it was readily extractable from isolated myofibrils of 1- and 2-day postmortem muscle. The extraction of component C by 1 mM Tris, pH 8.5 solution also appeared to increase in 1-day postmortem muscle sample. Postmortem increases in the percentage extractability of proteins and the extractability of α-actinin with 1 mM Tris, pH 8.5 solution indicated postmortem weakening and degradation of Z-disks and(or) I-Z bonds in myofibrils. The postmortem increase in the extraction of actin and α-actinin from isolated myofibrils by H-S solution or buffered 0.6M KCl solution, also suggested the occurrence of postmortem weakening and degradation of the Z-disk and(or) I-Z bonds in myofibrils.

It was further observed that postmortem storage of muscle at 23°C accelerated those biochemical changes observed at 2°C, i.e., troponin T was more rapidly degraded to the 30,000 dalton component. The degradation of component C, troponin I and 30,000 dalton component were also observed in the isolated myofibril of 10-day postmortem samples at 23°C, while these alterations in myofibrillar proteins were not seen in the isolated myofibrils of 10-day postmortem sample at 2°C. These results further substantiate the theory that the specific and limited proteolysis of myofibrillar proteins was caused by CAF activity endogenous to muscle
fibers since storage of muscle at 23°C accelerated those changes observed in muscle postmortem stored at 2°C.

These biochemical changes in myofibrils and their proteins observed for postmortem muscle stored at 2° and 23°C resemble the typical characteristics of CAF activity on isolated myofibrils and purified myofibrillar proteins. The studies of the relationship between postmortem biochemical changes associated with CAF activity and the availability of calcium ion in the muscle fibers were carried out by adding calcium ion and oxalate to at-death minced muscle samples and storing them at 2° and 23°C. The addition of 1 mM calcium ion potentiated the biochemical changes characteristic of postmortem muscle as evidenced by the more rapid appearance of 30,000 dalton component in the isolated myofibrils and by greater extractability of myosin by H-S solution and α-actinin by 1 mM Tris, pH 8.5 solution. On the other hand, addition of 10 mM oxalate retarded all postmortem biochemical changes. Hence, it is concluded that Ca$^{2+}$ is the major factor controlling the postmortem biochemical changes associated with CAF activity in muscle fibers.

Further experiments showed that incubation of at-death muscle in the presence of 1 mM Ca$^{2+}$ at 37°C activated postmortem biochemical reactions associated with CAF activity. These results suggest that pre- or post-slaughter (immediately after death of animal) administration of Ca$^{2+}$ to elevate
the Ca\textsuperscript{2+} concentration in muscle fiber may be advantageous in hastening the postmortem aging process.

The studies of heat-induced changes in myofibrillar proteins were conducted by heating small samples of meat in a controlled temperature water bath to simulate cookery conditions and using buffered 0.6M KCl solution to extract myofibrillar proteins. These proteins were further characterized with SDS-polyacrylamide gel electrophoresis. It was found that the major myofibrillar proteins responded to heating in different ways, myosin of the thick filaments was completely denatured at 55°C, α-actinin of Z-disk was completely denatured at 50°C and actin, tropomyosin of the thin filaments was completely denatured between 70° and 80°C. After heating of muscle samples to 55° or 60°C, the thin filament proteins, actin and tropomyosin, appeared to be completely extracted by buffered 0.6M KCl solution, thus suggesting that weakening of thin filaments attachments or I-Z bonds occur during heating. It is likely that thermal alterations of myofibrillar proteins are even greater than those alterations by postmortem aging. The weakening of Z-disks and(or) I-Z bonds and the alterations of thick filaments in postmortem aging may have additive effects to the thermal modifications on the myofibrillar proteins during heating.
CONCLUSIONS

1. The degradation of troponin T and the concurrent appearance of a 30,000 dalton component occurs without affecting any of the major myofibrillar proteins in myofibrils isolated from postmortem stored muscle. These changes became apparent at 2-days postmortem (2°C) and at 1-day postmortem (23°C).

2. Hasselbach-Schneider (H-S) solution is specific for extraction of the thick filament proteins, myosin, M proteins and component C and it extracted limited quantities of the thin filament proteins, actin, tropomyosin and troponin from isolated myofibrils.

3. Marked increase in extractability of isolated myofibrils with H-S solution was observed in 1- and 2-day postmortem muscle stored at 2°C and this increase was mainly due to the increase in extraction of myosin. It is therefore evident that alterations occur in the structure and in the integrity of thick filaments and their proteins during postmortem storage of muscle.

4. 1 mM Tris, pH 8.5 solution extracts the thin filament proteins, actin, tropomyosin and troponin and the thick filament proteins, component C and myosin light chains. Alpha-actinin is very resistant to extraction with 1 mM Tris, pH 8.5 solution from at-death myofibrils, but it becomes
readily extractable from postmortem samples. Thus, postmortem weakening and degradation of Z-disks occur during postmortem storage of muscle.

5. Room temperature (23°C) storage of muscle accelerates all postmortem biochemical changes observed at 2°C. Postmortem storage at room temperature caused degradation of component C, troponin I and the 30,000 dalton component.

6. Biochemical observations offer evidence for a limited and specific proteolysis of muscle proteins caused by CAF enzyme in muscle fibers. Addition or chelation of calcium ion in muscle fibers potentiates or retards all observed postmortem biochemical changes associated with CAF activity, that is, the degradation of troponin T to a 30,000 dalton component in isolated myofibrils, the increased extractability of myosin by H-S solution and α-actinin by 1 mM Tris solution from isolated myofibrils. Furthermore, storage of muscle at room temperature (23°C) and incubation of at-death muscle samples in the presence of 1 mM calcium ion at 37°C hasten the biochemical reactions associated with CAF. Calcium ion availability and CAF are major causal agents of postmortem muscle changes.

7. Calcium ion accelerates the rate of postmortem glycolysis and hence, muscle pH decline.

8. Myofibrillar proteins respond to heating temperatures in different ways. Proteins of the thick filaments
and Z-disks are more heat labile than proteins of the thin filaments. Myosin is completely denatured at 55°C, α-actinin at 50°C, and actin and tropomyosin between 70° and 80°C.

9. Thin filaments dissociate from Z-disks and possibly from thick filaments when muscle is heated to 55° ~ 60°C. Postmortem weakening of Z-disks and alterations of thick filaments are additive to the effects of thermal alterations in myofibrils during heating.

10. SuS-polyacrylamide gel electrophoresis is an outstanding technique for identification of small molecular subunit changes in myofibrils and their proteins which occur during postmortem aging and heating.
BIBLIOGRAPHY


Ritchey, S. J. 1965. The relationships of total, bound, and free water and fat content to subjective scores for eating quality in two beef muscles. J. Food Sci. 30:375-381.

Ritchey, S. J., and R. L. Hostetler. 1964. Relationship of free and bound water to subjective scores for juiciness and softness and to changes in weight and dimensions of steaks from two beef muscles during cooking. J. Food Sci. 29:413-419.


ACKNOWLEDGMENTS

The author expresses his sincere appreciation to Dr. F. C. Parrish, Jr. for his guidance, counsel and encouragement throughout the course of the graduate study and in the preparation of the dissertation.

The author is grateful to Dr. D. E. Goll for his advice and assistance throughout the graduate career and to Dr. M. H. Stromer for his invaluable assistance in the graduate study. Special appreciation is extended to Dr. D. R. Griffith and Dr. J. A. Thomas for their participation in the graduate program.

Thanks must also go to the other staff members, graduate students and technicians in the department for their help, consideration and friendship. Special recognition is given to Miss Maurine MacBride for her invaluable technical assistance in the research program.

To Mrs. Mary Smith, the author expresses his sincere appreciation for typing this dissertation.

A special thank you to my wife, Sue, for her encouragement and patience and to my daughters, Yin, Mei, and Alice for their patience during my graduate career.